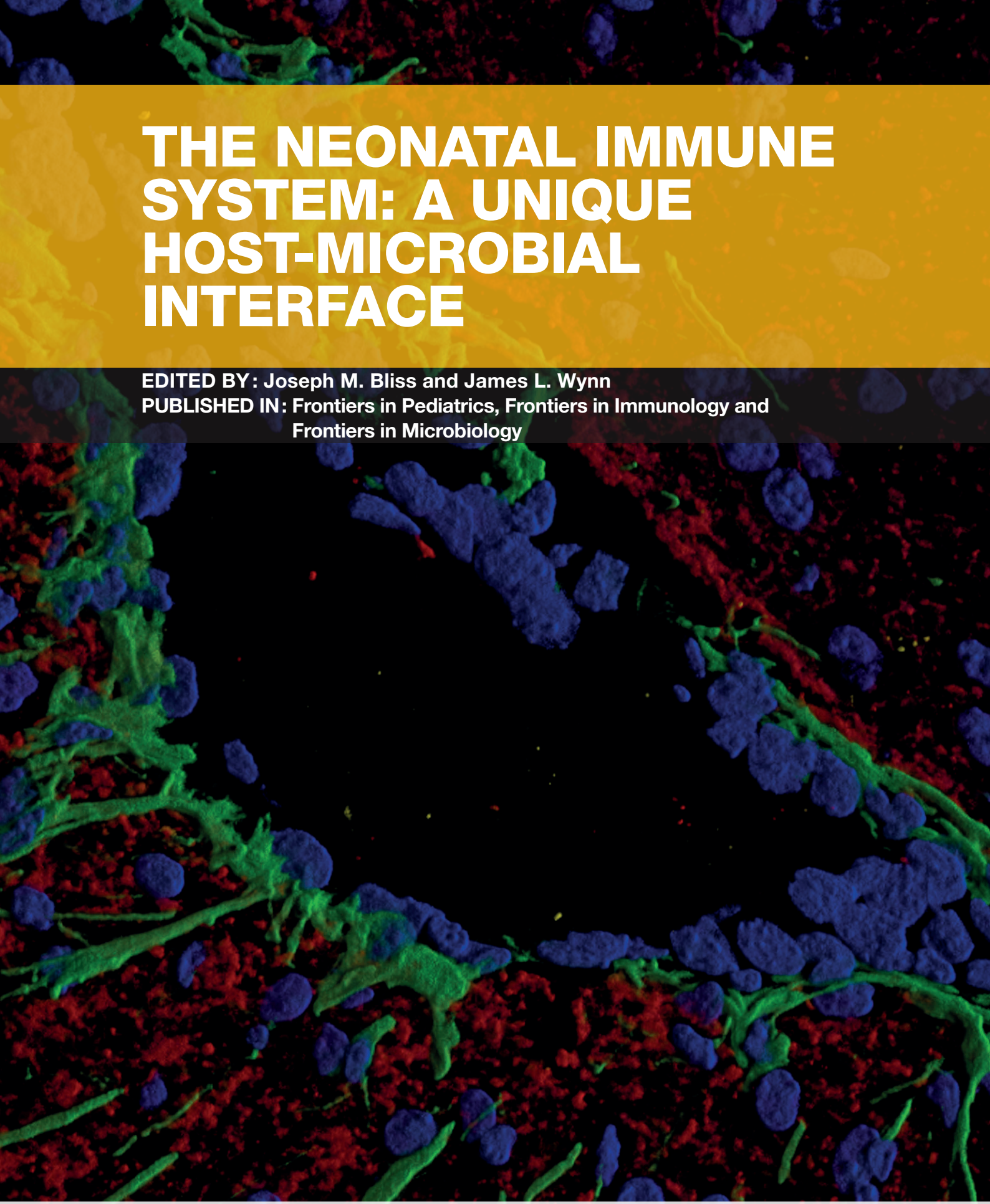

THE NEONATAL IMMUNE SYSTEM: A UNIQUE HOST-MICROBIAL INTERFACE

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THE NEONATAL IMMUNE SYSTEM: A UNIQUE HOST-MICROBIAL INTERFACE

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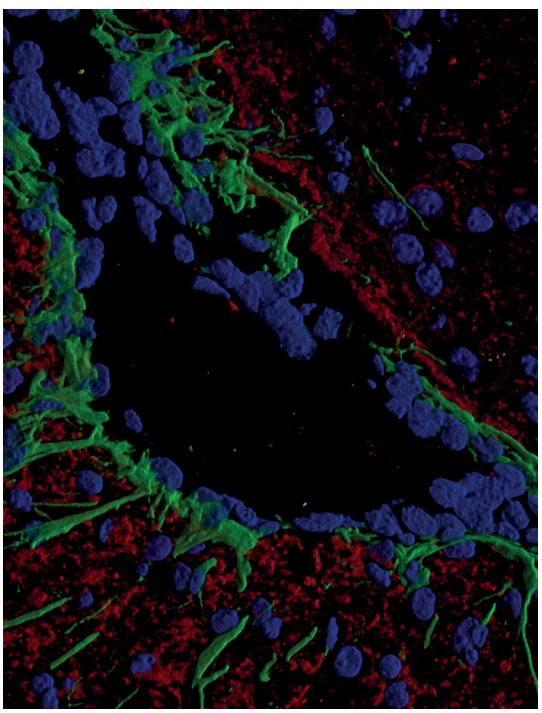

A representative 3D confocal immunofluorescence image of the mouse brain at 21 days of age after sepsis on day 7, highlighting the presence of periventricular scarring. Green: GFAP (Glial Fibrillary Acidic Protein); Red: beta-3-tubulin; Blue: DAPI (4',6-diamidino-2-phenylindole). Image: Dr. James L. Wynn.

Emerging from the protective environment of the uterus, the newborn is exposed to a myriad of microbes, and quickly establishes a complex microbiome that shapes the infant's biology in ways that are only now beginning to come to light. Among these exposures are a number of potential pathogens. The host responses to these pathogens in the neonatal period are unique, reflecting a developing immune system even with delivery at term. Preterm infants are delivered at a time when host defense mechanisms are even less developed and therefore face additional risk. As such, the organisms that cause disease in this period are different from the pathogens that are common in other age groups, or the disease they cause manifests in more severe fashion.

Developmental alterations in both innate and adaptive immune responses in neonates have been documented among many cell types and pathways over the last several decades. Contemporary insights into the human immune system and methodologies that allow an "omics" approach to these questions have continued to provide new information regarding the mechanisms that underlie the human neonate as an "immunocompromised host."

This Research Topic highlights studies related to this unique host-pathogen interface. Contributions include those related to the innate or adaptive immune system of neonates, their response to microbial colonization or infection, and/or the pathogenesis of microbes causing disease in neonates.

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EDITORIAL
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Editorial: The Neonatal Immune System: A Unique Host-Microbial Interface

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Keywords: neonate immune responses, neonatal infection, sepsis, host–pathogen interactions, microbiome and immune system

Editorial on the Research Topic

The Neonatal Immune System: A Unique Host-Microbial Interface

The human infant is delivered at a stage in which every organ system is very much in the process of rapid development. As such, this stage of life is unique relative to any other that the organism will subsequently experience. This Research Topic focuses on the immune system of the newborn as one such organ system. The articles herein are original research or review articles that discuss some aspect of the neonatal immune system and/or the pathogens against which the system has evolved to defend. The articles can be broadly classified into three overall themes. (1) The influence of early life microbial and nutritional exposures on the formation and function of the human microbiome and mucosal immunity. (2) Characteristics of specific immune cell subsets that differ in the neonatal period relative to other life stages. (3) Specific neonatal infections, neonatal host-defense mechanisms, and unique features of the inflammatory response including vaccines. (4) Fungal infections and host defense in the newborn.

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SECTION 1: THE MICROBIOME, FEEDING, AND MUCOSAL IMMUNITY

The complexity and importance of the microbiome on human development, health, and disease has only begun to be appreciated. In this section, the microbiome and the influence of feeding early in life are reviewed with an emphasis on its influence in developing mucosal immunity.

Early Life Host–Microbiome Interphase: The Key Frontier For Immune Development

Amenyogbe et al. summarize the interaction of the immune system with the microbiome in early life as a critical window of susceptibility for life long disease, as well as an opportunity to protect and promote life-long health.

Starter Feeding Supplementation Alters Colonic Mucosal Bacterial Communities And Modulates Mucosal Immune Homeostasis In Newborn Lambs

An original study performed by Liu et al. shows that starter feeding altered colonic mucosal bacterial composition and modulated mucosal immune homeostasis, including TLR4 transcript as well as TNF-α and IFN-γ, during the milk-feeding period in lambs.

Innate Immunity And Breast Milk

The ability of human milk to provide optimal growth and development to the newborn in ways that formula cannot has become increasingly apparent. In this review, Cacho and Lawrence focus on bioactive components of human milk that contribute to the developing innate immune system through unique and fascinating mechanisms including shaping the intestinal microbiome, diminishing inflammation, and providing pluripotent human milk stem cells that may have a role in tissue regeneration in the breastfed infant.

The Role Of Mucosal Immunity In The Pathogenesis Of Necrotizing Enterocolitis

In this review, Hodzic et al. describe the cellular components of the intestinal epithelium and mucosal immune system as well as their relationship to NEC. They also discuss the relationship between the gut microbiota and cell signaling that underpins disease pathogenesis and highlight notable therapeutic advancements in NEC that target intestinal mucosal immunity.

SECTION 2: NEONATAL INNATE AND ADAPTIVE IMMUNITY

Although historically considered immature, deficient, or dysfunctional, more recent study of individual cellular components of the innate and adaptive immune system suggest that the alterations in function that are characteristic of neonatal cells reflect an evolutionarily adaptive phenotype rather than an immature one. Additionally, interactions between the cellular players have blurred the traditional distinction between the innate and adaptive arms. This section combines reviews and some original research into these phenomena.

Age-Appropriate Functions And Dysfunctions Of The Neonatal Neutrophil

Lawrence et al. review that neutrophils are no longer viewed as short-lived, indiscriminate phagocytes of the immune system, but instead as essential components necessary for proper B and T cell function, antigen presentation, and tissue repair and regeneration. Unlike adult neutrophils, neonatal neutrophils are not simply "dysfunctional," but rather exhibit phenotypic and functional variances required to tolerate the hypoxic *in utero* condition without triggering inflammatory responses, yet, remain able to mount sufficient pro-inflammatory reactions if exposed to pathogenic organisms.

Rapid $CD8^+$ Function Is Critical For Protection Of Neonatal Mice From An Extracellular Bacterial Enteropathogen

New research from Siefker and Adkins demonstrates that neonatal $CD8^+$ cells, but not $CD4^+$ cells, increase rapidly in proportion and IFN-γ production to primary infection with *Y. enterocolitica,* and play an important early, innate-like role in survival. However, using knockout mice, they show $CD8^+$ cells were not necessary for the development of protective memory in neonates since they were dispensable for survival of secondary infection.

Comparison of The Functional Microrna Expression In Immune Cell Subsets of Neonates and Adults

Yu et al. present novel systematic and functional miRNA profiles of neonate cord blood and adult leukocyte subsets. These studies serve as a basis to further understand the altered immune response observed in neonates and advance the development of therapeutic strategies.

SECTION 3: NEONATAL SEPSIS, MENINGITIS AND VACCINES

Neonatal sepsis and meningitis continue to carry a high burden of mortality and morbidity among those affected. Although attention to infection prevention practices has been effective at reducing risk of acquiring some of these infections, our approaches to treatment: administration of antimicrobial drugs, and supportive care, have remained essentially the same for decades. This section reviews new information about this problem garnered from modern techniques and presents some original research related to vaccine development and responses in neonates. Finally, the unique susceptibility of neonates to fungal infection is reviewed.

Immunological Defects In Neonatal Sepsis And Potential Therapeutic Approaches

Raymond et al. review key distinctions of neonatal innate and adaptive immunity as compared to adults, summarize what has been learned using transcriptomic approaches on blood to uncover unique neonatal sepsis pathophysiology, and discuss why novel approaches unique to the neonate will be required for the development of both diagnostics and therapeutics in this population.

Neonatal Meningitis: Overcoming Challenges In Diagnosis, Prognosis, and Treatment With Omics

In this contemporary review of a vexing problem, Gordon et al. discuss how modern "omics" technologies have been applied to study and further elucidate the complex interplay between infecting bacterial pathogens and the central nervous system in the setting of neonatal meningitis. Data generated from these studies are likely to be key components to a better understanding of pathogenesis, which is needed to improve diagnostic and therapeutic strategies and ultimately outcomes of this devastating disease.

A Meningococcal Outer Membrane Vesicle Vaccine Incorporating Genetically Attenuated Endotoxin Dissociates Inflammation From Immunogenicity

In this original research article, Dowling et al. describe the unique and favorable profile of an outer membrane vesicle-based vaccine for *Neisseria meningitidis* derived from a mutant strain with

attenuated endotoxin. This vaccine generated a considerably less inflammatory profile from human neonatal and adult leukocytes in whole blood assays *in vitro* compared to several currently licensed vaccines while still inducing a robust antibody response *in vivo*. These data suggest that *in vitro* systems may be useful to reflect age-specific inflammatory and immunogenic profiles of vaccine candidates as well as the properties of adjuvants in vaccine design.

Immunization Of Newborn Mice Accelerates The Architectural Maturation of Lymph Nodes, But Aid-Dependent IgG Responses Are Still Delayed Compared to The Adult

This original research article by Munguía-Fuentes et al. describes the anatomic, cellular, and functional maturation that occurs in neonatal mouse lymph nodes following immunization on the day of birth. They describe recruitment of B cells and T cells, but less robust formation of germinal centers and reduced isotype switching compared to adults. The findings further define unique aspects of the murine antigen response in the early postnatal period that may be more accurately described as adapted to early life rather than immature.

SECTION 4: NEONATAL FUNGAL INFECTIONS

Antifungal Immunological Defenses In Newborns

Michalski et al. present a review of the developing innate and adaptive immune responses of newborns, specifically as they may relate to susceptibility to fungal infections. Although much has been learned about the developing immune system in neonates and its relation to risk for infections, much less has been devoted to fungi as targets of host-defense. While highlighting antifungal host defense, the authors also describe important limitations of current studies.

Investigation of Och1 In The Virulence of *Candida parapsilosis* Using A New Neonatal Mouse Model

Csonka et al. describe a method to investigate *C. parapsilosis* virulence in a newborn mouse model using intravenous infection *via* the facial vein in 2-day old mouse pups. This unique approach provides a novel contribution to available murine models of neonatal candidiasis. The similarity of this method to the well-established tail-vein method in adult mice allows more direct comparisons between the adult and neonatal response to bloodstream infection with *Candida* than previously described neonatal methods.

Candida parapsilosis Protects Premature Intestinal Epithelial Cells From Invasion And Damage By *Candida albicans*

Gonia et al. investigate the potential for *C. parapsilosis* to modulate pathogenic interactions of *C. albicans* with the premature intestine. In this original work, they demonstrate that *C. parapsilosis* is able to reduce invasion, damage, and virulence functions of *C. albicans via* secreted molecules as well as by physical contact with the *C. parapsilosis* cell surface. This discovery brings the possibility that virulence features of pathogens can be modulated by other closely related species in the microenvironment.

AUTHOR CONTRIBUTIONS

Both authors participated in the editorial process for the entire Research Topic and wrote the accompanying editorial.

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REVIEW
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Early-Life Host–Microbiome Interphase: The Key Frontier for Immune Development

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Human existence can be viewed as an "*animal in a microbial world*." A healthy interaction of the human host with the microbes in and around us heavily relies on a well-functioning immune system. As development of both the microbiota and the host immune system undergo rapid changes in early life, it is not surprising that even minor alterations during this co-development can have profound consequences. Scrutiny of existing data regarding pre-, peri-, as well as early postnatal modulators of newborn microbiota indeed suggest strong associations with several immune-mediated diseases with onset far beyond the newborn period. We here summarize these data and extract overarching themes. This same effort in turn sets the stage to guide effective countermeasures, such as probiotic administration. The objective of our review is to highlight the interaction of host immune ontogeny with the developing microbiome in early life as a critical window of susceptibility for lifelong disease, as well as to identify the enormous potential to protect and promote lifelong health by specifically targeting this window of opportunity.

Keywords: microbiome, immunity and infections, ontogeny, immune diseases, probiotics

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INTRODUCTION

A key function of the immune system is to interact with and respond to the environment (1). Microbes are a major part of this environment. In fact, all animals harbor diverse, host-specific microbial communities, each discretely assembled in organ-specific microhabitats across nearly all parts of the body (2). Not surprisingly then, the host–microbiome interphase was found to be key for optimal immune function in adults (3, 4). However, emerging data strongly indicate that the most formative period for this interaction occurs very early in life (5–8), possibly starting even before birth (9, 10). One of the first detailed longitudinal surveys of the intestinal microbiota in its first year of postnatal development found a rapidly changing succession of bacterial taxa beginning with aerobes such as *Streptococcus* and *Staphylococcus* in the first week of life that soon are replaced by obligate anaerobes like *Prevotella* and *Veillonella,* which then continue to feature prominently into adult life (11). A later seminal work compared the genetic potential, or microbiome, of babies compared to adults across geographically distinct populations and found that with dramatic shifts in colonization early in life also came functional shifts, while infant bacteria contain folate synthesis genes, those in adults contain more genes for folate metabolism and cobalamin, vitamin B7, and B1 synthesis (12). Since then, the development of microbiota throughout infancy has been the topic of numerous reviews (13–16).

Given the rapid changes of the microbiome in early life (16), even minor perturbances during this highly dynamic phase could have negative long-lasting consequences (17). For example, in atopy, an at least partly immune-mediated disease, alterations of the microbiota in early life appear to be the culprit (18). Specifically, atopic infants harbor fewer Bifidobacteria, Lactococci, and Enterococci as early as 1 week of age as compared to non-atopic controls (19); and infants diagnosed with atopy at 5 years of age had been less likely colonized at 1 week of age with *Bacteroides adolescentis* and Lactobacilli Group I as compared to non-atopic infants (20). Furthermore, colonization at 1 month of age with *E. coli* and *C. difficile* is associated with increased risk of eczema in first 2 years of life, and for *C. difficile* specifically with recurrent wheeze, allergic sensitization, and atopic dermatitis at 2 years (21). How such differences in the microbiota lead to clinically symptomatic atopic disease is not understood, but given the underlying immune pathogenesis of atopy, the mechanisms likely involve altered immune ontogeny (22). One recent study identified newborns with a distinct microbiota enriched in fungal species *Rhodotorula* and *Candida* together with decreased relative abundance of *Bifidobacterium*, *Lactobacillus*, and *Akkermansia* who suffered an increased risk for atopy at 2 years of age and physician-diagnosed asthma at 4 years of age. Moreover, sterile fecal waters from such at-risk infants induced a higher proportion of IL-4-secreting compared to IFNγ-secreting adult CD4+ cells, linking fecal metabolites to possible immune cell alterations that could play a role in increased asthma risk for these children (23). Other studies have linked particular early-life microbiota to variation in immune ontogeny later in infancy. For example, newborns colonized with *Bacteroides fragilis* express lower levels of TLR4 and TLR2 mRNA in their peripheral blood leukocytes at 1 year of age and produce lower levels of inflammatory cytokines (24). However, in another study, infants with a greater abundance of *Bacteroides dorei* in the microbiota during infancy displayed a higher incidence of inflammatory diseases (25). As these contrasting results involve distinct human populations as well as different strains of *Bacteroides*, they caution against generalizing properties of different, but related microbial species across populations.

Assigning molecular cause–effect relationships to alterations of the microbiome with impact on the developmental trajectory of the immune system is difficult given the complexity of the systems involved and the rapidity with which each changes (5, 26). However, *B. fragilis* is an exception as it provides one of the best studied examples of a human commensal driving immune ontogeny. Specifically, polysaccharide A (PSA), a sphingolipid specific to *B. fragilis,* was among the first bacterial products shown to induce maturation of CD4+ T cells in both the mucosa and spleens of germ-free mice (27). *B. fragilis* PSA in particular was shown to play a critical role in neonatal immune development, where colonization with PSA-expressing *B. fragilis* was necessary for regulatory T cell (Treg) development and invariant NKT cell inhibition in the intestine—the absence of which led to exacerbated inflammation in adulthood (28). Importantly, colonizing adult mice with *B. fragilis* failed to correct this defect (28), indicating critical early-life window of susceptibility for the microbiota to educate the immune system (5). This is again reflected on the clinical level, where differences in microbiota at 3 months of age better predict atopic outcome at 1 year than the microbiota collected at 1 year (29), and microbiota at 3 months predict milk allergy resolution at 8 years of age better than microbiota collected at 6–12 months (30). Further evidence for the existence of a critical early-life window was found when studying the effects of early-life microbial exposure on NK cell phenotypes, where conventionalization of germ-free mice at either 1 week or 3 weeks of life resulted in higher splenic IFNγ-expressing CD4 cells, and higher frequencies of NK and NKT cells compared to conventionally housed mice (31). Immune-regulatory genes were also underexpressed in ileal tissues of the same mice after conventionalization at 1 or 3 weeks of age (32). Clostridial species (specifically, *Clostridium* clusters IV and XIVa) have also been shown to induce Treg accumulation in mouse colons if present during specific early-life periods. Colonizing mice with these bacteria at two weeks of age protects them from colitis in adulthood and lowers their systemic IgE levels (33). On the other hand, exposure to segmented filamentous bacteria (SFB) in early life of mice is uniquely able to induce large numbers of Th17 cells (34) using a mechanism dependent on their adherence to the intestinal epithelium (35). Through induction of Th17 cells, SFB were also shown to exacerbate autoimmune arthritis in colonized germ-free mice (36). While the role of SFB, or similar bacteria in the neonatal period has yet to be defined, one survey of SFB abundance across species and ages found SFB to colonize humans by 2 years of age, but could no longer be found after the third year of life suggesting a possible early-life restricted colonization for these bacteria in humans (37).

While much of the necessary detailed knowledge is still amiss, current data clearly support the notion that perturbations of microbiota in the early-life imprint the host immune phenotype for a long time (maybe lifetime) and can manifest as immune-mediated disease later in life. We here extract overarching themes of how pre-, peri-, as well as early postnatal environmental modulators of newborn microbiota associated with changes in immune ontogeny that predispose to disease; given the little data there are on this topic, we focus on those disease states for which existing data suggest this to be a plausible if not reasonable connection. In doing so, we also begin to delineate the windows of opportunity, knowledge of which should help guide to target research efforts into mechanisms and interventions. The goal of this review then is to highlight the potential harm as well as benefit of early-life alteration of the host immune–microbiome interaction and its long-lasting impact on homeostasis and health.

IN UTERO COLONIZATION INFLUENCES IMMUNE DEVELOPMENT AFTER BIRTH

The dogma of a sterile intrauterine environment as necessary for normal, healthy term pregnancies was recently challenged when bacteria were found in human placental membranes (38, 39), amniotic fluid and umbilical cords (40) as well as meconium (41, 42) of healthy term newborns. Even more surprising was the finding that these fetal tissues contained not a random collection of microbes but an organ-specific microbiome. Specifically, the

human placenta harbors a unique microbiome with a taxonomic profile that is most similar to the oral cavity of the mother (41, 43). Previously, maternal oral flora had only been thought to be associated with preterm delivery or stillbirth, not healthy term feti (44–46). However, using *in situ* hybridization, bacterial organisms are detectable in 70% of the placental membranes that harbor no sign of inflammation (chorioamnionitis) (39). And in a cross sectional study designed to randomly sample the placental basal plates at delivery revealed *via* histological analysis that of a total of 195 human pregnancies, Gram-positive as well as Gram-negative bacteria of diverse morphologies were detectable in 27% (47). Transmission of bacterial DNA from the oral cavity of the mother to the fetus was directly proven when genetically labeled bacteria orally inoculated into pregnant mice could be detected by PCR in the meconium of the pups delivered by C-section (42). While transmission of maternal microbial products (not live microbes) during pregnancy across the murine placenta to the fetus can be enhanced by the presence of maternal antibodies (10), the mechanism that promotes transfer of live maternal oral flora across the placenta to the fetus has not yet been elucidated.

Given not all the human placentas of term pregnancies examined contained bacteria (39, 47), and the fact that germ-free mice deliver their litter at term (48), it is likely that a placental and fetal microbiome may not be necessary to carry normal pregnancies to term, but serve another function, such as shaping the development of host immune responses in the offspring (9, 49). For example, germ-free newborn mice born to mothers transiently colonized by *E. coli* during pregnancy are better able to avoid postnatal hyper-inflammatory responses and also more readily curtail systemic invasion with intestinal microbes than offspring born to non-colonized dams (10). Maternal colonization appeared to reprogram intestinal transcriptional profiles in the offspring including increased expression of genes encoding epithelial antibacterial peptides as well as metabolism of microbial molecules; gestational colonization also increased intestinal group 3 innate lymphoid cells as well as F4/80+CD11c+ mononuclear cells (10). The data of this study support the notion that the maternal microbiota and its products transferred to the fetus prepare the newborn for optimal host–microbial mutualism, rather than solely enhancing antibacterial immune responses (10).

The findings summarized above suggest that actively modulating the maternal microbiome *via* probiotics during pregnancy may provide avenues to modulate immunity in her offspring. For example, in a randomized double-blind placebo-controlled trial where 29 women who were to undergo an elective C-section at term received *Lactobacilli* and/or *Bifidobacterium lactis* 14 days prior to delivery, the presence of the specific probiotic administered orally to the mother was detectable in the placenta, the amniotic fluid, as well as the meconium of the offspring (50). Furthermore, administration of the probiotic to the mother was associated with changes in the expression of Toll-like receptors (TLRs) in the placenta and the infant meconium (50). In particular, a reduced TLR7 mRNA expression was detected in intestinal samples of infants whose mothers received *B. lactis*, while the combination of *B. lactis* with Lactobacillus GG was associated with decreased TLR6 mRNA expression in the fetal intestine (50). Moreover, oral supplementation with *Lactobacillus rhamnosus* or *B. lactis* probiotics during pregnancy significantly increased cord blood interferon-gamma (IFNγ) production as compared to the placebo group (51). However, given that the presence of bacterial products in fetal tissues was only recently discovered, the relevance of *in utero* colonization for clinical outcomes in humans has not yet been determined.

PERINATAL MEDICAL INTERVENTIONS PROFOUNDLY ALTER THE NEWBORN MICROBIOME WITH LASTING IMPACT ON IMMUNE DEVELOPMENT AND HEALTH OUTCOMES

Delivery mode (cesarean vs. vaginal delivery) and intrapartum antibiotic use represent two rather common perinatal events that significantly alter a newborn's microbiota, immune ontogeny, and health outcomes even later life (see **Tables 1** and **2**).

Cesarean Delivery (CD)

Cesarean deliveries have increased globally from 6.7% in 1990 to 19.1% in 2014, with rates above 30% in several countries such as the United States, Brazil, and China (52). While CD can certainly be lifesaving for indications such as placenta previa and uterine rupture, the growing use of CD has been under increasing scrutiny as data suggest that increased use of elective primary cesareans for low-risk pregnancies can be associated with increased morbidity and mortality to mother and child compared

TABLE 1 | Effect of perinatal perturbances on newborn's microbiota.

Perturbance	Sampling age	Microbiota trends
Cesarean delivery	First week of life	Fewer *Bifidobacteriaceae, Enterobacteriaceae, Bacteroides,* and *Lactobacilli,* and greater relative abundance of *Haemophilus, Veillonella, Clostridiaceae,* and *Klebsiella* (66)
	First 3 months of life	Fewer *Bacteroidaceae* and greater abundance of *Clostridiaceae* (with more striking differences for emergency vs. elective C-sections) (70)
	First 12 months of life	Fewer *Bacteroidales* other taxa like *Clostridiales* and more abundance of *Enterobacteriaceae* (68)
Intrapartum antibiotic exposure	Day 3 of life	Reduced *Bacteroides* and *Parabacteroides*, increased abundance of *Enterococcus* and *Clostridium* (70)
	Day 7 of life	Reduced bacterial diversity with lower levels of *Bifidobacteria* and *Bacteroides*, and higher levels of *Enterobacteriaceae* or *Streptococcaceae* (77, 78)
	Day 7 and 30 of life	Reduced proportions of *Bifidobacteria* and increased proportions of *Enterobacteria;* no changes in *Lactobacillus* and *Bacteroides* at any time (80)
Neonatal antibiotic exposure	First weeks of life	Increased abundance of *Enterococcaceae* (70, 77–80)
Formula feeding		Reduced abundance of *Bifidobacteria* and *Lactobacilli* (85)

TABLE 2 | Effect of perinatal perturbances on newborn's health.

Perturbance	Health condition and/or disease associated	Age at onset	Reference
Cesarean delivery	Type 1 diabetes, celiac diseases, childhood and adult obesity, asthma, allergic disease, bronchitis	First 2 years of life to adult life	(54–63)
Antibiotics exposure (before 6 months of age)	Increased risk for corticosteroid-treated wheezing, necrotizing enterocolitis, late-onset sepsis, early mortality, obesity, and exacerbation of hypersensitivity to pneumonitis	First year of life—school age	(108–113, 117)
Formula feeding	Increased risk for diarrheal disease, mortality, diabetes, and overweight. Possible association with a higher occurrence of early-onset inflammatory bowel disease, atopic disease, and ankylosing spondylitis when compared to breastfed infants	First year of life up to 8 years of life	(84, 97, 103)

to spontaneous vaginal delivery (53). Further, and more relevant to this review, CD has been associated with range of immune-mediated diseases in the offspring, such as an increased risk for type 1 diabetes (54, 55), celiac disease (55), childhood and adult obesity (56–58), asthma (59, 60), and allergic disease (61, 62). CD may also be associated with susceptibility to infections, as CD born infants are more likely to be hospitalized for bronchitis throughout the first 2 years of life (63). In all this, the microbiota has often been implicated as a driver of these various immune-mediated diseases.

The vagina provides vaginally delivered (VD) newborns with their first *ex utero* microbial inoculum. The skin and oral microbiota of VD newborns moments after birth, and rectum 24 h after birth, closely resembles the mother's vaginal microbiota (64). In contrast, CD infants' microbiota most closely resembles skin microbes and is no more like their mother's than to another women's skin microbiota. For example, in Swedish infants and their mothers, 72% of operational taxonomic units (a DNA sequence-based classification of bacteria) detected in stools of VD infants at 1 week of age could also be found in the mother's stool; this was reduced to only 40% for CD infants (65). In a recent meta-analysis, microbiota of CD newborns was found to be less diverse within the first week of life, harbored fewer *Bifidobacteriaceae, Enterobacteriaceae, Bacteroides,* and *Lactobacilli,* and greater relative abundance of *Haemophilus, Veillonella, Clostridiaceae,* and *Klebsiella* than VD infants (66). Furthermore, increased abundance of *Clostridiaceae* was detectable up to 2 months, and both lower diversity and relative abundances of *Bifidobacteria* and *Bacteroides* were detectable up to 3 months of age. However, the microbiota of CD and VD infants became increasingly less distinguishable over the first 3 months of life, suggesting an equalizing influence of the environment. This has again been noted in more recent studies, where the microbiota of infants differed by mode of delivery at birth for the nares, mouth, and skin but not for meconium, with few differences still seen at 6 weeks of age (67). And another survey of 24 VD and 19 CD newborns showed that while the stool microbiota of both groups converged by 2 years of age, CD infants were less colonized by *Bacteroidales* during the first year of life, while other taxa such as *Clostridiales* and *Enterobacteriaceae* became more abundant (68).

It is interesting to note that microbiota of elective vs. emergency cesarean deliveries can often be not distinguished. Only one small study reported lowest bacterial diversity among three infants delivered by elective CD compared to three infants delivered by emergency CD, which were more similar to VD infants (69). In another study, the skin, nares, mouth, and meconium or stool microbiota of infants were surveyed alongside their mothers at the same four sites in addition to the vagina at birth or 6 weeks of age (67). Here, the differences seen by delivery mode at birth were most apparent for CD infants born without labor, compared to CD or CD after labor onset. However, the sample size of this study also was limited, as only 13 mother–infant dyads were sampled at the 6-week time points for combined labored- and unlabored-CD compared to 40 VD diads. In a more highly powered study comparing 17 elective CD, 23 emergency CD, 40 VD infants born to mothers given intrapartum antibiotic prophylaxis (IAP), and 96 VD infants not exposed to any antibiotics found the opposite, namely, that both elective and emergency CD infants harbored fewer *Bacteroidaceae* and greater *Clostridiaceae* at 3 months of life compared to VD infants irrespective of IAP exposure, but these differences were more striking for emergency CD infants rather than elective. Moreso, these differences persisted up to 1 year of age more in emergency CD infants compared to any other group (70). As such contradictory findings may be due to sample size, larger cohorts are needed to provide more insight into colonizing differences between elective vs. emergency CD infants, together with changes due to antibiotic use alone—especially since the effect of CD on the microbiota overall has been minimal—explaining only 2% of total variance in the first year of life (68) and less than 4% even at birth (67). Large, well-defined cohorts will be necessary to capture these differences.

However, such equalization was not seen for immune responses, where differences between CD and VD infants remain detectable up to 2 years of age. For example, human CD newborns harbor fewer IgA, IgG, and IgM-secreting cells throughout the first year of life (71), as well as lower levels of Th1-supporting chemokines CXCL10 and CXCL11 (72), lower levels of IFNγ and IL-8, and lower CD4+ T-cell responses to tetanus toxoid (73) over the first 2 years of life. Mouse studies further support imprinting of immune differences in the immediate period after CD vs. VD. Mice delivered by CD display distinct microbiota at weaning, but not later in adulthood. On the contrary, immune differences persist from the newborn period into adulthood, where CD mice display a lower tolerogenic mucosal immune profile with fewer Tregs and *IL10* gene expression in their mesenteric lymph nodes as compared to VD mice (74).

In summary, while the epidemiological data regarding a causative links between CD and any of the aforementioned immune-mediated diseases were not drawn from randomized trials and have yet to be confirmed using relevant animal models, CD infants appear to display an increased risk to suffer from

several immune-mediated diseases. The evidence that CD born infants display an immune developmental trajectory that differs from VD born infants on the other hand is sound. Equally robust is the finding that microbiota of CD infants differs substantially from that of VD infants, but more so during the first 3 months of life, after which differences become increasingly less apparent. Thus, if the microbiota were to represent the mechanistic link between CD, altered immunity, and with that increased disease susceptibility, then early-life differences must have been imprinted during an early-life "window of susceptibility." However, such window and its associated mechanistic links have yet to be defined sufficiently well in the human setting to address it clinically.

Intrapartum Antibiotics

Intrapartum antibiotic prophylaxis is the intravenous administration of penicillin or ampicillin to women during labor, who were found to be vaginally or rectally colonized with group B Streptococcus (GBS). Prior to its routine use in the 1990s, early-onset GBS was a leading cause of newborn morbidity and mortality in the United States (75). Following implementation of IAP, early-onset GBS disease incidence fell from 1.7 per 1,000 live births in the 1990s to 0.37 per 1,000 live births by 2008 (76).

Beyond the clinical success of IAP in the prevention of GBS infection in the newborn, the impact of IAP on the newborn infant microbiome has barely been investigated, despite the obvious implications. Most surveys of microbiota alterations due to IAP have been conducted by on group (77–80). This group recruited a cohort of mothers receiving ampicillin for GBS prophylaxis alongside GBS negative mothers not receiving any antibiotics at or within a month of delivery. Stools from their newborns were collected at postnatal days 7 and 30. A fist set of studies compared microbiota of 10 IAP to 10 controls at postnatal day 7 using sequence-based approaches, finding that IAP infants displayed reduced bacterial diversity, lower levels of *Bifidobacteria* and *Bacteroides*, and higher levels of *Enterobacteriaceae* or *Streptococcaceae* (77, 78). In a follow-up study comparing effects of exclusive breast- to mixed-feeding at both 7 and 30 days, microbiota of 13 IAP and 13 controls were assessed with a sequence-based approach, finding that differences between IAP and controls were more prominent in exclusively breastfed (BF) compared to mixed-fed babies, and moreso at 7 days compared to 30. However, IAP infants had reduced proportions of *Bifidobacteria* and increased proportions of *Enterobacteria* regardless of feeding group. And while *Bifidobacteria* proportions equalized by day 30, exclusively BF IAP infants still had especially high proportions of *Enterobacteria* compared to unexposed infants (80). The highest-powered study compared 35 IAP to 49 control infants at days of life 7 and 30 using quantitative PCR for select bacterial taxa, finding fecal bacterial counts of *Bifidobacterium* alone were reduced at day of life 7 only, while *Lactobacillus* and *Bacteroides* were unaffected at any time (79). Only one other group has described alterations of the microbiota due to IAP, and did so for a cohort of Canadian infants receiving IAP either for GBS prophylaxis or CD, and compared their microbiota at 3 months and 1 year of life, finding that IAP was also associated with lower *Bacteroides* as well as lower levels of *Parabacteroides* but higher *Enterococcus* and *Clostridium* levels at 3 months of life among both vaginally and cesarean delivered infants (70). The major differences to persist to 1 year of life were among emergency CD infants only (as discussed above), whereby VD infants were now indistinguishable by IAP exposure aside from a minor increase in *Clostridiaceae*. There is only one study that assessed effects of IAP on the whole genetic content of the microbiota using whole-genome sequencing (67), with few differences found overall, yet functional pathways in the stool at 6 weeks of age revealing correlations to IAP among delivery mode, feeding, maternal weight, and gestational age.

The impact of IAP on immune ontogeny has to our knowledge not been addressed at all. Furthermore, despite the striking similarity of the changes of the microbiota in infants exposed to IAP and those born by CD, and the many health implications associated with CD, the clinical impact of IAP on health outcomes other than neonatal GBS infection has not been addressed at all. The first study addressing this serious knowledge gap is currently in progress, following 240 mother–infant pairs prospectively, assessing IAP and control infant microbiota at 3 months and 3 years of age (81).

In summary, IAP has undoubtedly prevented many newborn GBS-related deaths. However, given that 10–30% of women in North America are colonized with GBS and receive IAP during labor (82, 83), there has been a surprising lack of effort to address long-term effects of IAP on immune development and the health of their offspring.

EARLY POSTNATAL EVENTS DRAMATICALLY ALTER THE MICROBIOME WITH IMPACT ON LONG-TERM HEALTH OUTCOMES, BUT A CAUSATIVE ROLE OF IMMUNE CHANGES IN THIS REMAINS UNEXPLORED

Feeding mode (breast vs. formula) as well as antibiotic exposure during the neonatal period (here defined as up to day of life 28) have clearly been linked to changes in microbiota (**Table 1**); their causal relationship to immune development and clinical outcome have surprisingly not been well delineated (**Table 2**).

Feeding Mode

Differences in the microbiota of BF and formula-fed (FF) infants were first reported nearly 100 years ago with compounds in breast milk found to promote the growth of *Bifidobacteria* (this "bifidus factor" is now recognized as human milk oligosaccharides) (84). As a result, BF infants harbor more *Bifidobacteria* and *Lactobacilli* in their colons than FF infants (85). Interestingly, there is little effect of mixed vs. exclusive BF on the microbiome, as the profound shift in microbiota to an adult-like composition occurs not with the addition of solid food, but rather at cessation of BF (65).

Immune protective functions provided by breast milk were first reported in the 1970s (86). While immunoglobulins were among the first immune molecules recognized in breast milk,

breastfeeding has further profound anti-inflammatory influences mediated largely by high concentrations of TGF-β and IL-10, and other immunomodulatory influences mediated by molecules such as soluble CD14, defensins, lactoferrin, and lysozymes that survive passage to the intestinal tract and together act to maintain homeostasis in the colonizing gut (87–90). Indeed, one study has found higher concentrations of anti-inflammatory TGFβ and lower concentrations of pro-inflammatory TNFα and IL-2 in sera of BF compared to FF infants throughout the first year of life (91).

Breast milk contains its own microbiome, harboring a wide range of microbes from 100 to 10^5 CFU per ml depending on the study (92), with *Streptococcus* and *Staphylococcus* being most common, but others such as *Lactobacillus, Bifidobacterium, Enterococcus,* and *Propionibacterium* readily isolated from milk of healthy women (92). Many short-chain fatty acid producing bacteria such as *Veillonella, Propionibacterium,* and *Faecalibacterium* have also been isolated from breast milk (92). Breast milk itself contains lactic acid bacteria, double-stranded RNA from which stimulates intestinal dendritic cells *via* TLR3 to produce IFNβ, which in turn promotes an anti-inflammatory environment and protects mice against colitis (93, 94). However, the key mediator of the immune homeostatic function of breast milk is presumed to relate to its impact on gut microbiota. In mice, for example, the presence of maternal sIgA in early-life molds the composition of the gut microbiota long into in adulthood, with pups born to sIgA-deficient dams harboring more *Pasteurellaceae* and *Lachnospiraceae* than controls (95).

Many of breast milk's health-promoting properties were recognized starting over 100 years ago when BF infants were found to suffer less diarrheal disease and reduced mortality rates, and reduced risk for diabetes, and overweight compared to FF infants (84). These were recently reviewed in great detail (87). Beyond diarrhea, BF has since been found to protect from other infections, and in BF newborns who did not receive antibiotics prior to weaning, every additional month of breastfeeding is associated with a 5% decrease in number of postweaning antibiotic courses (96). A recent meta-analysis summarized the powerful evidence that BF is associated with decreased risk for infectious diseases and mortality (97). Specifically, BF infants have only 12% of the risk of FF infants to die in the first 6 months of life. Other data further support an immune-mediated mechanism of BF as one of the possibly responsible mechanisms. For example, BF is associated with lower risk for eczema and recurrent wheeze in first year of life (98), with exclusive BF for >4 months associated with reduced risk for asthma up to 8 years of age (99), and in another study breastfeeding for less than 4 months was associated with increased corticosteroid-treated wheezing episodes in the first year of life (100). However, it is important to note that while meta-analyses do detect a protective effect of BF on asthma and allergic rhinitis, these effects are weaker when limited to studies with the lowest risks of confounding (97, 101). And in a cohort of familial ankylosing spondylitis patients and their families, disease prevalence was 25% in children who were breast fed while it was 40% in the FF comparator (102). Finally, while BF is weakly associated with decreases in early-onset inflammatory bowel diseases but with non-significant differences found for ulcerative colitis and Crohn's disease separately (103), it is protective against Crohn's disease-related surgery later in life (104).

Despite the many documented clinical benefits of BF, as well as the known profound impact on immune ontogeny and the microbiome, direct cause–effect relationships between BF-induced changes in the microbiome leading to immune-mediated clinical benefit have not yet been provided.

Antibiotic Exposure in the Neonatal Period

Empiric antibiotic treatment (EAT) is often given to newborns at risk of developing early-onset sepsis (EOS). Clinical diagnosis of EOS is imprecise and based on non-specific signs and symptoms; rapid, sensitive tests to differentiate infected from uninfected newborns are also lacking (105). Therefore, EAT is administered to a very large number of newborns (106, 107). While this empiric approach can readily be justified given the potentially horrific outcome of treatment delay in EOS (105), the impact on the microbiome, immune development, and clinical outcome beyond sepsis has barely been investigated. The little that is known suggests a profound alteration of normal physiology may occur. For example, antibiotic administration in early life is associated with being overweight at age 12 years (108). Contrary to the previous study that only found associations between overweight and antibiotic use throughout the first year (108), in another study antibiotic use in infants less than 6 months was associated with obesity in childhood, but antibiotic use after 6 months of age was not (109). Moreso, antibiotic administration specifically in the neonatal period was associated with an increased risk for corticosteroid-treated wheezing in the first year of life (100) and allergic rhinitis in school age children (110). Longer duration of antibiotic use in premature infants has been associated with increased risk for necrotizing enterocolitis, late-onset sepsis, and death in early life (111, 112). Even the choice of antibiotic regimen has effects, where ampicillin combined with cefotaxime was associated with increased mortality as compared to ampicillin with gentamicin (113).

As early-life antibiotic use has become a topic of increasing interest, mouse studies have begun to reveal possible cause–effect relationship between early-life antibiotic and later life disease: administration of penicillin to pregnant dams right before birth and through weaning increases body mass of the pups in adulthood, and transferring such perturbed microbiota to germ-free mice is sufficient to replicate this phenotype (114). An association of early-life antibiotic use and altered immune ontogeny is suggested by findings in mouse models where mice exposed to antibiotics prenatally and shortly after birth had increased susceptibility to Vaccinia virus infection and altered CD8 T cell responses at 2 weeks of age (115). Antibiotic exposed infant mice also harbored a microbiota rich in *Enterococcus faecalis* (115), consistent with findings above where human newborns born to mothers given IAP had a microbiota enriched in *Enterococcaceae* (70, 77–80). Further, a series of studies exposing mice to vancomycin in drinking water through pregnancy and weaning exacerbated asthma in pups after weaning (116), an effect that was later linked to greater numbers of eosinophils and neutrophils in bronchoalveolar lavage fluid, increased serum IgE, and reduced frequency of colonic regulatory T-cells (117). While intranasally

administered streptomycin had little effect on asthma, it exacerbated hypersensitivity pneumonitis and increased IL-17 and IFNγ expression in the lung (117). It is important to note that while these mouse studies are informative, none of these capture the dose, frequency, or route of neonatal antibiotic exposure seen in humans. Furthermore, while these murine studies suggest a possible connection along the microbiome–immune–clinical outcome axis (which is the topic of this review), studies to investigate this in the human setting have to our knowledge not been conducted.

CONCLUSION

It has been over 100 years that Elie Metchnikoff has popularized the notion of a healthy microbiome as important for a healthy human existence (118, 119). Over the last decade in particular, it has increasingly been recognized that much of this health-promoting interaction is mediated *via* interaction of the microbiome with the human immune system (3, 4, 13, 120–122). Not surprisingly then, perturbations of this evolutionary conserved, beneficial interaction increase the risk for several immune-mediated diseases (17). Emerging now is the concept of an early-life window of increased susceptibility, during which perturbations of this immunity–microbiome interaction cause the most severe and long-lasting damage (5–10). In other words, perturbation of this host–microbiome interphase in early life has to be viewed as a "newborn disease with childhood/adult onset" (**Figure 1**). With this view in mind, and as reviewed here, it is disturbing to realize that many of these early-life disease-causing perturbations are in fact "man-made," such as CD, FF, IAP, and EAT. On the other hand, this realization provides us with the opportunity not only to take control and change these choices but also to design well-informed interventions to counteract these perturbations, which are often life-saving and cannot be avoided. In doing so, we can turn the window of susceptibility into a window of opportunity *via*, e.g., timely administration of probiotics (**Figure 1**) (29, 121–124).

The interaction of the developing microbiome with the host is clearly highly complex, and much of it is currently still unknown (5). But given that the impact of perturbations of the host–microbiome interaction affect clinical outcome far beyond the period, an altered microbiome is detectable suggests the mechanisms involved imprinted themselves into the host in ways beyond the microbiota. In part at least, this relates to the finding of such perturbations often manifesting themselves as immune-mediated diseases; the immune system after all is equipped with long-term memory within both the adaptive as well as innate immune system (125). Innate immune memory already is known to relate to epigenetic alterations (125). However, long-lasting changes in the epigenetic make up of the host in response to alterations of the microbiome extend even beyond the immune system to affect, e.g., metabolism, and connects the theme of this review to the developmental origin of health and disease (126). Specifically, this includes bacterial products that function as substrates for one-carbon metabolism (e.g., vitamins B2, B6, B9, and B12), and substrates for epigenetic modification (e.g., vitamin B7 for biotinylation and vitamin B5 for acetylation), or metabolites that interfere with the host epigenetic machinery (e.g., SCFA-mediated histone deacetylase inhibition) (127). Furthermore, pre- and early postnatal life is thought to be critical window for epigenetic modification specifically because growth and cell division are then at their highest rate. As such, dividing cells require larger amounts of methyl donors to retain cellular methylation patterns that would otherwise be diluted out. Furthermore, bacterial SCFAs such as butyrate and propionate can function as histone deacetylase inhibitors (128), and the *Bacteroides* genus is a major source of propionate in the gut (129). As outlined above, *Bacteroides* colonization is delayed in CD infants and their abundance is reduced in newborns of IAP-treated mothers. While SCFA levels in stools have yet to be investigated in term newborns, propionate levels were found to be reduced in colons of VD piglet colons compared to CD piglets (130). This supports the possibility of a far-reaching impact of the early-life microbiota on our epigenome. On the other hand, such a far-reaching and long-lasting impact also predicts that targeted interventions are likely to have broadly beneficial and long-lasting benefit. For instance, a small study has shown promise that inoculating neonates born by elective cesarean section with vaginal secretions from their mothers leaves them with a microbiota more similar to VD infants compared to infants born by CD that were not inoculated (131). And enteral probiotics administered to premature newborns reduce not only the risk of necrotizing enterocolitis but broadly reduce infection-related mortality (132–138).

From our review of this topic here, several overarching insights can be extracted that help guide future research and intervention efforts:

1. The earlier in life the perturbation, the more profound the impact (both in terms of range as well as duration) (**Figure 1**) (5–10, 17). This suggests that interventions (e.g., probiotics) would have the most beneficial impact administered as early as possible [e.g., prenatally to the mother (50, 51)].
2. Different perturbations (e.g., cesarean delivery, formula feeding, and intrapartum antibiotic prophylaxis) merge toward a similar final common that often is immune mediated. This suggests that interventions targeting these pathways will likely provide far-reaching, broadly beneficial benefit.

Future research priorities:

1. Impact of prenatal microbiota and viability of organisms found in placenta and amniotic fluid.
2. Understanding effects of cesarean delivery: elective vs. emergency, medical indications, primary vs. repeat, etc.
3. Antibiotic use: reasons for antibiotic administration, comparison to suitable control groups to minimize possible frailty bias. Animal models with comparable exposures to human use.
4. Impact of perinatal events such as chorioamnionitis, neonatal sepsis, and necrotizing enterocolitis on immune and microbiome development.
5. Long-term health impacts of probiotic use in preterm infants.
6. Not discussed in this review is the insight that this interaction of host–microbiome is not restricted to bacteria in the

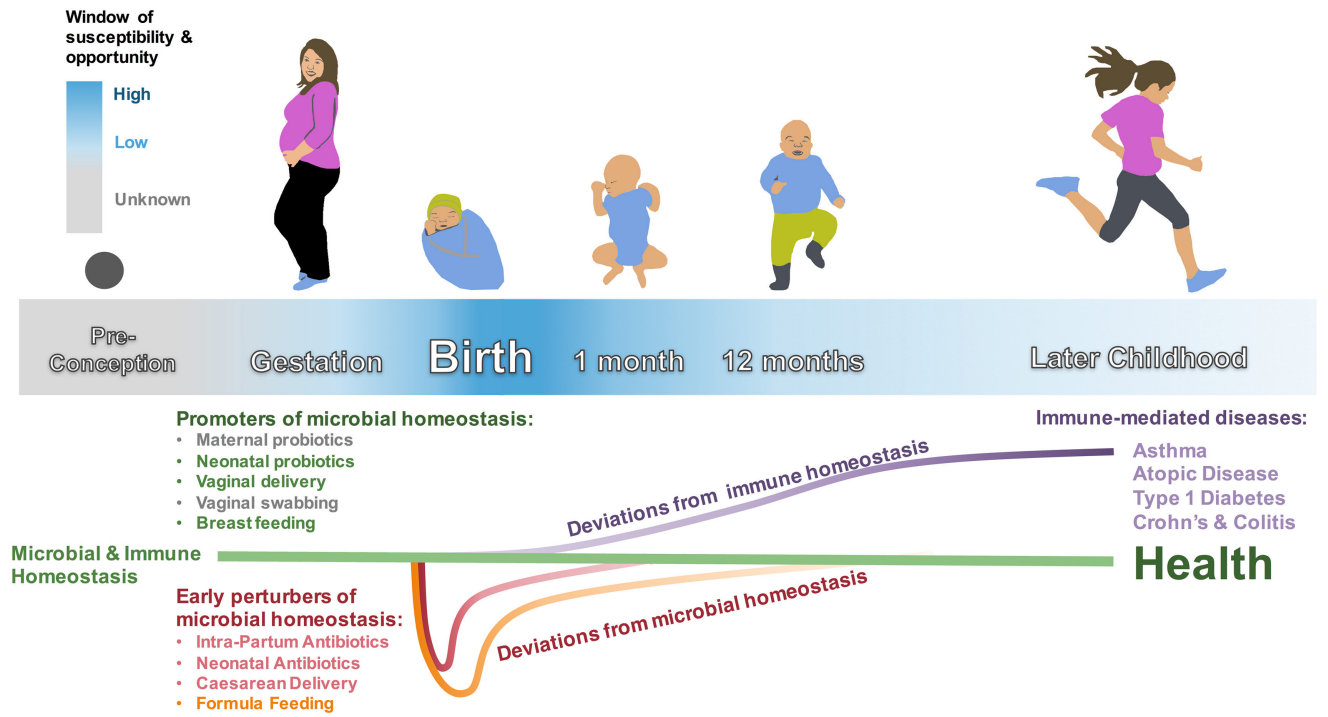

FIGURE 1 | Microbial and immune homeostasis from preconception to early childhood. Early-life perturbances (intrapartum antibiotics, neonatal antibiotics, cesarean delivery, and formula feeding) are associated with colonizing differences in the intestinal microbiota that are mostly evident in the first weeks to months of life—with the exception of feeding mode, which is associated with a unique microbiota until cessation of breastfeeding. These perturbers are also associated with immune dysfunction and immune-mediated diseases that manifest later in childhood. The window of susceptibility and opportunity represents the period around birth when promoters of microbial homeostasis may have the largest effect on correcting microbial dysbioses, with an unknown extension into gestation and possibly even preconception. Neonatal probiotics, vaginal delivery, and breastfeeding have strong associations with healthy colonization and decreased risk for immune-mediated disease. Maternal probiotics and vaginal swabbing are possible interventions that need to be further studied. Early-life microbiota and immune-mediated disease in later life need to be studied for cause and effect relationships.

gastrointestinal tract, but also includes fungi, viruses, and other microbes across many other body sites (2).

The complexity of the host microbiome–immunome interaction is astounding, but likely will be deciphered using modern tools of systems biology. The future of this field of study is poised to finally bring about the revolution that Elie Metchnikoff already brilliantly foreshadowed over a century ago (118, 119).

AUTHOR CONTRIBUTIONS

NA, TK, and RB-O have reviewed the literature and co-wrote the manuscript. TK and RB-O contributed equally.

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Starter Feeding Supplementation Alters Colonic Mucosal Bacterial Communities and Modulates Mucosal Immune Homeostasis in Newborn Lambs

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This study aims to investigate the effect of starter feeding supplementation on colonic mucosal bacterial communities and on mucosal immune homeostasis in pre-weaned lambs. We selected eight pairs of 10-day-old lamb twins. One twin was fed breast milk (M, $n = 8$), while the other was fed breast milk plus starter (M+S, $n = 8$). The lambs were sacrificed at 56 days age. Colonic content was collected to determine the pH and the concentrations of volatile fatty acids (VFA) and lactate. The colonic mucosa was harvested to characterize the bacterial communities using Illumina MiSeq sequencing and to determine mRNA expression levels of cytokines and toll-like receptors (TLR) using quantitative real-time PCR. The results show that starter feeding decreased luminal pH and increased the concentrations of acetate, propionate, butyrate, total VFA, and lactate in the colon. The principal coordinate analysis (PCA) and analysis of molecular variance show that starter feeding supplementation significantly affected the colonic mucosal bacterial communities with a higher relative abundance of the dominant taxa unclassified S24-7, *Oscillibacter*, *Prevotella*, *Parabacteroides*, *Bifidobacterium*, *Ruminobacter*, and *Succinivibrio*, and a lower proportion of unclassified Ruminococcaceae, *RC9_gut_group*, *Blautia*, *Phocaeicola*, *Phascolarctobacterium*, unclassified BS11_gut_group, unclassified family_XIII, and *Campylobacter* in lambs. Meanwhile, starter feeding decreased mRNA expression of TLR4 and cytokines TNF-α and IFN-γ in colonic tissue. Furthermore, the changes in the colonic mucosal mRNA expression of TLR and cytokines were associated with changes in mucosal bacterial composition. These findings may provide new insights into colonic mucosal bacteria and immune homeostasis in developing lambs.

Keywords: starter feeding, colonic mucosa, bacterial community, immune homeostasis, lamb

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INTRODUCTION

Gastrointestinal microbiota are integral to feed digestion, nutrient absorption and metabolism, immune response, and gastrointestinal development in ruminants (Yáñez-Ruiz et al., 2015). The gastrointestinal microbiome can be manipulated by nutritional interventions to improve productivity and health. However, the complexity and resilience of the ecosystem in adult

ruminants can preclude such alterations (Yáñez-Ruiz et al., 2015). Recent findings have indicated that early life, when there is unstable and fragile gastrointestinal microbial ecology, is an advantageous time to intervene and change the developmental profile of the gastrointestinal microbiota and impact adult health and performance (Abecia et al., 2013; Yáñez-Ruiz et al., 2015). Therefore, implementing nutritional interventions to affect gastrointestinal microbiota at an early age can improve lifelong health and performance in ruminants and other animals.

One such nutritional intervention used is supplementation of breast milk feeding with a concentrate starter in ruminants, which enhances gastrointestinal fermentation and promotes overall gastrointestinal development (Jiao et al., 2015a; Wang et al., 2016). Previous studies have demonstrated that compared with milk feeding only, concentrate starter feeding helps shape and diversify ruminal microbial composition in calves (Malmuthuge et al., 2013) and goat kids (Jiao et al., 2015a). Jiao et al. (2016) found that concentrate feeding decreased bacterial diversity in the colonic digesta of goat kids. Furthermore, Malmuthuge et al. (2014) reported a difference in the bacterial communities of colonic digesta and mucosa in preweaned calves, suggesting that colonic mucosal bacteria may serve some specific functions, e.g., host metabolism and immune response, in young ruminants. However, little information is available regarding the effect of starter feeding on the colonic mucosal bacterial community in young ruminants, despite the importance of this bacteria in animal health. Thus, more attention should be paid to the effects of starter feeding on the colonic mucosal bacterial community in preweaned ruminants.

Colonic mucosal microbiota are integral to host immune maturation. Toll-like receptors (TLR), as novel receptors mediating innate immune responses, can recognize microbiota and their products (Abreu, 2010). Recent studies have demonstrated that changes in ruminal epithelial bacterial diversity and some specific commensal microbes is associated with changes in the expression of TLR during high-concentrate diet feeding in steers (Chen et al., 2012) and goats (Liu et al., 2015). Furthermore, microbiota and their products bind to TLR and may subsequently initiate proinflammatory pathways (Abreu, 2010). Thus, understanding the impact of starter feeding supplementation on the gene expression of TLR and cytokines as well as the role of mucosal microbiota in host immune maturation in young ruminants is necessary for their health and performance in adulthood. In the present study, we hypothesized that concentrate starter feeding changes the colonic mucosal bacterial community, and that these alterations can modulate the immune response in lambs. Our first objective was to investigate the effect of starter feeding supplementation on the colonic mucosal bacterial community and expression of TLR and cytokines in preweaned lambs. Our second objective was to evaluate the relationship between the bacterial community and host immune response in the colonic mucosa of lambs.

MATERIALS AND METHODS

Animal Experimental Design

The experimental design and procedures were approved by the Animal Care and Use Committee of Nanjing Agricultural University. The experiment was carried out using Suzhou Hu sheep at a breeding farm in the Jiangsu province, China. Eight pairs of healthy, 10-day-old lamb twins (Hu sheep, a native Chinese sheep breed) were selected. One kid from each pair remained with the mother and received milk *ad libitum* without receiving starter feed (M group, $n = 8$), while the other kid was separated from the mother and received starter feed (M+S group, $n = 8$) from 4:00 a.m. to 7:00 p.m. every day in a separate pen. During this period, lambs in the M+S group were fed milk for 1 h at each fixed time point (6:30 a.m., 10:30 a.m., and 3:30 p.m.). When the dry matter intake (DMI) of the lambs' starter reached 200 g/animal^{-1}d^{-1}, the amount of starter did not rise any further. The eight lambs in the M+S group maintained a 200 g/animal^{-1}d^{-1} starter intake for an average of 14 days before sacrifice. All lambs received oat hay (10.05% crude protein, 28.71% crude fiber) and water *ad libitum*. The ewes were fed three times per day according to the farm's feeding management schedule. None of the lambs in the M and M+S groups had access to the ewes' feed. The DMI of the starter in the M+S group was recorded every day, and the body weights of each lamb was measured weekly (before morning feeding). The experimental starter diets were designed according to the nutrient requirements of Hu sheep lambs (NY/T816-2004; Ministry of Agriculture of China, 2004). The nutrient composition of the starter diet is presented in **Table 1** (Liu et al., 2017).

Sample Collection

Lambs were stunned by captive bolt and exsanguination at 56 days of age. A representative sample of colon digesta was collected from the proximal colon immediately after slaughter to determine the pH value. Colon digesta from each lamb were homogenized and mixed thoroughly with twice the amount of distilled water. The mixtures were then immediately centrifuged at 12,000 × g, and the supernatants were stored at −20°C until analysis for volatile fatty acids (VFA) and lactic acid. Within 5 min, a segment of the colon tissue was harvested and immediately washed three times in ice-cold, phosphate-buffered saline. A

TABLE 1 | Ingredient and chemical composition of the starter diet (DM[a] basis).

Ingredient	% DM	Component	
Maize starch	51.60	DM, %	88.78
Soybean meal	28.00	Crude protein, % DM	25.15
Corn gluten meal	15.00	Crude fat, % DM	3.80
Soybean oil	1.20	Crude ash, % DM	6.33
Limestone meal	0.80	Crude fiber, % DM	6.34
CaHPO4	1.80	Starch, % DM	45.92
Salt	0.60	Metabolic energy[c], MJ/kg DM	11.43
Premix[b]	1.00		

[a] *DM, dry matter.*

[b] *Contained 16% calcium carbonate, 102 g/kg of Zn, 47 g/kg of Mn, 26 g/kg of Cu, 1,140 mg/kg of I, 500 mg/kg of Se, 340 mg/kg of Co, 17,167,380 IU/kg of vitamin A, 858,370 IU/kg of vitamin D, and 23,605 IU/kg of vitamin E.*

[c] *Calculated value based on database of the nutrient requirement for lamb (NY/Y816-2004; Ministry of Agriculture of China, 2004).*

portion of the tissue sample was cut into smaller pieces (∼0.5 × 0.5 cm) and immediately frozen in liquid nitrogen for RNA extraction. Another portion of the tissue sample was cut to ∼1 × 1 cm and scraped from the underlying tissue using a germ-free glass slide, immediately transferred into liquid nitrogen, and then stored at −80°C until microbial DNA extraction. A final portion was immediately fixed in 4% paraformaldehyde (Sigma, St. Louis, MO, USA) and 2.5% glutaraldehyde for histomorphometric microscopy analysis.

Physiological Parameter Measurements

A portable pH meter (HI 9024C; HANNA Instruments, Woonsocket, RI, USA) was used to determine the pH of colonic digesta. Capillary column gas chromatography (GC-14B, Shimadzu, Japan; Capillary Column: 30 m × 0.32 × 0.25 mm film thickness; Column temperature = 110°C, injector temperature = 180°C, detector temperature = 180°C) was used to measure VFA concentration (Qin, 1982). Lactate concentration was detected using a method described by Barker and Summerson (1941).

Microbial DNA Isolation

One gram of colonic mucosal tissue was used for DNA extraction. The DNA was extracted by a PowerSoil DNA Isolation Kit (MOBIO Laboratories, Carlsbad, CA, USA, catalog 12888-100). The solution was precipitated with ethanol, and the pellets were suspended in a 50-μL Tris-EDTA buffer. DNA was quantified using PicoGreen dsDNA reagent kit (Invitrogen Ltd., Paisley, UK) with a Molecular Devices SpectraMax Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

PCR Amplification, Illumina MiSeq Sequencing, and Sequencing Data Processing

The V4 regions of bacterial 16S rRNA genes were amplified by PCR (Initial denaturation at 95°C for 2 min, 25 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, elongation at 72°C for 1 min, and final extension at 72°C for 5 min) using primers 515F (5′-barcode-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-barcode-GGACTACHVGGGTWTCTAAT-3′). Amplicons were purified using the Qiagen QIAquick PCR purification kit (Qiagen, Duesseldorf, Germany) according to the manufacturer's instructions and quantified using PicoGreen dsDNA reagent kit (Invitrogen, Paisley, UK). Purified amplicons were pooled in equimolar, and the amplicon size was determined by Aglient 2200 Bioanalyzer (Agilent Technologies, CA, USA). The pooled product was pair-end sequenced (2 × 300) on an Illumina MiSeq platform according to standard protocols.

For data analyses, raw Illumina fastq files were demultiplexed, quality filtered, and analyzed using Quantitative Insights into Microbial Ecology (QIIME, v.1.8.0), as described by Caporaso et al. (2010b) and with the following criteria, as described by Mao et al. (2015): Operational taxonomic units (OTU) were clustered with a 97% similarity cut-off using UPARSE (Edgar, 2013), and chimeric sequences were identified and removed using UCHIME (Edgar et al., 2011). The most abundant sequences within each OTU (representative sequences) were aligned to the Greengenes database using PyNAST (Caporaso et al., 2010a) with the default parameters set by QIIME. Taxonomy was assigned to representative sequences using QIIME (Wang et al., 2007) with a confidence value of 0.8 against the Greengenes 16S rRNA gene dataset (v.13.8) (DeSantis et al., 2006). Rarefaction curves and alpha and beta diversity calculations were also performed using QIIME. Principal coordinate analysis (PCA) was used to compare groups of samples based on unweighted UniFrac distance metrics (Lozupone and Knight, 2005), and an unweighted distance-based analysis of molecular variance (AMOVA) was conducted to assess significant differences among samples using the MOTHUR v.1.3.9 program (Schloss et al., 2009).

Histological Measurements

The colonic tissues were embedded in paraffin, sectioned into 6 μm, and stained with hematoxylin and eosin (H&E). During histomorphometric analyses, the microscopist was blinded to treatment conditions. For each lamb, two slides were prepared and two images were captured per slide, resulting in a total of 32 replicates per measurement per group. Predefined criteria described by Steele et al. (2011) were used to assess colonic injury using Image Pro Plus software (Media Cybernetics, Bethesda, MD, USA). The criteria were as follows: a score of one indicated no lesions or minor lesions; a score of five indicated minor lesions with mucosa sloughing; and a score of nine indicated severe, deep lesions with large amounts of mucosa sloughing.

The tissues were fixed with 2.5% glutaraldehyde for at least 24 h, postfixed in 1% osmium, and embedded in Epon araldite. A glass knife was used to cut semithin sections (0.25–0.5 μm) and ultrathin sections (70–90 nm). To stain semithin sections, 1% toluidine blue and 1% sodium borate were used, while uranyl acetate and lead citrate were used to stain ultrathin sections. A transmission electron microscope (H-7650; Hitachi Technologies, Tokyo, Japan) was used to examine and determine ultrastructures of the colonic tissue.

RNA Extraction and Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from the colonic tissue using TRIzol (Takara Bio, Otsu, Japan), as described by Chomczynski and Sacchi (1987). RNA concentrations were then quantified using a NanoDrop spectrophotometer (ND-1000UV-Vis; Thermo Fisher Scientific, Waltham, MA, USA). The absorption ratio (260/280 nm) of all of the samples was between 1.8 and 2.0, indicating high RNA purity. Aliquots of RNA samples were subjected to electrophoresis through a 1.4% agarose–formaldehyde gel to verify integrity. The concentration of RNA was adjusted to 1 μg/μL based on optical density and stored at −80°C. Total RNA (1 μg) was reverse-transcribed using a PrimeScript RT Reagent Kit with gDNA Eraser (Takara Bio, Otsu, Japan) according to the manufacturer's instructions.

The primers of cytokine (Liu et al., 2013), TLR (Charavaryamath et al., 2011), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Wang et al., 2009) genes used in the present study were described in previous studies. All of the primer sequences are listed in Table S1. The primers were synthesized by Invitrogen Life Technologies (Shanghai, China).

The ABI 7300 Real-time PCR System (Applied Biosystems, Foster City, CA, USA) with SYBR green dye fluorescence detection was used to perform qRT-PCR of the target genes and GAPDH. Amplification conditions were as follows: 95°C for 30 s followed by 40 cycles of 5 s at 95°C and 31 s at 57.5°C (for GAPDH) or 62°C (for the cytokines and TLR). Each sample contained 1–10 ng cDNA in 2 × SYBR Green PCR Master Mix (Takara Bio, Otsu, Japan) and 200 nmol/L of each primer in a final volume of 20 μL. All measurements were performed in triplicate. The negative controls were a reverse-transcription-negative blank of each sample and a no-template blank. The GAPDH (a housekeeping gene) mRNA level was used to normalize the relative amount of each studied mRNA, and the $2^{-\Delta\Delta CT}$ method was used to analyze the data (Livak and Schmittgen, 2001).

Statistical Analyses

Statistical analyses were performed using the SPSS software package (SPSS v.16, SPSS Inc.). The normality of the distribution of variables was assessed with the Shapiro-Wilk test. The data found to have a normal distribution were analyzed by the Independent Samples *t*-test procedure, according to the following model: $[Y = u + C + e]$, where u is the mean, C is the effect of diet, and e is the residual error. The Kruskal-Wallis test was used to analyze variables found to have a non-normal distribution according to the following statistical model: $H = \frac{12}{n(n+1)} \sum_{i=1}^{k} \frac{Ri^2}{n_i} - 3\,(n+1)$, where H is the Kruskal-Wallis test, n is the number of measurements, R_i is the sum of the ranks, and n_i is the number of experiments. Significance was declared at $P < 0.05$.

Correlation analysis was assessed by Spearman's correlation test using GraphPad Prism v.5 (GraphPad Software, San Diego, CA, USA). Significance was declared at $P < 0.01$.

RESULTS

Animal

We observed no significant differences in birth weights (3.43 ± 0.10 vs. 3.31 ± 0.07 kg, $P = 0.375$) and final body weights at 56 days age (14.89 ± 0.36 vs. 14.44 ± 0.34 kg, $P = 0.381$) between the M and M+S groups. During the feeding trial, the average total DMI of starter per lamb in the M+S group was 5.54 ± 0.16 kg.

pH, VFA, and Lactate Concentrations in Colonic Contents

As shown in **Table 2**, compared with milk-fed lambs, starter-fed lambs had a higher concentration of total VFA ($P = 0.001$), acetate ($P = 0.018$), propionate ($P < 0.001$), butyrate ($P < 0.001$), and lactate ($P < 0.001$), but had lower luminal pH ($P = 0.002$) and acetate to propionate ratio ($P < 0.001$) in colonic content. Starter feeding did not affect other VFA concentrations significantly ($P = 0.485$).

TABLE 2 | The effect of starter feeding on colonic fermentation in lambs at the time of slaughter[a].

Item	M[b]	M+S[c]	*P*-value
pH	6.99 ± 0.08	6.76 ± 0.15	0.002
Total VFA[d], μmol/g	48.30 ± 5.38	62.55 ± 7.18	0.001
Acetate, μmol/g	35.72 ± 3.44	41.90 ± 5.54	0.018
Propionate, μmol/g	7.63 ± 0.82	12.14 ± 2.33	<0.001
Butyrate, μmol/g	2.81 ± 1.10	6.09 ± 1.17	<0.001
Others[e], μmol/g	2.15 ± 0.69	2.42 ± 0.83	0.485
Acetate: Propionate	4.71 ± 0.44	3.54 ± 0.69	0.001
Lactate, μmol/g	1.85 ± 0.19	2.47 ± 0.25	<0.001

[a] *Values are means ± SD, n = 8.*
[b] *M, milk.*
[c] *M+S, milk plus starter.*
[d] *VFA, volatile fatty acid.*
[e] *Others, valerate+isobutyrate+isovalerate.*

Characterization of the Colonic Mucosal Bacterial Communities

After quality control, 697,630 valid reads were obtained in all samples with an average of 43,602 sequences per sample. MOTHUR analysis showed that 7,752 OTU at sequence divergences of 0.03 were classified based on these valid sequences. The average number of OTU was 485 ± 6, with an average coverage of 99.77 ± 0.01%. The Chao1 richness, abundance-based coverage estimator (ACE), and Shannon and simpson diversity indices were 580 ± 9, 573 ± 6, 4.30 ± 0.07, and 0.04 ± 0.01, respectively. We found a total of 18 phyla in all samples. The most dominant phyla were Firmicutes (48.58%) and Bacteroidetes (36.33%), and the next dominant phyla were Proteobacteria (4.00%), Verrucomicrobia (3.91%), and Actinobacteria (1.28%). Unclassified bacteria (3.22%) together with these five phyla represented 97.32% of total reads. The proportion of the phyla Tenericutes, Planctomycetes, Lentisphaerae, Spirochaetae, Cyanobacteria, Fusobacteria, and Fibrobacteres accounted for <1.00% of total sequences. We did not detect the phyla Candidate, Elusimicrobia, Synergistetes, Deferribacteres, and Chloroflexi in all of the samples. We found a total of 218 taxa (at the genus level) in all samples. The dominant bacterial taxa were unclassified Ruminococcaceae (18.20%), Bacteroides (9.96%), unclassified S24-7 (7.84%), and unclassified Lachnospiraceae (6.93%), followed by unclassified Christensenellaceae (5.23%), unclassified Bacteroidales (3.88%), *Akkermansia* (3.87%), *RC9_gut_group* (3.67%), *Alistipes* (3.66%), unclassified Clostridiales (2.73%), *Blautia* (2.62%), *Oscillibacter* (2.49%), *Phocaeicola* (1.86%), *Prevotella* (1.33%), *Phascolarctobacterium* (1.33%), and unclassified Defluviitaleaceae (1.16%). The proportion of other taxa was below 1.00% of total sequences. As was shown in Figure S1, the top 50 bacterial taxa of different samples were presented in the heat map.

Effect of Starter Feeding on Colonic Mucosal Bacterial Diversity

The rarefaction curves of colonic mucosal bacterial communities (Figure S2, at dissimilarity levels of 0.03) showed that all curves approached a plateau, suggesting that deeper sequencing was not responsible for an increase of OTU across all samples. We used the unweighted UniFrac metric in MOTHUR to evaluate β-diversity across the samples (**Figure 1**). As shown in the PCA figure, the plots of the M and M+S groups were distinctly

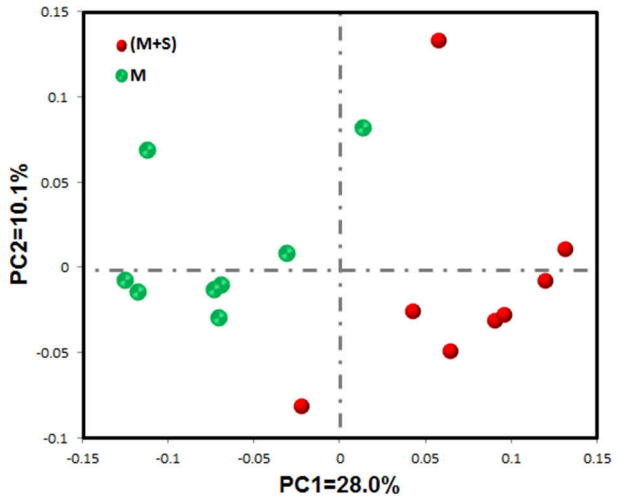

FIGURE 1 | Differences in colonic mucosal bacterial structures between the M and M+S groups. Unweighted UniFrac principal coordinate analysis (PCA) of colonic mucosal microbiota was based on the operational taxonomic unit (OTU) data. The marks relate to donor lambs of different groups: M group (●) and M+S group (●).

separated (**Figure 1**; axis 1 + axis 2 = 38.1%). The AMOVA analysis shows that starter feeding significantly affected the colonic mucosal bacterial communities (AMOVA, $Fs = 3.68$, $P = 0.001$). The effects of starter feeding on the α-diversity of colonic mucosal bacterial communities are shown in **Table 3**. The results show that starter feeding supplementation increased Chao1 richness ($P = 0.034$), and no significant difference in OTU numbers ($P = 0.203$), ACE ($P = 0.181$), and Shannon ($P = 0.373$) or simpson ($P = 0.331$) indices.

Effect of Starter Feeding on the Relative Abundance of Colonic Mucosal Bacteria

At the phylum level (**Table 4**), we found that compared with the M group, the M+S group had a higher relative abundance of Bacteroidetes ($P = 0.027$) and Actinobacteria ($P = 0.005$), with a lower relative abundance of Firmicutes ($P = 0.027$), unclassified Bacteria ($P = 0.046$), and Cyanobacteria ($P = 0.036$). We observed no significant difference in the proportions of the phyla Proteobacteria ($P = 0.600$), Verrucomicrobia ($P = 0.462$), Tenericutes ($P = 0.172$), Planctomycetes ($P = 0.141$), Lentisphaerae ($P = 0.294$), Spirochaetae ($P = 0.916$), and Fusobacteria ($P = 0.916$) between the M and M+S groups.

At the genus level (**Table 5** and Figure S3), starter feeding caused an increase in the relative abundance of the dominant taxa unclassified S24-7 ($P = 0.002$), *Oscillibacter* ($P = 0.046$), *Prevotella* ($P = 0.009$), *Parabacteroides* ($P = 0.002$), *Bifidobacterium* ($P = 0.002$), *Ruminobacter* ($P = 0.002$), and *Succinivibrio* ($P = 0.006$). Starter feeding also caused a decrease in the relative abundance of unclassified Ruminococcaceae ($P = 0.006$), *RC9_gut_group* ($P = 0.027$), *Blautia* ($P = 0.002$), *Phocaeicola* ($P = 0.036$), *Phascolarctobacterium* ($P = 0.009$), unclassified BS11_gut_group ($P = 0.027$), unclassified family_XIII ($P = 0.016$), *Campylobacter* ($P = 0.016$), unclassified Firmicutes ($P = 0.002$), *Pseudobutyrivibrio* ($P = 0.009$), *Barnesiella* ($P = 0.046$), *Lactobacillus* ($P = 0.001$), unclassified Gastranaerophilales ($P = 0.036$), *Butyrivibrio* ($P = 0.006$), *dgA-11_gut_group* ($P = 0.001$), and *Dorea* ($P = 0.012$).

TABLE 3 | Effects of starter feeding on the diversity of colonic mucosal bacterial communities at the 3% dissimilarity level[a].

	OTU[b]	ACE[c]	Chao 1 value	Shannon index	Simpson
M	477 ± 24	565 ± 28	562 ± 41	4.36 ± 0.16	0.03 ± 0.01
M+S	492 ± 22	582 ± 20	598 ± 13	4.23 ± 0.38	0.04 ± 0.04
P-value	0.203	0.181	0.034	0.373	0.331

[a] *Values shown are means ± SD, n = 8.*
[b] *OTU, operational taxonomic units.*
[c] *ACE, abundance-based coverage estimator.*

TABLE 4 | The effect of starter feeding on relative abundance of phylum level (% of total sequences) in colonic mucosa[a].

Phylum	M	M+S	*P*-value
Firmicutes	53.11 ± 5.61	44.04 ± 8.81	0.027
Bacteroidetes	31.35 ± 5.31	41.31 ± 10.02	0.027
Proteobacteria	3.86 ± 1.07	4.14 ± 2.33	0.600
Verrucomicrobia	4.11 ± 1.54	3.71 ± 3.05	0.462
Unclassified Bacteria	3.71 ± 0.55	2.72 ± 1.36	0.046
Actinobacteria	0.62 ± 0.27	1.93 ± 1.23	0.005
Tenericutes	1.03 ± 0.50	0.68 ± 0.55	0.172
Planctomycetes	0.61 ± 0.21	0.43 ± 0.39	0.141
Lentisphaerae	0.55 ± 0.58	0.27 ± 0.24	0.294
Spirochaetae	0.37 ± 0.28	0.40 ± 0.37	0.916
Cyanobacteria	0.44 ± 0.34	0.20 ± 0.15	0.036
Fusobacteria	0.16 ± 0.13	0.14 ± 0.11	0.916
Others	0.09 ± 0.06	0.02 ± 0.01	0.001

[a] *Values are means ± SD, n = 8.*

Morphology and Ultrastructure of Colon Tissues

For the lambs from the M group, we observed sloughing of the mucosal surface in the representative cross-sections of the colonic tissue (**Figure 2A**). For the lambs from the M+S group, we observed that the intercryptal surface was covered by an irregular layer of mucus (**Figure 2B**). The colonic injury scores of lambs in the M+S group were significantly lower than that of lambs in the M group (2.04 ± 0.16 vs. 4.63 ± 0.25, $P < 0.001$). The lambs from the M group had sparse and irregular microvilli in the ultrastructure of the colon tissue (**Figure 2C**), while the lambs from the M+S group had clear and organized microvillus clusters (**Figure 2D**). For the lambs in the M group, intercellular tight junction erosion, and mitochondrial swelling appeared in the cells (**Figure 2E**); for the lambs in the M+S group, we observed normal and clear cell organelles and tight junction bands in the cells (**Figure 2F**).

TABLE 5 | Effects of starter feeding on average relative abundance of genus level (% of total sequences) in colon mucosa, ranked by alphabetical order of first letter of phylum, family, and genus name.

Phylum	Family	Genus	Abundance (%)		*P*-value
			M	M+S	
Actinobacteria	Bifidobacteriaceae	*Bifidobacterium*	0.31 ± 0.17	0.95 ± 0.70	0.002
Bacteroidetes	BS11_gut_group	Unclassified BS11_gut_group	1.73 ± 1.64	0.16 ± 0.25	0.027
	Porphyromonadaceae	*Parabacteroides*	0.20 ± 0.09	1.70 ± 1.39	0.002
		Barnesiella	0.65 ± 0.80	0.18 ± 0.26	0.046
	Prevotellaceae	*Prevotella*	0.25 ± 0.25	2.41 ± 3.08	0.009
	Rikenellaceae	*dgA-11_gut_group*	0.57 ± 0.39	0.01 ± 0.02	0.001
		RC9_gut_group	5.01 ± 1.59	2.32 ± 3.21	0.027
	S24-7	Unclassified S24-7	0.60 ± 0.65	15.07 ± 14.06	0.002
	Unclassified Bacteroidales	Phocaeicola	3.40 ± 3.15	0.33 ± 0.39	0.036
Cyanobacteria	Unclassified Gastranaerophilales	Unclassified Gastranaerophilales	0.44 ± 0.34	0.20 ± 0.15	0.036
Firmicutes	Family_XIII	Unclassified Family_XIII	0.80 ± 0.25	0.50 ± 0.33	0.016
	Lachnospiraceae	*Blautia*	3.92 ± 1.63	1.31 ± 0.60	0.002
		Butyrivibrio	0.35 ± 0.04	0.23 ± 0.07	0.006
		Dorea	0.33 ± 0.11	0.21 ± 0.05	0.012
		Pseudobutyrivibrio	0.61 ± 0.50	0.22 ± 0.29	0.009
	Lactobacillaceae	*Lactobacillus*	0.71 ± 0.48	0.05 ± 0.05	0.001
	Oscillospiraceae	*Oscillibacter*	1.70 ± 0.90	3.28 ± 1.74	0.046
	Ruminococcaceae	Unclassified Ruminococcaceae	21.76 ± 2.63	14.65 ± 4.90	0.006
	Unclassified Firmicutes	Unclassified Firmicutes	0.77 ± 0.31	0.19 ± 0.23	0.002
	Veillonellaceae	*Phascolarctobacterium*	1.91 ± 0.64	0.75 ± 0.63	0.009
Proteobacteria	Campylobacteraceae	*Campylobacter*	0.93 ± 0.46	0.32 ± 0.26	0.016
	Succinivibrionaceae	*Ruminobacter*	0.01 ± 0.00	1.08 ± 2.43	0.002
		Succinivibrio	0.04 ± 0.04	0.61 ± 0.60	0.006

Only results obtained for the predominant bacterial taxa (Top 50 taxa) that were significantly affected by starter feeding ($P < 0.05$) are presented. Values shown are means ± SD, $n = 8$.

Changes in mRNA Expression of Cytokines and TLR with Starter Feeding Supplementation in the Colonic Mucosa

As shown in **Figure 3**, starter feeding decreased the mRNA expression of cytokines TNF-α ($P < 0.001$) and IFN-γ ($P < 0.001$) in the colonic mucosa. We found no significant differences in mRNA expression of IL-1β ($P = 0.759$), IL-6 ($P = 0.472$), IL-10 ($P = 0.068$), and IL-12 ($P = 0.986$) between the M and M+S groups. Meanwhile, starter feeding also decreased colonic mucosal TLR4 ($P = 0.017$) mRNA expression, and we observed no significant differences in mRNA expression of TLR2 ($P = 0.251$), TLR3 ($P = 0.938$), and TLR5 ($P = 0.223$).

Correlation Analyses

Figure 4 depicts the relationships between the relative abundance of colonic mucosal bacteria and TLR and cytokine expression. The relative mRNA expression of IL-6 was negatively associated with the relative abundance of the genus *Blautia* ($P = 0.0076$), while IL-10 mRNA expression was negatively linked with the relative proportion of the genus *Phocaeicola* ($P = 0.0079$). IL-12 mRNA expression was positively correlated with the relative abundance of the genus *Alistipes* ($P = 0.0057$), whereas the mRNA expression level of TNF-α was positively associated with the relative abundance of the taxa unclassified Ruminococcaceae ($P = 0.0022$), *dgA-11_gut_group* ($P = 0.0003$), *Blautia* ($P < 0.0001$), *Lactobacillus* ($P < 0.0001$), *Dorea* ($P = 0.0076$), unclassified Firmicutes ($P = 0.0012$), and *Butyrivibrio* ($P = 0.0017$), and negatively correlated with the abundance of the taxa unclassified S24-7 ($P = 0.0019$), *Prevotella* ($P = 0.0056$), *Parabacteroides* ($P = 0.0014$), *Ruminobacter* ($P = 0.0027$), and *Bifidobacterium* ($P = 0.0077$). IFN-γ mRNA expression was positively correlated with the relative proportion of the taxa *dgA-11_gut_group* ($P = 0.0049$), *Blautia* ($P = 0.0005$), *Lactobacillus* ($P = 0.0001$), *Phocaeicola* ($P = 0.0099$), *Pseudobutyrivibrio* ($P = 0.0054$), *Desulfovibrio* ($P = 0.0095$), unclassified Firmicutes ($P < 0.0001$), *Butyrivibrio* ($P = 0.0012$), and *Phascolarctobacterium* ($P = 0.0034$), and negatively associated with the abundance of the taxa unclassified S24-7 ($P = 0.0005$), *Parabacteroides* ($P = 0.0008$), *Ruminobacter* ($P = 0.0059$), and *Bifidobacterium* ($P = 0.0074$). The mRNA expression of TLR4 was positively associated with the taxa unclassified Ruminococcaceae ($P = 0.0075$), *Pseudobutyrivibrio* ($P = 0.0037$), and unclassified Firmicutes ($P = 0.0093$), and negatively linked with the taxa unclassified S24-7

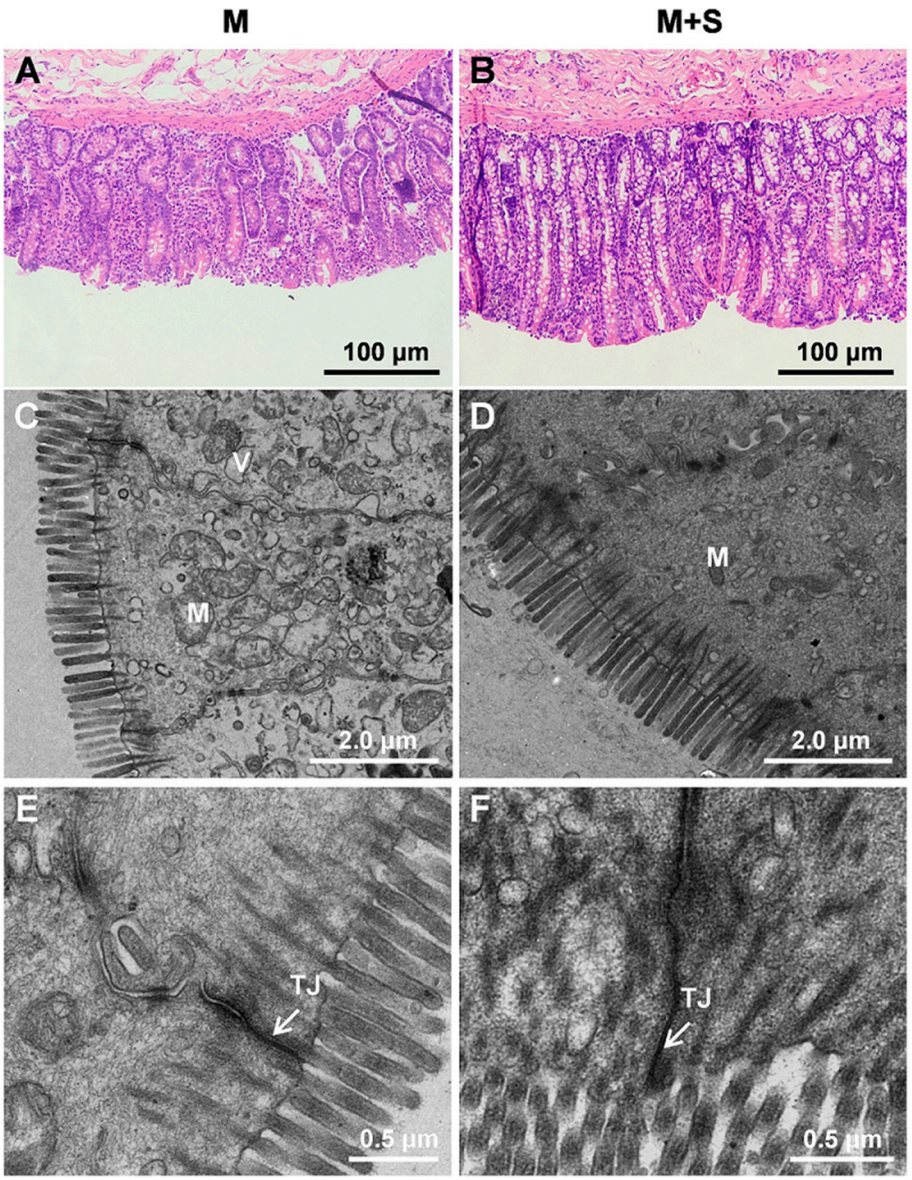

FIGURE 2 | Histology of colon tissue comparing M and M+S groups. Light microscopy cross-section of colon tissue in the M group (**A**, scale bar = 100 μm) and M+S group (**B**, scale bar = 100 μm). Comparison of colonic epithelial ultrastructure in lambs from the M group (**C**, scale bar = 2 μm) and M+S group (**D**, scale bar = 2 μm). Colonic epithelial ultrastructure of junctional complexes in representative lambs from the M group (**E**, scale bar = 0.5 μm) and the M+S group (**F**, scale bar = 0.5 μm). M, mitochondria; V, vacuole; TJ, tight junction.

($P = 0.0017$), *Parabacteroides* ($P = 0.0083$), and *Ruminobacter* ($P = 0.0080$).

DISCUSSION

Colonic mucosal microbiota play important roles in host metabolism and immune homeostasis, thus affecting the health of ruminants. Early nutritional intervention is an advantageous strategy for modulating gastrointestinal microbiota, and their development profile can impact host health. In the current study, we found that supplementation of breast milk with concentrate starter feeding can regulate colonic mucosal bacterial composition and structure, and that these changes were associated with variations in the mRNA expression of TLR and cytokines. These findings may provide new insights into colonic mucosal bacteria and immune homeostasis in developing lambs.

The similar final body weight gain in the M and M+S groups suggests that lambs in the two groups had a similar total nutrient intake. We found that concentrate starter feeding increased the bacterial richness of the colonic mucosal community as reflected by a higher Chao 1 value, which is somewhat inconsistent with findings on the colonic mucosal community of concentrate-fed

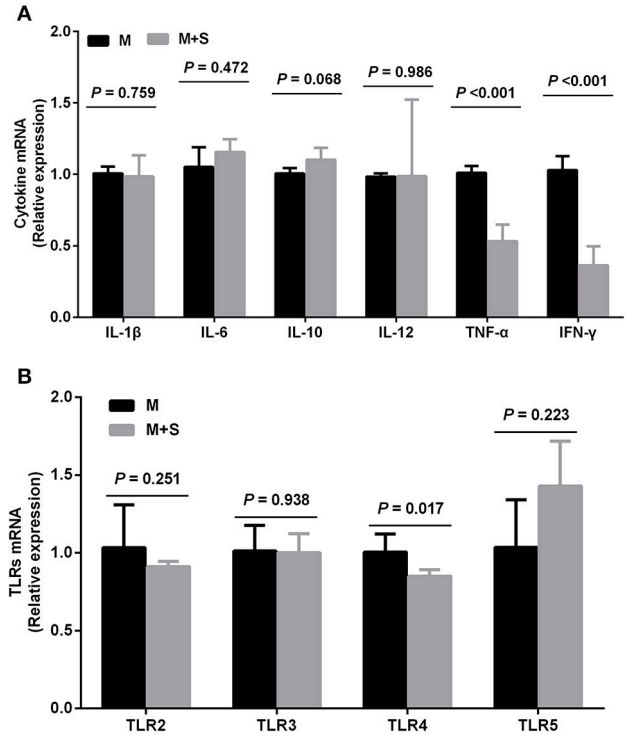

FIGURE 3 | Changes in the relative mRNA expression of cytokines (A) and TLR **(B)** in the colonic mucosa of lambs during starter feeding (means ± *SD*, $n = 8$). The relative amount of each was normalized to GAPDH mRNA levels as a housekeeping gene, and the data were analyzed according to the $2^{-\Delta\Delta CT}$ method.

adult goats (Ye et al., 2016). Moreover, the PCA and AMOVA analyses showed that concentrate starter feeding significantly affected colonic mucosal bacterial composition and structure. In this study, concentrate (especially starch) was supplemented in M+S lambs, but not M lambs, which resulted in the most remarkable difference between these two feeding strategies. Thus, more amount of starch substrates may flow into the colon of M+S lambs. As expected, we also found that M+S lambs had lower colonic pH and higher colonic VFA and lactate concentrations. Therefore, the changes in colonic luminal environment may contribute to the changes in colonic mucosal bacterial composition and structure.

At the phylum level, we found that Firmicutes, Bacteroidetes, and Proteobacteria were the dominant phyla associated with the colonic mucosa of lambs, which agrees with data found for preweaned calves (Malmuthuge et al., 2014) and goat kids (Jiao et al., 2015b). Meanwhile, compared with lambs in the M group, lambs in the M+S group had a lower relative abundance of Firmicutes and a higher proportion of Bacteroidetes. Similar results were observed in the colonic mucosa of high-concentrate diet-fed goats (Ye et al., 2016) and in the colonic digesta of concentrate-fed goat kids (Jiao et al., 2016).

At the genus level, starter feeding increased the relative abundances of unclassified S24-7 (family), *Prevotella, Ruminobacter*, *Oscillibacter, Parabacteroides,* and *Bifidobacterium*, but decreased the proportions of unclassified Ruminococcaceae (family), *Blautia*, *Campylobacter*, *Butyrivibrio*, *Pseudobutyrivibrio*, and *Lactobacillus*. On the one hand, the enrichment of starch degraders, like unclassified S24-7, *Prevotella*, *Bifidobacterium*, and *Ruminobacter*, may be due to greater starch availability in the colon during starter feeding. Other studies have demonstrated the presence of family S24-7 in dairy and beef cattle (McCann et al., 2014; Lima et al., 2015; Anderson et al., 2016); however, the role of S24-7 in the colon of ruminants remains poorly understood. Bacteria belonging to family S24-7 have also been identified in the colons of mice fed high-fat diets and gluco-oligosaccharides (Serino et al., 2012). Therefore, it is possible that the family S24-7 is capable of starch utilization (Serino et al., 2012; Anderson et al., 2016). As expected, concentrate starter feeding increased the proportion of *Prevotella* (a kind of starch degrader) in the colonic mucosa of lambs. Similarly, previous studies have demonstrated that high-grain diet feeding increases the abundance of *Prevotella* in the colons of adult goats (Metzler-Zebeli et al., 2013; Ye et al., 2016) and goat kids (Jiao et al., 2016). *Bifidobacterium*, a starch-hydrolyzing bacteria, can produce acetate and lactate fermentation end products (Xia et al., 2015). Other studies have found a higher abundance of *Bifidobacterium* in the rumen of high-concentrate-fed calves (Trovatelli and Matteuzzi, 1976) and dairy cows (Zened et al., 2013). This result also partly explains why the starter-fed lambs in our study had higher lactate concentrations in their colons. Additionally, the genus *Ruminobacter* is also involved in starch degradation (Halbrügge and Walter, 1990; Anderson, 1995). On the other hand, Ruminococcaceae and members of the Lachnospiraceae family are important fibrolytic bacteria in the guts of mammals (Biddle et al., 2013; Li et al., 2014). Thus, lower fibrous substrate availability in the colon may have contributed to a decrease in fibrolytic bacteria (unclassified Ruminococcaceae, *Blautia*, *Butyrivibrio*, and *Pseudobutyrivibrio*) in the M+S group.

Furthermore, changes in colonic mucosal bacterial composition may partly impact host immune homeostasis in the colon, and dysregulated immune responses to opportunistic commensals potentially affect host health (Donaldson et al., 2016). In the current study, we found that starter feeding increased the relative abundances of *Oscillibacter*, *Parabacteroides*, and *Bifidobacterium*, but decreased the proportions of unclassified Ruminococcaceae, *Blautia*, and *Campylobacter* in the colonic mucosa of lambs. Among these variation taxa, *Oscillibacter* is a bacteria found in the colonic mucosa of humans. Reports have shown that healthy people have a higher abundance of *Oscillibacter* in their colonic mucosa than patients diagnosed with Crohn's disease (Man et al., 2011; Mondot et al., 2011), which indicates that *Oscillibacter* may be beneficial for colonic health. Some species of *Parabacteroides* significantly reduce the severity of intestinal inflammation in murine models of acute and chronic colitis induced by dextran sulfate sodium (Kverka et al., 2011). Some species of *Bifidobacterium*, which produce acetate and lactate, are beneficial to the colonic health of both animals and humans (Gibson et al., 2004) and to the normalization of the ratio of anti-inflammatory to proinflammatory cytokines (O'Mahony et al., 2005). Thus,

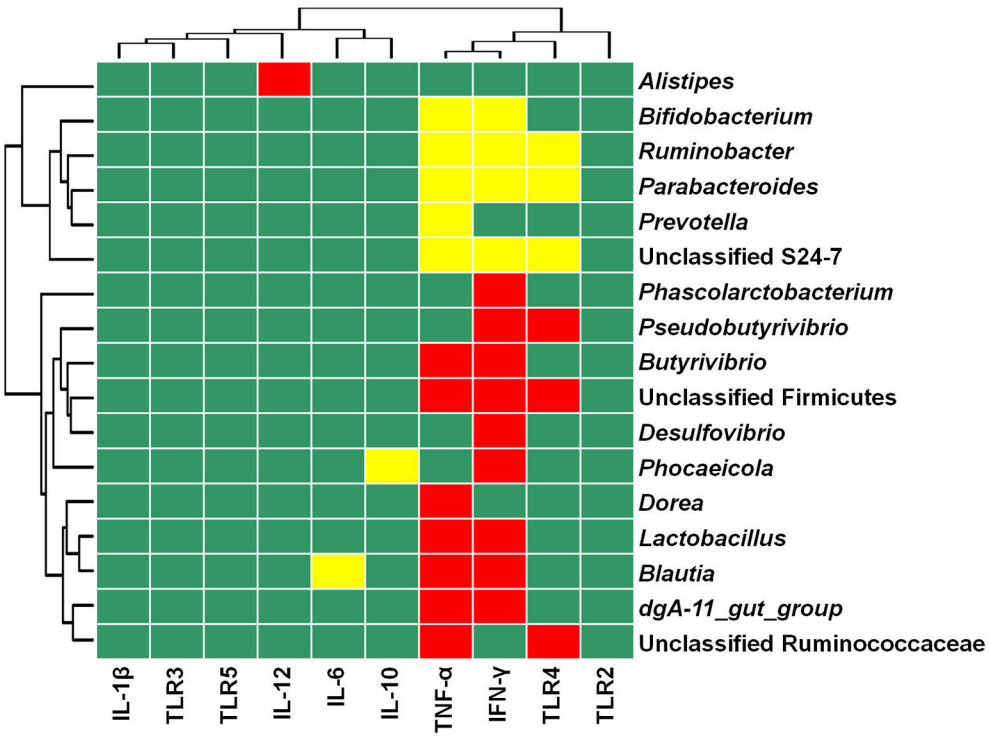

FIGURE 4 | Heat map showing the correlation between the relative abundances of bacterial taxa and mRNA expression in colonic mucosa. The top 50 bacterial taxa were selected to perform the correlation analyses, and those significantly associated with TLR and cytokines are shown. Cells are colored based on Spearman's correlation coefficient. Red represents a significant positive correlation ($P < 0.01$), yellow represents a significant negative correlation ($P < 0.01$), and green represents a non-significant correlation ($P > 0.01$).

our findings indicate that the higher relative abundances of some beneficial bacteria (*Oscillibacter*, *Parabacteroides*, and *Bifidobacterium*) during concentrate starter feeding may have beneficial effects on the colonic health of lambs in the milk-feeding period.

Ruminococcaceae is the dominant family in the colonic mucosa of mammals and has been associated with the maintenance of gut health (Donaldson et al., 2016). Previous studies have shown that the enrichment of this family is associated with colonic mucosal inflammation (Willing et al., 2010). Moreover, the enrichment of *Blautia* has been related to colonic inflammation in humans (Loh and Blaut, 2012). Thus, starter feeding-induced depression of *Blautia* may be beneficial for alleviating local inflammation in lamb colons. Our recent study showed that high-grain diet feeding increases the relative abundance of *Blautia* in the colonic mucosa of adult goats (Ye et al., 2016). This discrepancy indicates that colonic mucosal bacteria reflect different responses to concentrate diet feeding in preweaned and adult ruminants. Some studies have found *Campylobacter* in the colons of cattle (Inglis et al., 2005), goat kids (Jiao et al., 2016), and sheep (Stanley and Jones, 2003), while other studies have demonstrated that the enrichment of some specific species of *Campylobacter* is closely associated with local inflammation in the colons of humans and animals (Russell et al., 1993; Chen et al., 2006). Thus, these findings suggest that starter feeding-induced depression in the proportion of some pathenogens and potential pathenogens (unclassified Ruminococcaceae, *Blautia*, and *Campylobacter*) may also have beneficial effects on lamb health.

Colonic mucosal microbiota are integral for stimulating the innate immune response of the host (Abreu, 2010). In the current study, we found that concentrate starter feeding decreased TLR4 expression, which agrees with Jiao et al. (2016), who demonstrated that supplemental feeding (compared with grazing) decreases TLR4 expression. TLR4 can recognize Gram-negative bacteria and their products (Abreu, 2010). Surprisingly, the correlation analysis revealed that TLR4 expression is positively associated with some Gram-positive bacterial taxa (unclassified Ruminococcaceae, *Pseudobutyrivibrio*, and unclassified Firmicutes) and negatively associated with some Gram-negative bacterial taxa (unclassified S24-7, *Parabacteroides*, and *Ruminobacter*) in the colonic mucosa of lambs. The changes in Gram-negative bacterial products during starter feeding may contribute to this discrepancy.

It has been reported that TLR can recognize some specific commensal bacteria and their products and then initiate proinflammatory pathways (Abreu, 2010). Thus, the effect of starter feeding on proinflammatory cytokine expression was also investigated. The data show that concentrate starter feeding decreased mRNA expression of the cytokines TNF-α

and IFN-γ in the colonic tissue of lambs. These results were somewhat consistent with Jiao et al. (2016), who indicated that supplemental feeding (compared with grazing) decreased IL-6 expression. The correlation analysis further revealed that the depression of mRNA expression in cytokines is associated with some specific bacteria. In particular, TNF-α and IFN-γ are negatively correlated with *Parabacteroides* and *Bifidobacterium*, respectively, and positively associated with *Blautia* and unclassified Ruminococcaceae, respectively. As mentioned earlier, treatment with *Parabacteroides* prevented dextran sodium sulfate-induced increases in proinflammatory cytokines IL-6 and IFN-γ in mice colons (Kverka et al., 2011). Some species of *Bifidobacterium* are considered beneficial to the colonic health of animals and humans (Gibson et al., 2004). On the other hand, *Blautia* is related to colonic mucosal inflammation in humans (Loh and Blaut, 2012). Previous studies have also shown that the enrichment of the Ruminococcaceae family is associated with colonic mucosal inflammation (Willing et al., 2010). A high-fat, diet-induced increase of proinflammatory cytokine (IL-1β, IL-6, and TNF-α) expression has been associated with the enrichment of Ruminococcaceae in the colonic tissue of mice (Kim et al., 2012). Thus, the decreased expression of cytokines in our study may be partly due to enrichments of some beneficial bacteria (*Parabacteroides* and *Bifidobacterium*) and the depression of some pathenogens and potential pathenogens (*Blautia* and Ruminococcaceae family) during starter feeding in lambs. Our findings show that starter feeding increased the abundance of some beneficial bacteria while decreasing the proportion of some pathenogens and potential pathenogens, which could in turn protect colonic mucosal morphology and modulate immune homeostasis in preweaned lambs. Certainly, these starter feeding-induced responses may not be necessarily beneficial for postweaning health in ruminants. Many previous studies indicated that the upregulation of TLR and cytokine genes to a certain degree may faciliate gastrointestinal immune system development (Abreu, 2010; Chen et al., 2012). It is possible that the increase in TLR4, TNF-α, and IFN-γ levels in breast-milk-fed lambs are actually beneficial to the developing immune system and that the starter feeding could contribute to problems observed in later life. Thus, more studies are needed to investigate whether starter feeding affects postweaning health in ruminants.

CONCLUSION

We found that concentrate starter feeding increased colonic fermentation and significantly affected colonic mucosal bacterial communities by increasing the relative abundances of the dominant taxa unclassified S24-7, *Oscillibacter*, *Prevotella*, *Parabacteroides*, *Bifidobacterium*, *Ruminobacter*, and *Succinivibrio*, and decreasing the proportions of unclassified Ruminococcaceae, *RC9_gut_group*, *Blautia*, *Phocaeicola*, *Phascolarctobacterium*, unclassified BS11_gut_group, unclassified family_XIII, *Campylobacter*, unclassified Firmicutes, *Pseudobutyrivibrio*, *Barnesiella*, *Lactobacillus*, unclassified Gastranaerophilales, *Butyrivibrio*, *dgA-11_gut_group*, and *Dorea* in lambs. Meanwhile, starter feeding decreased the colonic mucosal mRNA expression of TLR4 and cytokines TNF-α and IFN-γ. Furthermore, the changes in mRNA expression of TLR and cytokines were associated with variations in the abundances of some specific bacteria in colonic mucosa. Collectively, our study shows that concentrate starter feeding can alter colonic mucosal bacterial composition and modulate mucosal immune homeostasis during the milk-feeding period in lambs.

AUTHOR CONTRIBUTIONS

DS and JL carried out the majority of the experiment including animal care, VFA analyses, RNA isolation, and real-time PCR. GB and JL were responsible for pyrosequencing data processing, analyses and interpretation. JL, SM, and WZ contributed to the conception of the project. The manuscript was prepared by JL and SM.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00429/full#supplementary-material

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REVIEW
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Innate Immunity and Breast Milk

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Human milk is a dynamic source of nutrients and bioactive factors; unique in providing for the human infant's optimal growth and development. The growing infant's immune system has a number of developmental immune deficiencies placing the infant at increased risk of infection. This review focuses on how human milk directly contributes to the infant's innate immunity. Remarkable new findings clarify the multifunctional nature of human milk bioactive components. New research techniques have expanded our understanding of the potential for human milk's effect on the infant that will never be possible with milk formulas. Human milk microbiome directly shapes the infant's intestinal microbiome, while the human milk oligosaccharides drive the growth of these microbes within the gut. New techniques such as genomics, metabolomics, proteomics, and glycomics are being used to describe this symbiotic relationship. An expanded role for antimicrobial proteins/peptides within human milk in innate immune protection is described. The unique milieu of enhanced immune protection with diminished inflammation results from a complex interaction of anti-inflammatory and antioxidative factors provided by human milk to the intestine. New data support the concept of mucosal-associated lymphoid tissue and its contribution to the cellular content of human milk. Human milk stem cells (hMSCs) have recently been discovered. Their direct role in the infant for repair and regeneration is being investigated. The existence of these hMSCs could prove to be an easily harvested source of multilineage stem cells for the study of cancer and tissue regeneration. As the infant's gastrointestinal tract and immune system develop, there is a comparable transition in human milk over time to provide fewer immune factors and more calories and nutrients for growth. Each of these new findings opens the door to future studies of human milk and its effect on the innate immune system and the developing infant.

Keywords: human milk, breast milk, innate immunity, colostrum, preterm

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INTRODUCTION

The innate immune system is the first line of defense against infection and is activated within minutes, reacting in a nonspecific, preprogrammed, and patterned manner to various infectious or foreign (non-self) stimuli (1). The infant's immune system is immature at birth, and this immaturity is pronounced for the premature infant placing the infant at increased risk of infection (2). Important developmental immune deficiencies at birth include incomplete physical and chemical barriers, poor innate effector cell function, limited and delayed secretory immunoglobulin A (IgA) production, incomplete complement cascade function, and insufficient anti-inflammatory mechanisms of the respiratory and gastrointestinal (GI) tracts.

Human milk is the everchanging secretions of the human breast, an evolving composition of nutrients and active factors. Just as nutrition and protection of the fetus occurs through the mutable

nature of the uterus, placenta, and amniotic fluid, the evolution of human milk from colostrum through transitional milk to mature milk provides nutrition and protection appropriate for the time-affected development of the infant (3). There is a large body of evidence documenting the benefits of human breast milk for human infants, in diminishing morbidity and mortality and protecting against specific infections during the period of breastfeeding (4–6). Additional data demonstrate long-term health benefits for the infant (and the mother) beyond the period of lactation (7, 8) and led to the current recommendations for duration of exclusive breastfeeding from the American Academy of Pediatrics (9) and the World Health Organization. Although research into the specific factors in human breast milk, which lead to the remarkable health benefits of exclusive breastfeeding, has been ongoing for decades; there are still intriguing mysteries of how human milk contributes to the development and regulation of both the infant's innate (10) and adaptive immune function (11–13).

Elucidating the relationship between the innate immune system and human milk, as well as their individual and interactive transition over time, remains challenging. Many components in milk are multifunctional, serving as enzymes, antimicrobial proteins/peptides (AMPs), growth factors, chemokines, antioxidants, anti-inflammatory elements, prebiotics, probiotics, and nutrients for the growing infant (14, 15). The use of modern molecular approaches such as microbial genomics, metabolomics, proteomics, and glycomics has led to novel discoveries in both composition and function of human milk components. This review will focus on the current understanding of the critical interactions between human breast milk and the infant's developing innate immune system (**Figure 1**) (12, 16–18).

CHEMICAL BARRIERS OF INNATE IMMUNITY

The chemical barrier of the intestine is predominately the mucus layers lining the GI tract. These mucus layers minimize antigenic contact between epithelial cells and commensal bacteria as well as potentially pathogenic bacteria. Antimicrobial peptides produced by Paneth cells, released into the mucus layer, bolster this chemical barrier innate effect by neutralizing microbes *via* various mechanisms. The multifunctionality of individual human milk factors adds another layer of complexity to the innate protection effected within the intestinal mucus layers.

Human milk oligosaccharides (HMOs) are the predominant glycans and important nutrients in human milk. They function in direct pathogen binding and as prebiotics facilitating the establishment of a healthy infant microbiome (2). The human milk glycoproteins (HMGPs) vary in size, structure, and amount in human milk and can be classified based on their location relative to the cell (secreted or attached to the cell membrane) and the different mucus layers. Mucin 1 (MUC1) and Mucin 4 (MUC4), gangliosides (GM1, GM3, and GD3), and glycoproteins similarly function by binding pathogens and do so without stimulating an inflammatory response (19). MUC1 and MUC4 have been described as binding to specific pathogens including HIV, rotavirus, *Escherichia coli*, and *Salmonella*. Other HMGPs have demonstrated binding to *H. influenzae*, Streptococci, *Helicobacter pylori*, Reovirus, *E. coli*, and *Burkholderia cepacia*. The specific molecular mechanism of this binding by glycans and interference with infection by the pathogen requires additional clarification. Unraveling the complex glycoprotein–ligand interactions will require application of newer technologies such as nanosurface plasmon resonance and glycan microarrays (20). Another challenge is to demonstrate whether, how, and when this occurs in a mother–infant dyad exposed to a specific pathogen through changes in the glycan composition of the mother's milk (21).

Lactoferrin (LF), another glycoprotein abundant in colostrum and transitional milk and ubiquitously expressed in most exocrine secretions, is one of the best studied glycoproteins in human milk. LF has multiple functions in host defense through binding iron, binding to bacterial membranes, inhibition of tumor necrosis factor-alpha (TNF-α) and interleukin-1β (IL-1β), stimulating the activity, maturation of lymphocytes, and contributing to an anti-oxidizing mileau (22). Peptide breakdown products of LF, lactoferricin and lactoferrampin, have specific direct antibacterial and antifungal effects (23).

There are numerous other HMGPs that have been described but the extent and specificity of their immune function need to be elucidated. Lactadherin can inactivate viruses and limits inflammation by increasing the effective phagocytosis of apoptotic cells. The sialic acid component of lactadherin seems to directly interact with rotavirus while the protein backbone of the molecule demonstrates a proangiogenic effect on neovascularization. The list of bioactive glycoproteins in human milk is still expanding, and their individual multifunctional nature is just being described. Butyrophilin, leptin, adiponectin, bile salt-stimulated lipase lysozyme, lactoperoxidase (LP), xanthine dehydrogenase, α-lactalbumin, κ-casein, and β-casein are just a few of these glycoproteins requiring additional study (19).

CELLULAR CONTRIBUTORS TO INNATE IMMUNITY

The cellular layers of the infant's intestine include the barrier of epithelial cells with the tight junctions, specialized goblet, Paneth and microfold cells, the lamina propria and Peyer's patches with macrophages, neutrophils, and dendritic cells. The immunity provided by these cellular interactions is partially innate and partially the beginning of adaptive immunity (24). Immaturity of the intestinal barrier function, limitations in production of AMPs, and ineffective response and action of epithelial cells and phagocytes place the infant at risk for infection in the neonatal period (25, 26).

Within fresh human milk, there is a remarkable repertoire of heterogeneous living cells. Cells originating in the breast include lactocytes (secretory cells), myoepithelial cells from the ducts as well as progenitor cells and mammary stem cells, and a small number of squamous epithelial cells from the nipple and skin of the breast. Cells originating from the blood, which are in human milk, include immune cells (macrophages, neutrophils, and lymphocytes), hematopoietic progenitor cells, and hematopoietic stem cells (27, 28). The role of live breast cells in human milk in the infant remains uncertain. New theories are being formulated to explain the fact that these breast milk epithelial cells are

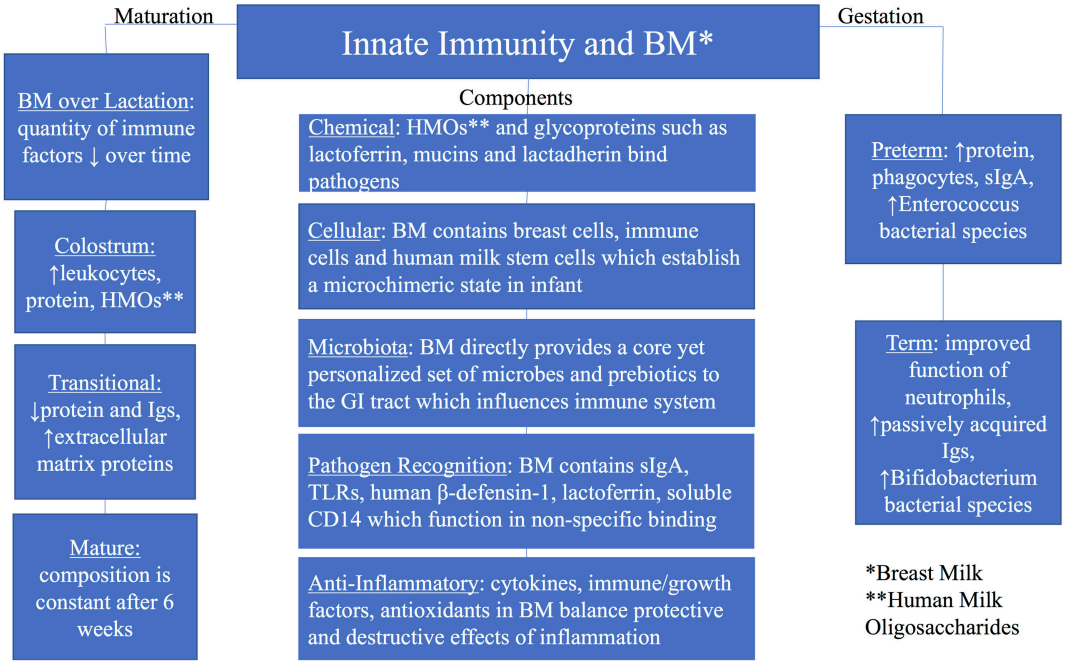

FIGURE 1 | Summary of innate immunity and breast milk with groups including components of the innate immunity, maturation of breast milk over lactation, and breast milk by gestation (preterm and term).

capable of motility and can in primary culture form functioning mammospheres (29, 30). Hassiotou et al. (29) explain the fact that these cells are in different stages of differentiation as a continuum of mammary development, albeit that does not answer the question why they are in such large numbers in expressed human breast milk.

There are large numbers of macrophages present in early lactation that decrease with the maturation of the milk (31, 32). These macrophages appear to function by phagocytizing pathogens without initiating a significant, unregulated inflammatory response. An increase in milk leukocytes, above the number usually present at the specific stage of lactation, occurs with infection in either the mother or the infant and suggests a functional role for milk leukocytes in protecting the infant (21, 33, 34). Breast milk from mothers of infants with severe bronchiolitis demonstrated an increased number of live cells. Those live cells produced a specific cytokine profile response when stimulated with live respiratory syncytial virus, a common cause of bronchiolitis (35). These data directly support the concept of mucosal-associated lymphoid tissue and that cells and/or activating factors are transferred from the mother in her breast milk to the infant at the time of infection or exposure to an infection. Additional studies measuring changes in the bioactive factors in human milk of mothers with sick infants or who are sick themselves for various specific infections should provide more insight into the essential bioactive factors in human milk for protection against specific infections.

The human milk stem cells (hMSCs) likely play an important role in the "regeneration" of the breast in preparation for lactation and theoretically in the infant's tolerance of maternal cell antigens as these cells from human milk do establish a microchimeric state in the infant. Hassiotou et al. (29) have demonstrated the pleuripotential differentiation nature of hMSCs. hMSCs were able to differentiate into the three germ layer cell lineages, in *in vitro* testing. Expression of octamer-binding transcription factor 4, related to "self-renewal" functions, is upregulated in hMSCs isolated from human milk (33). Using milk from genetically modified mice, Hassiotou et al. (36) demonstrated the persistence of modified milk stem cells within the brain, thymus, pancreas, liver, spleen, and kidneys of non-modified mice (36). These data specifically suggest a potential role for hMSCs in tissue regeneration in the breastfed infant and perhaps regeneration of cells of the infant's innate immune system. Beyond that, the hMSCs in human milk may provide a ready source of patient-specific stem cells with a true multilineage potential for the study of such stem cells and variables related to breast cancer, tissue regeneration, and even bioengineering.

MICROBIOTA

In the past, human milk was considered sterile, but that is far from the truth. Using culture techniques, the majority of bacteria identified as facultative anaerobes in human milk belong to the *Staphylococcus* and *Streptococcus* species and other species in smaller numbers (*Propionibacterium*, *Rothia*, *Enterococcus*, and *Lactobacillus* species) (37, 38). Obligate anaerobic bacteria were later identified, *Bifidobacterium* and *Bacteroides* species (39–41). Culture-independent techniques (sequencing and metagenomics analysis) have demonstrated a significantly more complex and diverse group of bacteria in human milk (42, 43). There is a large

degree of interindividual variability in the milk microbiome. Similar factors affecting the infant's or the mother's intestinal microbiomes also affect the milk microbiome (genetics, mode of delivery, geographic area, gestational age, maternal diet and nutrition, antibiotics, lactation stage, etc.) (42, 44). Nevertheless, most samples from healthy women appear to contain a "core" microbiome (34, 45). Hunt et al. name nine bacterial groups and Jimenez et al. reported seven bacterial groups. These two analyses shared just three common bacterial groups: *Staphylococcus*, *Streptococcus*, and *Propionibacterium* (34, 45). Variability between these two studies and others (43) can be attributed to the use of different primers, types of sequencing, comparison with different microbial reference libraries, possible variation due to geographic regions, and timing of the milk collection as well as technique and sterility of milk collection (43, 46, 47).

The actual origin of the milk microbiota remains uncertain (48). Postulated origins include mammary gland itself, skin flora of the breast, an "entero-mammary pathway" where intestinal bacteria translocate and home to the breast and retrograde flow from the infant's mouth into the breast (12, 49–51). Tracing the origins of milk microbiota and the relative influence of environmental factors on its makeup should inform our understanding of the role of the common groups of bacteria or their interaction in symbiosis with the infant's innate immune system and developing intestine.

Human milk directly contributes to the establishment of the intestinal microbiota and facilitates a symbiosis between that microbiota and the infant by providing essential nutrients, in particular milk glycans or HMOs, for microbial metabolism (52). Nanthakumar et al. (53) proposed that specific microbiota colonizing the gut early on in the infant induce the expression of intestinal epithelial cell (IEC) fucosyltransferase 2. This leads to fucosylation of surface markers on GI epithelial cells, which enhances the growth of microbes utilizing fucose as part of their metabolism (53). Microorganisms utilize the available glycosaminoglycans, glycoproteins, glycolipids, and oligosaccharides (milk glycans) as prebiotics to facilitate growth (54). The intestinal microbiota limits the growth of pathogenic bacteria by competition for nutrients and receptors. Specific microbes facilitate the formation of the intestinal mucus layer and the development of the IEC barrier and submucosal lymphoid structures. Separately, the HMOs bind to surface molecules of bacteria and viruses preventing binding to the intestinal epithelium and appear to diminish intestinal inflammation *via* signaling pathways (54). Newburg and Morelli (55) describe the symbiosis of the microbiota and the infant's intestinal development as dependent on HMOs in the mother's milk. The "commensal" microbes, specifically *Bacteroides* and *Bifidobacterium*, induce mucosal glycan production, which further supports microbial growth, and the microbes convert indigestible milk glycans to absorbable short-chain fatty acids (55). He et al. (56) describe two different HMOs (3′-galactosyllactose and 2′-fucosyllactose) present in high amounts in colostrum. These HMOs affect pathogen-associated molecular pattern signaling pathways [Toll-like receptor (TLR) 3, TLR5, IL-1β, or cluster of differentiation 14 (CD14) expression and binding] decreasing cytokine production and the inflammatory response (56, 57). There are now even clinical trials examining the effect of probiotics added to the diet of lactating women, which increased the levels of secretory immunoglobulin A (sIgA) in the infant's stool and on IL-6 mean values in colostrum and on IL-10 and TGF-β1 mean values in mature breast milk. This demonstrates the possibility of potentially beneficial microbes influencing the content of the woman's breast milk and subsequently the infants' GI tract *via* specific bioactive factors reaching the infant. The ultimate benefit to the infant remains to be studied (58). Human milk is not simply adding additional bacteria to the infant's gut and intestinal microbiome but providing both bacteria and prebiotics to function in a symbiotic relationship creating the milieu in which the infant's innate intestinal immunity functions and the intestine develops. How that symbiotic interaction influences the infant's health in the future, as it relates to immune protection and immune reactivity (allergy or autoimmune disease) will require careful research involving not only genomics, metabolomics, proteomics, and glycomics but also epigenetics and techniques still need to be developed.

INNATE MECHANISMS OF PATHOGEN RECOGNITION

There are specific TLRs present in human milk, including TLR2, TLR3, TLR5 as well as soluble CD14 (sCD14), and human β-defensin-1 (hBD-1), which function as pattern recognition receptors (PRRs) and AMPs (59). Chatterton et al. (59) discuss the potential role of PRRs in human milk affecting the protective response in the intestine balanced with other bioactive factors in milk affecting an anti-inflammatory mileau. There is evidence that TLR responses in the infant are modified by soluble TLRs (sTLRs) and sCD14. The interaction of both sTLRs and sCD14 with other bioactive factors in human milk upregulates and downregulates the action of various TLR-mediated inflammatory responses (60, 61). LeBouder et al. (61) demonstrated the effect of human milk on TLR-mediated microbial recognition. They describe specific responses on epithelial cells, monocytes, dendritic cells, and peripheral blood monocytic cells. The responses were different based on which TLRs were activated. Infant formulas did not exhibit such effects.

Immunoglobulins are the most recognized immune protective component in human breast milk. As preformed Igs from the mother, they constitute a discrete group of proteins capable of pathogen recognition. sIgA is the principal Ig in human milk (>90% of the Ig fraction), immunoglobulin M (IgM) in the pentameric form is next most abundant. There is a small amount of immunoglobulin G (IgG) in colostrum and transitional milk, with IgG becoming a much larger proportion of human milk Igs in mature milk (62). Secretory IgA binds pathogens blocking infection without stimulating a significant inflammatory response. In a largely innate immune-like action, sIgA simply blocks the pathogens contact with the intestinal epithelial layer and traps the pathogens within the mucin layers. The action of sIgA in the extracellular space is different from sIgA's intracellular neutralization of viruses and bacterial lipopolysaccharides within epithelial cells. The glycan sugar component (galactose, fucose, and mannose) of sIgA contributes to sIgA resistance to proteolysis in the intestine and functions through a broad spectrum of

binding of pathogenic bacteria when compared with the antigen specific binding of the variable region of the Ig structure. The broad spectrum binding related to the glycan sugar component of sIgA is more consistent with an innate immune response which would explain the absence of an increase in specific sIgA in the milk of mothers with sick infants (21).

Immunoglobulin M causes agglutination of recognized pathogens and complement activation as well as innate immune-like activities. Immunoglobulin G (IgG) activates phagocytosis with antigen transport to the lamina propria for B-cell activation affecting the infant's adaptive response. The list of pathogens (viruses, bacteria, fungi, and parasites) recognized by human milk Igs is extensive (63). Gao et al. (64) report data from proteomic analysis of human milk, which demonstrate increased amounts of sIgA and IgM in transitional milk with IgG predominating in mature milk. They suggest that this transition fits with the infant's developing immunity and evolving adaptive immunity to produce increasing amounts of IgG.

There are various other AMPs, in human milk, active in microbe killing, which can supplement the protection of the immature neonatal intestine (14, 65). hBD-1 is one example of an AMP in human milk, which affects pathogen membrane permeability and cytokine stimulation in the intestine (66). Other proteins [lysozyme, LF (and peptide derivatives of it—lactoferricin and lactoferrampin), α-lactalbumin, transferring, and osteopontin (OPN)] within human milk are recognized as important AMPs functioning *via* various mechanisms some of which enhance the anti-inflammatory effects of human milk (59, 64, 67). LF and its derivatives demonstrate a wide variety of actions on various targets including iron deprivation, destabilization of microbial membranes, binding microbial receptors, affecting chemokine production, stimulating epithelial cell growth, stimulating T-cell growth and differentiation, and production of reactive oxygen species (ROSs). LF also interacts with other components in human milk such as OPN, ceruloplasmin, and neutrophil peroxidase, although the exact significance and function of these interactions remain uncertain (22, 68). Xanthine oxidoreductase, another protein found in large amounts in milk and upregulated in mature milk, affects mammary epithelium, generation of milk fat droplet membranes, and adds to the bactericidal effect of human milk by synthesizing ROS (64, 66). These different AMPs do not simply act in microbial recognition and inactivation; each have different secondary functions within the intestine. Equally important is the limited inflammatory response generated by these AMPs in the gut.

ANTI-INFLAMMATORY FACTORS AND EFFECTS

Maintenance of a homeostasis between protective inflammation and modulation of inflammation is essential to protecting the infant against infection at the same time as limiting the tissue damage due to inflammation (59). Oxidative stress through the production of free radicals does have some potentially beneficial effects for the host in terms of antibacterial action, immune defense, and signal transduction. The oxidative activity within the infant must be maintained in equilibrium with antioxidant capacity of tissues (69). Enterocytes and immune cells produce anti-inflammatory cytokines including transforming growth factor-beta (TGF-β), IL-10, IL-11, and IL-13. These factors act in an innate manner in the intestine.

Human milk caseins, LF, LP, OPN, Igs, superoxide dismutase (SOD), platelet-activating factor acetylhydrolase, and alkaline phosphatase each have both infection protective and anti-inflammatory effects. Specific hormones or growth factors predominantly exert their anti-inflammatory effects on intestinal innate immunity through their action on the proliferation and differentiation of IECs and immune cells (lymphocytes and macrophages) and modulating the inflammatory cytokine response. Transforming growth factors-β2 and -β1 upregulate tight junction proteins (caludin-1 and claudin-4) and downregulate TNF-α and IL-1β. In addition to growth stimulation, TGF-β has anti-inflammatory properties through stimulation of epithelial cell migration and repair of the epithelium after mucosal damage (70). Similarly, insulin-like growth factors, milk fat globule epidermal growth factor-8 (MFG-E8), and epidermal growth factor (EGF) influence growth and proliferation of IECs. Both MFG-E8 and EGF diminish the activation of nuclear factor kappa-light-chain enhancer of activated B cells (59). Trefoil factor 3 (TFF3) is an effector molecule that is present in the intestine and in large amounts in human breast milk. Generally, this molecule improves healing in the GI tract. The TFF3 present in breast milk produces downregulation of cytokines and promotes hBDs expression in IECs (71). Glucagon-like peptide 1 (GLP-1) is secreted from the enteroendocrine cell in the distal intestine and plays a role in regulating glucose metabolism and food intake. GLP-1 likely acts through vagal afferent pathway ultimately influencing feeding behavior (72). Recently, the first study to report GLP-1 in human milk showed that it was higher in hindmilk compared to foremilk and was correlated with infant weight gain during the first 6 months of life (73). Alternative forms of neonatal nutrition such as formula and TPN do not contain these anti-inflammatory properties, which may put these infants at a disadvantage by creating a relative deficiency of anti-inflammatory factors and activity.

Any enteral feeding directly stimulates growth of the neonatal gut. Human milk contains specific direct growth factors including platelet-derived growth factor, hepatocyte growth factor (HGF), vascular endothelial growth factor, and insulin (74–76). Each of these is important in angiogenesis, cell development, and tissue proliferation. HGF is expressed in the intestinal tissues and is present in human milk. HGF may play a role in mucosal growth and repair (77). Animal studies have shown that HGF given after intestinal resection or colitis has improved gut proliferation and nutrient transport (78, 79). Weiss et al. (80) describe declining levels of anti-inflammatory, proresolving lipoxin A4 (LXA4), and resolvin D1 (RvD1) and D2 (RvD2) in the lipid profile of human milk from colostrum through the first month of life. The average amount of LXA4 in human milk was two times the amount of proinflammatory leukotriene B4 (LTB4) (80). This highlights the importance of minimizing inflammation in the early period of an infant's life.

Specific antioxidants in human milk include vitamins A, E, C, LF, lysozyme, glutathione peroxidase, SOD, catalase,

ceruloplasmin, coenzyme Q10, thioredoxin, leptin, adiponectin, and trace elements—iron, copper, zinc, and selenium (69). These antioxidants act by reacting directly with a free radical before damage occurs or by interfering with the ongoing oxidation in liquid phase or in cell membranes. The total antioxidative capacity of human milk is highest in colostrum and declines over lactation with variability from person to person and time to time (69). Adiponectin, leptin, LF, and lysozyme each have antioxidant effects, and their concentrations in human milk also vary over time (81). Vitamin A or its derivatives bind to radicals of oxygen, thiol, or peroxide, limiting their oxidative damage on cells. There are higher concentrations of vitamin A in colostrum than mature milk (82). Higher concentrations of α-tocopherol (the predominant form of vitamin E) are in colostrum than transitional or mature milk (83). Vitamin E forms part of the milk fat globule membrane and constitutes the major portion of antioxidative function of breast milk at 1 month of age (84). Vitamin C is a hydrosoluble vitamin in human milk and an effective antioxidant in extracellular fluids.

Nucleotides, nucleosides, and nucleic acids are essential to cellular metabolic function. They constitute approximately 15–20% of the non-protein nitrogen or total potentially available nucleosides in human milk (85). Nucleotides function predominantly in cellular energy metabolism related to adenosine triphosphate, as messengers and coenzymes in metabolic pathways, and in nucleic acid production and salvage. In the face of infection, nucleotides are essential to the immune response (cellular activation, proliferation and action, and cellular signal transduction) and the repair of intestinal inflammation and damage. Brunser et al. (86) reported on the benefit of nucleotide supplementation for infants less than 6 months old at decreasing the severity and incidence of diarrhea. They postulated that the effect was due to effects on the intestinal integrity and repair as an example of an anti-inflammatory effect.

EVOLUTION OF BIOACTIVE FACTORS IN HUMAN MILK OVER LACTATION

The composition of human milk is dynamic with significant change from colostrum, transitional to mature milk, between preterm and term milk and with interindividual and intraindividual variation. Specifically, HMOs show interindividual variation relative to the total number of HMOs and individual HMOs varying with mother's Lewis blood group and secretor status.

Colostrum is produced between birth through the first 5 days of lactation, transitional milk is from 5 days to 2 weeks postpartum and maturation of the milk continues until it is "fully mature" at 4–6 weeks postpartum. There is only a small volume of colostrum produced, rich in leukocytes, protein, HMOs, and bioactive factors—IgA, LF, EGF, TGF-β, colony-stimulating growth factor, and antioxidants (31). Transitional milk has decreasing amounts of protein and Igs and increasing lactose and fat and water-soluble vitamins resulting in a higher caloric density of the milk to meet the infant's growth demands while the quantities of bioactive factors declines over time. The composition of mature milk remains constant after 6 weeks through the remainder of the lactation period. The amount of Igs and LF in milk decreases over the first 3–4 months, while the amount of lysozyme increases (87). Tregoat et al. (88) described declining amounts of mannan-binding lectin concentrations in transition from colostrum to mature milk. More recent published studies using proteomic analysis of human milk (16, 64, 89) continue to facilitate our understanding of the complex nature of human milk and its role related to immune function and intestinal development. Gao et al. (64) describe a similar transition of the various Igs, from colostrum to mature milk using proteomic analysis of the milk. They describe a large percentage of milk proteins having to do with immune function including complement factors and serine protease inhibitors important in regulating the complement system. The quantities of these factors declined in transition from colostrum to mature milk. They also identified proteins associated with the extracellular matrix including cytokines, fibronectin, tenascin, and OPN. These proteins were more prevalent in transitional milk. Glutathione and antioxidant activity-related proteins were more common in mature milk. Overall, the quantity of immune factors and immune effects of human milk diminish over time in parallel with the developing immune system of the infant. Nevertheless, it will be essential to understand the specific roles of the various bioactive components of human milk and how the change in milk composition over time influences the evolving effects of human milk on the intestine and innate immunity.

DIFFERENCES IN PRETERM AND TERM HUMAN MILK

Preterm babies require additional nutrition and immune protection compared to term infants. Interestingly, preterm breast milk has been found to contain increased nutrients such as protein (90) and higher concentrations of certain immune factors. Preterm human milk also has higher amounts of phagocytes and secretory IgA (24). These increased amounts may serve a protective role since premature infants have poorly functioning neutrophils, limited production of Igs, and lower levels of passively acquired Igs.

A few studies reported a difference in breast milk microbiome when comparing preterm and term milk. Some trends include more *Bifidobacterium* in term milk (91) and more Enterococcus in preterm milk (91). A study looking at preterm infants, testing stool and breast milk samples, found a high proportion of antibiotic-resistant high-risk clones in both fecal and milk samples during the neonatal intensive care unit (NICU) admission (92). Differences are also seen in gut microbiome between term and preterm infants. Variations in microbiota of preterm infants have been described as predisposing to development of necrotizing enterocolitis (NEC) (93–97). Hormones and cytokines also vary by gestational age. EGF has anti-inflammatory properties and is higher in preterm milk compared to full-term milk (24) The cytokine IL-10, with anti-inflammatory properties, was detected in lower amounts in breast milk for infants with increased risk of NEC (98). Trend et al. reported that TGF-β2 concentrations in human milk were significantly higher in the extreme premature infant group compared to the term infant group (99). Castellote et al. also reported that TGF-β2 was higher in preterm infants compared with term infants (100). A study by Maheshwari et al.

demonstrated that this anti-inflammatory cytokine suppresses endotoxin-induced cytokine responses of gut macrophages in the preterm infant *in vitro* and protects rat pups from gut injury *in vivo* (101). They also reported that percentages of IL-10 and TNF-α were lower in preterm milk compared to term milk.

Trend et al. found that AMPs can limit the *in vitro* growth of bacteria associated with neonatal sepsis (52). This is particularly important in preterm infants who are at increased risk of late-onset sepsis. The hBD-1 was higher in preterm colostrum compared to term colostrum (99). Wang et al. (102) also found that hBD-1 and hBD-2 were in higher concentration in mature preterm milk than mature term milk. Armogida et al. and Trend et al. both found that human α-defensin 5 levels were not affected by preterm birth, which suggests that these defensins are differentially regulated (99, 103). The amount of LF in human milk does not seem to be affected by gestational age (99) but is more dependent on milk volume expressed. There are conflicting results for concentrations of lysozyme in preterm and term milk.

It has been proposed that the increased levels of immune factors in preterm milk may be a result of a compensatory mechanism whereby in the mother during preterm labor, the breast shifts the immune content of the milk to provide more protection. Another explanation suggested by Goldman et al. (62) is that increased immune factors in preterm milk may be due to increased maternal systemic inflammation, a postulated condition leading to preterm delivery. The mechanisms for the differences between preterm and term milk remain unknown. Interestingly, most immune factors decrease over the first month regardless of gestational age, thus term and preterm milk become more similar over time as the chronological age of the baby increases.

SUMMARY

Feeding an infant human breast milk is not a matter of filling the infant with an "appropriate" amount of important nutrients and a protective level of bioactive factors. Although the various factors do complement and supplement the innate immunity of the infant, they actively affect the ongoing development of the infant's immunity and intestinal development. As Lars Bode et al. declared, "Human milk is alive, …" (27). The cells, the microbes, and the bioactive factors make milk alive, and the interactions of human milk with its natural host, the infant, create a symbiotic commensal relationship. This is the challenge to explain and understand the complexity and dynamic relationship between the everchanging secretion, human breast milk and the developing, evolving human infant.

AUTHOR CONTRIBUTIONS

All authors have made substantial, direct, and intellectual contribution to the work and approved it for publication. NC is primarily responsible for **Figure 1**.

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REVIEW
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The Role of Mucosal Immunity in the Pathogenesis of Necrotizing Enterocolitis

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Necrotizing enterocolitis (NEC) is the most devastating gastrointestinal disease of prematurity. Although the precise cause is not well understood, the main risk factors thought to contribute to NEC include prematurity, formula feeding, and bacterial colonization. Recent evidence suggests that NEC develops as a consequence of intestinal hyperresponsiveness to microbial ligands upon bacterial colonization in the preterm infant, initiating a cascade of aberrant signaling events, and a robust pro-inflammatory mucosal immune response. We now have a greater understanding of important mechanisms of disease pathogenesis, such as the role of cytokines, immunoglobulins, and immune cells in NEC. In this review, we will provide an overview of the mucosal immunity of the intestine and the relationship between components of the mucosal immune system involved in the pathogenesis of NEC, while highlighting recent advances in the field that have promise as potential therapeutic targets. First, we will describe the cellular components of the intestinal epithelium and mucosal immune system and their relationship to NEC. We will then discuss the relationship between the gut microbiota and cell signaling that underpins disease pathogenesis. We will conclude our discussion by highlighting notable therapeutic advancements in NEC that target the intestinal mucosal immunity.

Keywords: necrotizing enterocolitis, innate immunity, mucosa, intestine, toll-like receptor 4, human milk oligosaccharide, microbiota, prematurity

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INTRODUCTION

Necrotizing enterocolitis (NEC) is notably the most lethal gastrointestinal disease of premature infants. The disease prevalence is approximately 7% of infants born between 500 and 1,500 g in the United States and Canada (1–3). Unfortunately, both the treatment approach and mortality have remained unchanged for decades with a mortality rate as high as 42% (4). At the same time, NEC represents a significant economic burden on the health system with an estimated annual cost upwards of one billion dollars in the United States (2, 5–7). These challenges fuel the need to understand disease pathogenesis so as to develop novel therapeutic and preventative strategies. Presently, it is believed that the immature intestine of premature infants exists in a hyper-active state due to abnormal bacterial colonization, which ultimately results in a robust inflammatory response and impairment of intestinal perfusion, thereby predisposing infants to NEC (2, 7, 8). Current research suggests the intestinal immune system is intricately involved in this process, which is comprised of the intestinal epithelium, immune cells, and commensal bacteria that maintain gastrointestinal homeostasis. Here, we will review the current knowledge of intestinal mucosal immunity in relation to NEC. First, we

will discuss the cell types that comprise the intestinal immune system with attention to how these cells are involved in NEC. We will then describe the role of the innate immune system with specific attention to toll-like receptor 4 (TLR4) signaling in the pathogenesis of NEC. We will then review the role of gut microbiota in our current understanding of this disease. Finally, we will describe advancements in potential treatment strategies rooted in our current understanding of the relationship between mucosal immunity and the development of NEC.

CELLS OF THE INTESTINAL IMMUNE SYSTEM

In order to understand the pathogenesis of NEC, it is important to appreciate the role of the immune system in the maintenance of gastrointestinal homeostasis. We will first describe the role of epithelium and immune cells in mucosal immunity and then describe their role in the development of NEC with specific attention to the interplay of these cell types and the signaling pathways involved.

The Intestinal Epithelium

The epithelium represents the first layer of defense, comprised of at least seven differentiated cell types that together maintain barrier integrity and provide defense against pathogens with the presence of tight junctions (9). The epithelium has two distinct structures: the villus and the crypt. The villus contains enterocytes, goblet cells, enteroendocrine cells, and tuft cells, whereas the crypt houses transit amplifying cells, Paneth cells, and stem cells (**Figure 1**) (10). Stem cells expressing leucine-rich containing G protein-coupled receptor 5 (Lgr5) are capable of generating all cell types of the epithelium (11, 12). Together, these cells comprise the epithelium, which we will now discuss in further detail.

Enterocytes

Enterocytes (IECs) are the predominant absorptive cells of the epithelium defined by the presence of microvilli, but the role of enterocytes is not limited to nutrient absorption; rather, they are important cells in the preservation of intestinal integrity and mucosal immunity (13–16). IECs, as the most numerous cells of the epithelium, provide the physical barrier between the lumen of the gastrointestinal tract and the lamina propria *via* the maintenance of tight junctions (14). They originate from within the intestinal crypts and migrate along the villi, at which point they undergo apoptosis, renewing the epithelium every 3–5 days in a continuous cycle of IEC proliferation, migration, and apoptosis in mouse studies (17). This cycle is crucial for intestinal homeostasis; however, aberrancy in this process can lead to disastrous effects, such as bacterial translocation, which we will discuss in the context of NEC in a later section (18). IECs are notable for the presence of several pattern-recognition receptors (PRRs), such as toll-like receptors (TLRs) and nucleotide-binding oligomerization domains (NODs), thereby aiding in the clearance of pathogenic bacteria while maintaining a population of commensal bacteria (15, 16, 19, 20). We will describe TLRs and NODs more extensively in a later section with specific attention to the relationship between TLR4 expression by enterocytes and gut barrier integrity. Moreover, IECs also express major histocompatibility class (MHC) I and II molecules and non-classical MHC molecules, allowing IECs to process and present antigens to the immune cells of the intestine (21, 22). In doing so, there is a direct communication between the antigens within the lumen and the cells of the lamina propria (**Figure 1**). Accordingly, enterocytes are vital cells of the epithelium with the roles in the maintenance of the gut barrier and commensal bacteria, absorption of nutrients, and communication with the immune cells of the lamina propria.

Goblet Cells

Goblet cells are particularly important with their role in generating the mucus layer of the intestine, preventing the interaction between pathogenic bacteria and the epithelium, while providing support for commensal bacteria, antimicrobial peptides (AMPs), and secretory immunoglobulin A (IgA) (23). Moreover, goblet cells are also capable of delivering luminal antigens to a subset of underlying dendritic cells (DCs), $CD103^+$ lamina propria DCs, which have tolerogenic properties, thereby assisting with the maintenance of commensal bacteria and intestinal homeostasis (24).

Goblet cell differentiation is determined by the activity of the Notch signaling pathway (25). Sodhi et al. (26) determined that the innate immune receptor TLR4 regulates Notch signaling and subsequent goblet cell development in the small intestine, such that TLR4 signaling prevented goblet cell differentiation independent from the influence of the microbiota. Furthermore, Notch signaling was found to be increased in mice as well as premature infants with NEC, whereas inhibition of the Notch pathway led to an increased number of goblet cells and attenuated experimental NEC in mice (26). This study highlights the regulation by TLR4 and Notch signaling in NEC pathogenesis.

Goblet cells secrete glycoproteins called mucins, of which, the Muc2 mucin is of critical importance in maintaining an inner mucus layer impervious to pathogenic bacteria, while simultaneously creating an outer mucus layer and providing an ideal habitat for commensal bacteria (27). Notably, ileal Muc2 is decreased in NEC and depletion of intestinal goblet cells increases susceptibility and severity of experimental NEC (28, 29). This subsequent decrease in mechanical defenses increases the vulnerability of the epithelium to pathogenic bacteria (26, 30), which can be further exacerbated by decreased intestinal motility in the setting of prematurity (31, 32). Abnormal goblet cell function is implicated in the development of NEC and mechanisms to enhance goblet cell production and/or function may provide a unique way to prevent the disease.

Paneth Cells

Paneth cells also provide a unique source of protection in the maintenance of the intestinal barrier. Paneth cells produce AMPs, lysozyme, secretory phospholipase A2, C-lectin RegIIIγ, α- and β-defensins, and angiogenin-4 to protect the host from pathogenic bacteria while shaping the composition of the microbiota (33, 34). Paneth cells and their AMPs, particularly α-defensins, have been implicated in diseases of the intestine through the use of several animal models (33). One noteworthy study utilized two genetic mouse models to study the role of α-defensins, including *DEFA5*-expressing transgenic mice and also mice that are deficient in

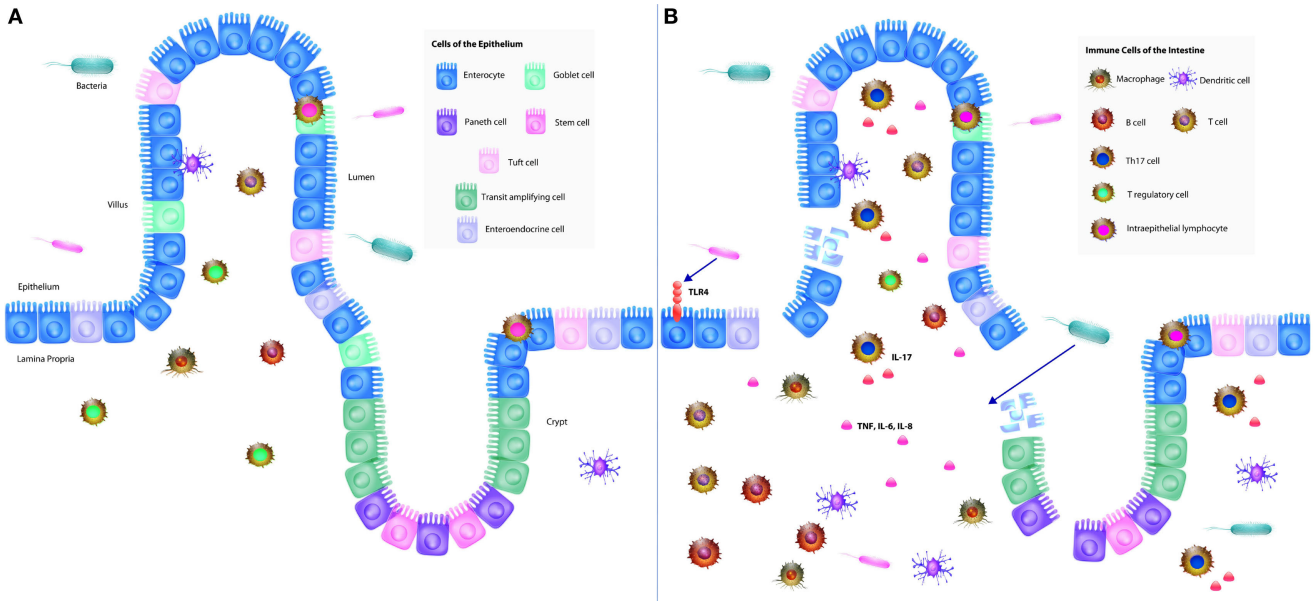

FIGURE 1 | The neonatal intestinal immune system and its interaction in the pathogenesis of necrotizing enterocolitis (NEC). **(A)** The intestinal mucosal immune system is comprised of the cells of the epithelium, immune cells, and commensal bacteria. The epithelium consists of villi and crypts. Enterocytes, goblet cells, enteroendocrine cells, and tuft cells exist within the villi, whereas Paneth cells and stem cells occupy the base of the crypts. Immune cells consist of intraepithelial lymphocytes, T regulatory cells (Tregs), T cells, B cells, macrophages, and dendritic cells, which reside predominantly in the lamina propria underlying the epithelium. Commensal bacteria inhabit the lumen of the gut, which constantly interact with the epithelium and immune cells to help maintain protection against pathogenic bacteria. **(B)** In NEC, lipopolysaccharide from Gram-negative bacteria interact with toll-like receptor 4 (TLR4) expressed by predominantly by enterocytes, which results in the breakdown of the gut barrier, allowing for pathogenic bacterial translocation. A pro-inflammatory response follows resulting in increased production of pro-inflammatory cytokines (IL-6, IL-8, and TNF) as well as increased Th17 cells and decreased Tregs. The combination of these cellular responses with TLR4 signaling results in a profound inflammatory response and subsequently NEC.

matrix metalloproteinase 7 (*MMP7*), which is required to activate α-defensins (35). Using 16S rRNA sequencing, they found there was an α-defensin-dependent change in the composition of the microbiota, such that there was a correlation between α-defensin deficiency, decreased populations of bacteria from the phylum Bacteroidetes, and increased populations of bacteria from the phylum Firmicutes (35). Moreover, in *DEFA5* transgenic mice, there was a loss of segmented filamentous bacteria and interleukin 17 (IL-17)-producing lamina propria T cells, substantiating the role of α-defensins and thus, Paneth cells in influencing the microbiota of the gut and modulating the intestinal immunologic response to pathogens (35).

The role of Paneth cells in disease has been well characterized in the studies on inflammatory bowel disease (IBD), specifically ileal Crohn's disease (36). There are several susceptibility genes associated with Crohn's disease, such as nucleotide-binding oligomerization domain-containing protein 2 (*NOD2*), which is expressed predominantly by Paneth cells in the small intestine (37–39). One such study evaluated known *NOD2* loss-of-function gene mutations known to be risk factors for IBD in a group of very low birth weight infants (40). They found that the presence of two or more *NOD2* genetic risk factors was an independently associated with the development of NEC and focal intestinal perforation (40). Moreover, *NOD2*-deficient mice develop ileal granulomatous inflammation in response to inoculation with *Helicobacter hepaticus* characterized by increased inflammatory cytokines and expression of Th1-related genes (41). This was restored with transgenic expression of α-defensin in Paneth cells (41). Moreover, in ileal Crohn's disease, there is decreased expression of α-defensins, suggesting a relationship between the secretion of α-defensins by Paneth cells and the pathogenesis of Crohn's disease. The involvement of Paneth cells in mediating protection against NEC is not well defined. However, animal models utilizing a Paneth-cell depletion method in the presence of *Klebsiella pneumoniae* have been shown to produce NEC-like intestinal injury, suggesting Paneth cells may have a role in NEC pathogenesis (42–44).

Enteroendocrine Cells

Enteroendocrine cells encompass several cell types that are located throughout the gastrointestinal system and primarily act to secrete hormones in response to food stimuli (45). The role of enteroendocrine cells in the promotion of mucosal immunity is incompletely understood; however, there is interplay between the immune system and enteroendocrine cells (46). For example, enteroendocrine cells express TLRs, and accordingly, can release chemokines and defensins in response to bacterial antigens, suggesting these cells have a role in the maintenance of intestinal homeostasis with respect to bacterial colonization (47, 48). Interaction with the gut microbiota is not limited to the expression of TLRs by enteroendocrine cells. Rather, there are several mechanisms by which these cells cooperate with the microbiota

(49). For example, L cells are a subset of enteroendocrine cells that secrete glucagon-like peptides 1 and 2 (GLP-1 and GLP-2) (45). Short-chain fatty acids (SCFAs) derived from gut microbiota can activate specific receptors of L cells, thereby influencing energy metabolism and gut barrier function *via* secretion of GLP-1 and GLP-2 (49). Accordingly, enteroendocrine cells have a unique interaction with the gut microbiota.

Furthermore, enteroendocrine cells have also been implicated in IBD. Friedrich et al. (50) found that patients have elevated serum and colonic pro-inflammatory IL-17c and discovered that enteroendocrine and to a lesser extent goblet cells were the main producers of IL-17c. This demonstrates a relationship between the neuroendocrine system of the gut and the Th17 pathway of the immune system (50). Nonetheless, more studies are necessary to understand the role of enteroendocrine cells during intestinal inflammation and more specifically NEC.

Tuft Cells

Finally, there is a unique cell type of the intestinal epithelium called tuft cells, which are thought to be taste-chemosensory cells found in both the respiratory tract and gastrointestinal tract (51, 52). Tuft cells were initially identified by electron microscopy due to their unique morphology with their tubulovesicular system and a tuft of long, blunt microvilli (51, 52). There is increasing evidence for tuft cell involvement in immunity (53). More specifically, there is a newly discovered role of tuft cells in parasitic infections, whereby, they generate type 2 helper T cell responses to promote immunity against such infections (54–56). This is accomplished by the ability to secrete IL-25 in a transient receptor potential cation channel subfamily M member 5 (Trpm5)-dependent mechanism (51, 54–56). IL-25 promotes the expansion of type 2 innate lymphoid cells, an important source of IL-13 (56). This is crucial because IL-13 acts to promote differentiation of both tuft and goblet cells contributing to the "weep and sweep" response of the intestine to parasitic helminth infections, which consists of goblet cell hyperplasia and increased smooth muscle contractility (56). Although there is currently no known role of tuft cells in the pathogenesis of NEC, these studies provide evidence that these cells are actively involved in the mucosal immunity of the intestine in addition to their chemosensory roles.

Immune Cells

Immune cells that compose the innate and adaptive immunity of the intestine exist in the epithelium and lamina propria (57). The epithelium houses intraepithelial lymphocytes (IELs) and the cytoplasmic extensions of DCs that interdigitate between epithelial cells (23, 57). Several cell types inhabit the lamina propria, including but not limited to DCs, macrophages, neutrophils, immunocompetent T and B cells, and T regulatory cells (Tregs) in addition to mesenchymal cell types, such as endothelial cells (23, 57). We will now discuss the various immune cells implicated in NEC, including T cells, Tregs, Th17 cells, IELs, B cells, macrophages, and DCs.

T Cells

There is increasing evidence that immune cells have a primary role in the development of NEC, including but not limited to T cells. In one study, NEC was induced in recombination activating gene 1-deficient (*Rag1*$^{-/-}$) mice, which lack functional T and B cells (58, 59). Compared to wild-type counterparts, these animals exhibited significantly decreased intestinal injury and mucosal cytokine production, suggesting they are protected from NEC development (59). Demonstrating that these cells have a causative role in NEC pathogenesis, susceptibility to NEC was restored with the adoptive transfer of naïve CD4$^+$ T cells (59). Furthermore, recruitment and differentiation of T cells were found to be mediated by activation of TLR4 *via* cognate chemokine ligand 25 (59). Thus, this study demonstrates the principal role of immune cells in NEC, specifically CD4$^+$ T cells, and their relationship to TLR4 signaling.

Relationship between Tregs and Th17 Cells

The roles of immune cells in NEC are dynamic, and there is an imbalance of immune cells that favor a exaggerated pro-inflammatory state. This is remarkably demonstrated by the relationship between Tregs and Th17 cells. Forkhead box P3 (Foxp3)-expressing Treg cells are essential for the maintenance of a stable equilibrium in the gut (57, 60). Tregs are important in counterbalancing inflammation and promoting antigen-specific IgA responses so as to maintain commensalism with gut microbiota (57, 61). In humans, intestinal Tregs are present as early as 23 weeks of gestation, suggesting an early role in intestinal homeostasis (60). In the intestinal lamina propria of both mice and premature infants with NEC, there is decreased production of Tregs and increased production of CD4$^+$ Th17 cells mediated by STAT3 (59). Additionally, expression of the IL-17 receptor, IL-17RA is significantly increased in a TLR4-mediated manner (59). Importantly, IL-17 release by CD4$^+$ Th17 cells results in intestinal mucosal injury, as demonstrated by impaired enterocyte tight junctions, increased enterocyte apoptosis, and decreased enterocyte proliferation, all of which are hallmark features of NEC (59). Similarly, in a study analyzing the T cell populations in human NEC, there was decreased Foxp3$^+$ Tregs relative to effector T cells compared to controls, which correlated with a mucosal cytokine expression profile indicative of inhibited Treg expression (62). With NEC resolution, Treg cell populations recovered, suggesting either the inflammatory response observed in NEC decreases Treg populations acutely or inflammation is attenuated as Treg populations increase with mucosal healing (62).

The premature human gut is notable for increased expression of pro-inflammatory cytokines, specifically IL-6, which is involved in T cells differentiating toward a Th17 phenotype during NEC development (59). Importantly, in a mouse model of NEC, oral administration of all-trans retinoic acid (ATRA), which binds to the nuclear retinoic acid receptor to stabilize transcription of Foxp3 and repress transcription of RAR-related orphan receptor γt, resulted in decreased NEC severity, increased Tregs, decreased CD4$^+$ Th17 cells, and attenuation of IL-17 expression (59). This suggests that the Tregs serve to provide a protective role in intestinal homeostasis, which is disrupted in NEC.

Further studies demonstrated that Th17 recruitment and Treg depletion resulted in apoptosis of Lgr5$^+$ intestinal stem cells within the crypts of Lieberkühn in an experimental model of NEC (63).

After exposure to recombinant IL-17, enteroids derived from intestinal stem cells exhibited decreased proliferation, decreased differentiation, and increased apoptosis (63). In addition, depletion of Tregs led to increased intestinal crypt apoptosis, exacerbating the experimental murine NEC model (63). Importantly, these effects were reversed upon administration of ATRA with its ability to modulate lymphocyte populations toward a protective Treg phenotype (63). This highlights the potential use of retinoic acid as a therapeutic in the treatment and prevention of NEC. Taken together, NEC is an immune cell-mediated disease defined by an imbalance of lymphocytes shifted toward a Th17 cell phenotype with a decrease in Tregs, which is destructive in this specific setting.

Intraepithelial Lymphocytes

Intraepithelial lymphocytes are a group of heterogeneous lymphocytes of the innate immune system in the distinctive position to interact dynamically with the local gut environment and the mucosal adaptive immune system to maintain intestinal homeostasis (64). This unique population of IELs bears the T cell receptor γδ (65). They differ from TCRαβ T-cells in that they do not require antigen processing for effector function (65). They are important in the discussion of the infant gut due to their early development in embryogenesis (66, 67). IELs are involved in a multifaceted approach in the maintenance and repair of the epithelium through tight junction preservation, recognition of stressors, and regulation of inflammation (65). They are also involved in a dynamic cross talk with commensal bacteria, as commensal bacteria direct expression of key immunomodulatory and antibacterial responses by influencing gene transcription of IELs, while IELs prevent opportunistic commensal bacteria overgrowth in the setting of mucosal injury (68). Weitkamp et al. (66) determined that γδ IELs are the predominant IEL subtype in the immature intestine of mice and premature infants. Furthermore, they showed that in the intestine of infants with NEC, there was a reduction in the γδ IEL subset as compared to non-NEC controls (66). This study highlights the important role of γδ IELs in intestinal barrier protection and suggests a potential cellular target for NEC prevention.

B Cells and Immunoglobulins

B cells are both important antigen-presenting cells (APCs) and integral members of adaptive immunity with their ability to secrete immunoglobulins as plasma cells, most notably IgA in the context of neonatal immunity. IgA can neutralize pathogenic bacteria to maintain intestinal homeostasis (9). The maternal IgA from colostrum and milk is an important source of immunity in the neonatal period. Secretory IgA (sIgA) is detected weeks after birth and increases progressively; however, preterm infants have decreased concentrations of sIgA compared to full-term infants (69, 70). One study found higher levels of sIgA from the colostrum and milk of mothers of preterm infants, suggesting immunological adaptation occurs in the setting of prematurity to enhance immunity with IgA and reinforces the benefits of breastfeeding in this context (71). There are both early and long-term benefits of early exposure to maternal sIgA (72). In one study, they found weanling mice exposed to sIgA-deficient breast milk displayed increased colonization of draining lymph nodes with bacteria, specifically aerobic bacteria and the opportunist pathogen *Ochrobactrum anthropi* (72). At both weaning and adulthood, there were significant differences in the gut microbiota and the pattern of epithelial gene expression of the intestine, including genes associated with IBD, between these mice compared to mice exposed to sIgA (72). Accordingly, exposure to sIgA in breast milk promotes intestinal homeostasis in the neonatal period with long-term implications (72). Taken together, IgA is important in intestinal immunity, and maternal IgA is crucial given the immaturity of the immune system during the neonatal period.

Macrophages

Macrophages are important phagocytic and bactericidal cells of the immune system. Macrophages are activated after exposure to lipopolysaccharide (LPS) and interferon-γ, which then release pro-inflammatory cytokines and nitric oxide (57). To counteract excessive inflammation, intestinal macrophages are inhibited by tumor growth factor-β (TGF-β) (57) and have been implicated in NEC pathogenesis (73). Intestinal macrophages from healthy term infants have increased phagocytic and bactericidal activity as well as minimal inflammatory cytokine production when exposed to bacterial products, which is attributed to the anti-inflammatory effects of transforming growth factor β_2 (TGF-β_2) (73). In contrast, the phenotype of macrophages during NEC is strongly inflammatory as demonstrated by increased expression of the gene, mothers against decapentaplegic homolog 7 (*Smad7*), an inhibitor of TGF-β_2 (74, 75). Consequently, this interrupts TGF-β-mediated downregulation of the pro-inflammatory response by macrophages in NEC and thereby sensitizes macrophages to bacterial products, leading to a significant pro-inflammatory response (74). Taken together, the imbalance of macrophage effector responses toward an inflammatory state increases mucosal injury in NEC.

Dendritic Cells

Dendritic cells are specialized APCs that have the delicate role of mediating protective adaptive immunity in response to pathogens while maintaining intestinal homeostasis and commensal bacteria (76). Although they are important in intestinal immunity, the role of DCs in NEC has not been fully elucidated. However, in one study using *Cronobacter sakazakii* to induce NEC in newborn mice, DCs were recruited from the lamina propria, resulting in intestinal barrier dysfunction *via* tight junction disruption and increased enterocyte apoptosis *via* TGF-β production (77). The study concluded that the presence *C. sakazakii* was able to modulate the activity of DCs to increase TGF-β production, which subsequently resulted in damage to the mucosal barrier integrity (77). This highlights the sensitive interaction between DCs and bacteria, which can be detrimental in the setting of NEC; however, more studies are necessary to further understand the role of DCs in NEC pathogenesis.

Neutrophils

Neutrophils are important effector cells of the innate immune system distinguished by their ability to respond rapidly and robustly to tissue trauma, including the intestine. The role of neutrophils

in NEC pathogenesis has not been fully elucidated, but there are several studies to note. Small for gestational age (SGA) neonates are more likely to have neutropenia during the first few days after birth, and these infants with neutropenia were at increased risk for developing NEC compared to SGA infants without neutropenia (78). Moreover, in a model of *C. sakazakii*-induced experimental NEC, depletion of neutrophils and macrophages in the lamina propria resulted in increased production of pro-inflammatory cytokines and increased enterocyte apoptosis, thereby exacerbating the disease (79). This suggests both macrophages and neutrophils are important in early infection and their absence intensifies the inflammatory response observed in NEC. One study utilizing TNBS-induced enterocolitis to model NEC found the leukocyte infiltrate relatively devoid of neutrophils in the intestinal tissue (80). Taken together, impairment of neutrophil function is one component of NEC; however, more studies are necessary to determine whether this is a primary observation in NEC or secondary to the disease.

Peyer's Patches

Peyer's patches are unique structures found primarily in the distal intestine that serve as secondary lymphoid tissue. Compared to other secondary lymphoid tissues, Peyer's patches are distinguished by their significant exposure to a diverse group of antigens, from the gut microbiota to food antigens (81). Antigens are delivered by M cells of the epithelium, and upon activation, generate B cells and plasma cells to maintain mucosal immunity (81). Peyer's patches appear in the intestine at 11 weeks, but continue to develop throughout gestation (82). Moreover, their numbers increase in proportion to gestational maturation, and thus, premature infants have less Peyer's patch numbers and decreased maturation (82). It is unknown if this has clinical significance in the setting of NEC. Accordingly, more studies are necessary to determine if Peyer's patches have a role in NEC as a consequence of prematurity.

CELL SIGNALING

The innate immune system of the intestine is intricately involved in the pathogenesis of NEC. An important aspect of the innate immune system is the expression of PRRs, including TLRs and NOD proteins, that can interact with the local gut environment and initiate several signaling pathways. We will review these essential components and their cellular responses with specific attention to aberrant TLR4 signaling, which is well studied in the pathogenesis of NEC.

PRRs: TLR and NOD

Pattern-recognition receptors are expressed throughout the cells of the body as a means of detecting threats to local homeostasis. The cells of the intestinal epithelium and immune cells express TLRs and NOD proteins, which can detect pathogen-associated molecular patterns such as the LPS of Gram-negative bacteria and flagella and consequently initiate an appropriate response to these bacterial stressors (23).

Specifically, in the case of TLR4 activation, signaling results in nuclear factor-κB (NF-κB) activation, subsequent cytokine production, resulting in an acute inflammatory response (23). TLRs are expressed by intestinal epithelial cells and immune cells of the lamina propria and are involved in epithelial cell proliferation, IgA production, maintenance of tight junctions, and AMP expression (23, 57, 83). TLR4 is of specific interest in the understanding of NEC pathogenesis, as its overexpression in the setting of prematurity yields a significant pro-inflammatory response and dysfunction of the epithelium (84). TLR4 is expressed by intestinal epithelial cells (85), which we will discuss in greater detail with respect to epithelial TLR4 expression and the development of NEC.

However, other TLRs have also been implicated in NEC pathogenesis. For example, TLR2 is expressed throughout the intestinal epithelium, including enterocytes, as well as various immune cells found in the lamina propria. Gram-positive bacteria in the intestine can engage TLR2, which can induce MyD88, which is a major adaptor for TLR2/TLR4, with subsequent NF-κB activation (86). TLR2 through the regulation of tight junctions is able to maintain gut barrier integrity, and thus, its deficiency can predispose the intestine to stress-induced injury (86). The role of TLR2 in NEC has not been fully elucidated thus far. However, in a rat model of NEC, investigators have found upregulation of ileal TLR4 and TLR2 in several studies, which precedes histological evidence of mucosal injury, suggesting a causative role in NEC pathogenesis (87, 88). Moreover, certain therapeutic interventions that reduced NEC severity in animal models downregulated the expression of TLR2 and TLR4, including glutamine (88) and probiotic *Bifidobacterium* (89). Thus, aberrancy in TLR4 signaling is not solely responsible for the pro-inflammatory response observed in NEC.

Moreover, NOD1 is also expressed by intestinal epithelial cells, which in response to the Gram-negative bacterial peptidoglycan, elicits an immune response to induce the formation of gut-associated lymphoid tissue, specifically Peyer's patches (9, 57). Conversely, NOD2 is expressed highly by monocytes and Paneth cells at baseline, and by enterocytes when under stress (9, 57). NOD2 is important in the cross talk between T-cells and intestinal epithelium to downregulate inflammation by inhibiting TLR signaling (9, 57). One study demonstrated that NOD2 activation inhibited TLR4 in enterocytes, thereby decreasing enterocyte apoptosis and attenuating the severity of experimental NEC (90). In this study, NOD2 provided protection from TLR4-mediated enterocyte apoptosis *via* a novel SMAC-diablo pathway (90). This suggests that modulation of this cross talk may provide a potential means of decreasing NEC severity (83, 90). Furthermore, NOD2 is important in regulating commensal bacteria such that NOD2-deficient mice had decreased ability to prevent colonization by pathogenic bacteria in the intestine (91). Conversely, germ-free mice had significantly decreased NOD2 expression, but this expression was inducible with the introduction of commensal bacteria, demonstrating the feedback mechanism by which NOD2 regulates the gut microbiota (91). Upon ligand binding, both NOD1 and NOD2 activate NF-κB, and independently function by activating this signaling pathway, but can also modulate TLR4 signaling in this way (92). Thus, TLR and NOD signaling exemplify the delicate interaction between luminal bacteria and the innate immune system of the gut and how perturbations in these pathways may lead to NEC development.

TLR4 Signaling

One of the major cornerstones in understanding the development of NEC is the role that TLR4 signaling plays in the pathogenesis (59, 85, 93–100). Activation of TLR4 within the intestinal epithelium in the setting of prematurity results in decreased enterocyte proliferation, increased enterocyte apoptosis, disruption of intestinal barrier integrity, and bacterial translocation, resulting in a systemic inflammatory response (26, 84, 95, 97). As a result of bacterial translocation, TLR4 is activated on the endothelium of premature gut, leading to impaired blood flow and subsequent intestinal ischemia *via* reduction of endothelial nitric oxide synthase (eNOS) (100, 101). The differential response to stress between premature and full term neonates rests in the increased expression of TLR4 in prematurity (96). TLR4 is expressed at high levels in the developing intestine as it is involved in normal gut development in both mice and humans (8). In the setting of prematurity, TLR4 expression remains elevated, resulting in a hyper-active, exaggerated response to stressors upon colonization by bacteria (8).

Furthermore, TLR4 is necessary for the development of NEC, and its enhanced expression is not a consequence of the disease. In studies utilizing TLR4-mutant mice strains (C3H/HeJ mice), lack of functional TLR4 was protective against the development of NEC, such that wild-type mice had increased NEC severity, increased enterocyte apoptosis, reduced enterocyte proliferation, and impaired restitution compared to TLR4-mutant mice (97). These results were further supported utilizing mice with either global TLR4 deletion or intestinal-specific TLR4 deletion (26). Both groups of mice were protected from the development of experimental NEC with preservation of mucosal integrity and minimal elevation of pro-inflammatory cytokines (26). These studies highlight the causative role of aberrant TLR4 signaling expressed by IECs in NEC pathogenesis.

Aberrant TLR4 signaling also has a direct role in the breakdown of the gut barrier in NEC. In healthy mucosa, healing of the epithelium occurs in two phases, intestinal restitution followed by enterocyte proliferation, whereby healthy IECs migrate to injured mucosa followed by increased generation of IECs from stem cells of the intestinal crypts (18). TLR4 signaling impairs IEC migration, thus impairing restitution (102, 103). Moreover, enterocyte proliferation is significantly decreased in NEC, diminishing the ability to heal in the setting of mucosal injury (18, 95, 97–99). Importantly, autophagy is a response to cellular stress and has been found to be upregulated in NEC (104). Furthermore, TLR4 signaling induces autophagy of enterocytes in both mouse and human studies (105). An experiment utilizing intestinal epithelial-specific autophagy gene ATG7 conditional knockout mice demonstrated autophagy *via* ATG7 was required for NEC development as these mice were protected from NEC (105). TLR4-induced autophagy leads to impaired enterocyte migration, demonstrating the effects of TLR4 signaling on the epithelium are multifaceted and interconnected (105). Ultimately, the deficiency in mucosal repair *via* enterocyte restitution and proliferation in the setting of NEC weakens the integrity of gut, allowing for bacterial translocation and the downstream inflammatory response observed in this disease.

Genetic Risk Factors

The study of genetic risk factors in NEC is an important component in understanding the signaling pathways that involve the innate immune system. There are several genetic association studies evaluating the relationship between specific genetic risk factors and the development of NEC (106). Genetic variation that affects TLR signaling increases the predisposition of the premature intestine to inflammatory aberrancy. The gene *SIGIRR* is important in the inhibition of LPS-induced inflammation (107). Loss-of-function mutations of *SIGIRR* result in unregulated TLR signaling, thereby predisposing infants to NEC (107). One study specifically evaluated the roles of single nucleotide polymorphisms of important genes in TLR signaling. They found in studying the blood samples of very low birth weight infants a relationship between *NFKB1* and *NFKBIA* variants and the development of NEC, such that *NFKB1* increased susceptibility to NEC, whereas *NFKBIA* decreased susceptibility to NEC (108). However, there was no association between *TLR2*, *TLR4*, *TLR5*, *TLR9*, *IRAK1*, and *TIRAP* genes and the development of NEC in this patient population (108). Genetic risk factors for NEC have not just been limited to TLR and NOD signaling pathways. For example, one study evaluating the autophagy gene ATG16L1 in premature infants found that hypomorphic variants conferred protection for the development of NEC (109). This study highlights the breadth of possible genetic risk factors in the NEC. Taken together, genetic predisposition to NEC is valid, and further large scale studies will likely reveal more genetic relationships and disease development.

GUT MICROBIOTA

The intricate relationship between gut microbiota and mucosal immunity is central to the discussion of NEC pathogenesis. The gut is exposed to a multitude of microbes, and it is the role of the intestinal immune system to distinguish commensal bacteria from pathogenic bacteria, particularly as the gut flora develops early in life. There are several variables that influence the microbial composition of the intestine; however, new evidence suggests that disruption of normal bacterial flora is involved in NEC pathogenesis. We will now explore the role of gut microbiota in the setting of prematurity.

Factors Influencing Gut Microbiota

There is a significant interest in understanding the composition of the gut microbiota and its relationship to the pathogenesis of NEC. Several factors influence the neonatal microbiota, including gestational age, mode of delivery (vaginal vs. cesarean section), antibiotic treatment, and diet (breast milk vs. formula feedings) (110). Colonization occurs in two waves, and the first wave is dependent on the mode of delivery (111). For example, one study found that compared to infants born vaginally, infants born *via* cesarean section had decreased populations of *Bifidobacteria* and *Bacteroides*, while there was an increased population of *Clostridium difficile* (112). The second wave is dependent on feeding method, which often differs between premature and term infants, as breast milk and formula feedings have different bacterial compositions and access to breast milk can be limited in

prematurity with delayed initiation of enteral feeding in preterm newborns (113). More specifically, formula-fed infants have increased populations of *Enterobacteriaceae*, *Bacteroides* species, and *C. difficile* in the stool compared to infants fed breast milk (112, 114).

Moreover, duration of antibiotic exposure is associated with the development of NEC (115, 116). In one retrospective study investigating the association between antibiotic exposure and subsequent diagnosis of NEC in infants, they found that antibiotic exposure duration is associated with increased risk of developing NEC (115). When sepsis was eliminated as a potential confounder, the probability of developing NEC was increased 20% per day of antibiotic exposure (115). Strikingly, antibiotic exposure greater than 10 days in neonates resulted in an approximately threefold increased risk of developing NEC (115). Another retrospective study aiming to assess the association between initial antibiotic therapy in extremely low birth weight infants and NEC, found there was an increased risk of developing NEC after initial empiric antibiotic treatment in the first three postnatal days (116). Empiric antibiotic treatment for greater than 5 days, which the study defined as prolonged antibiotic treatment, was associated with the development of NEC and death in extremely low birth weight infants in the setting of sterile blood cultures (116). These studies highlight the need to be judicious in the implementation of empiric antibiotic treatment in preterm infants.

A recent study utilizing 16S rRNA gene pyrosequencing suggests the most important variable influencing the composition of premature gut microbiota is the degree of prematurity (117). Using 922 specimens from 58 subjects, they found an ordered, tightly controlled microbial progression of bacterial classes: Bacilli to Gammaproteobacteria to Clostridia (117). Other factors, including antibiotics, mode of delivery, and age influenced the pace of the progression of bacterial classes, but the particular sequence of development remained the same (117). This is important as more studies demonstrate the significance of dysbiosis and the development of NEC (113, 118–122).

Dysbiosis in NEC

The differences in microbial colonization between preterm infants and term infants suggests the possible role of dysbiosis on the pathogenesis of NEC. The onset of NEC occurs 2–6 weeks of life with the highest risk of NEC occurring at a corrected age of 29–33 weeks (123), only after microbial colonization of the gut (2, 124, 125), highlighting the role of gut microbiota in disease pathogenesis. To date, no specific microbial pathogen has been identified to be responsible for the development of NEC (119, 124, 125). However, resected intestinal tissue with active NEC demonstrated increased microbial burden and an abundance of strict anaerobes with a decrease in community diversity (126). Moreover, until recently, it was unclear if abnormal gut microbiota is a cause or consequence of NEC (127). A prospective case–control study by Warner et al. (127) helps to define the role that the microbiota plays in NEC development. In 166 very low birth weight infants, 3,586 stool samples were prospectively collected, and of these subjects, 46 developed NEC (127). Differences between the NEC cases and matched controls emerged after 1 month with a predominance of Gammaproteobacteria and decreased quantities of Negativicutes and Clostridia in infants that went on to develop NEC, with the strongest correlation between bacteria composition and development of NEC occurring prior to 27 weeks gestation (127). This study suggests an increased concentration of Gram-negative facultative bacteria is detrimental, possibly due to their ability to activate TLR4 *via* LPS (84, 122). Conversely, infants with NEC had significantly decreased obligate anaerobes (127), which interestingly produce anti-inflammatory SCFAs (128). These results suggest that interventions focused on modulation of the gut bacteria may play a role in preventing NEC.

PROTECTIVE STRATEGIES AGAINST NEC

With advancements in our knowledge of NEC, there are several potential preventative and therapeutic strategies that did not exist decades ago. We will review several of these strategies with respect to how they influence the intestinal immune system (**Figure 2**). These advancements target several aspects of the intestinal immune system, such as TLR4 signaling modulation, gut barrier integrity, immune cell composition, and the gut microbiota, all of which have been described with their individual roles in NEC. There have been several studies that demonstrate the importance of providing breast milk to premature infants instead of formula. Human breast milk contains several bioactive components beneficial to the neonate some of which include: epidermal growth factor (EGF), heparin-binding EGF-like growth factor, platelet-activating factor (PAF) acetylhydrolase, human milk oligosaccharides (HMOs), nitrates/nitrites, L-arginine, lactoferrin, and probiotics (8, 129, 130). We will review several breast milk components and the evidence supporting the mechanisms by which they impact the intestinal mucosal immunity during NEC. We will also discuss the use of antenatal corticosteroids and delayed umbilical cord clamping in the prevention of NEC.

Breast Milk

Breast milk has a well-established role in the prevention of NEC and clinically represents one of the most effective strategies in decreasing the incidence and progression of NEC (130). New preclinical evidence has demonstrated that breast milk is capable of attenuating the TLR4-mediated pro-inflammatory response integral to NEC pathogenesis by activating the receptor for epidermal growth factor (EGFR), revealing the interplay between the EGF pathway and the hallmark TLR4 signaling of NEC (95). These inhibitory effects on TLR4 signaling were mediated by breast milk preventing the activity of the downstream target glycogen synthase kinase 3β, resulting in enhanced mucosal healing, intestinal stem cell proliferation and decreased enterocyte apoptosis (95). In seeking to determine the particular component in breast milk that mediated the protective effects, when EGF was either removed or its receptor, EGFR was inhibited, the protection on experimental NEC and TLR4 signaling was abolished (95). Taken together, these mechanistic studies describe one of the ways breast milk protects the intestinal epithelium against NEC.

Epidermal Growth Factor

Several other studies have looked at the protective role of EGF itself against NEC. In a neonatal rat model of NEC, administration of

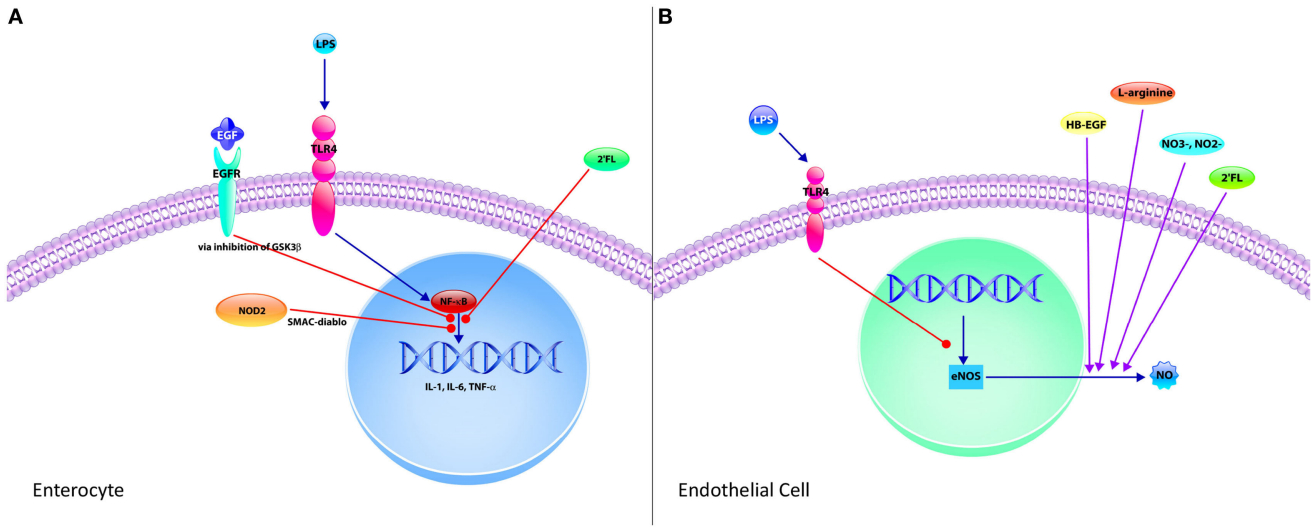

FIGURE 2 | Mechanisms of protective strategies in necrotizing enterocolitis (NEC). **(A)** The enterocyte is the predominant cell type of the epithelium and significantly contributes to the exaggerated pro-inflammatory response observed in NEC upon activation of toll-like receptor 4 (TLR4) by Gram-negative bacteria lipopolysaccharide (LPS). TLR4-mediated activation of nuclear factor-κB (NF-κB) results in increased expression of pro-inflammatory cytokines, such as IL-1, IL-6, and TNF-α. However, this signaling pathway can be attenuated through various mechanisms. Epidermal growth factor (EGF), which is found in breast milk and amniotic fluid, inhibits this pathway *via* inhibition of glycogen synthase kinase-3 beta (GSK3β). The human milk oligosaccharide 2′-fucosyllactose (2′FL) also attenuates the TLR4 pro-inflammatory signaling pathway. Finally, intracellular pattern recognition receptor nucleotide-binding oligomerization domain-containing protein 2 upon stimulation by pathogen associated molecular patterns *via* second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI (SMAC-diablo). **(B)** Endothelial cells have an integral role in the pathogenesis of NEC. Endothelial cells express TLR4, and upon stimulation by LPS following gut barrier dysfunction, decrease the expression of endothelial nitric oxide synthase (eNOS), thereby reducing the formation of nitric oxide (NO), an important vasodilator. This results in intestinal ischemia and subsequent NEC. However, several protective strategies increase the production of NO, including heparin-binding EGF-like growth factor (HB-EGF) and 2′FL by increasing expression of eNOS. Nitrates (NO_3^-) and nitrites (NO_2^-) can be found in breast milk and are precursors to NO, thus increasing NO production. Finally, L-arginine is required for NOS-dependent formation of NO. Supplementation with HB-EGF, nitrates, nitrites, and L-arginine are protective in NEC with their ability to improve intestinal blood supply *via* NO-mediated vasodilation.

EGF alone reduced both the incidence and severity of the disease (131). EGF administration resulted in significantly decreased epithelial permeability, normalized expression of tight junction proteins, increased goblet cells (28), and inhibited enterocyte apoptosis (132), all of which are protective hallmarks in NEC (26, 59, 95). Moreover, another mechanism by which EGF provides protection against NEC is inhibition of autophagy (133), a lysosomal pathway of self-digestion that has been shown to be activated in mice and infants with NEC (105). Additionally, a recent study demonstrates that a hypomorphic variant in an autophagy-related gene, ATG16L1 is associated with NEC in premature infants (108). This further underlies the importance of determining which infants are the most susceptible to NEC, so that protective strategies including EGF administration may be tailored to those individuals.

Amniotic Fluid

As discussed above, EGF administration mediates several protective effects on the intestine and is found in breast milk as well as amniotic fluid. We demonstrated that amniotic fluid inhibited TLR4-mediated inflammatory signaling in the fetal and neonatal intestinal epithelium in a manner dependent on EGFR (94). Intestinal EGFR expression was low in premature infants with NEC compared to that of a fetus or at the time of reanastamosis after NEC had resolved (94). The protective effects of amniotic fluid have been described in experimental NEC models in several species including neonatal mice (94), rats (134), and piglets (135). Other investigators have examined amniotic fluid outside the setting of EGF in the study of NEC. In a neonatal rat model of NEC, administration of amniotic fluid stem cells attenuated NEC by increasing enterocyte proliferation and decreasing apoptosis in a cyclooxygenase 2 dependent mechanism (136). In other studies, the administration of amniotic fluid resulted in differences in gut microbiota and reduced intestinal permeability compared to controls (137). Taken together, the ingredients in both breast milk and amniotic fluid provide important protective mechanisms to counter-regulate the detrimental pro-inflammatory effects in the intestine afflicted by NEC.

Heparin-Binding EGF-Like Growth Factor (HB-EGF)

Heparin-binding EGF-like growth factor is found in breast milk and amniotic fluid and provide numerous protective effects in models of intestinal injury and NEC (138). In mice, overexpression of the HB-EGF gene is protective against the development of NEC (139), whereas deletion of HB-EGF increases susceptibility to experimental NEC (140). As described previously, an important component in the pathogenesis of NEC is the disruption of gut perfusion and decreased expression of the vasodilatory molecule eNOS (100). In neonatal mice subjected to experimental NEC,

impairment of intestinal microvascular blood flow was improved, and there was decreased epithelial injury with administration of HB-EGF (141). Moreover, HB-EGF promotes angiogenesis with upregulation of eNOS and subsequent production of nitric oxide *via* the PI3K pathway (142, 143).

Heparin-binding EGF-like growth factor is also unique in its ability to modulate the intestinal immune system. For example, there are subtypes of macrophages, which can either be pro-inflammatory (M1) or anti-inflammatory (M2) (144). Macrophage infiltration in NEC was marked by a predominance of M1 pro-inflammatory macrophages and treatment with HB-EGF resulted in increased M2 anti-inflammatory macrophages, thereby protecting against experimental NEC. Moreover, HB-EGF also helps in maintaining gut barrier integrity with increased enterocyte proliferation (145) and migration (146) as well as decreased apoptosis of enterocytes (147), which is a common theme among the potential protective strategies in NEC.

Platelet-Activating Factor Acetylhydrolase (PAF-AH)

Platelet-activating factor is a potent phospholipid inflammatory mediator involved in the pathogenesis of NEC in human and animal studies (148, 149). Activation results in epithelial cell damage, increased apoptosis, increased mucosal permeability, disruption of tight junctions, leukocyte and platelet aggregation, and vasoconstriction, disrupting mucosal integrity (148, 149). Importantly, PAF-AH is capable of catabolizing PAF and decreases the destructive properties of PAF in the intestine (150). Additionally, breast milk contains PAF-AH, which is one component in breast milk thought to provide protection against the development of NEC (150, 151). The clinical relevance of PAF is demonstrated by the findings that compared to controls, infants with NEC display increased plasma and stool PAF concentrations and decreased plasma PAF-AH, which is responsible for PAF breakdown (152–154). In neonatal mouse and rat models, NEC susceptibility decreased with inhibition of PAF and increased with PAF-AH depletion (155–157). Of note, PAF was found to induce TLR expression in the intestine, providing a connection between two pathways involved in NEC pathogenesis, thereby suggesting luminal PAF in the preterm intestine may upregulate TLR expression and subsequently promote a pro-inflammatory state (158). Taken together, PAF-AH may serve as an important target by inhibiting intestinal inflammation and epithelial disruption.

Human Milk Oligosaccharides

Human milk oligosaccharides are carbohydrates recently heralded as protective components within breast milk. There are several means by which HMOs are protective in the setting of NEC. We recently demonstrated that 2′-fucosyllactose (2′FL) is an abundant HMO that is protective against NEC in mice *via* modulation of the vasodilatory molecule, eNOS expression and subsequently enhancing intestinal perfusion (101), which has been previously shown to be impaired in NEC (100). Moreover, 2′FL is capable of attenuating inflammation, and we demonstrated that 2′FL decreased the expression of several pro-inflammatory markers including IL-6, IL-1β, inducible nitric oxide synthase, and TLR4 (101). Importantly, HMOs have been shown to influence bacterial colonization in the intestine during the critical neonatal period in several studies (159–165). In another study, the HMO 2′FL inhibited the release of IL-8 by IECs in the setting of bacterial infection by attenuating the expression of CD14 (166). However, this effect was not observed in IECs that were not exposed to the bacterial pathogen, showing that HMOs are capable of modulating specific inflammatory pathways during infection (166). Another HMO, disialyllacto-N-tetraose, was capable of reducing the NEC severity in neonatal rats (167). Taken together, these studies advance our understanding of the effects of HMOs in preventing against NEC and may provide a nutritional preventative strategy worth pursuing.

The breast milk composition of HMOs varies among mothers, which in turn influences the microbiota of their offspring. Specifically, mothers with inactive alleles of the gene *fucosyltransferase 2* (FUT2) are referred to as non-secretor mothers (168), and this mutation leads to changes of the microbiota of their infants. Specifically, infants in this setting had delayed establishment of the genus *Bifidobacterium*, which are early colonizers in breastfed infants (168). This suggests HMOs can enrich certain beneficial populations of bacteria. Moreover, since the individual composition of breast milk varies widely, attention to these details may provide a novel strategy of personalized breast milk fortification to prevent NEC in preterm infants.

Nitric Oxide

One of the hallmarks of NEC pathogenesis is the disruption of the microcirculatory perfusion to the gut, which is regulated by endothelial expression of TLR4 (100). Specifically, endothelial TLR4 signaling impairs intestinal perfusion and decreases eNOS expression and accordingly nitric oxide (NO), such that mice with selective endothelial TLR4 deletion exhibited preserved mucosal integrity and decreased intestinal ischemia (100). In this context, TLR4 signaling promotes bacterial translocation with the disruption of the epithelial barrier, intensified by impaired intestinal perfusion by decreased expression of eNOS with the activation of endothelial TLR4.

Human breast milk contains sodium nitrate, which is a precursor to nitrite and NO production, a key vasodilatory molecule involved in maintaining intestinal perfusion (100). Interestingly, the concentration of sodium nitrate is higher in breast milk as compared to formula (100), and when compared to adults, infants in the NICU receive significantly less dietary nitrites and nitrates (169). Bacterial conversion of nitrates to nitrites first occurs in saliva, which can then be converted to nitric oxide *via* both NOS-independent mechanisms in the stomach and NOS-dependent mechanisms in the intestine from the amino acid L-arginine (170). This illustrates the interaction of bacteria and the gastrointestinal system and also highlights the importance of having dietary nitrates and nitrites in the form of breast milk during the critical neonatal period. This further suggests there is the opportunity to improve the deficits in dietary nitrites and nitrates inherent to neonates to prevent NEC. Moreover, studies have shown that enteral supplementation of L-arginine was not only safe but was able to reduce incidence and severity of NEC in very low birth weight preterm infants (171, 172). Accordingly, L-arginine, nitrates, and nitrites in the neonatal period are not

only important but given their ability to modulate the microcirculation of the preterm intestine, they have utility as a preventative strategy for NEC.

Lactoferrin

Lactoferrin is found in secretory fluids including breast milk and colostrum, where it acts an important member of mucosal immunity with its antimicrobial properties (173), ability to modulate the gut microbiota (174), and maturation of the intestine by inducing enterocyte growth and proliferation (175). Accordingly, the use of lactoferrin supplementation in preterm infants to prevent complications has been studied. The most recent Cochrane Review suggests that there is utility in the administration of oral lactoferrin prophylaxis due to the decrease in the development of NEC and late-onset sepsis without adverse effects (176–180). Another study evaluated the use of recombinant human lactoferrin (talactoferrin or TLf) in infants with a birth weight of 750–1,500 mg as means of reducing infection (181). There was no associated toxicity in the administration of TLf, and there was a trend toward less infectious morbidity in infants treated with TLf (181). Thus, lactoferrin administration in low birth weight infants is a promising preventative strategy against NEC with no adverse effects reported thus far.

Probiotics

Probiotics have generated significant interest in the prevention of NEC given the increasing data regarding the relationship between the gut microbiota and disease development. Prophylactic enteral probiotics show significant promise in the prevention of NEC. A Cochrane Review concluded probiotics in the setting of prematurity prevent severe NEC and decrease all-cause mortality (182). A more recent systematic review and meta-analysis utilizing additional studies supported these findings (183). As probiotics continue to be investigated, it is important to recognize there is significant heterogeneity with organisms chosen and dosing regimens across the studies. *Bifidobacterium lactis* has been studied in isolation in clinical studies. In a prospective randomized case–control study, administration of *B. lactis*-supplemented formula resulted in decreased intestinal permeability as measured by a sugar absorption test, suggesting *B. lactis* is important in maintaining epithelial integrity (184). In another clinical study, *B. lactis* Bb12 supplementation influenced the gut microbiota such that supplementation increased the number of *Bifidobacterium* spp. and reduced the number of *Enterobacteriaceae* and *Clostridium* spp., which may be implicated in NEC pathogenesis, in stool samples of preterm infants (185). It is important to note that the largest clinical trial for a specific probiotic, *Bifidobacterium breve* BBG-001, found no benefit in the administration of this probiotic in preterm infants to prevent NEC (186). More clinical studies evaluating the utility of specific organisms within probiotic regimens are necessary.

Moreover, there are several mechanisms by which probiotics can prevent the development of NEC (187). Two studies highlight our present knowledge of NEC pathogenesis as related to mucosal immunology. *Bifidobacterium adolescentis* is able to alter TLR4, TOLLIP, and SIGIRR expression in preterm neonatal rats, suggesting it is able to downregulate the TLR4-mediated pro-inflammatory response observed in NEC (188). Oral administration of *Lactobacillus rhamnosus* HN001 attenuated NEC severity in both premature piglets and newborn mice and the mechanism mediating this effect was *via* TLR9 activation (189). *Lactobacillus reuteri* in neonatal mice was able to increase enterocyte migration, enterocyte proliferation, and crypt height of the epithelium. This counters the impairment in enterocyte migration and proliferation that serve as hallmarks of NEC-mediated gut barrier dysfunction (190). These studies suggest probiotics are able to interact with the mucosal immunity of the gastrointestinal tract, thereby offering a viable preventative strategy in clinical NEC management.

Antenatal Steroids

Corticosteroids are often administered in the antenatal period to reduce mortality and morbidities associated with prematurity. Based on a Cochrane Review, the use of a single course of antenatal corticosteroids is recommended for all women at risk for preterm birth (191). In addition to accelerating fetal lung maturation, antenatal corticosteroids are associated with decreased likelihood for the development of necrotizing enterocolitis analyzing 8 studies with 1,675 infants total (191). A more recent clinical study supports these findings, in which there were significant differences between infants that received full course antenatal steroids and infants that did not, such that prompt administration of a full course of antenatal corticosteroids decreased the incidence of NEC and mortality (192). Presently, it is unknown the mechanism by which corticosteroids are protective in NEC. However, with our knowledge of the primary role of immune cells in the pro-inflammatory response in NEC, corticosteroids serve as an interest of study with their ability to modulate the immune system.

Umbilical Cord Clamping

There is debate on the optimal interval of time between infant delivery and umbilical cord clamping in the setting of prematurity. Delayed cord clamping provides more placental transfusion between the placenta and the newborn, whereas immediate cord clamping allows for immediate resuscitation of the premature newborn by a neonatologist (193). A Cochrane Review found that delayed cord clamping decreased the incidence of NEC (193); however, larger studies are necessary to evaluate this finding as well as the long-term effects of immediate vs. delayed cord clamping. In a more recent study, umbilical cord milking, which utilizes the same principle behind delayed umbilical cord milking also conferred protection against the development of NEC in preterm infants (194). The mechanisms by which umbilical cord clamping and umbilical cord milking provide protection against NEC are incompletely understood and warrant further investigation.

Immunoglobulins

The roles of B cells and immunoglobulins in the setting of NEC are not fully understood in the context of disease development and preventative strategies. A trial of oral immunoglobulins administration has been tried for their presumed immunoprotective effects as prophylaxis in preterm infants (195). However, based on a recent Cochrane Review of three randomized trials, there

is no protective role against NEC with the oral administration of immunoglobulins in the neonatal period, specifically with IgG or a combination of IgG/IgA, as these studies did not demonstrate a significant reduction in NEC incidence (195–198). A trial of oral IgA alone powered for the prevention of NEC in low birth weight neonates would be beneficial given the known beneficial properties of sIgA in breast milk (195). Nonetheless, more studies are necessary to appreciate the role of IgA in NEC pathogenesis and as a viable prophylactic or treatment strategy.

CONCLUSION

Necrotizing enterocolitis is a devastating disease of prematurity and recent evidence demonstrates incredible promise for prevention with continuous advancements in the field. These advancements are not limited to mucosal immunology as recent attention to the interplay of intestinal immune system, which includes epithelial integrity, immune cell composition, TLR4 signaling, intestinal microbiota, has yielded expansion of our understanding of the disease and how to best prevent its development. One of the most important themes of this review is the dynamic interaction of the components that comprise the mucosal immunity of the intestine. Several aspects are integral to maintain intestinal homeostasis, and there is no one component singularly responsible for the development of NEC. Rather, it is an intricate balance of many variables, which yield numerous therapeutic potentials in treating and preventing this disease. The goal of this review was to highlight these recent innovations and mechanistic insights into how administration of various treatments impacts the intestinal immune system and may alleviate NEC in premature infants.

AUTHOR CONTRIBUTIONS

All authors listed have made substantial, direct, and intellectual contribution to the work and approved it for publication.

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Age-Appropriate Functions and Dysfunctions of the Neonatal Neutrophil

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Neonatal and adult neutrophils are distinctly different from one another due to well-defined and documented deficiencies in neonatal cells, including impaired functions, reduced concentrations of microbicidal proteins and enzymes necessary for pathogen destruction, and variances in cell surface receptors. Neutrophil maturation is clearly demonstrated throughout pregnancy from the earliest hematopoietic precursors in the yolk sac to the well-developed myeloid progenitor cells in the bone marrow around the seventh month of gestation. Notable deficiencies of neonatal neutrophils are generally correlated with gestational age and clinical condition, so that the least functional neutrophils are found in the youngest, sickest neonates. Interruption of normal gestation secondary to preterm birth exposes these shortcomings and places the neonate at an exceptionally high rate of infection and sepsis-related mortality. Because the fetus develops in a sterile environment, neonatal adaptive immune responses are deficient from lack of antigen exposure *in utero*. Newborns must therefore rely on innate immunity to protect against early infection. Neutrophils are a vital component of innate immunity since they are the first cells to respond to and defend against bacterial, viral, and fungal infections. However, notable phenotypic and functional disparities exist between neonatal and adult cells. Below is review of neutrophil ontogeny, as well as a discussion regarding known differences between preterm and term neonatal and adult neutrophils with respect to cell membrane receptors and functions. Our analysis will also explain how these variations decrease with postnatal age.

Keywords: neonates, neutrophils, granulopoiesis, innate immunity, phagocytosis, chemotaxis, neutrophil extracellular traps, transmigration

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Abbreviations: AP, adaptor protein complex; BPI, bactericidal/permeability-increasing protein; CGD, chronic granulomatous disease; CL, chemiluminescence; EOS, early-onset sepsis; fMLP, *N*-formylmethionine-leucyl-phenylalanine; GA, gestational age; GBS, group B *Streptococcus*; GGA, Golgi-localized γ-adaptin ear homology ARF-binding protein; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; HIF, hypoxia-inducible factor; HSC, hematopoietic stem cell; ICAM, intercellular adhesion molecule; IVIG, intravenous immunoglobulin; LAD, leukocyte adhesion deficiency; LPS, lipopolysaccharide; LFA-1, lymphocyte function-associated antigen-1; NET, neutrophil extracellular trap; nNIF, NET-inhibitory factors; NBT, nitroblue tetrazolium; NRP, nHIF-related peptides; PMA, phorbol myristate acetate; RAGE, receptor for advanced glycation endproducts; ROS, reactive oxygen species; SGA, small for gestational age; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; TLR, toll-like receptor; VCAM-1, vascular cell adhesion molecule 1.

INTRODUCTION

The creation of life through human pregnancy is an astonishing achievement of nature, by which the allogeneic fetus is protected from maternal rejection through placental separation of fetal and maternal vascular systems as well as immunosuppression resulting from high levels of maternal progesterone and placental production of glucocorticoids (1). Intriguingly, this protection ensues even if the fetus is genetically diverse from its carrier, as observed with surrogate pregnancies, indicating a similarly tolerant fetus. Although suppressed, maternal immunity provides primary protection to the developing fetus against *in utero* infections and helps safeguard the newborn during the first year of life. This protection is achieved not only through placental transport of maternal immunoglobulins during the last trimester of pregnancy (2) but also by the newborn's consumption of breast milk, rich in antimicrobial proteins, immunoglobulins, and beneficial oligosaccharides (3). If maternal defenses are breeched by pathogens, resulting in chorioamnionitis or neonatal infection, a detrimental inflammatory cascade may be initiated in the neonate with the potential for devastating long-term neurodevelopmental sequelae (4, 5) and/or perturbations in the normal development of the immune system (6).

Because the fetus develops in a sterile milieu, neonatal adaptive immune responses are naïve from lack of antigen exposure *in utero*. Newborns must, therefore, rely on innate immunity to protect against early infection (7–9). Neutrophils are a vital component of innate immunity because they are the first circulating immune cells to respond to and defend against bacterial, viral, and fungal infections. However, notable phenotypic and functional disparities exist between neonatal and adult cells. The severity of these impairments is inversely related to gestational age (GA), revealing progressive neutrophil maturation throughout pregnancy, from the earliest hematopoietic precursors in the yolk sac to the well-developed myeloid progenitor cells in the bone marrow around the seventh month of pregnancy. These deficiencies place our most vulnerable patients at risk for infection and sepsis-related mortality.

Environmental factors also directly impact neutrophil phenotype and function, and differ considerably between the maturing fetus and adult. The intrauterine environment is exceedingly hypoxic with oxygen concentrations measured in the range of 1–5%, compared to 21% in the Earth's atmosphere. This low oxygen content necessitates cellular suppression mechanisms to counteract hypoxia-inducible factor 1α (HIF-1α)-mediated pro-inflammatory gene expression. Similarly, immune tolerance is vital during and after parturition, when the neonate is newly exposed to trillions of microorganisms that will become important components of its healthy microbiome.

For more than 50 years, scientists have been striving to understand the intrinsic mechanisms underpinning the normal transition of the suppressed *in utero* neutrophil into the fully functional postpartum cell capable of combating pathogenic organisms. This quest is even more urgent for extremely premature neonates, who are born at the limits of viability, and join the world before the immune developmental program is properly executed. As a consequence, these vulnerable neonates experience a profound compromise of both innate and adaptive immune responses. In this review, we explore differences between neonatal and adult neutrophils, describe neutrophil maturation throughout pregnancy, and highlight therapies trialed in neonates to enhance neutrophil function.

DEVELOPMENT

Hematopoiesis

Fetal hematopoiesis, or the creation of all blood cells, is an evolutionarily conserved process that originates in the extra-embryonic yolk sac around the third week of embryogenesis and gives rise to a transient population of primeval erythroid cells, macrophages, and megakaryocytes (10, 11). Around the seventh to eighth week of gestation, genuine hematopoietic stem cells (HSCs) are derived from specialized intra-embryonic endothelial cells located in the ventral wall of the descending aorta (12–14). These self-renewing primitive HSCs, with increased proliferation potential (15), will seed the liver, thymus, and spleen, where hematopoiesis will continue until the seventh month of gestation (10, 16). After this time, hematopoiesis will transition to the bone marrow, such that by the end of term gestation, the bone marrow becomes the primary source of red cells, white cells, and platelets (17, 18).

Neutrophils first appear in the human clavicular marrow at 10–11 weeks post conception (19). By the end of the first trimester, neutrophil precursors are detected in the peripheral blood, while mature cells appear by 14–16 weeks of fetal development (20, 21). HSCs that generate neutrophils are situated in specialized niches in the trabecular regions of long bones near the endosteum, or the interface between the bone and bone marrow, in proximity to osteoblasts (22–24). To exit the bone marrow, neutrophils must traverse the bone marrow endothelium through tight-fitting pores by a process known as transcellular migration, whereby the cells pass through the cell bodies of the endothelium rather than through cell junctions (25, 26).

Neutrophils reside in three different groups, or pools, known as the proliferative, circulating, and marginating pools, with numbers in each influenced by the maturational development of the cell and the individual's state of health. A delicate balance between neutrophil maturation, bone marrow storage and release, intravascular margination, and migration into peripheral tissues is closely regulated by conventional dendritic cells through the controlled production of granulocyte colony-stimulating factor (G-CSF), CXCL1, CCL2, and CXCL10 (27).

Proliferative Bone Marrow Pool

The proliferative pool comprised mitotic neutrophil precursors, including myeloblasts, promyelocytes, and myelocytes, which maintain their ability to multiply in order to replenish neutrophil numbers (28, 29). In human adults, the proliferative pool is estimated to contain between 4 and 5×10^9 cells/kg bodyweight (30, 31). In term neonates, however, this pool is greatly diminished at only 10% of adult values, with more than two-thirds of their cells residing in an active cell cycle, resulting in substantial cell turnover (20, 32). The absolute neutrophil cell mass per gram body weight in term neonates is also considerably less, calculated

to be only one-fourth that of adult levels (33), while preterm infants <32 weeks of gestation exhibit even lower values (~20% adult numbers) (34) (**Table 1**).

Neutrophils are activated from the early phases of bloodstream or deep tissue infection, causing their numbers in circulation to rapidly rise (35). Hence, bone marrow reserves of mature neutrophils are rapidly depleted due to limited stores, necessitating the release of immature granulocytes (IGs), known as a "left shift," which is often used to assess a person's probability of serious infection or sepsis (36). Because newborns, particularly very low birth weight premature neonates, have an exceptionally limited ability to recruit or generate significant neutrophil numbers, they are more likely to develop neutropenia when confronted by a pathogenic challenge, thereby increasing sepsis-associated morbidity and mortality (16, 20, 37). By contrast, adults maintain a large number of quiescent neutrophil progenitors that can be rapidly recruited into the cell cycle during times of sepsis (16, 20, 37, 38), coupled to a sizable bone marrow reserve of near-mature and mature neutrophils that can be quickly mobilized in early inflammatory responses (20 times that found in circulation) (30). Ultimately, neutrophil numbers in term and preterm infants will rise over the first few weeks of life to achieve adult values by 4 weeks of age (34).

TABLE 1 | Variances between neonatal and adult neutrophils.

Variable	Preterm	Term	Matures	Comment
Neutrophil cell mass (per gram BW)	↓↓	↓	Yes	Adult levels achieved by 4 weeks of age (20, 34)
Storage pool	↓↓	↓	Yes	Reduced storage pools lead to increased risks for neutropenia if infection occurs postnatally (20, 37)
Number in circulation	↑/↑	↑	Yes	Increases noted for all gestational age (GA) infants in the first 24 h after birth. Quantities return to adult levels by 72 h of life. The highest levels are found in neonates <28 weeks of GA (64)
Number of IG in circulation	↑	↑	Unknown	Neutrophil composition approximates that of adults by 72 h of life (65, 90)
Granule protein levels				
BPI	↓↓	↓	Yes	(68)
Lactoferrin	↓↓	↓	Yes	(75)
Chemotaxis	↓	↓	No	Factors include reduced mobilization of intracellular calcium (88) and anomalies in cytoskeletal organization (89)
Rolling and firm adhesion				
L-selectin levels	↓↓	↓	Yes	(8, 93)
L-selectin shedding	↓↓	↓	Yes	(8, 93)
CR3	↓↓	↓	Yes	(38, 93)
Transmigration	↓↓	↓	Yes	Decreased secondarily to reduced levels of CR3 and diminished release of chemokines and cytokines from tissue neutrophils and macrophages (38, 93)
Neutrophil extracellular trap (NET) production	↓↓	↓	Yes	Neonatal neutrophils only produce NETs in a ROS-independent manner (152, 153, 157)

↑, *increased;* ↓, *decreased; IG, immature granulocyte; BPI, bactericidal permeability-increasing protein.*

Diminished Neutrophil Production and Neonatal Neutropenia

Neonates who are small for gestational age (SGA) at birth, or have a birthweight <10th percentile, also have higher rates of neutropenia (absolute neutrophil count of <1,000/mL) compared to non-SGA infants, with an incidence of 6 vs. 1%, respectively. SGA neutropenia usually persists for the first week of life and is associated with thrombocytopenia in more than 60% of neonates (39). Previous conclusions of a direct correlation between neutropenia and preeclampsia (40), or related placental deficiency, have since been disproven with regression models demonstrating no higher incidence of low neutrophil counts over and above that calculated for SGA alone (39, 41). SGA neutropenia most likely results from *in utero* growth restriction rather than high maternal blood pressure because the severity of neutropenia is directly correlated with the number of circulating nucleated red blood cells (39). Diminished neutrophil production, rather than accelerated neutrophil destruction or excessive margination, is considered the primary mechanism underlying this phenomenon, as a normal immature to total (I:T) neutrophil ratio is maintained (39). Additionally, findings of decreased neutrophil production from pluripotent hematopoietic progenitors, reduced concentrations of granulocyte-macrophage progenitors, diminished bone marrow neutrophil proliferative and storage pools, and absence of evidence for excessive margination have been found in experimental models (40). Impaired neutrophil production is therefore thought to result from (1) downregulation of neutrophil growth or transcription factors due to high concentrations of erythropoietin (42), (2) inadequate G-CSF production, or (3) a placental inhibitor of neutrophil production that has yet to be identified (39). Notably, neutropenic SGA infants have an increased probability of being diagnosed with late-onset sepsis, as well as a fourfold increased risk of developing necrotizing enterocolitis for reasons that remain unclear (39).

Intriguingly, extremely low birth weight (ELBW) infants, or those born less than 1,000 g at birth, experience the highest frequency of neutropenia of any other neonatal group without an identified cause (43). Unlike older gestational aged neonates, neutropenia in ELBW infants is usually not associated with sepsis (44). Therefore, ELBW infants with and without neutropenia experience similar mortality rates in the NICU (43).

Trials investigating the clinical use of recombinant G-CSF and granulocyte-macrophage colony-stimulating factor (GM-CSF) to increase neutrophil numbers in preterm infants have yielded disappointing results, granted that sample sizes have been small.

Although both drugs increased overall neutrophil numbers, no differences in mortality were observed by day 14 from the start of therapy in preterm infants with suspected or proven systemic infection who received concurrent antibiotic therapy (45, 46). The PROGRAMS trial, which specifically studied the prophylactic use of GM-CSF in SGA preterm infants in the first 5 days of life, also found no benefit in sepsis-free survival to day 14 from trial entry compared to the control group (47). In this study, GM-CSF was preferentially chosen because of its ability to illicit a T_H1 immune response, prime neutrophils and monocytes to enhance bactericidal activity, and stimulate proliferation of neutrophil progenitors. A subgroup analysis by the Cochrane Group, however, identified 97 preterm infants from three studies, who suffered from both neutropenia and systemic infection at time of enrollment and received either drug. Remarkably, this defined group experienced a significant reduction in mortality by day 14 [RR 0.34 (95% CI 0.12–0.92); NNT 6 (95% CI 3–33)] (46), signifying that further appropriately powered studies should be undertaken to determine efficacy in this specific patient population (48).

Granulopoiesis

Neutrophil development is defined by the formation of granules within the maturing cell, known as granulopoiesis. This process begins between the myelocyte and promyelocyte stages of development and proceeds over the subsequent 4–6 days to produce mature, segmented neutrophils (**Figure 1**) (49–51). Formation of neutrophil granules occurs in a continuum by a process known as "*targeting by timing*," whereby granule proteins are sequentially packaged as they are produced, so that azurophilic granules are synthesized in promyelocytes, specific granule proteins in myelocytes, and gelatinase granule proteins in metamyelocytes and band cells, after which granule formation concludes and secretory vesicles form (50, 52–54). Recent findings reveal that direct sorting of granule components is also imperative for proper neutrophil granule formation. This is exemplified by the proteoglycan serglycin, which is essential for the shuttling and packaging of α-defensin and elastase into azurophilic granules (55–57). Additionally, the discovery of adaptor protein complexes and the monomeric Golgi-localized γ-adaptin ear homology ARF (GGA)-binding protein have shown that together, these substances recognize, organize, and traffic granule proteins from the *trans*-Golgi network to their respective granule compartments based on complex co- and posttranslational processing similar to that of lysosomal sorting of other cell types (58, 59).

Neutrophil granules are exocytosed in the reverse order of their formation in a hierarchical fashion based on the function

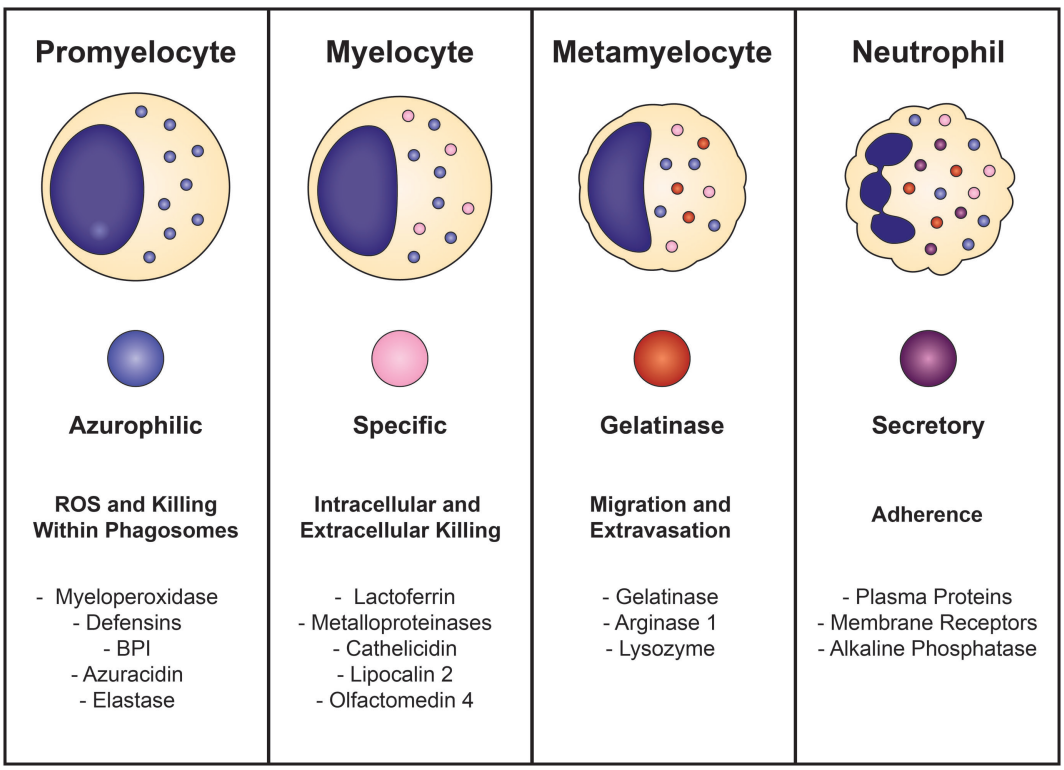

FIGURE 1 | **Neutrophil granulopoiesis**. Neutrophil maturation is defined by the sequential formation of three different granules and secretory vesicles, as well as nuclear segmentation. Granulopoiesis begins with the development of azurophilic granules in myeloblasts/promyelocytes and concludes after creation of secretory vesicles in mature, segmented cells. Azurophilic granules, which contain acidic hydrolases and microbicidal proteins, fuse with phagosomes to form highly toxic enclosures for oxidative reactions, necessary for pathogen destruction. Specific granules participate in both extracellular and intracellular microbial killing and are rich in antibiotic substances. Gelatinase granules are mobilized when the neutrophil establishes rolling contact with inflamed endothelium and contain matrix-degrading enzymes (gelatinase) and membrane receptors. Secretory vesicles are the first to be mobilized after minimal neutrophil stimulation and contain membrane-associated receptors that are key for chemotactic-directed migration and the establishment of firm contact with activated vascular endothelium.

of their contents and the magnitude of the stimulus (50, 51, 60). Thus, secretory vesicles are the first to be extruded after minimal cellular stimulation or activation. Although not considered a true neutrophil granule, secretory vesicles are mobilized in the earliest stages of neutrophil-mediated inflammatory responses and are vital reservoirs of membrane-associated receptors that allow the neutrophil to establish firm contact with activated vascular endothelium and transmigrate into inflamed tissues. Gelatinase granules follow and contain matrix-degrading enzymes (gelatinases) and membrane receptors that are important for extravasation into inflamed tissues during early inflammatory processes. Specific granules are mobilized next and release their antimicrobial contents extracellularly or discharge their substances into phagosomes. Phagosomes provide lethal enclosures for the intracellular killing of phagocytosed microorganisms through the creation of confined spaces that allow for exposure to high concentrations of microbicidal proteins and hydrolytic enzymes. Azurophilic granules are the last to be mobilized and are unique among the neutrophil granules because they can only degranulate their contents into phagosomes after activation by very powerful stimuli. Because azurophilic granules contain acidic hydrolases and microbicidal proteins that can be harmful to surrounding tissues if released extracellularly, close regulation is necessary. Recently identified variations in soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complexes explain these differences: whereas all neutrophil granules contain syntaxin 4 and SNARE complexes within their membranes, specific and gelatinase granules have SNARE complexes with high concentrations of VAMP-1, VAMP-2, and 23-kDa synaptosome-associated protein 23, while azurophilic granules have increased levels of VAMP-1 and VAMP-7 (61, 62).

Circulating and Marginating Neutrophil Pools

More mature neutrophils that reside outside of the proliferative pool (metamyelocytes, bands, and segmented cells) are found in equilibrium between the free flowing circulating pool and marginating pool (63). Remarkable fluctuations of the circulating pool occur in nearly all neonates after birth due to a surge in neutrophil numbers in the first 6–24 h of life to levels never again encountered in one's lifetime while healthy (64). This rise occurs earlier in neonates ≥28 weeks of GA with peak levels of 25–28,000 cells/μL noted around 6–12 h of life, while in those <28 weeks of GA experience a more gradual but dramatic rise, with maximum numbers of up to 40,000 cells/μL being achieved around 24 h of life (64). Irrespective of GA at birth, all newborns will subsequently undergo a gradual decline in neutrophil numbers over the next 72 h, with neutrophil composition and counts closely approximating that of adults by the third day of life (64).

Differences in neutrophil composition also exist, as term neonates have an increased number of IGs (promyelocytes, myelocytes, and metamyelocytes) when compared to adults (12 vs. 5%, respectively) (65). The higher quantity of IGs, which are deficient in vital early pro-inflammatory proteins and receptors due to incomplete or absent development of gelatinase granules or secretory vesicles, may increase a neonate's risk of infection after birth while simultaneously guarding against inappropriate inflammatory responses during the creation of its microbiome. Because neonates have a limited proliferative pool, neutrophils involved in this early surge are theorized to accrue from the marginating pool in response to birth-related stress hormones produced in the neonate, although the exact source and mechanism involved remain unknown.

FUNCTIONAL DIFFERENCES OF NEONATAL NEUTROPHILS

Microbicidal Proteins and Activity

Degranulation capabilities are similar between term neonatal and adult neutrophils, while those from preterm infants have considerable impairments in the release of bactericidal/permeability-increasing protein (BPI), elastase, and lactoferrin when compared to either term neonatal or adult cells (66, 67). Additionally, neutrophils from term healthy newborns and adults contain equal concentrations of the azurophilic granule proteins myeloperoxidase and defensin, while BPI is decreased threefold in unstimulated term neonatal neutrophils compared to adult controls (68). Interestingly, term infants with early-onset sepsis (EOS) experience a rise in plasma levels of BPI comparable to those of older children with sepsis syndrome (69) and adults with bacteremia (70) or pneumonia (67, 71). In laboratory studies using the stimulus phorbol myristate acetate (PMA), Nupponen and colleagues demonstrated that term and adult neutrophils generated similar concentrations of BPI, while its production remained significantly diminished in preterm neonatal cells. This finding suggests that BPI mobilization within the neutrophil exhibits an age-dependent maturational effect (67). BPI has a high affinity for the lipid A portion of lipopolysaccharide (LPS; the endotoxin of Gram-negative bacteria), thereby neutralizing its pro-inflammatory properties (68, 72). BPI also enhances phagocytosis of Gram-negative bacterium by acting as an opsonin (68, 73). These factors may explain why preterm infants deficient in BPI mobilization are more likely to become septic with Gram-negative bacteria, such as *Escherichia coli*, the leading cause of EOS in preterm infants. Additionally, the specific granule protein lactoferrin has direct bacteriostatic and bactericidal activities against viruses, Gram-positive bacteria, Gram-negative bacillis, and fungi (74). Lactoferrin measured in term neonatal neutrophils was half of adult concentrations, while preterm cells had even lower quantities (75).

Galectin-3, a S-type lectin receptor, is a non-traditional neutrophil membrane receptor with pro-inflammatory autocrine/paracrine effects on neutrophil phagocytosis, particularly of *Candida* species (76, 77). This receptor recognizes and binds to β-(1-2) oligomannan, thereby allowing the cell to distinguish between pathogenic and non-pathogenic fungi (76). Following its release extracellularly by activated or damaged neutrophils, galectin-3 binds to the neutrophil cell membrane. This binding results in the co-ligation of CD66a and CD66b, which leads to receptor clustering, integrin-mediated adhesion, and enhanced phagocytic capabilities (77). Galectin-3 has also been shown to increase reactive oxygen species (ROS) production, enhance

neutrophil degranulation, and inhibit apoptosis (77–79). Although contrasting data exist regarding serum and plasma levels in term neonates and adults, serum levels appear to be lower in preterm compared with term infants and rises throughout gestation (76, 80). Galectin-3 levels have also been demonstrated to be higher in neonates delivered vaginally compared to cesarean without labor, which may prime labor exposed neutrophils and render them more responsive to challenges with Gram-negative bacteria (79) or fungi (76, 80).

Chemotaxis and Migration

Neutrophils comprise the majority (60%) of leukocytes in humans (51) and are the "police force" of the immune system because they are the first immune cells to respond to and combat invading pathogens. During the earliest stages of infection or inflammation, chemoattractants derived from either the host (e.g., chemokines, cytokines, leukotrienes) and/or pathogen (e.g., LPS, fMLP) are released into the bloodstream, causing stimulation and activation of quiescent neutrophils. These agents also create a biochemical gradient that is sensed by specialized G protein-coupled receptors and induce intracellular signaling cascades that result in cell polarization, cytoskeletal rearrangement, and adhesion molecule clustering. These changes are necessary to enable the activated neutrophil to hone in and migrate toward the site of infection in the process known as chemotaxis (8, 81).

Neonatal neutrophils have similar chemotactic abilities, irrespective of GA (63, 82), but demonstrate reduced responsiveness when compared to adult cells (83–87). Though the number and affinity of cell surface receptors are comparable, deficiencies in neonates are attributed to reduced mobilization of intracellular calcium that result in aberrations in chemoattractant-induced signaling (88), as well as anomalies in microfilamentous cytoskeletal organization from delayed F-actin induction (89). IGs are also inept at chemotaxis and are found in higher numbers in neonates after birth as compared to adults (90, 91). In general, though, neutrophils from term infants achieved similar chemotactic abilities to adult cells by around 4 weeks of age. By contrast, deficiencies persisted in nearly half of preterm infants at 42 weeks of GA for reasons that remain unclear (84, 85), indicating that birth and extrauterine environmental factors do not fully correct the developmental program of maturation.

Once the stimulated neutrophils arrive at the site of infection, extravasation into the tissue requires four mechanisms: (1) capture and rolling, (2) firm adhesion, (3) crawling, and (4) diapedesis or transmigration (**Figure 2**). Initial contact between the neutrophil and vascular endothelium occurs during rolling and capture, which depends upon the interaction of L-selectin on the neutrophil and P- or E-selectin on the endothelium. Once contact is established, chemokines located on the inflamed endothelium bind to specific chemokine receptors on the neutrophil cell surface, thereby triggering a conformational change of the neutrophil, shedding of L-selectin (92), and induction of β_2 integrin expression, including lymphocyte function-associated antigen 1 (LFA-1) and CR3 (CD11b/CD18, $\alpha_M\beta_2$, MAC-1), by inside-out signaling (93). L-selectin is then shed from the neutrophil, and the β_2 integrins establish firm adhesion with the endothelium by binding to members of the immunoglobulin superfamily of

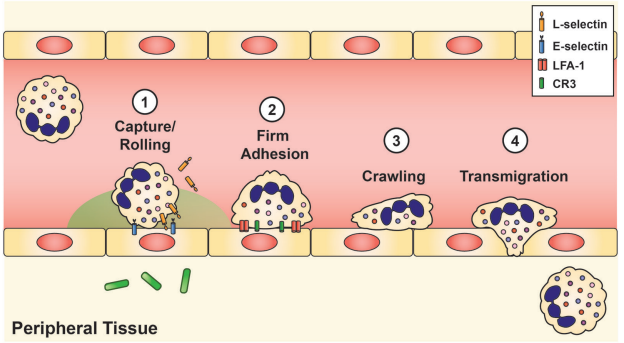

FIGURE 2 | Neutrophil recruitment and tissue extravasation. Chemoattractants derived from the host and/or invading pathogens activate quiescent neutrophils and provide a chemical gradient for stimulated neutrophils to hone onto and migrate toward the site of infection. Once at the inflamed site, initial contact between the neutrophil and vascular endothelium occurs during rolling and capture, which is facilitated by L-selectin on the neutrophil and E-selectin on the inflamed endothelium. This initial contact causes shedding of L-selectin and triggers the induction of lymphocyte function-associated antigen-1 and CR3, which establishes firm adhesion. Neutrophils then exit the vasculature by paracellular migration at the endothelial borders (70–90%) or *via* transcellular passage (not shown).

adhesion molecules, such as intercellular adhesion molecule 1 and 2 (ICAM-1 and ICAM-2), vascular cell adhesion molecule 1 (VCAM-1), and receptor for advanced glycation endproducts (8, 50, 60).

Neonatal neutrophils exhibit marked impairments in slow rolling and adhesion. One reason is reduced expression and shedding of L-selectin (94). This cell membrane receptor first appears on neonatal neutrophils around 21 weeks of fetal development (38, 95), and its concentration on the neutrophil cell surface increases in an age-dependent manner (38, 93). Although L-selectin shedding improves with fetal maturation, overall quantity and ease of release remains far below that of adult levels at term gestation, with substantial deficits noted in neonates <30 weeks of GA (8, 38, 93, 96).

Another reason for deficient slow rolling and adhesion in neonates is diminished upregulation of CR3 following chemotactic stimulation. Intriguingly, levels of CR3 in mid-gestational aged neonates are similar to those of patients with leukocyte adhesion deficiency type 1 syndrome (38, 93). When cytosolic and cell membrane measurements of CR3 stores were analyzed, investigators found that neonates had only 10% of adult levels at 27 weeks GA, increasing to 48% at 36 weeks GA, and then to 57 ± 4% at term (38). Of note, adult levels of CR3 were not attained by neonatal neutrophils until 11 months of age (38). Conversely, the appearance of LFA-1 in the neutrophil cell membrane is not dependent on maturation, with equivalent abundance in adults and neonates, regardless of GA (8, 38, 97) (**Table 1**).

The ability of the neonatal vascular endothelium to upregulate its expression of adhesion molecules following exposure to LPS is also greatly reduced in an age-dependent manner (93, 98). Neonatal neutrophils also have impairments of transmigration through the vascular endothelium due to decreased quantities of CR3 and diminished release of chemokines and cytokines from

tissue neutrophils and macrophages (47, 99). Thus, only about half as many term neonatal neutrophils transmigrate through the vascular endothelium in response to infection or inflammation when compared to adult cells (38).

Infant health has also been shown to affect chemotaxis. Remarkably, neonates who are ventilated for severe respiratory distress syndrome (but otherwise healthy) (84) and those with superficial infections (100) had neutrophils that exhibit enhanced chemotactic abilities when compared to those of stable preterm and term infants (84). Conversely, septic infants with Gram-negative bacteremia had poorly chemotactic neutrophils (82, 100). Likewise, intrapartum exposure to magnesium sulfate reduced neonatal neutrophil chemotactic abilities with impairment directly correlated with maternal serum magnesium levels (101), and insufficiencies related to antenatal betamethasone exposure were attributed to reductions in endothelial expression of adhesion molecules, including E-selectin, ICAM-1, and VCAM-1 (102).

Phagocytosis

Neutrophils generally exit the vasculature by paracellular migration at the endothelial borders (70–90%), but a few will travel through the endothelial cell *via* a transcellular passage (103). Once within the tissue, oxygen deprivation created by the pathological conditions of the infection drives HIF-dependent activation of prosurvival pathways in the neutrophil (104, 105) to enhance its bactericidal activity (106). Neutrophils then continue to migrate along the chemotactic gradient toward the site of microbial invasion. When encountered, neutrophils engulf the pathogens using receptors for complement and the Fc domain of immunoglobulins, including CR1, CR3, FcγRII, and FcγIII (38). While the number of CR1 (107) and both Fcγ receptors (108) are the same between term neonates and adults, CR3 receptors are decreased as previously discussed (38, 109). Conversely, preterm infants at birth express 80–88% of adult levels of FcγRII but only 50% of FcγIII (109). FcγIII levels, however, rise rapidly postnatally to reach adult levels by 2 weeks of life. Together, these four receptors mediate binding, ingestion, and killing of bacteria (38) (**Table 2**).

Neonatal neutrophils from term, healthy infants opsonize and ingest both Gram-negative (91, 110) and Gram-positive bacteria with equivalent efficiency to adult cells (111). By contrast, phagocytosis was less proficient in preterm infants <33 weeks of GA, who experienced both slower uptake and ingestion of bacteria compared to term neonates and adults. Curiously, these impairments persisted at 1–2 months of age, despite no apparent maturational defect (112). These deficits are believed to result from low circulating levels of opsonization factors, particularly maternal immunoglobulins that are actively transported across the placenta in the last trimester of pregnancy. Supportive evidence comes from the observation that administration of intravenous immunoglobulins (IVIG) normalizes phagocytosis capacity in preterm infants <32 weeks of GA (113). The use of IVIG in the treatment of neonatal sepsis, however, failed to reduce either (1) mortality during the hospital stay or (2) death or major disability at 2 years of age in infants with suspected or proven sepsis in a large cohort of neonates (114, 115). In the face of infective or non-infective clinical stress, the ability of neonatal neutrophils to phagocytose Gram-positive bacteria remained intact, but was impaired for Gram-negative bacteria (116).

Conversely, phagocytosis of *Candida* not only depends upon the size and form of the pathogen (i.e., hyphae or yeast) but also upon the species (76, 117). Destin and coworkers concluded that neutrophils from preterm and term infants as well as adult controls all failed to phagocytose unopsonized *Candida albicans* yeast, yet were similarly capable of phagocytosing unopsonized *Candida parapsilosis* (77, 117). Likewise, oxidative burst was equally robust when challenged with *C. albicans* hyphae in all groups, but non-existent against *C. parapsilosis* and attenuated against *C. albicans* yeast forms (117). Others have also demonstrated reduced phagocytosis and killing of *C. albicans* by preterm neonatal neutrophils due to deficiencies of opsonization factors (66, 118–120). Likewise, galectin-3, mentioned earlier in this review, is critical for the neutrophil's ability to recognize, engulf, and kill pathogenic *Candida* species. This finding is most likely due to the ability of galectin-3 to co-ligate neutrophil cell membrane receptors CD66a and

TABLE 2 | Similarities between neonatal and adult neutrophils.

Variable	Preterm	Term	Matures	Comment
Degranulation capabilities	↓	N	Yes	Only known for BPI, elastase, and lactoferrin (66, 67)
Granule protein levels				
Myeloperoxidase	N	N	No	(68)
Defensin		N	Unknown	(68)
Rolling and firm adhesion				
Lymphocyte function-associated antigen-1 levels	N	N	No	(8, 38, 97)
Phagocytosis	↓	N	Yes	Reduced in neonates with sepsis or non-infective clinical stress for some organisms (116)
CR1		N	Yes	(107)
FcγRII	↓	N	Yes	(108, 109)
FcγIII	↓	N	Yes	Adult levels in preterm infants reached by 2 weeks of age (108)
Respiratory burst	N/↑	N/↑	No	Decreased in stressed neonates or those with perinatal distress (134)
Chemiluminescence	N/↓	N/↑	No	Reduced in critically ill neonates and those challenged with large bacterial loads (139, 142)

N, normal; ↑, increased; ↓, decreased.

CD66b, resulting in cell receptor clustering and integrin-mediated adhesion (77). Galectin-3 also primes/activates the neutrophil, thereby enhancing ROS production, prolonging cell survival, and increasing degranulation of microbicidal proteins and substances (77).

Phagocytosis-Associated Respiratory Burst and Chemiluminescence (CL)

Microorganisms, engulfed by neutrophils, are trapped within phagosomes that fuse with azurophilic and specific granules to form phagolysosomes, which are small, confined spaces designed for toxic, oxidative reactions vital in destroying pathogens while protecting the host tissue against harmful metabolites (121, 122). The formation of phagolysosomes is associated with an increase in hexose monophosphate shunt metabolism of glucose and, in turn, a proportional rise in molecular oxygen consumption that is known as the respiratory burst (121). NADPH oxidase, localized on the membrane of the phagolysosome, is also activated by phagocytosis and is essential in driving the respiratory burst *via* the reduction of oxygen (O_2) to yield hydroxydioxylic acid (HO_2) and hydrogen peroxide (H_2O_2) (121, 123). Inactivating mutations in NADPH oxidase result in chronic granulomatous disease (CGD), which is characterized by recurrent bacterial and fungal infections, as well as granulomas that result from the neutrophils' inability to completely kill and eliminate pathogens (124). HO_2 and H_2O_2 are weakly bactericidal (125) and lead to considerable acidification of the phagolysosome (95, 121). Myeloperoxidase, also released from azurophilic granules, catalyzes oxidation reactions between H_2O_2 and chloride (Cl^-) to form hypochlorous acid (HOCl) (126), hydroxyl radicals (·OH), and chloramines, all of which are potent oxidants (127) that further contribute to the microbicidal capabilities of neutrophils (125). The resulting reactions between microorganisms and oxygenation radicals produce electronic excited products that cause light emission in the visible spectrum, known as CL (128).

The generation of O_2^- can be detected using the nitroblue tetrazolium (NBT) test (129), which remains negative (clear) in patients with CGD, but produces a positive (or blue) reaction in neutrophils from healthy term newborns. Neonatal neutrophils induce an intensely positive reaction that corresponds to enhanced oxygen consumption in the initial phase of the respiratory burst and normal or elevated production of H_2O_2 (130). The NBT is also more intense from cord blood neutrophils exposed to labor than those without, suggesting that parturition primed these cells for increased activity (131, 132). Quantitative differences in the kinetic activity of the neonatal NADPH oxidase system may explain variances in NBT results (131, 133). Comparable bactericidal activity has been demonstrated between healthy neonatal and adult neutrophils toward *Staphylococcus aureus*, *E. coli*, *Serratia marcescens*, *Pseudomonas* species, and groups A and B streptococci (127), while neutrophils from stressed preterm and term infants demonstrated significantly decreased bactericidal activity against both Gram-positive and Gram-negative bacteria (127). Neutrophils from neonates with perinatal distress also exhibited respiratory burst suppression (134–136). Even though the respiratory burst normalized to adult cellular function in preterm infants by 2 months of age, it remained depressed in ill infants receiving intensive care (137).

The interaction between ROS and microorganisms is the foundation of CL. In healthy, term neonates, CL is normal or enhanced for group B *Streptococcus* (GBS) and opsonized zymosan (111, 138) but is generally dampened in neutrophils from healthy preterm neonates <34 weeks of GA (111, 130, 134, 139). Moreover, critically ill preterm or term infants have significantly reduced CL (140), as do neutrophils challenged with large bacterial loads (141). Although neonatal deficiencies in CL persist throughout the course of serious infection, it tends to normalize to adult levels by 2 months after birth (142).

Neutrophil Extracellular Traps (NETs)

By extruding chromatin material loaded with antimicrobial molecules including citrullinated histones, elastase, myeloperoxidase, lactoferrin, and defensins extracellularly (143) through formation of NETs, neutrophils can entrap and kill bacteria, fungi, and protozoa (144). Neutrophils can produce NETs by two distinct pathways. The first is initiated in response to LPS, TNF-α, or IL-8 (145, 146) and requires activation of NADPH oxidase (147), ROS production (148, 149), and induction of the RIPK3–MLKL cascade (150, 151). Neonatal neutrophils from term and preterm infants fail to form NETs in this manner, even though they have the ability to generate endogenous ROS and have NADPH activity equivalent to adult cells (152). Hence, Lipp and coworkers demonstrated significantly less NET formation, with reduced NET area, from neonatal cord blood neutrophils compared to adult cells following stimulation with *N*-formylmethionine-leucyl-phenylalanine (fMLP), PMA, and LPS, with even lower numbers and NET area noted in preterm neonatal neutrophils (153). This may be due to NET-inhibitory factors (nNIF) or nHIF-related peptides, which appear to be unique to neonatal neutrophils and function as important regulators of fetal and neonatal inflammation (154).

Neonatal neutrophils, irrespective of GA at birth, produce NETs *via* the second, ROS-independent pathway after exposure to certain pathogens and following activation by the complement system (155, 156), TLR2, and/or fibronectin (150). Byrd and colleagues recently demonstrated that preterm and term infants as well as adult controls produced NETs equally well when exposed to fibronectin together with either purified β-glucan or *C. albicans* hyphae in a ROS-independent manner but did not form NETs when exposed to fibronectin or β-glucan independently (157).

NEUTROPHILS AND THE MICROBIOME

During parturition, the once naïve fetus passes through the vaginal canal where it is exposed to trillions of microorganisms that comprise the maternal microbiota. Neutrophil tolerance is imperative during this period to prevent the induction of pro-inflammatory reactions as the newborn is colonized with commensal microbes that harbor a variety of nucleic acids, proteins, and antigens. Once established, a symbiotic relationship is created between the host microbiota and neutrophils to ensure proper neutrophil function and numbers. Interventions that hinder the natural development of the newborn's microbiota, such as

cesarean delivery or exposure to intrapartum and/or postpartum antibiotics, may place the infant at an increased risk for late-onset sepsis, necrotizing enterocolitis, prolonged length of stay, and/or death (4, 158, 159). Recent discoveries by Deshmukh and colleagues help explain this association. Using a murine model, this group demonstrated that the pup microbiota induced IL-17 production by group 3 innate lymphoid cells in the intestine, which increased G-CSF and, hence, neutrophil production in a TLR-4- and MyD88-dependent manner (160). This interaction also increases the number of aged circulating neutrophils in adult mice, which possess enhanced $\alpha_M B_2$ integrin and are more proficient at NET production under inflammatory conditions (161). Similar studies on aging neutrophils, however, are lacking in neonatal models.

NEONATAL NEUTROPHILS AND IMMUNOLOGIC *QUID PRO QUO*

After birth, cord blood neutrophils appear "primed" because they demonstrate increased concentrations of pro-inflammatory chemokines and cytokines, as well as experience prolonged survival secondary to deficient programmed apoptosis (162). Shortages of crucial cell membrane receptors, diminished intracellular signaling, and impaired cellular functioning, however, can result in neutrophil dysfunction leading to increased susceptibility to sepsis, tissue damage, and shock. Well-documented examples of enhanced neutrophil inflammatory responses with associated inhibitory mechanisms are outlined below.

Unstimulated cord blood neutrophils from term, healthy neonates have higher concentrations of pro-inflammatory cytokines such as IL-1β, TNF-α, and IFN-γ compared to adult controls, irrespective of labor exposure (91). In addition, neonatal neutrophils experience heightened IL-1β expression following stimulation by TNF-α and LPS as compared to adult cells (163). When toll-like receptors (TLRs) 1–9 are directly stimulated, however, neonatal neutrophils exhibit a global decrease in the production of these T_H1-polarizing cytokines, which are vital in protecting the newborn against intracellular viral and bacterial infections. Instead, neonatal neutrophils secrete greater amounts of T_H2-polarizing cytokines, including IL-6 and IL-10, which are adapted to defend against parasitic infections but can also increase the newborn's future risk of developing atrophy, allergy, and asthma (9, 164, 165). The reasons for their decreased responsiveness to T_H1-mediated responses may include reduced intracellular mediators of TLR signaling (166) or increased levels of plasma adenosine (9).

It has been proposed that adenosine, by binding G protein-coupled A3 adenosine receptors (A3ARs), increases intracellular cAMP levels, thereby preserving generation of T_H2-polarizing cytokines, including IL-6, which have anti-inflammatory properties and can impede neutrophil migration to site of inflammation (9). It is known, however, that A3ARs couple to the G protein G_i, which inhibits adenylyl cyclase and lowers cellular cAMP levels (167). Thus, it is possible that alternate G protein coupling may occur in neutrophils or that adenosine may facilitate IL-6 generation through a different member of this receptor family. Although adenosine suppresses neutrophil cell membrane levels of CD11b in neonatal and adult neutrophils, their ability to phagocytose microorganisms remains unaffected. Unlike adult cells (168, 169), however, adenosine does not alter chemotaxis or production of ROS by neonatal neutrophils (170). Nonetheless, adenosine increases susceptibility to infection by intracellular pathogens through TH2-polaring pathways while facilitating colonization of commensal microorganisms after birth by limiting excessive inflammation.

As previously noted, cell membrane levels of galectin-3 are greatly increased in neonatal compared to adult neutrophils (80). Additionally, unstimulated neonatal cells also produced larger amounts of the potent chemoattractant IL-8 (171). Following labor exposure, both galectin-3 and IL-8 concentrations are notably higher in neonatal neutrophils, leading investigators to conclude that fetal neutrophils reside in a "pre-primed" state and become reactive following labor. When stimulated with LPS, however, no differences in neutrophil galectin-3 or IL-8 levels were found, whether or not the neonate was exposed to labor. Importantly, neonatal neutrophils also did not demonstrate improved L-selectin shedding (80). Yost and colleagues also showed similar lack of upregulation of pro-inflammatory mediators after TLR 1–9 stimulation but did demonstrate elevated IL-8 levels using the TLR1/2 heterodimer agonist, PAM_3CSK_4 (172). While not directly tested in neutrophils, TLR 1/2 activation by PAM_3CSK_4 in monocytes is associated with significantly increased production of IL-10 (173), thereby blunting pro-inflammatory T_H1 reactions in favor of T_H2 immune responses. Thus, elevated levels of IL-8 should result in the recruitment of additional activated neutrophils to area of inflammation or infection, amplifying the acute inflammatory response with the potential to propagate local tissue damage. Without the shedding of L-selectin, however, neutrophils experience impaired rolling, firm adhesion, and endothelial transmigration, limiting their accumulation in inflamed tissue. Nonetheless, this dysregulated local response may lead to tissue damage that could result in chronic lung disease or necrotizing enterocolitis, although more research is needed to investigate this association *in vivo* (169, 174).

Neonatal neutrophils also have exaggerated pro-inflammatory responses to the major cell component of Gram-positive bacteria, peptidoglycan. Exposure to peptidoglycan stimulates neonatal neutrophil expression of CD11b, TNF-α, and IL-8, which improves chemotaxis capabilities and increases ROS production. These actions are facilitated *via* heat shock proteins, including HSPA1A and OLR1 (175). Conversely, CR3 (CD11b/CD18, MAC-1), an essential pathogen recognition receptor for Gram-negative bacteria, can bind LPS, thereby enhancing neutrophil phagocytic capabilities of these microorganisms. At baseline, however, cell membrane quantities of CR3 are decreased in neonatal cells, particularly in preterm infants, which may impair their ability to detect Gram-negative pathogens. Furthermore, once activated, neonatal neutrophil CR3 levels remain reduced compared to adult cells, which not only inhibits neutrophil activation but may also limit their accumulation at sites of inflammation (97).

Group B *Streptococcus* remains the leading cause of neonatal EOS and can elicit variable immunologic responses in the host.

For example, GBS hemolysin and inflammasome components can trigger pro-IL-1β processing and IL-1β release by neonatal neutrophils, amplifying their recruitment to sites of infection (176). Alternatively, molecular mimicry by GBS capsular sialic acid and β protein attenuates innate immune responses by binding to inhibitory sialic acid-binding immunoglobulin-like lectin receptors (Siglecs) on neutrophils, triggering SHP-2 phosphatase-dependent signaling to impede neutrophil activation and phagocytic killing (177, 178). Finally, GBS can induce IL-10 production in murine pups compared to adult animals, which impairs neutrophil recruitment to inflamed tissues and reduces bacterial clearance. Attenuation of this response, however, is exhibited by TLR2-deficient murine pups, which experience improved survival by limiting GBS bacterial dissemination through enhanced GBS phagocytosis (179).

Anti-inflammatory neutrophil granular proteins, such as olfactomedin-4 located in specific granules, can also attenuate neutrophil bacterial killing and host innate immunity against Gram-negative and Gram-positive bacteria (180). Its expression is significantly upregulated in unstimulated cord blood neutrophils from healthy term newborns compared to adult cells (91) and levels are dramatically higher in septic neonates (181). OLFM-4 restricts neutrophil cathespsin c-mediated protease activity and Nod-like receptor-mediated NF-κB activation, thereby restricting antimicrobial killing (180). Elevated OLFM-4 expression is associated not only with decreased levels of IL-1β, IL-6, IL-12p40, CXCL2, G-CSF, and GM-CSF but also with greatly increased sepsis-related mortality (180, 182).

Finally, bacterial and host pro-inflammatory mediators can prolong neutrophil survival by delaying apoptosis. While this response is crucial for competent early innate immune responses that facilitate bacterial clearance, it may also promote excessive tissue injury resulting in poor neonatal outcomes (183). Allgaier and colleagues (184) have demonstrated that the overexpression of IL-1β and IL-8 cause activation of NF-κB and induction of anti-apoptotic genes. Additionally, neonatal neutrophils have diminished cellular expression of Siglec-9 and its downstream signaling protein SHP-1, an inhibitory tyrosine phosphate with proapoptotic functions (185). Inflammation resolution and tissue repair are generally facilitated by removal of toxic neutrophils through pathogen-induced programmed cell death *via* apoptosis with subsequent clearance by tissue macrophages and monocytes. The consequences of delayed neutrophil apoptosis and turnover are clearly demonstrated by Grigg and colleagues (186) who showed direct correlation with higher rates of chronic lung disease and pulmonary injury.

CONCLUSION

Annually, an estimated 400,000 neonates, or 1 in 10 newborns, are born prematurely in the United States (187, 188), and prematurity-related health issues account for an astonishing 36% of all infant deaths (189). Infectious disease is the second leading cause of neonatal mortality worldwide, preceded only by complications related to preterm birth (190). Technological advancements in ventilator management, thermoregulation, nutrition, and medical therapies (such as surfactant) have permitted survival of extremely preterm infants to the limits of viability, now considered 22–23 weeks of GA or slightly beyond the halfway point of normal gestation. Nature, however, did not intend fetal survival outside the womb at this young age as evidenced by the underdevelopment of all organ systems, which increases the neonate's risk for infection, intraventricular hemorrhage, retinopathy of prematurity, patent ductus arteriosus, necrotizing enterocolitis, chronic lung disease, and impaired neurodevelopmental outcomes. While adaptive responses are generally deficient, certain aspects of innate immunity may be absent, including the vernix that develops around 28 weeks' GA and the stratum corneum in the third trimester (191). As with other organ systems, postnatal neutrophil deficits are exacerbated in the most immature neonates, resulting in a 10-fold greater risk for early infection compared to term infants and 30% mortality rate in those infected (191–194).

Neonatal neutrophils have been extensively studied and are often inappropriately characterized as "dysfunctional" when compared to adult cells. Because considerable differences between fetal and adult physiology exist, phenotypic and functional variances are vital. Neonatal neutrophils have adapted and evolved over time to endure extremely hypoxic *in utero* environmental conditions without triggering HIF-1α-mediated pro-inflammatory responses (195–197). Furthermore, neutrophil suppression is essential for the naïve newborn to establish a healthy microbiome in the postpartum period, but detrimental if unable to mount sufficient pro-inflammatory reactions if exposed to pathogenic organisms.

The evolution of laboratory techniques over the last 50 years has enabled researchers to substantially expand our knowledge of neutrophil biology. Neutrophils are no longer viewed as short-lived, indiscriminate phagocytes of the immune system, but instead as essential components necessary for proper B and T cell function, antigen presentation, and tissue repair and regeneration. Differences in laboratory methods and variations in neonatal populations over time may make comparisons between past and present data difficult and yield potentially contrasting results. The rapid evolution of neonatal care, from its first appearance in the 1960s to the present, may also challenge current concepts of neonatal neutrophil biology as younger, sicker babies are resuscitated, pushing the limits of viability to even lower GAs. As scientists continuously strive to discover novel therapies to enhance neutrophil function during neonatal sepsis, efforts and resources must also be dedicated to unraveling the mysteries of neutrophil biology during fetal development, taking into account environmental and compositional influences.

AUTHOR CONTRIBUTIONS

SL researched and composed this review. RC created the figures and edited the final version of the review. VN helped compose and edited the final version of the manuscript.

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Rapid CD8+ Function Is Critical for Protection of Neonatal Mice from an Extracellular Bacterial Enteropathogen

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Both human and murine neonates are characteristically highly susceptible to bacterial infections. However, we recently discovered that neonatal mice are surprisingly highly resistant to oral infection with *Yersinia enterocolitica*. This resistance was linked with activation of both innate and adaptive responses, involving innate phagocytes, CD4+ cells, and B cells. We have now extended these studies and found that CD8+ cells also contribute importantly to neonatal protection from *Y. enterocolitica*. Strikingly, neonatal CD8+ cells in the mesenteric lymph nodes (MLN) are rapidly mobilized, increasing in proportion, number, and IFNγ production as early as 48 h post infection. This early activation appears to be critical for protection since B2m$^{-/-}$ neonates are significantly more susceptible than wt neonates to primary *Y. enterocolitica* infection. In the absence of CD8+ cells, *Y. enterocolitica* rapidly disseminated to peripheral tissues. Within 48 h of infection, both the spleens and livers of B2m$^{-/-}$, but not wt, neonates became heavily colonized, likely leading to their deaths from sepsis. In contrast to primary infection, CD8+ cells were dispensable for the generation of immunological memory protective against secondary infection. These results indicate that CD8+ cells in the neonatal MLN contribute importantly to protection against an extracellular bacterial enteropathogen but, notably, they appear to act during the early innate phase of the immune response.

Keywords: neonatal, enteropathogens, CD8+ T cells, immunity, innate, IFN-gamma

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INTRODUCTION

Neonates and infants are commonly highly susceptible to infectious diseases. Many of these diseases are caused by bacterial pathogens, which are largely naturally acquired by oral ingestion. Because of the many similarities in immunity in early life between mice and humans (1, 2), neonatal mice provide a reasonably faithful and experimentally convenient model system for studying infection with bacterial enteropathogens. Indeed, as in humans, mouse neonates are very sensitive to oral infection with a number of bacterial enteropathogens, including *Salmonella typhimurium* (3, 4), *Helicobacter pylori* (5–7), *Shigella flexneri* (8–10), *Vibrio cholera* (11, 12), and the Enteropathogenic *E. coli*-related *Citrobacter rodentium* (13–16). Often, these susceptibilities are linked to quantitative or qualitative

differences in neonatal and adult responses involving both the innate and adaptive gastrointestinal immune systems.

In contrast to all other descriptions of infections in neonatal mice, our laboratory found that 7-day-old murine neonates are highly resistant to orogastric infection with the extracellular enteropathogen *Yersinia enterocolitica* (17). *Y. enterocolitica* disseminates to the mesenteric lymph nodes (MLN), and analyses of immune function in that site revealed robust responses involving both the innate and adaptive arms of immunity (18, 19). Proinflammatory cytokine gene expression was highly induced, and innate phagocytes infiltrated the MLN in high numbers. Mature or supra-mature adaptive responses were also detected. We demonstrated that CD4 cell Th1 and Th17 function were both critical for protection of neonates and serum antibody responses of similar magnitude and avidity to those in adults were observed. Finally, and strikingly, neonates developed protective immunity against subsequent exposure as adults to a lethal dose of the bacterium.

These results indicated that protective responses against *Y. enterocolitica* in early life involve multiple innate and adaptive cell types. In these initial experiments, we largely ignored CD8 cells since these cells are more commonly associated with viral or intracellular bacterial infections. However, in the course of these studies, we noted that a large proportion of CD8 cells in the MLN of uninfected neonates expressed the proliferative antigen Ki67. Upon infection, $CD8^+$, but not $CD4^+$ cells, increased rapidly in proportion and IFNγ production. $B2m^{-/-}$ neonates were more susceptible to infection, compared with wt neonates, indicating that $CD8^+$ cells were required for survival of primary infection. The susceptibility of $B2m^{-/-}$ neonates was linked to the early dissemination of the bacteria to peripheral organs. Last, although required for primary infection, $CD8^+$ cells were dispensable for survival of secondary infection. These results indicate that neonatal $CD8^+$ cells may play an important early, innate-like role in survival to primary infection with *Y. enterocolitica* but they are not necessary for the development of protective memory in neonates.

MATERIALS AND METHODS

Mice

Adult C57BL/6 and B2m-deficient mice (B6.129P2-$B2m^{tm1\text{-}Unc}$/J) were purchased from Jackson Laboratories. All mice were bred and housed under barrier conditions in the Division of Veterinary Resources of the University of Miami Miller School of Medicine. Mice were regularly screened for specific common pathogens. Adult mice (6–10 weeks of age) and neonatal mice (7 days of age) were used in experiments. All mice were handled in compliance with the Institutional Animal Care and Use Committee (IACUC) of the University of Miami Miller School of Medicine, Miami, FL, USA.

Bacterial Infections

Wild-type high-virulence *Y. enterocolitica* A127/90 serotype 0:8 biotype IB was originally provided by G. R. Cornelis (Universität Basel, Basel, Switzerland). For infection, bacterial frozen stocks (17) were washed twice with Hank's Balanced Salt Solution (HBSS, Gibco, Grand Island, NY, USA), diluted to the desired concentration, and inoculated with the indicated doses. Adults were inoculated orogastrically with a 22-gauge, round-tipped feeding needle (Fine Science Tools, Foster City, CA, USA) attached to a 1-ml syringe (Becton Dickinson, Franklin Lakes, NJ, USA). Neonates were inoculated orogastrically with PE-10 tubing (polyethylene tubing with an outside diameter of 0.61 mm) (Clay Adams, Sparks, MD, USA) attached to a 30-gauge needle and Hamilton syringe (20). The actual administered dose was determined by plating serial dilutions of the suspensions on Luria Broth plates and incubating for 48 h at 27°C.

Cell Staining, Antibodies, and Flow Cytometry Analysis

Individual MLN from neonates and adults were harvested and placed in cold HBSS containing 1% calf serum (Gibco), 10 mM HEPES (Gibco), and 4 mM sodium azide. Cell suspensions were prepared by mincing tissues with scissors and pressing cells through wire mesh with 74 μm pore size (Compass Wire, Westville, NJ, USA). Cells were incubated in mouse Fc Block (CD16/CD32; BD Pharmingen, San Diego, CA, USA) for 5 min on ice, followed by a 30-min incubation with fluorochrome-conjugated antibodies specific for CD4, CD8, Ki67, or TCRαβ (BD Pharmingen). For intracellular cytokine staining, cells were activated with 50 ng/ml PMA and 0.5 μM ionomycin in the presence of 5 μg/ml brefeldin A, fixed and permeabilized, and stained with fluorochrome-conjugated anti-IFNγ (BD Pharmingen). Samples were run on a Becton Dickinson LSR II flow cytometer and analyzed with FlowJo flow cytometry analysis software.

Bacterial Enumeration from Organs of Infected Mice

To measure *Y. enterocolitica* titers, tissues were weighed and homogenized in HBSS using a Seward Biomaster 80 Stomacher (Brinkman, Westbury, NY, USA) for 4 min at high speed. For small intestine mucosa-associated *Y. enterocolitica* titers, the small intestine contents were flushed with HBSS prior to homogenization. Individual MLN were homogenized in 400 μl (neonates) or 500 μl (adults) of HBSS using a VWR disposable pellet mixer with cordless motor (VWR International). *Y. enterocolitica* titers were enumerated by plating dilutions of homogenates on Difco Yersinia Selective Agar Base plates (Becton Dickinson, Sparks, MD, USA).

Statistical Analyses

All experiments were performed at least two times. Statistical tests were performed using GraphPad Prism software, as follows: unpaired *t* test for the Ki67/CD4/CD8/IFNγ staining experiments; Mann–Whitney test for the bacterial colonization experiments; Log-rank (Mantel–Cox) test for the survival experiments. The significant threshold was $P \leq 0.05$.

RESULTS

Rapid Responses of Neonatal MLN CD8$^+$ Cells following Oral *Y. enterocolitica* Infection

In the course of characterizing immune responses to *Y. enterocolitica* infection, we compared MLN cells from uninfected neonates and adults for expression of the proliferation antigen Ki67. Approximately 10% of both CD4$^+$ and CD8$^+$ cells in adult MLN expressed the Ki67 antigen. A much greater proportion of both T cell types were Ki67$^+$ in neonatal MLN (**Figures 1A,B**). This is consistent with previous studies in which neonatal T cells in mouse spleen and cord blood were found to be spontaneously proliferating at a higher rate than in adults (21, 22). However, the frequency of proliferating cells in the MLN, especially among the CD8$^+$ population, is markedly higher than in blood or spleen.

The substantial endogenous proliferation of CD8$^+$ cells in uninfected neonates indicated that this population may pre-exist in a state poised for rapid responsiveness to invasive intestinal pathogens. Indeed, within 24 h of infection with *Y. enterocolitica*, there was a significant increase in the proportion of neonatal CD8$^+$ cells while percentages of neonatal CD4$^+$ and adult CD4$^+$ and CD8$^+$ did not increase (**Figure 1C**). The absolute numbers showed a similar pattern, although neonatal CD4$^+$ cells also increased by fivefold while CD8$^+$ cell numbers increased eightfold. Neither population increased in absolute numbers in adults.

These results indicated that the neonatal CD8$^+$ population was able to expand rapidly in response to *Y. enterocolitica* infection. The next question was whether this expansion was accompanied by effector function. A prominent effector function of CD8$^+$ cells is the production of IFNγ. Our previous studies had shown that IFNγ is required for survival of neonates to *Y. enterocolitica* infection (18) and that IFNγ production by CD4$^+$ cells was an important component of protection. However, IFNγ-producing CD4$^+$ cells most likely act in the later stages of primary infection, during the adaptive phase of the response, since death in CD4-deficient neonates is delayed until ≥15 days post infection. In striking contrast, IFNγ-deficient neonates die rapidly, within 7 days of infection. Together, these results suggest that IFNγ may provide important protection both early and late post infection. Thus, we proposed that early activated CD8$^+$ cells may be a rapid source of IFNγ production. We first examined early IFNγ production among total MLN cells and found an increase in total cytokine-producing cells in neonates, but not adults (**Figure 2A**). Indeed, a significant increase in IFNγ-production was selectively observed in the neonatal CD8$^+$ population (**Figure 2B**). Thus, *Y. enterocolitica* infection of neonates is characterized by the rapid mobilization of CD8$^+$ MLN populations with IFNγ-producing effector function.

Neonatal CD8$^+$ Cells Are Essential for Survival to Primary Infection and for Preventing Early Systemic Dissemination of *Y. enterocolitica*

Our results thus far indicated that neonates infected with *Y. enterocolitca* mount early CD8$^+$ responses. To test whether CD8$^+$ cells were essential for protection against infection, survival of wt and B2m$^{-/-}$ neonates to a threshold lethal dose of *Y. enterocolitica* was compared. As shown in **Figure 3**, B2m$^{-/-}$ neonates were more susceptible to lethal infection and, notably, most deaths occurred prior to 14 days post infection—i.e., well before the time that CD4$^{-/-}$ neonates begin to succumb (18). In these experiments, we used female and male neonates indiscriminately but subset analyses showed that there were no differences in survival of male or female neonates in either wt or B2m$^{-/-}$ neonates. Therefore, CD8$^+$ cells appear to play an important role in the survival of neonates, regardless of gender.

Yersinia enterocolitica replicates in the small intestines and routinely invades to the MLN but only systemically disseminates

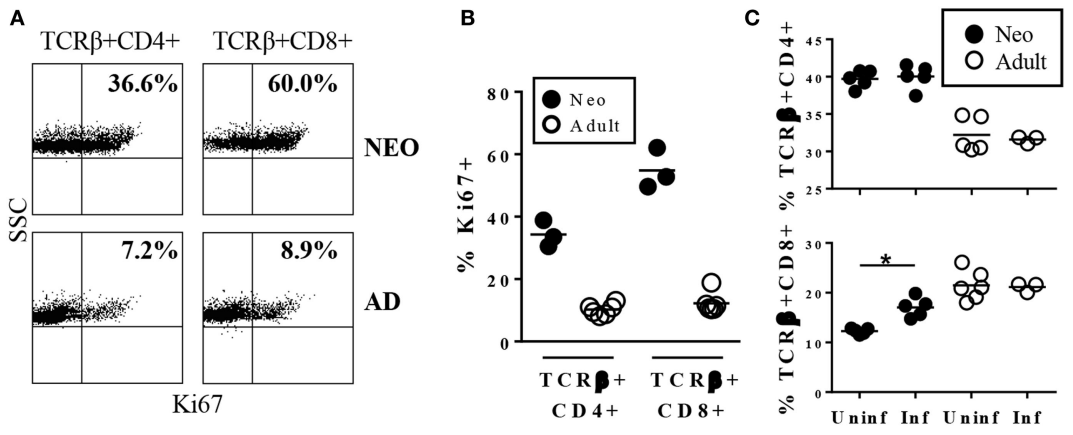

FIGURE 1 | Neonatal TCRβ$^+$CD8$^+$ cells in the mesenteric lymph nodes (MLN) are cycling in uninfected animals and rapidly increase in response to infection with *Yersinia enterocolitica*. (A) Representative profile of MLN cells from uninfected 7-day-old or adult mice stained for TCRβ, CD4, CD8, and Ki67. **(B)** The percentages of Ki67$^+$ among TCRβ$^+$CD4$^+$ or TCRβ$^+$CD8$^+$ cells in uninfected individual neonatal or adult MLN. **(C)** The percentages of TCRβ$^+$CD4$^+$ or TCRβ$^+$CD8$^+$ MLN cells in individual mice 1 day post infection with 5 × 10^8 CFU *Y. enterocolitica*.

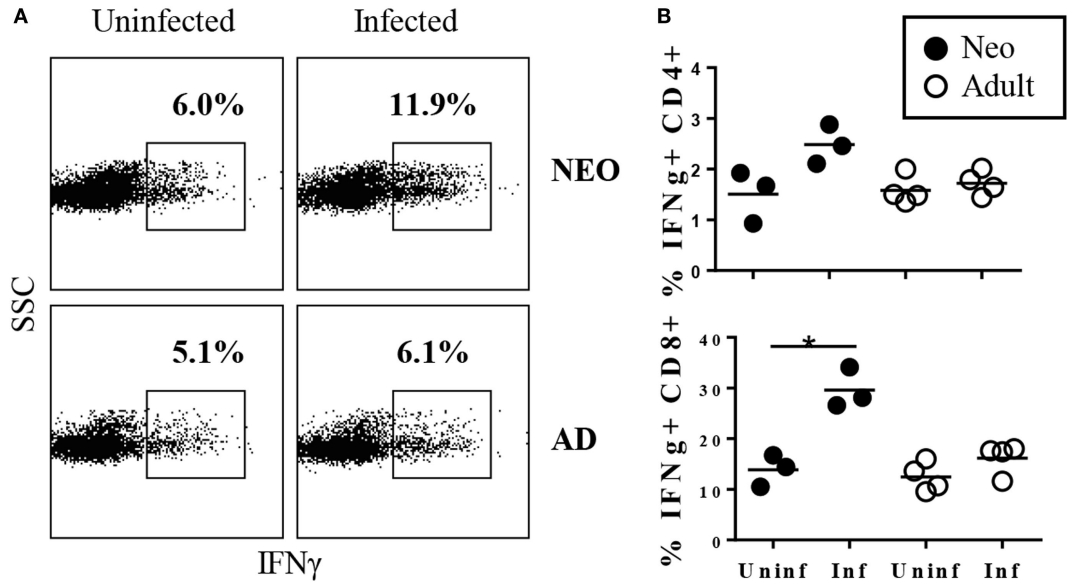

FIGURE 2 | Rapid increase in the percentages of TCRβ⁺CD8⁺ cells producing IFNγ in neonatal mesenteric lymph nodes (MLN) after infection with *Yersinia enterocolitica*. Neonatal and adult mice were infected with 5×10^8 CFU *Y. enterocolitica*. Forty-eight hours later, MLN cells were prepared, stimulated with PMA + ionomycin, in the presence of brefeldin A, for 4 h, and then subjected to intracellular cytokine staining. **(A)** Frequencies of IFNγ⁺ cells among total lymph node cells in uninfected and infected neonatal and adult animals. Gates for IFNγ were determined by comparison with isotype-matched control stains. **(B)** The frequencies of IFNγ-producing cells within the TCRβ⁺CD4⁺ or TCRβ⁺CD8⁺ populations. Each point for neonates represents a pool of 2–4 animals; a total of eight neonates were examined. Each point for adults represents an individual animal. Differences between uninfected and infected animals are non-significant unless indicated with *.

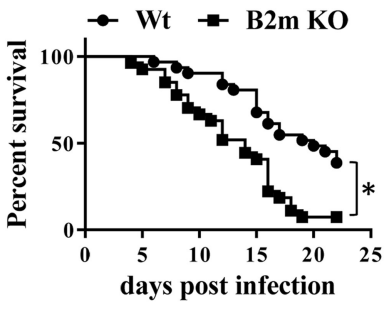

FIGURE 3 | CD8⁺ cells are critical for survival of neonatal mice to primary *Yersinia enterocolitica* infection. The 7-day-old wt or B2m$^{-/-}$ pups were orally infected with 1×10^7 CFU *Y. enterocolitica* and survival was monitored. A total of 33 wt and 27 B2m$^{-/-}$ pups was analyzed in ≥3 separate experiments. $P = 0.0004$ in the Log-rank (Mantel–Cox) test.

if the dose of infection is very high in wt animals or if animals are immunocompromised (23). Significant systemic dissemination most likely leads to death *via* septic shock. To investigate the potential importance of CD8⁺ cells in confining *Y. enterocolitica* to the intestinal tissues, we compared early colonization of intestinal and systemic organs in infected wt and B2m$^{-/-}$ neonates. 48 h post infection, colonization levels of the small intestines and MLN were similar in wt and B2m$^{-/-}$ neonates (**Figures 4A,B**). However, at this relatively early time point, both the liver and spleen showed massive bacterial infiltration only in the B2m$^{-/-}$ neonates (**Figures 4C,D**). These results indicate that the early activation of CD8⁺ cells in the neonatal MLN may be critical for preventing the systemic spread of *Y. enterocolitica*.

Protective Memory Responses Are Generated in Neonates in the Absence of CD8⁺ Cells

In adult mice, CD8⁺ cells appear to be essential for protection against secondary infection with the closely related bacterium *Yersinia pseudotuberculosis* (24). To test whether this was also the case in neonates, B2m$^{-/-}$ neonates were infected with a sublethal dose (1×10^4 CFU) of *Y. enterocolitica*. Uninfected littermates were kept as control mice. Eight weeks later, all mice were challenged with 1×10^5 CFU of *Y. enterocolitica*, and survival was monitored. At this challenge dose, approximately 50% of the control, previously uninfected, mice survived (**Figure 5**). However, 100% of the B2m$^{-/-}$ mice previously infected as neonates survived the infection. Therefore, CD8⁺ cells are not required for either the development of immunological memory in neonates or for its manifestation in response to secondary challenge.

DISCUSSION

The results presented here demonstrate that CD8⁺ cells are critical for protection of neonates against oral exposure to an

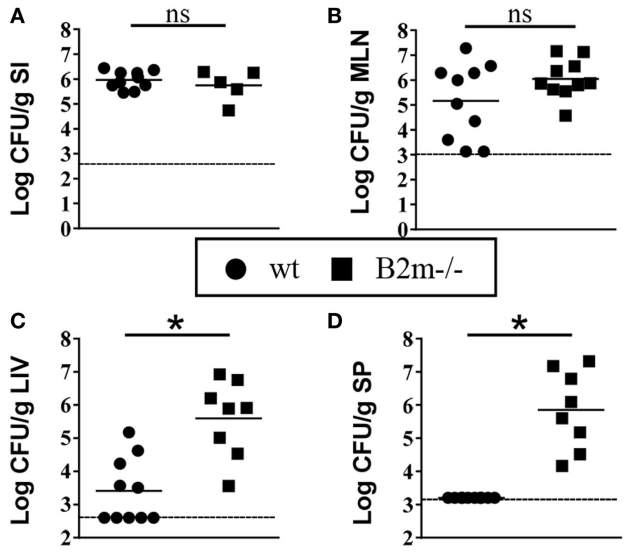

FIGURE 4 | Enhanced early dissemination to peripheral tissues in B2m$^{-/-}$ neonates. 7-day-old wt and B2m$^{-/-}$ pups were orally infected with 5×10^8 CFU *Yersinia enterocolitica*, and CFU in **(A)** small intestines (minus contents) (SI), **(B)** mesenteric lymph nodes (MLN), **(C)** liver (LIV), and **(D)** spleen (SP) were measured 48 h post infection. Each point represents an individual mouse; the dashed lines indicate the limits of detection in each assay.

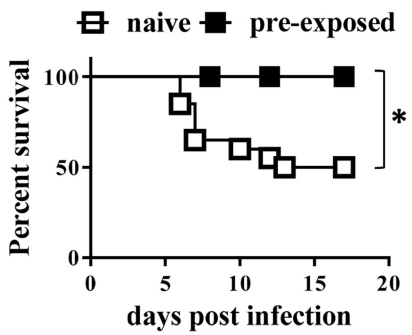

FIGURE 5 | Neonatal mice develop protective memory responses in the absence of CD8$^+$ cells. 7-day-old B2m$^{-/-}$ mice were infected with 1×10^4 CFU *Yersinia enterocolitica*. Eight weeks later, the mice were challenged with 1×10^5 CFU *Y. enterocolitica*; in parallel, naïve age-matched B2m$^{-/-}$ controls were similarly challenged. A total of 6 pre-infected and 20 naïve mice were analyzed in two separate experiments. $P = 0.0239$, using the Log-rank (Mantel–Cox) test.

extracellular bacterial enteropathogen. The protective effects appear to manifest early after infection since CD8$^+$ cells in the MLN selectively increase within 24 h of infection and over ¼ of the cells are producing IFNγ just 48 h post infection. Moreover, CD8$^+$ cells appear to be important for the early containment of bacteria within the intestines; the spleens and livers of B2m$^{-/-}$ neonates were heavily colonized by bacteria as early as 48 h post infection. Therefore, unlike in their typical adaptive role, neonatal CD8$^+$ cells in intestinal lymphoid tissues act during the early, innate phase of the response, providing an important rapid antibacterial function.

CD8$^+$ cell function in neonates has been mostly studied in response to infection with viruses, including influenza virus (25, 26), herpes simplex virus (27–29), respiratory syncytial virus (30), cytomegalovirus (31, 32), lymphocytic choriomeningitis virus (33), adenovirus (34), and Cas-Br-E murine leukemia virus (35–37). Most often, these viruses have been introduced either systemically or through a pulmonary route. Common, although not exclusive, observations are that the CD8 cell response is delayed relative to that of adults, lytic and/or IFNγ-secreting activities are diminished, the repertoire appears to be limited, and neonatal CD8$^+$ memory responses are poor. In all cases, however, neonatal CD8$^+$ cell activity was measured during the adaptive phase of the response. There are far fewer studies on neonatal CD8 cell function in response to bacterial infection and those are limited to systemic infection of neonates with the intracellular pathogen *Listeria monocytogenes* (38, 39). Thus, we believe that the neonatal response to *Y. enterocolitica* represents the first description of neonatal CD8$^+$ cells acting in a protective manner during the innate phase of the response against an extracellular bacterial enteropathogen.

Although CD8 cells had not previously been implicated in enterobacterial infection in neonates, a role for these cells in adult *Yersinia* infection has been described (24). Like our observations in neonates, it was reported that oral infection with *Y. pseudotuberculosis* led to increased colonization of peripheral tissues in B2m$^{-/-}$ adults. However, we found rapid (≤48 h) increased colonization in neonatal B2m$^{-/-}$ mice whereas the increase in adults was not detected until 14 days post inoculation, during the adaptive phase of immunity. In addition, we found that CD8 cells were not required for protective memory responses whereas in adults, anti-CD8 treatment greatly compromised memory responses. Overall, while CD8 cells may contribute to immunity against *Yersinia* infection in both neonates and adults, the roles these cells play and when they act differ in early life and adulthood.

The mechanisms underlying the early responses of neonatal CD8 cells in *Yersinia enterocolitica* infection are not yet fully understood. One finding that may provide some insight is the observation that >50% of neonatal MLN CD8$^+$ cells express proliferating antigens in uninfected, resting animals. Due to the lymphopenic state of neonates, homeostatic proliferation of both CD4$^+$ and CD8$^+$ cells has been previously described in neonatal spleen (21) and in human cord blood (22). In both cases, as we see here, a greater proportion of CD8$^+$ cells are cycling relative to CD4$^+$ cells. However, the percentages of CD8$^+$ cells in cycle in the MLN is approximately fivefold higher than that in the blood or spleen, perhaps due to the proximity of ongoing colonization by the commensal microbiota (40). Proliferation in lymphopenic hosts leads to the capacity for rapid induction of IFNγ expression (41, 42)—in our case, the IFNγ appears to be elicited upon exposure to bacterial enteropathogen. In that regard, human neonatal CD8$^+$ cells have been shown to rapidly respond to TLR2 or TLR5 stimulation with increased proliferation and cytokine production (43), and

it is possible that murine neonatal CD8$^+$ cells in the MLN are similarly responding to PAMPs expressed by *Y. enterocolitica*. Thus, neonatal CD8 cells may perform both adaptive functions, in response to virus or intracellular bacteria, and innate functions when exposed to extracellular pathogens, especially at mucosal sites.

AUTHOR CONTRIBUTIONS

DS performed many of the experiments in partial fulfillment of his doctoral degree. He contributed significantly to the intellectual development of the overall project and the manuscript. BA, the principal investigator, oversaw the scholarly and technical progress of the project and wrote the manuscript.

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Comparison of the Functional microRNA Expression in Immune Cell Subsets of Neonates and Adults

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Diversity of biological molecules in newborn and adult immune cells contributes to differences in cell function and atopic properties. Micro RNAs (miRNAs) are reported to involve in the regulation of immune system. Therefore, determining the miRNA expression profile of leukocyte subpopulations is important for understanding immune system regulation. In order to explore the unique miRNA profiling that contribute to altered immune in neonates, we comprehensively analyzed the functional miRNA signatures of eight leukocyte subsets (polymorphonuclear cells, monocytes, $CD4^+$ T cells, $CD8^+$ T cells, natural killer cells, B cells, plasmacytoid dendritic cells, and myeloid dendritic cells) from both neonatal and adult umbilical cord and peripheral blood samples, respectively. We observed distinct miRNA profiles between adult and neonatal blood leukocyte subsets, including unique miRNA signatures for each cell lineage. Leukocyte miRNA signatures were altered after stimulation. Adult peripheral leukocytes had higher let-7b-5p expression levels compared to neonatal cord leukocytes across multiple subsets, irrespective of stimulation. Transfecting neonatal monocytes with a let-7b-5p mimic resulted in a reduction of LPS-induced interleukin (IL)-6 and TNF-α production, while transfection of a let-7b-5p inhibitor into adult monocytes enhanced IL-6 and TNF-α production. With this functional approach, we provide intact differential miRNA expression profiling of specific immune cell subsets between neonates and adults. These studies serve as a basis to further understand the altered immune response observed in neonates and advance the development of therapeutic strategies.

Keywords: microRNA, cord blood, leukocyte subsets, let-7b, monocytes

INTRODUCTION

Differences in the expression of biological molecules in the immune cells of newborns and adults contribute to diverse cell function and atopic properties (1–5). These immune differences are reflected in varied immune responses, cellular subset composition, cytokine production, and cellular/humoral protein levels (3, 4, 6, 7). Other mediators including interleukin (IL)-10,

prostaglandin E2, and progesterone produced by the placenta can also upregulate Th2 differentiation, resulting in down-regulation of Th1 responses (8–10). In the previous studies, we found that neonates have selectively impaired IFNα-mediated Th1 immune responses (3, 11). By using proteomic tools, we identified at least 34 differentially expressed proteins between adult peripheral blood mononuclear cells (PBMCs) and cord blood mononuclear cells (CBMCs). There were also validated cytoskeletal differences between PBMCs and CBMCs (12). Moreover, we observed a decrease in adenosine deaminase and an increase in arginase-1 in neonatal mononuclear cells (MNCs), which was associated with impaired immune function (6). Different modulatory effects of adenosine and L-arginine on neonatal and adult leukocytes have also been investigated (4).

microRNAs (miRNAs) are small (19–22 nt) single-stranded non-coding RNA molecules derived from hairpin-structured precursors (13). These miRNAs function by directly binding to the indicated 3′-untranslated region of specific target mRNA, leading to target mRNA degradation or translational repression. miRNAs have been shown to play important roles in human development, cellular differentiation and homeostasis, adaptation, oncogenesis, and host cell interactions with pathogens (14–16). miRNAs are also involved in the regulation of immune systems, indicating that they modulate many aspects of the immune response, such as differentiation, proliferation, and activation of intracellular signaling pathways (17–19).

Recently, the essential regulatory roles of specific miRNAs in neonatal immune responses have also been noted. miR-184 was reported to regulate NFAT1 in neonatal CD4 T cells (20), while miR-146a and miR-155 downregulated toll-like receptor (TLR) signals in neonatal monocytes and plasmacytoid dendritic cells (pDCs), respectively (21, 22). We also found that miR-125b negatively regulates LPS-induced TNF-α expression in neonatal monocytes (23). It is possible that there is greater diversity in miRNA expression in neonatal leukocytes, which may contribute to the unique immunity of neonates.

Accumulating evidence demonstrates that miRNAs show specific signatures in different blood cell lineages and various stages of cellular differentiation (24–26). Since different cell types have unique functions and correspondingly, distinct gene expression profiles, determining the specificity of miRNA expression profiles in different leukocyte subpopulations is very important for both understanding the biology of the immune system and for characterization. Our hypothesis is that distinct miRNA profiles of different leukocyte subpopulations from neonatal and adult samples contribute to their relatively different immune responses. To exhibit proper immune functions, the immune cells must undergo activation, proliferation, and cytokine production upon encountering antigens (27). The miRNA profiles of leukocytes from neonates and adults have been widely reported. However, an important limitation of these studies was that they did not directly compare activated leukocytes of cord blood (CB) with those from adult peripheral blood, but instead used resting leukocytes. This approach is not as informative for identifying true differences in the functional transcriptome. In other studies, total leukocytes were investigated rather than unique leukocyte subsets (28). This approach is also information limiting because leukocyte subsets have distinct functions. In this study, we set out to comprehensively analyze the miRNA expression signatures of eight leukocyte subsets [polymorphonuclear cells (PMNs), monocytes, $CD4^+$ T cells, $CD8^+$ T cells natural killer (NK) cells, B cells, pDCs, and myeloid dendritic cells (mDCs)] between neonatal and adult samples. The miRNA profile of activated and resting leukocytes was also analyzed. With this functional approach, we provide differential miRNA expression profiling of specific immune cell subsets between neonates and adults. This provides a basis for further understanding of the altered immune response in neonates and can guide the development of novel therapeutic strategies.

MATERIALS AND METHODS

Collection of Human Umbilical Cord Blood and Adult Peripheral Blood and Cell Separation

Human umbilical CB was collected in heparinized tubes (10 U/ml) by cordocentesis at the time of elective Cesarean section or normal spontaneous delivery of healthy mothers, following the receipt of informed consent. About 20–50 ml of CB was obtained from each case. The peripheral blood samples were obtained from healthy adult volunteers aged 20–40 years. Approximately 50–100 ml of blood was obtained from each healthy adult depending on the experimental design. Heparinized blood samples were collected, and the plasma was stored at −80°C before analysis. The leukocyte separation protocol was utilized as previously described (11, 12). Briefly, whole blood was mixed with 4.5% (w/v) dextran (Amersham Pharmacia Biotech, Uppsala, Sweden) sedimentation at a ratio of 1:5 to separate leukocytes from red blood cells (RBCs) for 30 min. Leukocytes were then separated into PMNs and MNCs by density gradient centrifugation in Ficoll-Plaque™ (Amersham Pharmacia) at a ratio of 2:1 at 1,500 rpm for 30 min at 20°C. After centrifugation over a Ficoll cushion, MNCs were washed and counted on a hemocytometer by trypan blue staining. The PMN fraction in MNCs was less than 1% in adult and neonate samples. The study protocol was approved by the institutional review board of the study hospital.

Cell Separation and Enrichment

$CD4^+$ T-cells, $CD8^+$ T-cells, $CD14^+$ monocytes, or $CD56^+$ NK cells were separated from MNCs using the IMag Cell Separation Systems (BD Biosciences, San Jose, CA, USA) following the protocol supplied by the manufacturer. In brief, per 2×10^7 of MNCs pellet was suspended in 100 µl of anti-human CD4, CD8, CD14, or CD56 magnetic particles (BD Biosciences) and incubated at room temperature for 30 min. Then, the labeled cells were resuspended in 1× BD IMag™ buffer, and the tubes were placed in the BD Imagnet™ (BD Biosciences) for 10 min. With the tube in the BD IMagnet™, the supernatant was removed to discard the undesired leukocytes. To maximize purity, the

process was repeated three times. Then, the indicated cell fraction was carefully washed and suspended in PBS or medium. For pDCs isolation, dead cells were initially depleted by magnetic negative selection with Dead Cell Removal Kit (Miltenyi. Biotec, Bergisch Gladbach, Germany), then per 5×10^7 of MNCs pellet was isolated with 50 µl of CD304 (BDCA-4)-microbeads (Miltenyi Biotech) and magnetic columns according to the manufacturer's instructions. The mDCs were isolated by magnetic positive selection with CD1c (BDCA-1)-microbeads and a magnetic column (Miltenyi Biotech) following the depletion of $CD19^+$ cells. The purity of isolated cells was confirmed by flow cytometry, and all isolated cells demonstrated greater than 90% purity (12).

Cell Culture and Stimulation

Approximately 1 ml of leukocytes (2×10^6 cells/ml) suspended in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum were co-cultured with and without the indicated

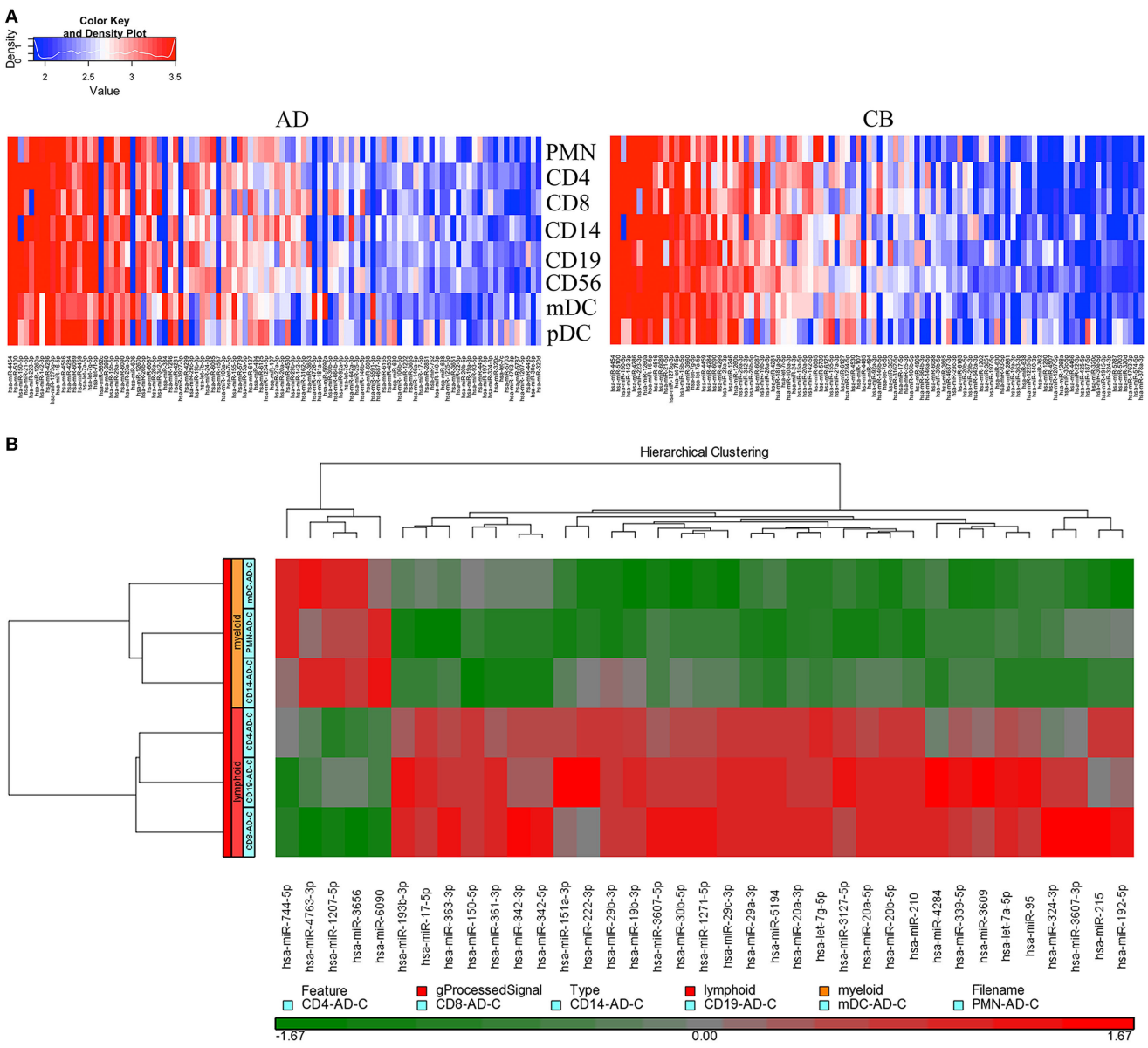

FIGURE 1 | **Distinct microRNA (miRNA) expression between adult and neonatal leukocytes**. **(A)** Neonate leukocyte subsets show distinct miRNA expression compared to adult leukocytes. Heat map showing distinct expression of miRNAs from different leukocyte subsets of adult peripheral blood (left) and cord blood (right). Each heat map lane represents the top 100 expressed miRNAs in each leukocyte subset. Higher intensity is shown in red and lower intensity in blue. **(B)** Myeloid and lymphoid leukocytes have unique miRNA profiles. Heat map showing distinct expression of miRNAs from myeloid (polymorphonuclear cells, $CD14^+$ monocytes, and myeloid dendritic cells) and lymphoid ($CD4^+$ T cells, $CD8^+$ T cells, and $CD19^+$ B-cells) leukocyte subsets. Thirty-seven miRNAs with the highest transcript levels across all tested cell lineages were shown. Higher intensity is shown in red and lower intensity in green.

stimulatory agents in 24-well culture plates. The cells were incubated in a humidified atmosphere of 5% CO_2 at 37°C. PMNs, monocytes, and mDCs were stimulated with LPS (100 ng/ml) for 6 h and $CD4^+$ and $CD8^+$ T cells, B-cells, and NK cells were stimulated with PHA (5 μg/ml) for 24 h. The stimulation condition for pDCs was CpG-ODN 2,216 (5 μg/ml) for 16 h as previously reported (29). Cells were harvested after culture and subjected to miRNA expression analysis.

RNA Isolation

Total RNA was extracted using the TRIzol® Reagent (Invitrogen, USA) according to the manufacturer's instructions. Purified RNA was quantified at OD260 nm using a ND-1000 spectrophotometer (Nanodrop Technology, USA) and qualitatively analyzed using a Bioanalyzer 2100 (Agilent Technology, USA) with RNA 6000 nano lab-chip kit (Agilent Technologies, USA).

RNA Labeling and Hybridization

Total RNA (0.1 μg) was dephosphorylated and labeled with pCp-Cy3 using the Agilent miRNA Complete Labeling and Hyb Kit (Agilent Technologies, USA, microRNA Spike-In Apply). Hybridization buffer (2×) (Agilent Technologies, USA) was added to the labeled mixture at a final volume of 45 μl. The mixture was heated for 5 min at 100°C and immediately cooled to 0°C. Each 45 μl sample was hybridized onto an Agilent human miRNA Microarray R19 (Agilent Technologies, USA) at 55°C for 20 h. After hybridization, slides were washed for 5 min in Gene Expression Wash Buffer 1 at room temperature and then washed for 5 min in Gene Expression Wash Buffer 2 at 37°C. Microarrays were scanned using the Agilent microarray scanner (Agilent Technologies, model G2505C) at 535 nm for Cy3. Feature Extraction (Agilent Technologies) software version 10.7.3.1 was used for image analysis. If both channels produced intensities less than 100 for a given miRNA, that spot was filtered out. For spots with one channel intensity less than 100 and the other 100 or greater, the signal that was less than 100 was set to 100 prior to calculation of the signal ratio. Hierarchical clustering was performed using the Genesis software (Graz University of Technology), and Pearson's correlation was calculated as the distance metric.

miRNA Array

In this study, we used Agilent SurePrint Human miRNA Microarray chips (Release 19.0, Agilent Technologies, Santa Clara, CA, USA) to globally evaluate the miRNA expression

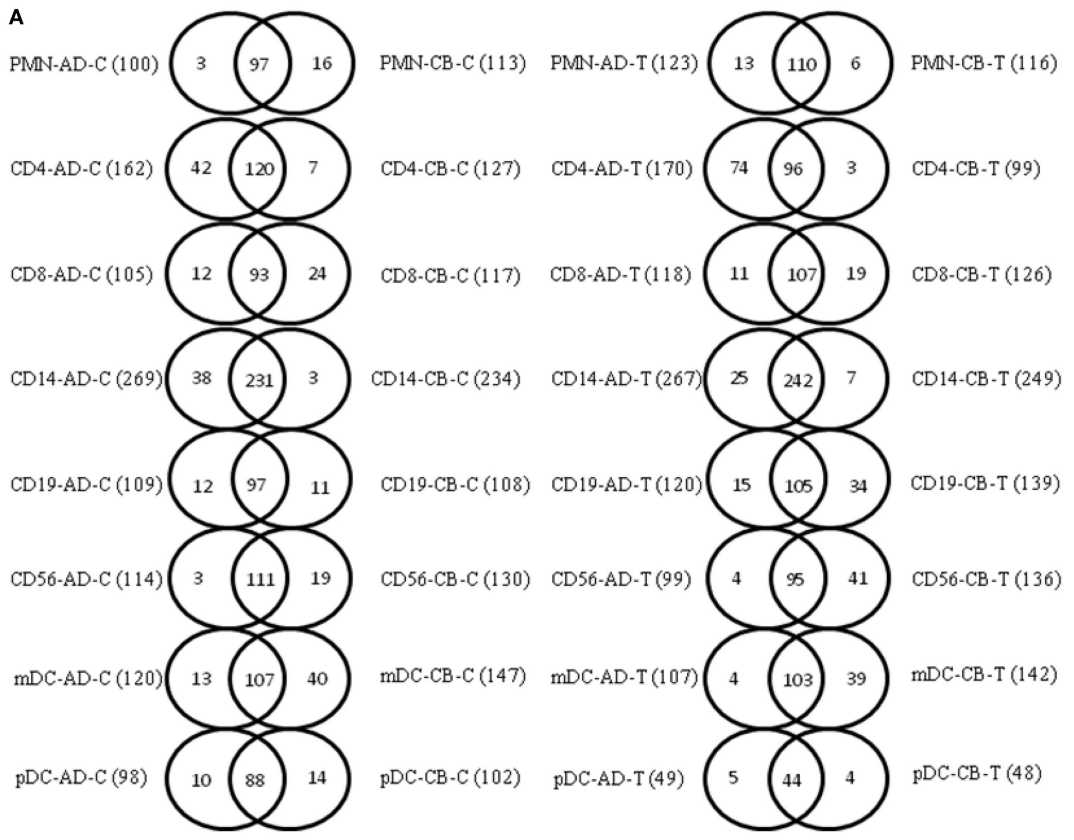

FIGURE 2 | Common and unique microRNA (miRNA) transcripts exist in adult and neonatal leukocyte subsets.

(*Continued*)

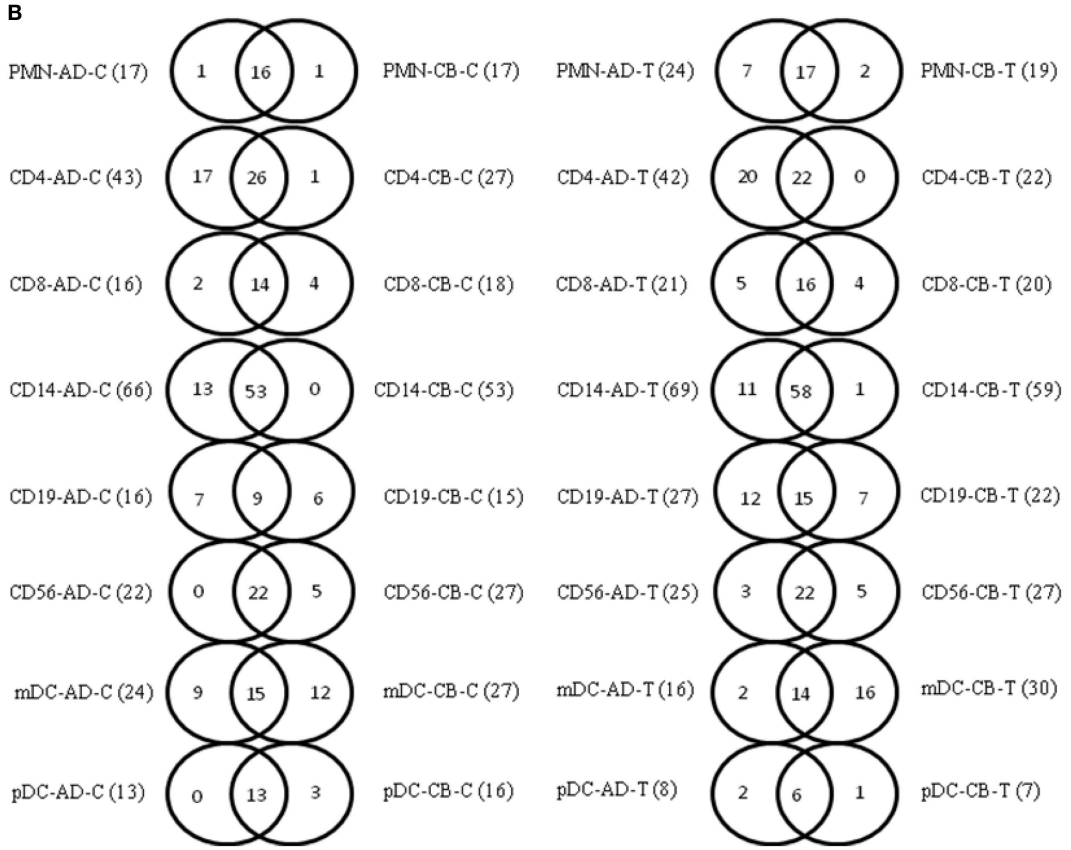

FIGURE 2 | **Continued**

(A) Venn diagrams showing comparative analysis of common and unique miRNA transcripts in the corresponding adult and cord blood (CB) leukocyte subsets (array intensity log 2 greater than 6). **(B)** Venn diagrams showing comparative analysis of common and unique miRNA transcripts in the corresponding adult and CB leukocyte subsets (array intensity log 2 greater than 10). Abbreviations: AD-C, adult blood cells without stimulation; CB-C, cord blood cells without stimulation; AD-T, adult blood cells with stimulation; CB-T, cord blood cells with stimulation.

profiles of the samples. The extracted RNA samples were first subjected to quality examination with a Bioanalyzer to check whether they passed the criterion, RIN $\geq$ 7.0. Then, 0.5 μg of the qualified RNA samples were prepared using the Agilent SurePrint Human miRNA Microarray kits, followed by microarray chip assays. The generated microarray data were analyzed with Partek (Partek Inc., St. Louis, MO, USA) under quantized normalization and log 2 transformation. Total 32 miRNA array chips were used for 8 kinds of leukocytes with/without indicated stimulation from CB and adult peripheral blood.

Quantitative Reverse Transcription PCR (qRT-PCR) Analysis of miRNAs

Total RNA was extracted from the cell pallet using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. qRT-PCR analysis was performed based on two-step qRT-PCR (30). Briefly, in the reverse transcription (RT) step, each RNA sample was reverse transcribed to cDNA using the TaqMan® miRNA Reverse Transcription Kit (#PN 4366597, Applied Biosystems, Foster City, CA, USA). Each reaction contained 10 ng of RNA, 50 U of Multiscribe Reverse Transcriptase, 100 mM of deoxyribonucleotide triphosphate, 1× reaction buffer, 4U of RNase inhibitor, and 1× specific miRNA primers (TaqMan MicroRNA Assay, PN 4427975, Applied Biosystems) with nuclease-free H_2O to 15 μl of final volume. The reaction mixture was then incubated at 16°C for 30 min, followed by incubation at 42°C for 30 min. The enzyme was then inactivated at 85°C for 5 min. In the TaqMan real-time PCR step, 2 μl of cDNA solution was amplified using 1× specific miRNA primers (TaqMan MicroRNA Assay), 1× TaqMan Universal Master Mix II (#PN 44440040, Applied Biosystems, Darmstadt, Germany), and nuclease-free H_2O to 10 μl of final volume. Quantitative PCR was performed on a 7500 System quantitative PCR machine (Applied Biosystems, Darmstadt, Germany) using a two-step PCR protocol with a denaturation step at 95°C for 10 min, followed by 40 cycles with a denaturation step at 95°C for 15 s, and an annealing/elongation step at 60°C for 60 s. The cycle threshold (Ct) values were calculated using the SDS 2.1 software (Applied Biosystems, Darmstadt, Germany). The relative amount of miRNA to small nuclear U6 snRNA (the internal control) was calculated using the equation $2^{-\Delta CT}$, where ΔCt = (Ct microRNA—Ct U6RNA) data

were analyzed as previously described (31). The target miRNA primer sequences were as follows: hsa-let-7b-5p (002619): 5′-UGA GGU AGU AGG UUG UGU GGU U-3′; hsa-miR-29a-3p (002112): 5′-UAG CAC CAU CUG AAA UCG GUU A-3′; hsa-miR-29b-3p (000413): 5′-UAG CAC CAU UUG AAA UCA GUG UU-3′; hsa-miR-130a-3p (000454), 5′-CAG UGC AAU GUU AAA AGG GCA U-3′. hsa-U6 snRNA (001973).

Transfection of let-7b-5p Mimic and Inhibitor with Functional Validation

A total of 2.5×10^5 cells/0.5 ml primary human $CD14^+$ monocytes were maintained in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml Amphotericin B (GIBCO) at 37°C in a humidified incubator containing 5% CO_2. For functional analysis, Has-let-7b-5p mimics

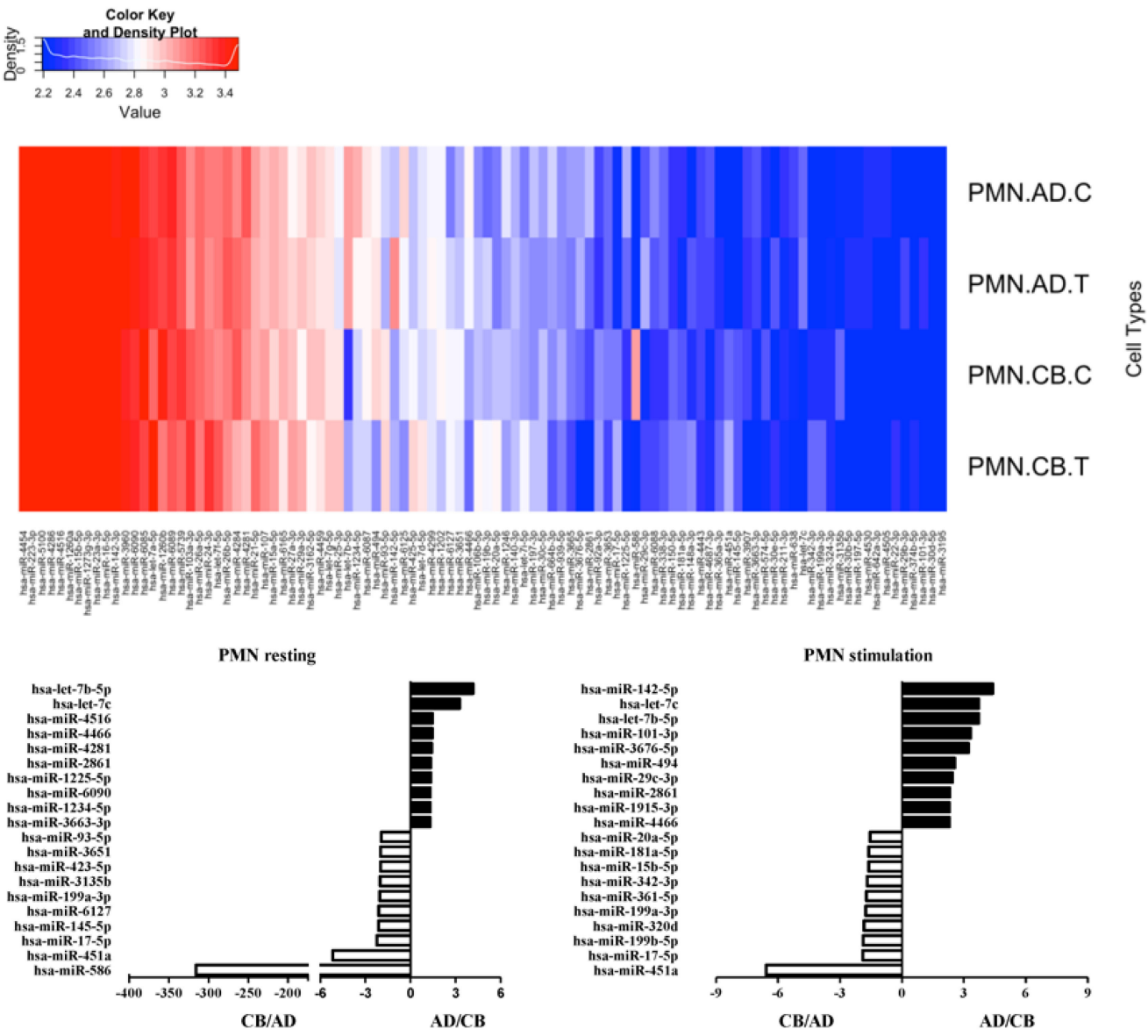

FIGURE 3 | Differential microRNA (miRNA) profiles between adult and neonatal polymorphonuclear cells (PMNs). Upper heat map showing distinct miRNA expression in activated and resting leukocyte subsets of adult peripheral blood and cord blood. Each heat map lane represents the top 100 expressed miRNAs in each leukocyte subset. Higher intensity is shown in red and lower intensity in blue. Lower bar charts showing the top 10 differentially expressed miRNAs in adult (black bar) or (white bar) neonate cells in specific leukocytes at resting (left) or activated states (right). The expression of miRNAs is represented as relative folds. Has, *Homo sapiens*.

(#47-775, EXIQON, TGAGGTAGTAGGTTGTGTGGTT), Has-let-7b-5p miRCURY LNA™ microRNA inhibitor (#4100945, EXIQON, ACCACACAACCTACTACCTC), cel-miR-39-3p miRCURY LNA™ microRNA Mimic Negative Control (#479902, EXIQON, TCACCGGGTGTAAATCAGCTTG), and miRCURY LNA™ microRNA inhibitor scramble control (#199900, EXIQON, ACGTCTATACGCCCA) were used. The miR mimics and LNA inhibitors were transfected into $CD14^+$ cells using TransIT-X2 (Mirus, Madison, WI, USA) according to the manufacturer's instructions. Briefly, cells were plated in a 48-well plate. miR mimics (50 µl) or LNA inhibitors and 1.5 µl of TransIT-X2 Transfection Reagent were added separately to 450 µl of RPMI-1640 medium (Invitrogen). The solution was then mixed to form the transfection complex. The transfection complex was added to cells with a final working concentration 20 or 40 nM and incubated at 37°C for 24 or 48 h, as indicated. After incubation with the transfection complex, the transfected cells were stimulated with or without LPS (100 ng/ml) for 16 h. The culture supernatants were then collected for quantification of IL-6, IL-8, and TNF-α production by ELISA (R&D Systems, Minneapolis, MN, USA, catalog number DY260,208 and DY210).

Statistics

Data are expressed as the mean ± SEM. The Mann–Whitney *U* test was used when two groups were analyzed. Results with a *p*-value of less than 0.05 were considered to be statistically significant. All

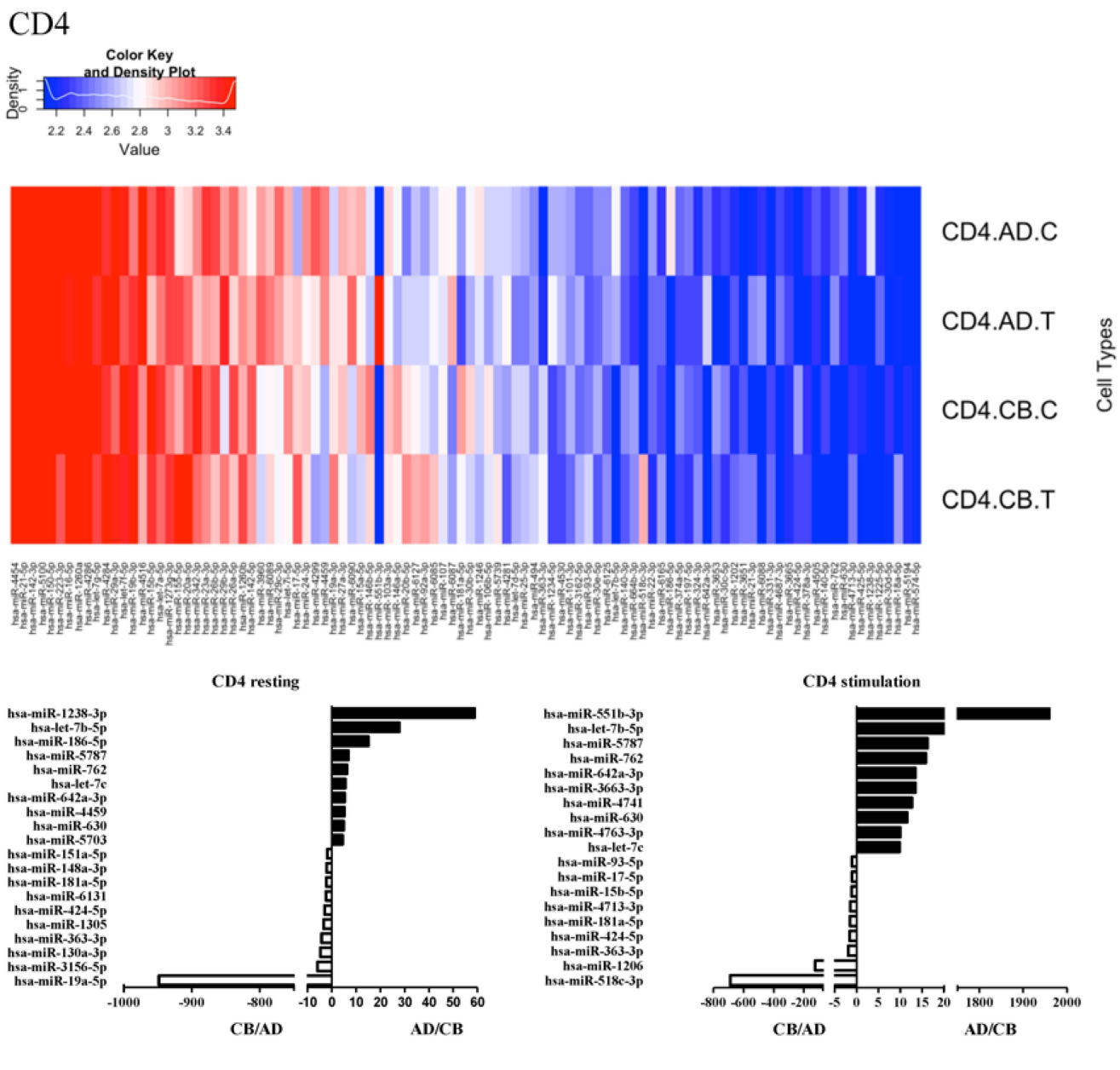

FIGURE 4 | Differential microRNA profiles between adult and neonatal $CD4^+$ T cells.

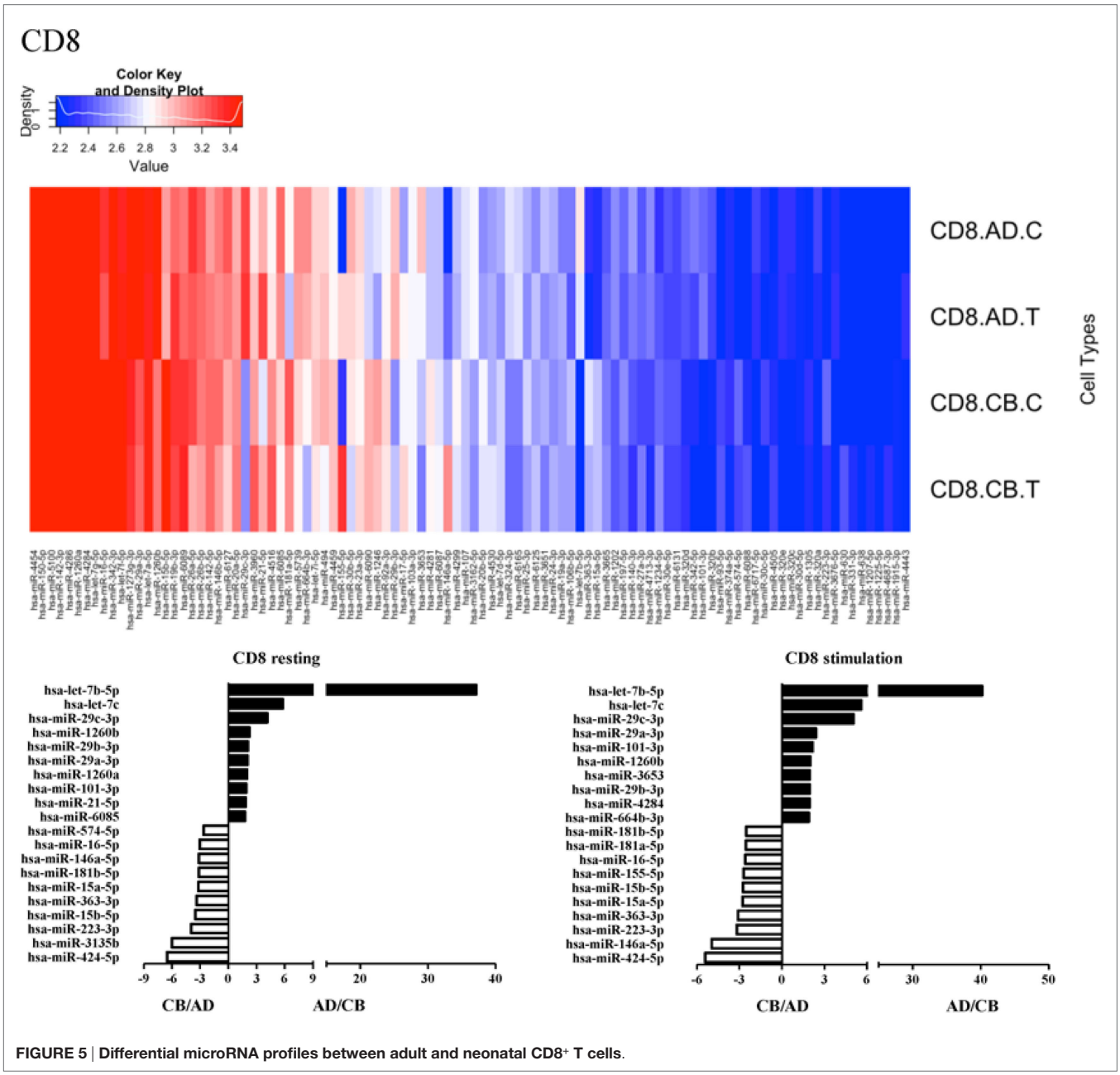

FIGURE 5 | **Differential microRNA profiles between adult and neonatal CD8⁺ T cells**.

statistical tests were performed using the SPSS 19.0 for Windows XP (SPSS, Inc., Chicago, IL, USA).

RESULTS

Hierarchical Clustering of Adult Peripheral Blood and Cord Blood Leukocyte Subpopulations Based on miRNA Expression

According to the manufacturer's design, probe sets displaying a log 2 signal value greater than 6 were linear and therefore reliable for miRNA analysis. Using data from the pooled samples, specific probe sets displayed an average log 2 signal value greater than 6 in at least one cell type and were deemed to be expressed above background. PMNs, CD4⁺ T cells, CD8⁺ T cells, CD14⁺ monocytes, and CD56⁺ NK cells were isolated from healthy adult peripheral or CB obtained from pools of five donors each. The BDCA1⁺ mDCs were obtained from pools of 10 healthy adult peripheral or CB donors. The BDCA4⁺ pDCs were obtained from pools of 19 healthy adult peripheral blood and 31 CB donors for its little amount. Subjects were randomized across pools for gender. We chose to pool samples in order to get sufficient cell numbers for rare cell types and to decrease sample variation. Because of the rare amount of dendritic cells, normalized miRNA expression data from one chip array with

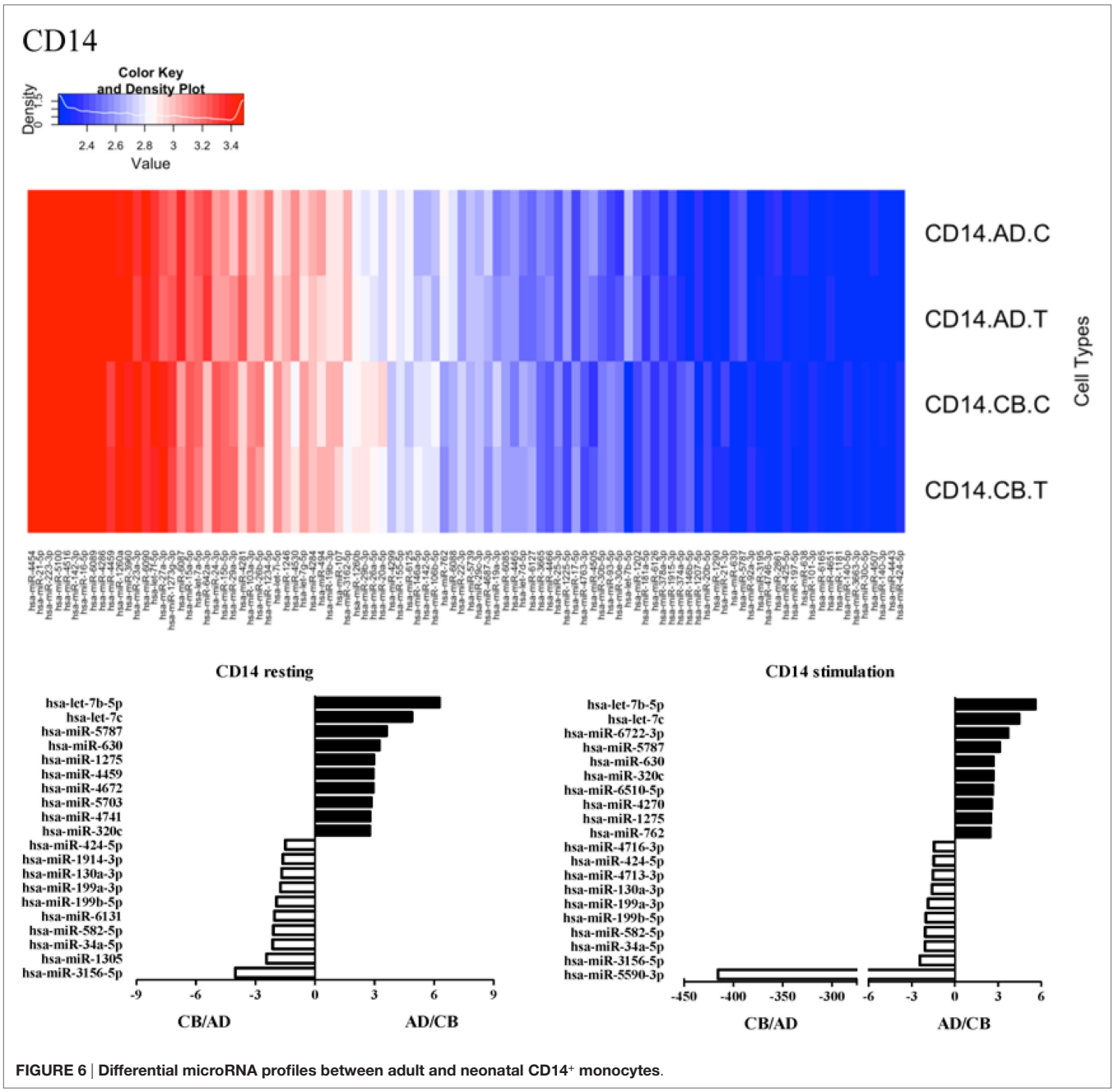

FIGURE 6 | **Differential microRNA profiles between adult and neonatal CD14⁺ monocytes**.

pooled leukocytes were analyzed in this study. The array data have been submitted to GEO and assigned an *accession number* "*GSE89853*." The raw data are now available and open for the researcher community. Full lists of normalized miRNA expression are shown in Table S1 in Supplementary Material. An unsupervised hierarchical cluster analysis of the top 100 most abundant miRNAs revealed discrete miRNA profiles for adult and CB leukocyte subsets, including unique miRNA signatures for each cell lineage (**Figure 1A**).

In order to characterize the specific miRNA expression between myeloid and lymphoid leukocytes, adult leukocyte subsets were divided into myeloid (PMNs, monocytes, and mDCs) and lymphoid groups (CD4⁺, CD8⁺ T cells, and CD19⁺ B-cells). The miRNA profiles of these two groups were compared as shown in **Figure 1B**. Samples from the same origin (myeloid or lymphoid) had similar miRNA expression profiles and were clustered together. Myeloid and lymphoid leukocytes have unique miRNA profiles that demonstrate the difference in their development. miR-744-5p, miR-4763-3p, miR-1207-5p, miR-3656, and miR-6090 were predominantly expressed in myeloid cells. In contrast, let-7g-5p (especially in CD4⁺ T cells), miR-342-3p, miR-324-3p, miR-3607-3p, miR-215 (especially in CD8⁺ T cells), let-7a-5p, miR-95, miR-151a-3p, miR-222-3p, miR-3127-5p, miR-4284, and miR-3609 (especially in B-cells) were expressed mainly in

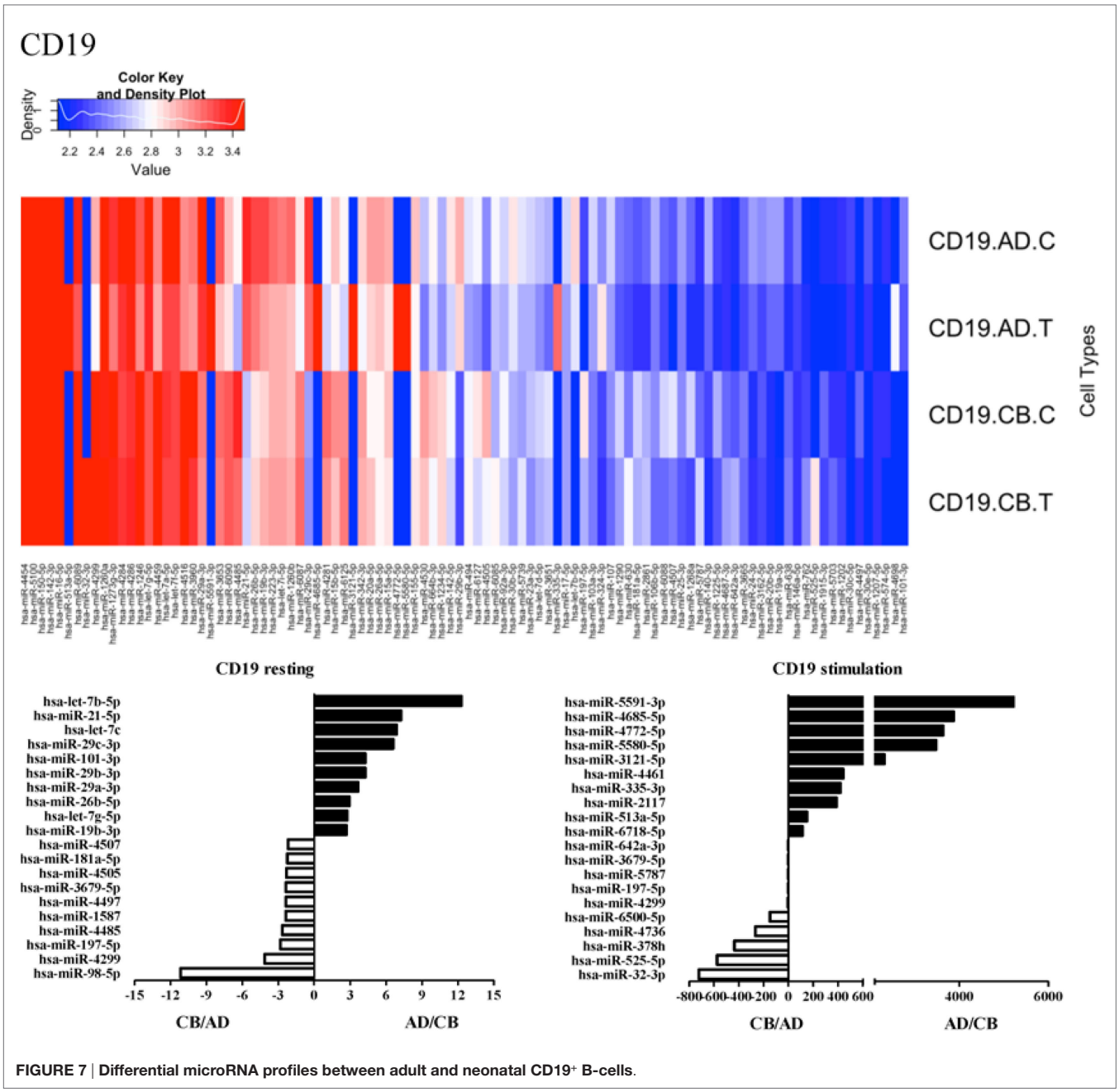

FIGURE 7 | Differential microRNA profiles between adult and neonatal CD19⁺ B-cells.

lymphoid cells. Interestingly, two members of the let-7 family (let-7a-5p and let-7g-5p) were present in the set of abundantly expressed miRNAs in lymphoid cells.

miRNA Signature Distinguished Adult and Neonatal Leukocyte Subsets

A lower filter threshold (log 2 signal value greater than 6) was initially set for miRNA analysis. Adult CD14⁺ monocytes demonstrated higher miRNA expression (269 miRNAs) than other leukocyte subsets, while adult pDCs had the lowest miRNA expression (98 miRNAs) (Table S2 in Supplementary Material). When comparing adult and CB leukocyte subsets, the Venn diagrams also showed common and unique miRNA transcripts in indicated leukocytes before and after stimulation (**Figure 2**). There were significant overlaps between adult and neonatal leukocyte subsets. We also applied a higher filter (log 2 signal value greater than 10) to identify the highly expressed miRNA transcripts among the different leukocyte subsets. With the higher filter, adult CD14⁺ monocytes had the highest number of miRNAs (66 miRNAs) compared to other leukocyte subsets, while adult pDCs had the lowest number of miRNAs (13 miRNAs) (Table S2 in Supplementary Material). **Figure 2B** demonstrates the common and unique miRNA transcripts in indicated leukocytes

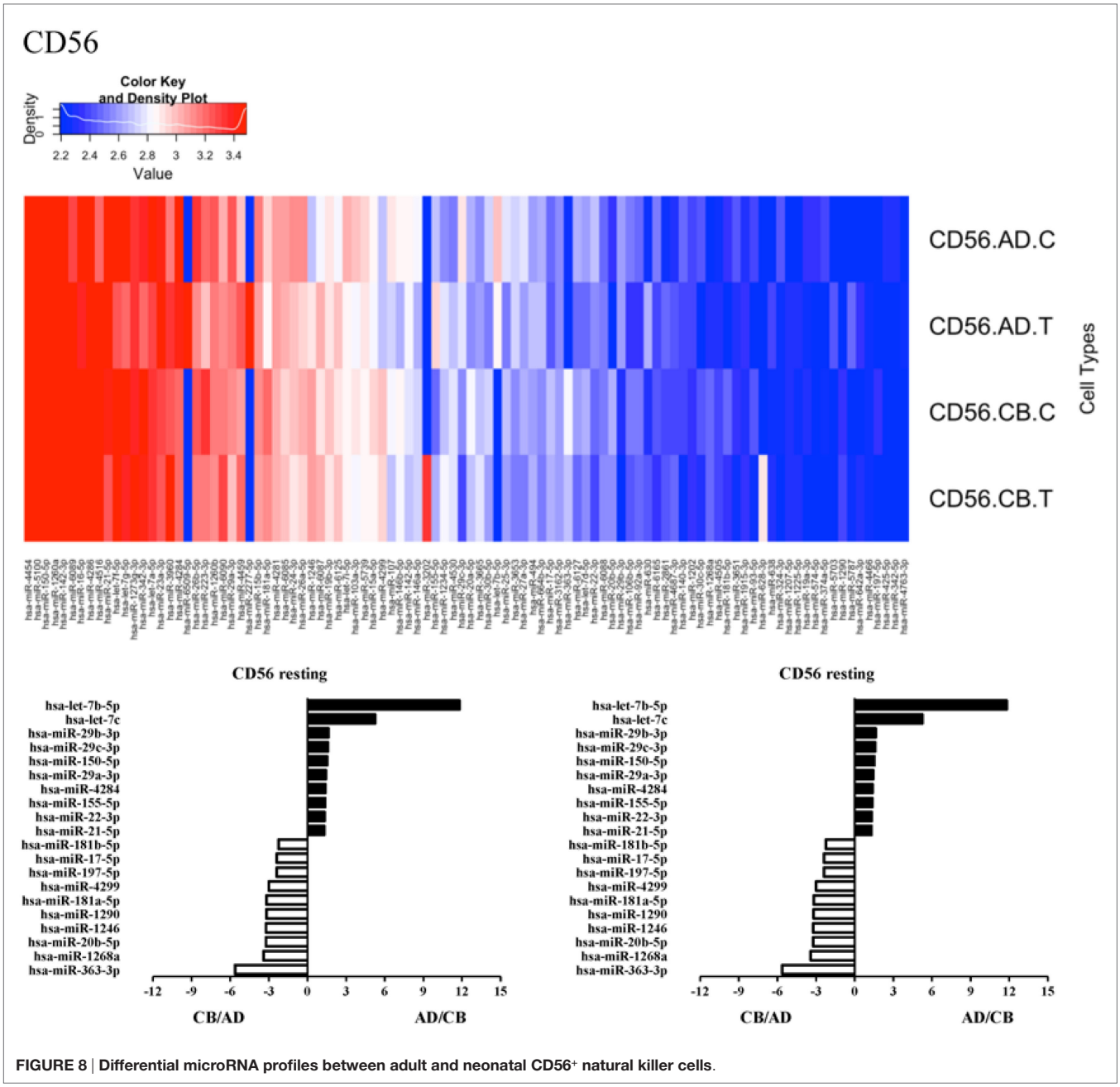

FIGURE 8 | **Differential microRNA profiles between adult and neonatal CD56⁺ natural killer cells**.

subsets between adult and CB with the higher filter. Significant overlaps between the adult and neonatal leukocyte subsets were also observed with the higher filter range. Using a higher filter, mDCs had the least overlap of miRNAs between adult and neonatal leukocytes (5/26; 19.2% overlap). These common and unique miRNA transcripts detail between indicated adult and CB leukocyte subsets in Table S3 in Supplementary Material.

Leukocytes miRNA Signature Altered after Stimulation

Since the major roles of immune cells are to defend the body against foreign substances, stimulation is needed to activate immune responses. Therefore, we also compared the miRNA profiles of leukocyte subsets between adult and neonate samples upon stimulation. Among the differentially expressed miRNA, the top 20 miRNAs expressed in specific leukocytes from adult (more than or less than neonate) samples were chosen and illustrated. The differentially expressed miRNA profiles of PMNs, CD4⁺ T cells, CD8⁺ T cells, CD14⁺ monocytes, CD19⁺ B-cells, CD56⁺ NK cells, mDCs, and pDCs are shown in **Figures 3–10**, respectively. Under resting conditions, compared to adult counterparts, the top 10 underexpressed miRNAs in CB PMNs were let-7b-5p, let-7c, miR-4516, miR-4466, miR-4281, miR-2861, miR-1225-5p, miR-6090, miR-1234-5p, and miR-3663-3p. The

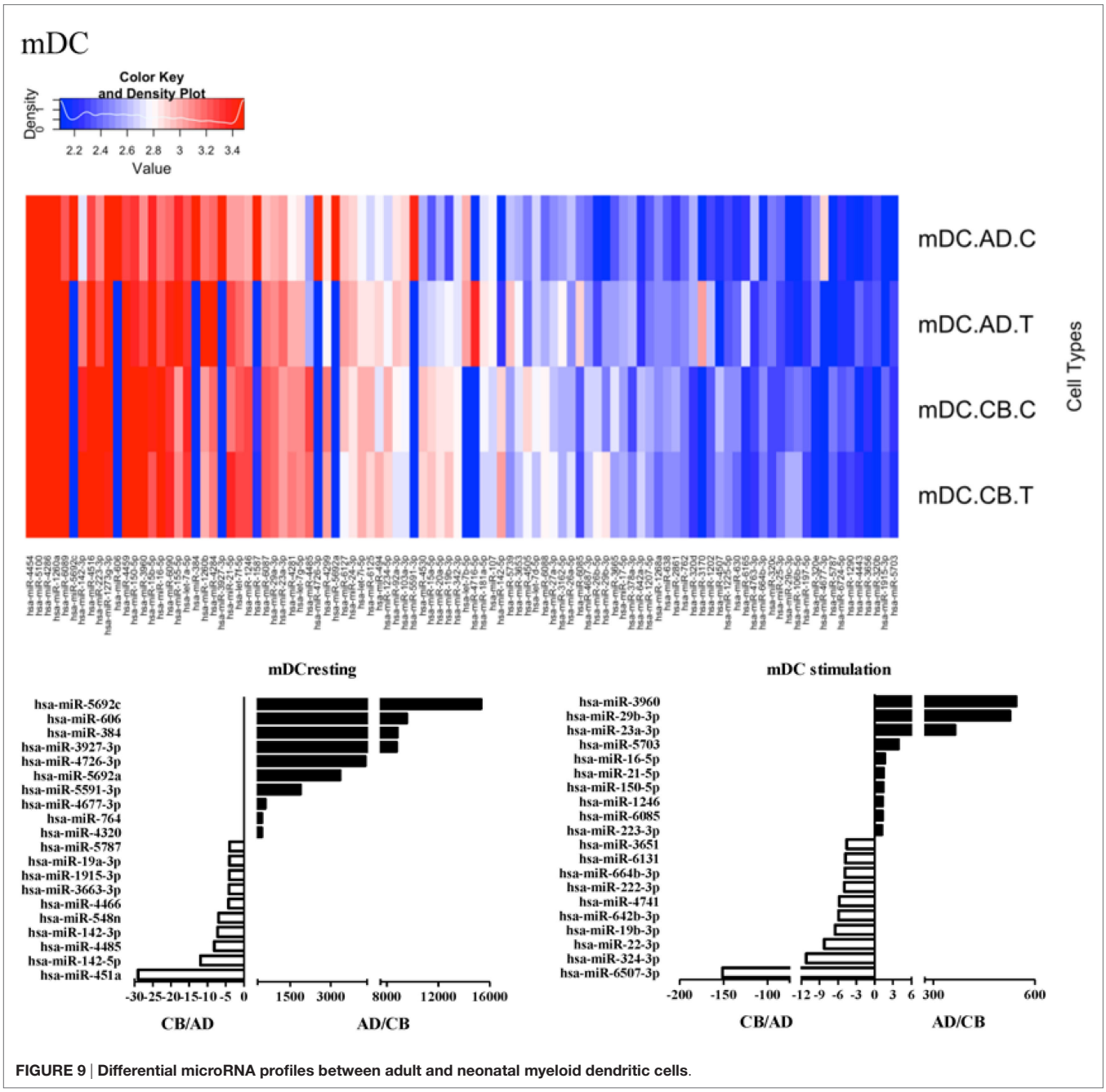

FIGURE 9 | **Differential microRNA profiles between adult and neonatal myeloid dendritic cells**.

top 10 miRNAs (miR-586, miR-451a, miR-17-5p, miR-145-5p, miR-6127, miR-199a-3p, miR-3135b, miR-423-5p, miR-3651, and miR-93-5p) were overexpressed in CB PMNs (**Figure 3**). Upon LPS stimulation, compared to adult counterparts, the top 10 underexpressed miRNAs in CB PMNs were miR-142-5p, let-7c, let-7b-5p, miR-101-3p, miR-3676-5p, miR-494, miR-29c-3p, miR-2861, miR-1915-3p, and miR-4466, while the top 10 miRNAs overexpressed in CB PMNs were miR-451a, miR-17-5p, miR-199b-5p, miR-320d, miR-199a-3p, miR-361-5p, miR-342-3b, miR-15b-5p, miR-181a-5p, and miR-20a-5p (**Figure 3**). With LPS stimulation, miR-142-5p exhibited the highest expression in adult PMNs compared to CB PMNs and miR-451a showed the highest expression in neonatal PMNs compared to adult PMNs. let-7b-5p and miR-142-5p exhibited the highest expression in adult PMNs compared to neonatal PMNs at resting and activated states, respectively. miR-586 and miR-451a exhibited the highest expression in cord PMNs compared to adult PMNs at resting and activated states, respectively. These dynamic changes in miRNA profiles correspond to the functional roles of miRNAs in immune cells during stimulation. Similarly, miR-1238-3p exhibited the highest expression in adult $CD4^+$ T cells compared to neonatal $CD4^+$ T cells at resting state (**Figure 4**). miR-19a-5p

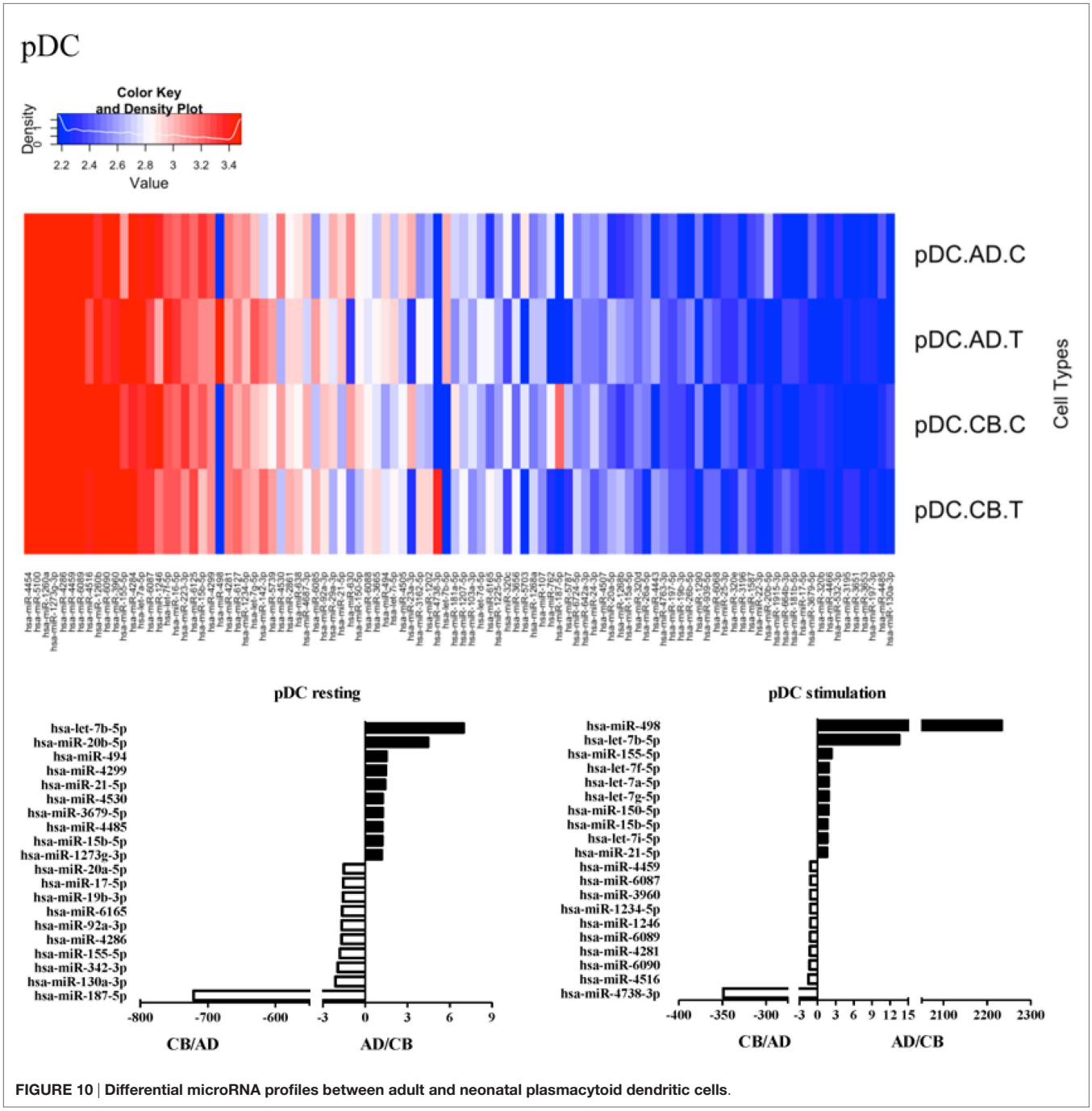

FIGURE 10 | **Differential microRNA profiles between adult and neonatal plasmacytoid dendritic cells**.

had the highest expression in neonatal CD4+ T cells compared to adult CD4+ T-cells at resting state. With PHA stimulation, miR-551b-3p exhibited the highest increase in expression in adult CD4+ T cells. miR-518c-3p showed the highest expression in neonatal CD4+ T cells compared to adult CD4+ T cells after PHA stimulation. Interestingly, let-7b-5p exhibited higher expression in adult monocytes compared to neonatal monocytes with or without stimulation (**Figure 6**). Likewise, we identified unique miRNA profiles of other leukocyte subsets between adults and neonates with or without stimulation. These unique miRNA profiles between adult and neonate leukocytes likely contribute to the altered immune function of neonates. It is noteworthy that adult peripheral leukocytes consistently demonstrated higher let-7b-5p and lower miR-181a-5p expression than CB cells across multiple subsets, with or without stimulation.

Since the differentially expressed miRNAs were selected based on signal intensity from a chip array with pooled leukocytes, we selected let-7b-5p, miR-29a-3p, miR-29b-3p, and miR-130a-3p to validate the array results by qRT-PCR in PMN, CD4+ T cells, and CD14+ monocytes. As shown in **Figure 11**, the qRT-PCR

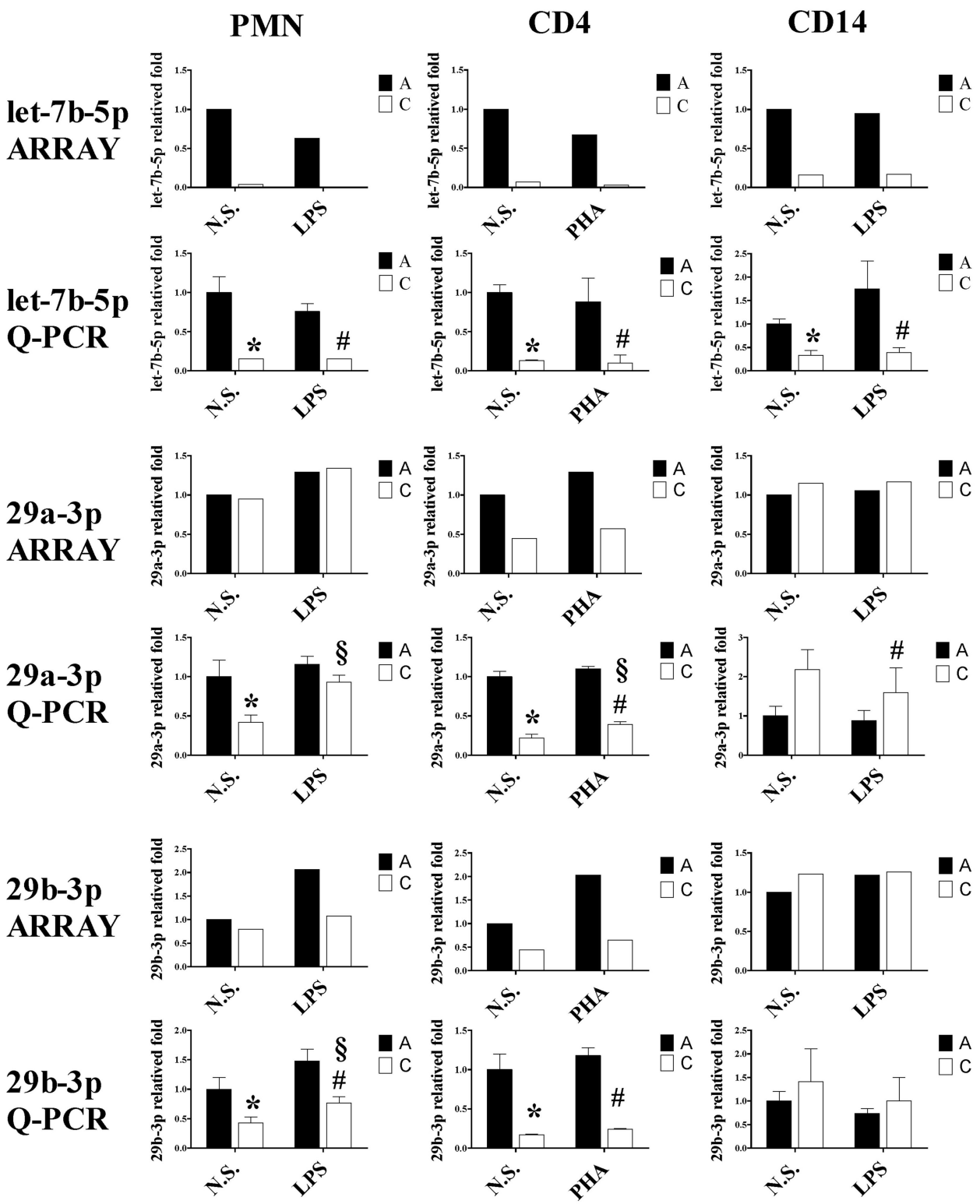

FIGURE 11 | Quantitative reverse transcription PCR (qRT-PCR) validation of let-7b-5p, 29a-3p, 29b-3p, and miR-130a-3p expression in various leukocyte subsets from cord blood (CB) and adult peripheral blood.

(*Continued*)

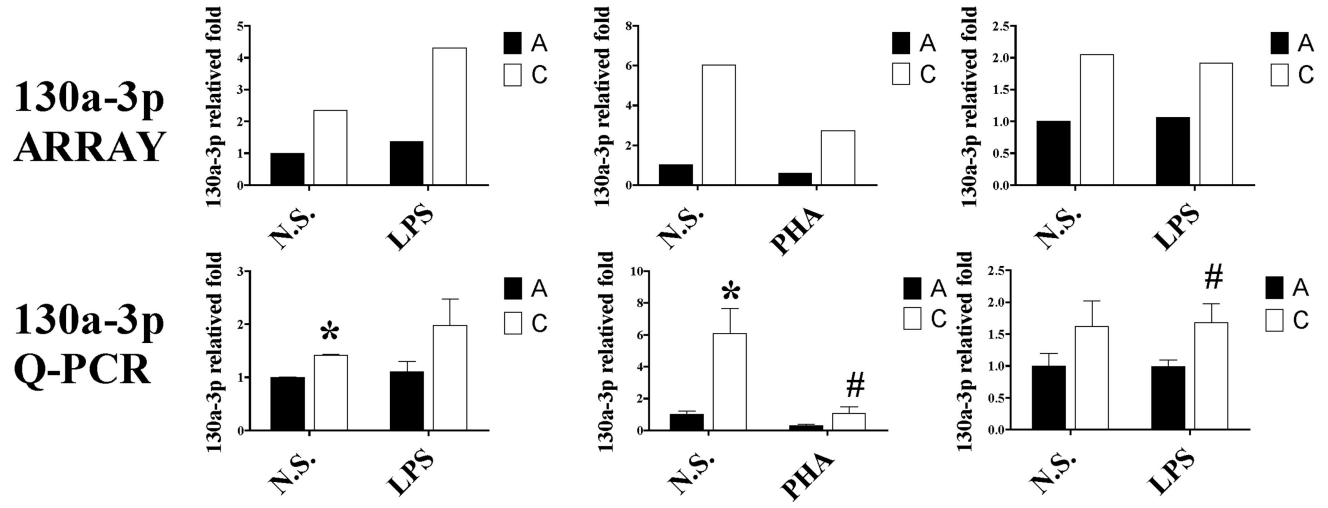

FIGURE 11 | **Continued**

To validate the data derived from our microarray analysis and literature references, we subjected RNA extracts harvested from stimulated or unstimulated polymorphonuclear cell, $CD4^+$ T cells, and $CD14^+$ monocytes of neonatal and adult samples for qRT-PCR analysis of let-7b-5p, miR-29a-3p, miR-29b-3p, and miR-130a-3p expression $^*p < 0.05$ between indicated adult and CB leukocytes without stimulation; $^\#p < 0.05$ between indicated adult and CB leukocytes with stimulation; $^\S p < 0.05$ between indicated CB leukocytes without and with stimulation (by ANOVA with the LSD *post hoc* test, $n = 4$).

results demonstrated a decrease in the expression of miR-29a-3p in PMNs and $CD4^+$ T cells of CB compared to adult cells, as opposed to $CD14^+$ monocytes. A similar trend to miR-29a-3p was observed with the expression of miR-29b-3p, whereas the expression of miR-130a-3p was higher in neonatal PMN, $CD4^+$ T cells, and $CD14^+$ cells. These data were consistent with the microarray results.

Functional Validation of let-7b-5p on Pro-inflammatory Cytokine Production by Monocytes

In the next study, monocytes were selected for further functional validation of let-7b-5p. Because adult monocytes had higher let-7b-5p expression than CB, we transfected a let-7b-5p inhibitor and let-7b-5p mimic into adult monocytes and CB monocytes, respectively. The transfection efficiency of the let-7b-5p inhibitor and mimic were determined by qPCR. As shown in Figure S1 in Supplementary Material, transfection of the let-7b-5p inhibitor into adult monocytes for 24 h suppressed let-7b expression 0.05-fold (at 20 and 40 nM of let-7b-5p inhibitor), white transfection of the let-7b-5p mimic into CB monocytes enhanced the expression of let-7b-5p 16,000- (at 20 nM of let-7b-5p mimic) and 20,000-fold (at 40 nM let-7b-5p mimic).

The function of let-7b-5p on the pro-inflammatory cytokine production was determined. Upon LPS stimulation, the monocytes produced large amounts of IL-6, IL-8, and TNF-α. Cord blood monocytes showed higher IL-6 (12,265 ± 1,387 vs. 4,623 ± 1,474 pg/ml) and TNF-α (2,436 ± 706 vs. 536 ± 54 pg/ml) production than adult monocytes (**Figure 12**). Transfection of the let-7b-5p inhibitor (20 nM) into adult monocytes for 24 h enhanced IL-6 production (**Figure 13A**). Transfecting the let-7b-5p mimic into CB monocytes for 24 h did not significantly suppress IL-6 production, although there was a trend. After transfecting let-7b-5p mimic into CB monocytes for 48 h, IL-6 production was suppressed with 40 nM of let-7b-5p mimic (**Figure 13B**). Transfection of the let-7b-5p inhibitor at 20 and 40 nM for 24 h enhanced TNF-α production by adult monocytes (**Figure 14A**). However, transfection of let-7b-5p mimic at 20 nM for 48 h was needed to suppress TNF-α production by CB monocytes (**Figure 14B**). These results suggest that the modulatory effects of CB monocytes could be achieved with let-7b mimic, but a longer incubation time is needed. Either transfection with the let-7b-5p inhibitor or let-7b-5p mimic for 24 or 48 h had no influence on IL-8 production by adult or CB monocytes (**Figures 15A,B**).

DISCUSSION

Blood contains different leukocyte subsets owning distinct gene expression profiles corresponding to distinct immune functions upon stimulation. Since miRNA behavior at the molecular level is context and cell type dependent, knowing the distinct functions of specific mRNAs in adult and neonatal leukocyte subsets can help to clarify the mechanisms leading to altered immunity in newborns. In this study, we conducted a systematic and comprehensive analysis of functional miRNAs in purified PMN, monocytes, T cells, NK cells, B cells, pDCs, and mDCs from neonates and adults. Transfection of the let-7b inhibitor into adult monocytes significantly enhanced IL-6 and TNF-α production, while transfection of a let-7b mimic into CB monocytes significantly suppressed the production of these cytokines. Accordingly, functional validation identified higher IL-6 and TNF-α production by CB monocytes than adult monocytes upon

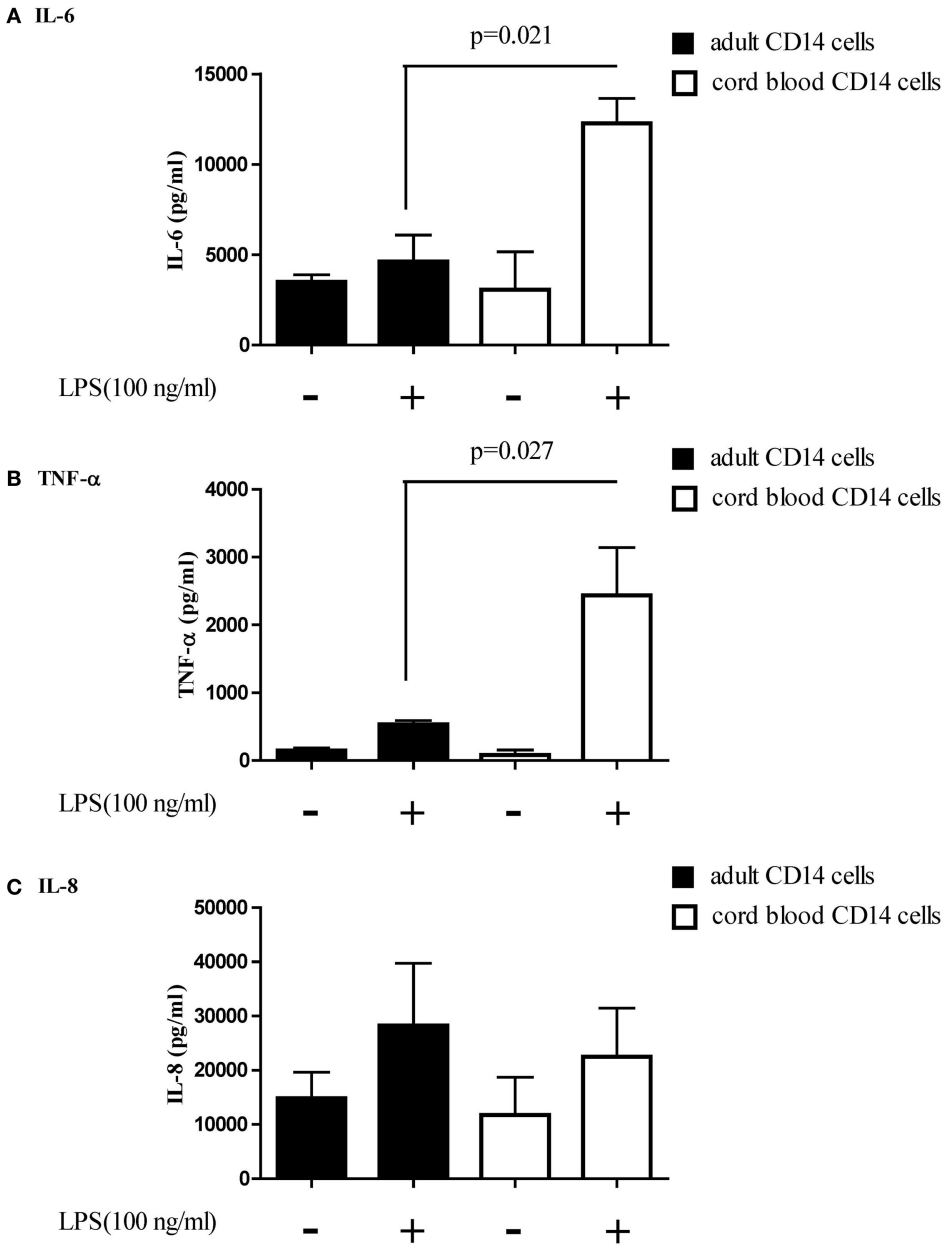

FIGURE 12 | Adult and cord blood (CB) monocytes produced large amounts of pro-inflammatory cytokines upon LPS stimulation. Adult and CB monocytes were stimulated with LPS for 16 h, and then the supernatant was collected for quantification of **(A)** interleukin (IL)-6, **(B)** IL-8, and **(C)** TNF-α secretion. *$p < 0.05$ between adult and CB monocytes with Mann–Whitney *U* test ($n = 5$).

LPS stimulation. This is, at least partly, due to their lower let-7b expression. Our results demonstrate the essential roles of specific miRNAs in regulating neonatal immune functions and provide insight into the molecular mechanism.

Understanding of the immune response in disease is an important issue for living organisms. The immune system is a complex system of interacting cells whose primary purpose is to identify foreign substances. To exhibit proper immune function, the immune cells must undergo activation, proliferation, and cytokine production upon encountering antigens (27). Therefore, activated leukocyte subsets have more biological transcripts than resting cells (3, 23). In order to investigate the corresponding innate or adaptive immune response, leukocyte subsets were isolated and stimulated with LPS, PHA, or CpG ODN as indicated in this study. LPS is a component of Gram-negative bacteria and is used in *in vitro* studies to assess innate immune responses to Gram-negative bacterial infection (32, 33). PHA is a lectin that binds to the sugars on glycosylated surface proteins, including

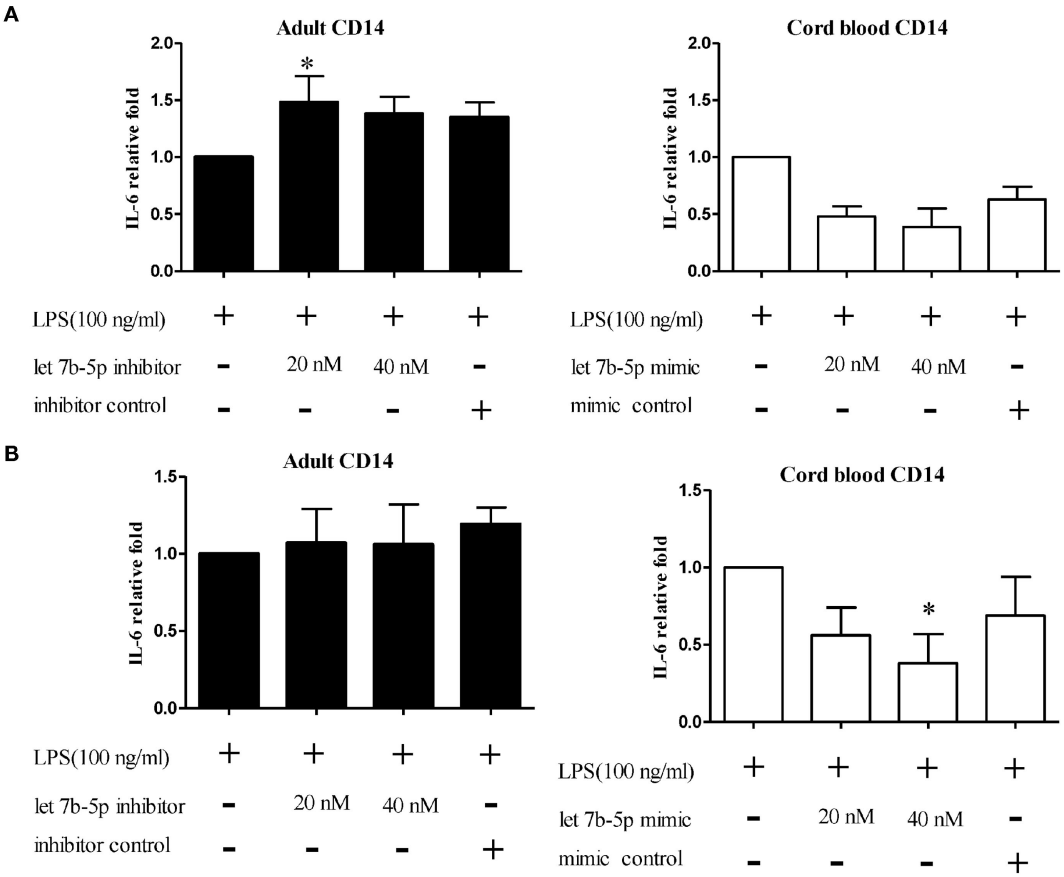

FIGURE 13 | The effect of let-7b-5p on interleukin (IL)-6 production by monocytes. let-7b-5p inhibitor transfected or untransfected adult monocytes and let-7b-5p mimic transfected and untransfected CB monocytes were subjected to detection of the IL-6 protein after LPS stimulation for 16 h. The transfection times were **(A)** 24 h and **(B)** 48 h. IL-6 production is represented as relative fold expression compared to the LPS stimulation control. *$p < 0.05$ as compared with LPS stimulation only with Mann–Whitney *U* test ($n = 5$).

the T cell receptor, and thereby crosslinks T cell activation signals. PHA has been widely used to stimulate human lymphocytes (34), whereas unmethylated CpG ODN is a ligand for TLR9 of pDCs (29). We chose LPS as a stimulus for PMN, monocytes, and mDCs. PHA was used to stimulate lymphocytes and NK cells, while CpG ODN was used to stimulate pDCs. With this approach, we provide functionally differential miRNA expression profiling of specific immune cell subsets between neonates and adults.

Although some studies have reported differences in miRNA expression between cord and adult blood immune cells, most focused on the miRNA profiles of a single resting leukocyte subpopulation (20, 22, 35). Several studies have provided miRNA profiles for limited leukocyte subsets without comparing to adults, thus providing only limited array information (28, 36, 37). Our study identified many previously reported miRNAs as well as unidentified miRNAs in all leukocyte subsets. We determined that all adult leukocyte subsets show higher let-7b-5p expression than the corresponding CB leukocytes, irrespective of their activation state. The let-7 miRNA family has been reported to consistently demonstrate increased abundance in adult erythroid cells (38).

To the best of our knowledge, this study is the first to demonstrate the abundance of let-7b-5p in all adult leukocyte subsets compared to neonates. Our data also demonstrate that neonatal $CD4^+$ T cells have more abundant miR-181a expression than adult $CD4^+$ T cells as previously reported by Palin et al. (35). Furthermore, our data show that all neonatal leukocyte subsets have more abundant miR-181a expression than relevant cells in adults, besides $CD4^+$ T cells. Our findings also correlate with that of Charrier et al. showing that neonatal pDCs have more abundant TLR signal-related miR-155-5p (called miR-155 previously) expression than adult pDCs at the resting state (22). Moreover, we found that miR-155-5p expression in adult pDCs is upregulated and compatible with neonatal pDCs after CPG ODN stimulation. Our study confirms the overexpression of miR-15b, miR-181a, miR-363, and miR-424 in CB $CD4^+$ and $CD8^+$ T cells, as previously reported (37). However, we did not observe significant change in miR-155 expression after LPS stimulation in our array data, as reported by Takahashi et al. (37). The dynamic changes of miR-155 presented in our previous study could explain this inconsistency (23). Higher miR-184 expression in CB $CD4^+$ T cells that regulate the abundance of NFAT1 has been reported

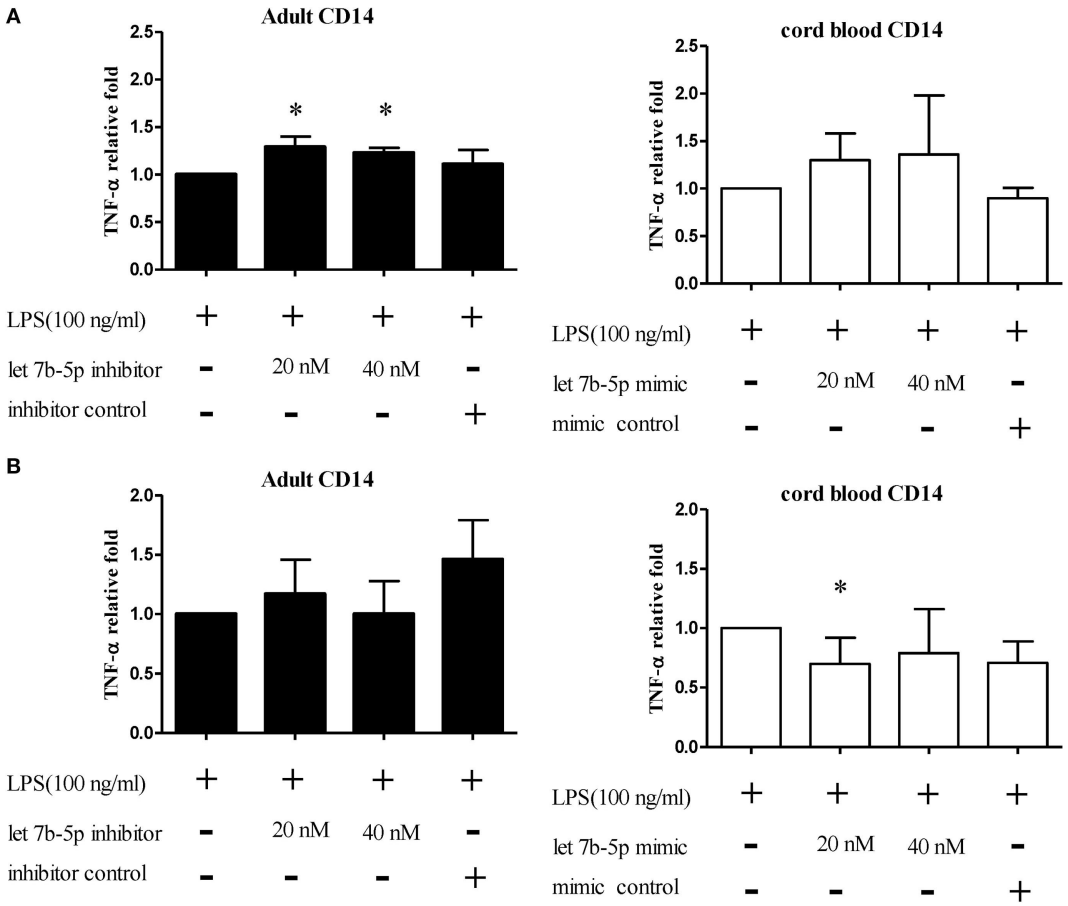

FIGURE 14 | The effect of let-7b-5p on TNF-α production by monocytes. Adult monocytes with or without let-7b-5p inhibitor transfection and cord blood monocytes with or without let-7b-5p mimic transfection were subjected to detection of TNF-α protein after LPS stimulation for 16 h. The transfection times were **(A)** 24 h and **(B)** 48 h. TNF-α production is represented as relative fold expression compared to the LPS stimulation control. Data presented are calculated from five replicate measurements. *$p < 0.05$ as compared with LPS stimulation only with Mann–Whitney *U* test ($n = 5$).

(20). However, our data showed low miR-184 expression below the threshold setting, in adult and cord $CD4^+$ T cells. Three small-RNA transcripts were expressed specifically in one cell type: miR-378 in monocytes, miR-31 in T cells, and miR-143 in neutrophils (25). In our study, we did observe specific miR-143 in neutrophils, although miR-378 and miR-31 were selectively expressed in adult and neonatal monocytes and T cells, respectively. These inconsistent results in miRNA expression among the different studies might be due to the differences of array or isolation kits used for study.

Based on our data, all adult leukocyte subsets illustrated higher let-7b-5p expression than the corresponding CB leukocytes. The higher let-7b-5p expression in adult leukocyte subsets was 3.7- to 63.0-fold that of neonates (Table S1 in Supplementary Material). PHA-stimulated adult $CD4^+$ T cells and $CD8^+$ T cells had higher let-7b-5p expression than neonates (63.0- and 40.2-fold, respectively).

let-7 is an important miRNA family consisting of 13 members. They are highly conserved across several animal species (39). The role of let-7 family members in tumor suppression is well established, and their role in the regulation of innate immunity is slowly being unveiled. Teng et al. have shown that let-7b regulates the expression of TLR4 *via* posttranscriptional suppression and subsequently influences the activation of NF-κB and downstream gene expression (40). Type 1 IFN production and antiviral signaling cascades are an important defense mechanism in the innate immune response to viral infections. let-7b has also been shown to regulate type 1 IFN production and can inhibit HCV replication and viral protein translation through insulin-like growth factor 2 mRNA-binding protein 1 (41–43). Whether diminished let-7b expression in neonatal leukocyte subsets can lead to altered type 1 IFN production and the susceptibility of newborns to viral infections need further investigation (1, 29). In our study, let-7b-5p modulated IL-6 and TNF-α production by monocytes. It has been suggested that, with LPS stimulation, hepatocarcinoma cells transfect let-7b enriched microparticles into macrophages, resulting in IL-6 downregulation in macrophages (44). We observed that more abundant let-7b expression in adult monocytes corresponded to lower IL-6 production than in CB monocytes with LPS stimulation. Transfection of adult monocytes

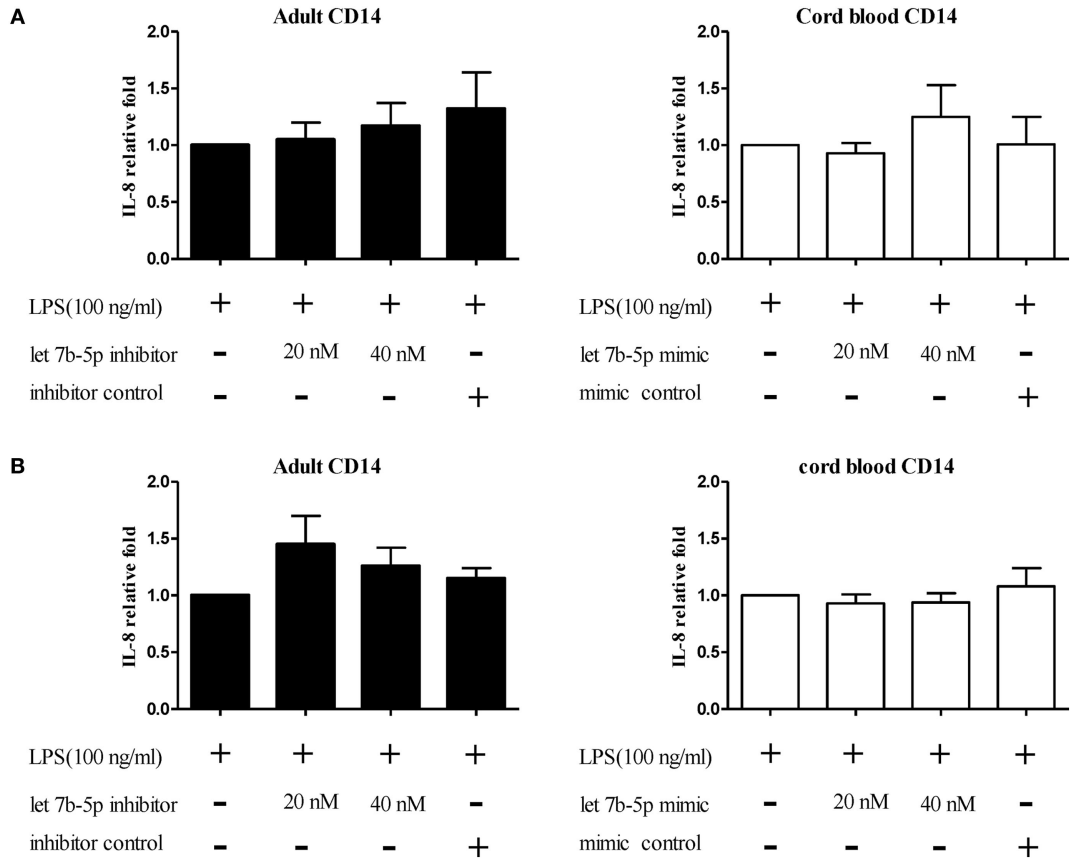

FIGURE 15 | The effect of let-7b-5p on interleukin (IL)-8 production by monocytes. Adult monocytes with or without let-7b-5p inhibitor transfection and cord blood monocytes with or without let-7b-5p mimic transfection were subjected to detection of the IL-8 protein after LPS stimulation for 16 h. The transfection times were **(A)** 24 h and **(B)** 48 h. IL-8 production is represented as relative fold expression compared to the LPS stimulation control. *$p < 0.05$ as compared with LPS stimulation only with Mann–Whitney *U* test ($n = 5$).

with a let-7b-5p inhibitor enhanced IL-6 production, while transfection of a let-7b-5p mimic into CB monocytes suppressed IL-6 production. This suggests that reduced let-7b expression in CB monocytes contributes to higher IL-6 production, compared to adult monocytes. Since IL-6 can modulate the recruitment and differentiation of T and B lymphocytes (45), let-7b plays an important modulatory role in the altered immune responses in newborns.

Interleukin-6 is a multifunctional cytokine secreted by many kinds of cells. IL-6 is produced in response to TLR ligands and other pro-inflammatory cytokines through the NF-κB signaling pathway (45). Although let-7b was also reported to target TLR4 and suppress IL-8 production through NF-κB regulation in gastric epithelial cells with *Helicobacter pylori* infection (40), there are likely different mechanisms for IL-6/IL-8 production control in specific cells, as let-7b cannot influence IL-8 production in both adult and CB monocytes.

Neonates are susceptible to infection, and neonatal sepsis is a significant cause of mortality and morbidity in newborns (46). Inflammatory responses are necessary for the host to respond to components of invading organisms. At the early stages of infection, TNF-α, IL-1β, IL-6, and IL-8 pro-inflammatory cytokines are produced and stimulate immune cells leading to activation of an inflammatory cascade (47). The subsequent inflammatory cascade includes many biologically active mediators and can induce a systemic inflammatory response. The inflammatory response is thereafter counteracted by anti-inflammatory mediators to restore immunological homeostasis. Excessive pro-inflammatory mediators or inadequate anti-inflammatory responses will lead to sepsis (47). In addition to antibiotics, other adjuvant therapies primarily target the dysregulated innate immune response. Pentoxifylline, a xanthine-derived phosphodiesterase inhibitor that decreases TNF-α and IL-6 production, has been reported to improve the clinical outcome of preterm sepsis (48). Recently, anti-cytokine agents in early sepsis with excessive uncontrolled hyper-inflammatory state have been addressed (49). Riedemann et al. demonstrated that an anti-IL-6 antibody could significantly improve survival through reduction of C5aR in various organs in a cecal ligation/puncture model in mice (50). Remick et al. also showed that the combination of IL-1 receptor antagonist and TNF soluble receptor is also effective in this clinically relevant mouse model of sepsis (51). Although each strategy has certain benefits,

there is still controversy regarding the general clinical application and potential side effects. Since transfection of let-7b-5p mimic could suppress IL-6 and TNF-α production of CB monocytes, regulation of let-7b-5p has the potential to play a role in neonatal sepsis control.

In conclusion, we demonstrate systematic and functional miRNA profiles of neonate and adult leukocyte subsets. We show that all adult leukocyte subsets have higher let-7b-5p expression than the corresponding CB leukocytes. let-7b-5p negatively regulates the production of IL-6 and TNF-α by human monocytes. Our study supports a comprehensive miRNA transcriptome database of neonatal immune cells and serves as a valuable resource for elucidating the role of miRNA-mediated regulation in neonatal immunity.

AUTHOR CONTRIBUTIONS

H-RY, T-YH, H-CH, H-CK, and S-CL contributed to designed the work; H-RY, T-YH, H-CH, H-CK, S-CL, and K-SH contributed to data acquisition; H-RY, T-YH, H-CH, H-CK, and S-CL performed data analysis and interpretation; H-RY, KY, and K-SH drafted the manuscript; H-RY and T-YH finalized the article. All the authors have read and approved the final manuscript and agreed to be accountable for all aspects of the work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2016.00615/full#supplementary-material.

FIGURE S1 | Transfection of let-7b-5p inhibitor and mimic into adult and cord blood (CB) monocytes, respectively. **(A)** Transfection of the let-7b-5p inhibitor into adult monocytes suppressed the expression of let-7b-5p. **(B)** Transfection of let-7b-5p mimic into CB monocytes enhanced the expression of let-7b-5p.

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MINI REVIEW
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Immunological Defects in Neonatal Sepsis and Potential Therapeutic Approaches

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Despite advances in critical care medicine, neonatal sepsis remains a major cause of morbidity and mortality worldwide, with the greatest risk affecting very low birth weight, preterm neonates. The presentation of neonatal sepsis varies markedly from its presentation in adults, and there is no clear consensus definition of neonatal sepsis. Previous work has demonstrated that when neonates become septic, death can occur rapidly over a matter of hours or days and is generally associated with inflammation, organ injury, and respiratory failure. Studies of the transcriptomic response by neonates to infection and sepsis have led to unique insights into the early proinflammatory and host protective responses to sepsis. Paradoxically, this early inflammatory response in neonates, although lethal, is clearly less robust relative to children and adults. Similarly, the expression of genes involved in host protective immunity, particularly neutrophil function, is also markedly deficient. As a result, neonates have both a diminished inflammatory and protective immune response to infection which may explain their increased risk to infection, and their reduced ability to clear infections. Such studies imply that novel approaches unique to the neonate will be required for the development of both diagnostics and therapeutics in this high at-risk population.

Keywords: inflammation, innate immunity, shock, infection, host response, genomics, transcriptomics

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INTRODUCTION

Neonatal sepsis is a major health-care burden worldwide, accounting for approximately 1.4 million neonatal deaths annually (1). Preterm infants are particularly susceptible to sepsis and have a higher risk of long-term complications and mortality than term infants (2). Despite advances in the delivery of neonatal intensive care, improving outcomes as well as diagnostic and prognostic accuracy in neonatal sepsis have been challenging (3).

The diagnosis of neonatal sepsis relies on the subjective interpretation of each case. This stems from a lack of specificity for clinical signs and symptoms (4) and suboptimal predictive ability of routine laboratory tests (5). Even blood cultures, the "gold-standard", result positive in fewer than 10% of cases of suspected sepsis (6, 7). The subjectivity of the clinicians approach to neonatal sepsis is further demonstrated by the observation that intraregional variation of antibiotic usage in the neonatal intensive care unit can range up to 40-fold (8). Underlying the diagnostic and prognostic challenges for this population is the absence of a consensus definition of neonatal sepsis to align research efforts (2).

The classification of neonatal sepsis is dependent on timing of onset; early-onset neonatal sepsis (EOS) occurs in the first 72 h of life, while late-onset neonatal sepsis (LOS) occurs after the first 72 h and is considered to have been contracted postnatally (9). The primary etiologies of EOS remain group B streptococcal (GBS) infection, despite a reduction in vertical transmission by intrapartum antibiotic prophylaxis, and *Escherichia coli*, which is increasing in very low birth weight (VLBW, <1,500 g birth weight) preterm neonates (3). In contrast, LOS is most commonly caused by coagulase-negative staphylococci and occurs primarily in VLBW neonates (3). The risk factors for EOS are more closely related to vertical maternal transmission (GBS colonization, premature rupture of membranes, maternal urinary tract infection, etc.), while LOS tends to be associated with the shortcomings of long-term critical care hospitalization (invasive procedures, intubation, prolonged indwelling catheters, interruption of natural barriers, etc.), as may be expected in very preterm and extremely preterm neonates (1).

The neonatal adaptive immune system lacks the capacity to support a robust response to infection (10) (**Figure 1**). The adaptive immune response in neonates differs dramatically from that of children and adults. Neonatal T cells have been categorized as being both anti-inflammatory and toleragenic, a functional phenotype that appears to be programed into the hematopoietic stem cell (HSC) development of neonates (11). Mold and colleagues have shown that different populations of HSCs are active at various stages of development, and neonates possess HSCs whose T cell lineage is biased toward tolerance (12). This premise

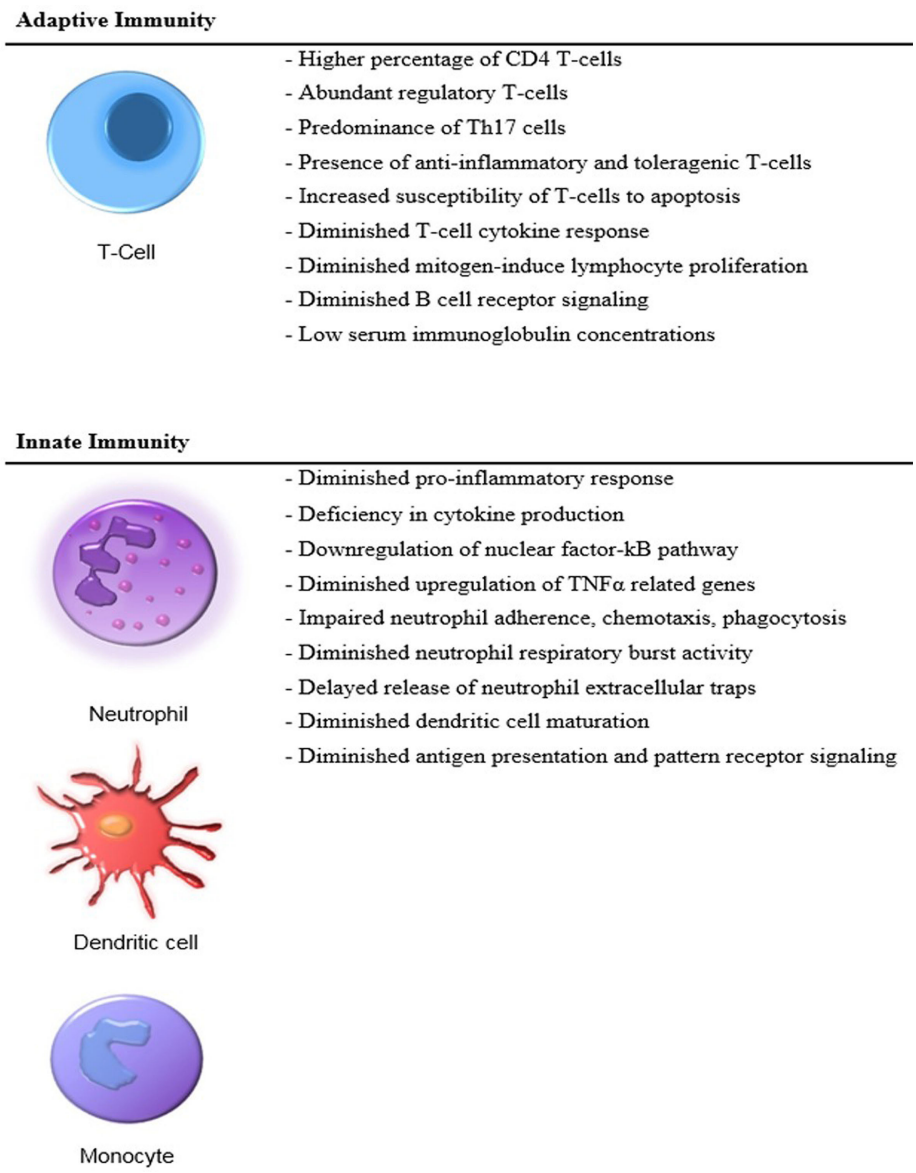


FIGURE 1 | **Distinct features of the neonatal immunity**.

is also consistent with the findings of Elahi who demonstrated that immature erythroid populations ($CD71^+$) found in neonates are immunosuppressive and concluded that these cells increase the risk of infection (13). However, Wynn et al. demonstrated that $CD71^+$ erythroid cell depletion, adoptive transfer of $CD71^+$ cells, or both (depletion followed by adoptive transfer) had no impact on murine neonatal polymicrobial sepsis survival, and $CD71^+$ cells in human neonates were revealed to be enucleated reticulocytes (14).

Because an adaptive immune response favors tolerance and contributes little to host protective immunity, the neonate predominantly relies on an immature innate immunity (15). These deficiencies are linked to the developmental age of the neonate and place the preterm infant at the greatest risk of developing sepsis (16). Despite a significant dependence upon innate immunity, neonates have an underdeveloped innate response to infection including decreased cytokine production and reduced neutrophil and dendritic cell function as compared to adults, which further increases the risk of developing bacterial, fungal, and viral infections (17, 18).

TRANSCRIPTOMIC RESPONSE TO NEONATAL SEPSIS

Since 1990 and the original sequencing of the human genome, the growth of genomic research has greatly expanded our understanding of the underlying response to infection in neonates. In the past decade alone, genome-wide association studies and transcriptomic analyses have become routine and are currently being used for precision medicine initiatives. Genome-wide transcriptomics in particular have become routine discovery tools to better understand tumor and host tissue responses, either by microarray or ribonucleic acid (RNA) sequencing.

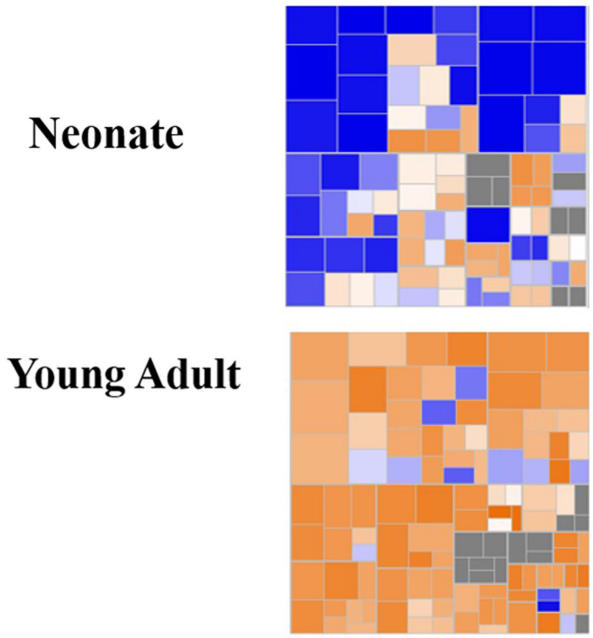
With these new tools have come new challenges (19). Bioinformatics have had difficulty keeping up with both the statistical approaches used, as well as the extraction of useable information from these vast amounts of data. Development of biomarkers based on these technologies has been especially challenging due to the initial inability to replicate the findings in confirmatory studies (20). There have been some novel approaches to overcoming the hurdles associated with high-throughput studies conducted in small data sets (21) and the difficulty in replicating the findings. Sweeney et al. have taken a meta-analysis approach using publically available datasets of septic adults and children to extract robust transcriptomic changes that are seen across a large number of individual smaller studies (22, 23).

Investigations into the transcriptomic responses to infection have led to identification of genomic alterations associated with both infection and developmental age (24, 25) and provided new approaches to diagnostics, prognostics, and therapeutics in sepsis (26). In this narrative review, we focus on the current understanding of the neonatal transcriptomic response to sepsis and how this knowledge may be used to improve our investigative and clinical approach to neonatal sepsis.

Among pediatric patients with septic shock, neonates demonstrate significant reduction in the change of gene expression representing key inflammation and immunity signaling pathways compared to infants, toddlers, and children (25). As expected, septic neonates have a significant upregulation, albeit attenuated, of several genes involved in innate immunity compared to uninfected neonates (24, 27, 28). The neonatal innate immune response to sepsis is driven by an increased expression of neutrophils and monocytes (28). By contrast, there is a net suppression of the adaptive immune response as characterized by a decrease in expression levels by T and B cells (28). Genome-wide expression profiles of neonates differ significantly between early- and late-onset sepsis (27), suggesting that the host immune response is determined in part by the postnatal age at the onset of sepsis. Interestingly, genome-wide expression profiles of VLBW neonates with sepsis also differ between Gram-positive and Gram-negative sepsis, suggesting that the neonatal response to sepsis varies depending on the inducing pathogen (24). Similar responses have been seen in adults when exposed *ex vivo* to Gram-negative and Gram-positive pathogens (29).

Studies on the genomic response of whole blood or enriched blood leukocytes from neonates confirm this attenuation of early inflammatory responses. Compared to infants and children, neonates with septic shock downregulate expression of key nuclear factor kappa B pathway-related genes (25). Neonatal mice likewise have an attenuated early inflammatory response to infection compared to adults, including a diminished upregulation of tumor necrosis factor alpha (TNFα)-related genes (30). This raises an interesting paradox. If neonates have a weakened early inflammatory response compared to adults, but early mortality is more common, there can only be a limited number of explanations. The first is that neonates are markedly more sensitive to the inflammatory signals produced early in response to microbial infection. Despite a diminished inflammatory response, that response is much more likely to result in endothelial injury, circulatory failure, organ injury, and death. An alternative explanation is that the attenuation in host inflammatory responses parallels an attenuation of host protective mechanisms. In this case, an early failure to control bacterial growth leads to rapid colonization of organs, cardiovascular failure, and death. Support for this latter hypothesis comes from genomic and functional studies on innate and adaptive immunity in neonates (25, 31). It is likely, however, that both processes are active.

The transcriptomic differences seen in human neonates are in agreement with transcriptional changes seen in murine models of polymicrobial sepsis in which neonatal mice exhibit increased mortality compared to young adult mice. These models demonstrate that neonates do not depend on an intact adaptive immune system to provide host protective immunity (15). In response to early sepsis, neonates also have decreased ability to recruit innate immune effector cells to the source of infection, with recruited cells having decreased ability to produce reactive oxygen species compared to adult mice (30). This is best revealed in **Figure 2** which examined whole blood leukocyte gene expression from young adult and neonatal mice. Young adult mice had the characteristic increase in the gene expression of proteins involved predominantly in inflammation and innate immunity. Not only were these increases absent in



Function	Predicted Activation State Z-score
Quantity of T lymphocytes	-4.449
Quantity of lymphocytes	-4.013
Quantity of mononuclear leukocytes	-3.493
Development of blood cells	-3.43
Development of lymphocytes	-3.417
Quantity of hematopoietic progenitor cells	-3.411
T cell development	-3.32
T cell homeostasis	-3.273
Development of mononuclear leukocytes	-3.225
Development of leukocytes	-3.215

Activation of neutrophils	3.75
Activation of granulocytes	3.675
Differentiation of phagocytes	3.291
Adhesion of immune cells	3.15
Chemotaxis of leukocytes	3.123
Proliferation of mononuclear leukocytes	2.91
Homing of leukocytes	2.874
Quantity of hematopoietic progenitor cells	2.866
Proliferation of B lymphocytes	2.856
Differentiation of myeloid cells	2.809

FIGURE 2 | Pathway analysis of gene expression from blood leukocytes of neonates and young adults subjected to sepsis. Modified from Gentile et al. (30). Copyright 2014. The American Association of Immunologists, Inc. Heat maps show the gene expression of the functional category hematological systems development and function. Blue represents pathways with an overexpression of genes leading to downregulation of the pathway, whereas orange represents pathways with an overexpression of genes whose activation will lead to upregulation of the pathway. The corresponding tables show the top 10 pathways and their corresponding *z*-score within the functional category. Blue indicates the pathway is significantly downregulated, and red indicates the pathway is significantly upregulated.

neonatal mice, but there was also a downregulation of genes involved in the development of an adaptive immune response.

Likewise, significant reductions in proinflammatory plasma cytokine concentrations exist among neonatal mice as compared to adult mice including interleukin (IL)-1α, IL-1β, IL-12p40, IL-13, TNFα, granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage inflammatory protein-1 beta, regulated on activation normal T-cell expressed and secreted, and interferon gamma (32). Analysis 24 h after sepsis reveals neonatal plasma cytokine concentrations are equivalent to young adults except for IL-1β and IL-12p40 which remain diminished. Based on functional pathway analysis, isolated leukocytes from neonatal mice have significant downregulation of pathways 24 h after sepsis corresponding to the development of leukocytes, quantity of T lymphocytes, and proliferation of immune cells (30). In addition to transcriptomic differences in the response to infection, the baseline transcriptome differed significantly between neonatal and adult mice leukocytes by 5,798 probe sets. (30). The representative genes demonstrate functional suppression of the adaptive immunity and down-regulation of genes in pathways involved with the activation of the innate immunity.

When evaluating these studies of sepsis in mice, it is important to note key differences between murine and human immunity. Whereas the predominating circulating leukocyte in humans is the neutrophil (50–70%), the lymphocyte is the predominating circulating leukocyte in mice (75–90%) (33). Also, neutrophils are an abundant source of defensins in humans, while mice lack these antimicrobial peptides (34). In addition to differences in innate immunity, humans and mice also exhibit differences in adaptive immunity. These differences include, but are not limited to, expression of Fc receptors, immunoglobulin isotypes and antibody class switching, and T cell receptor signaling and costimulation (35).

Many of these changes in gene expression are recapitulated phenotypically. Neonates exhibit defective neutrophil function compared to adults including adherence, chemotaxis, phagocytosis, neutrophil respiratory burst activity (NRBA), and release of neutrophil extracellular traps (NETs) (36–40). Merry and colleagues demonstrated that neutrophils from healthy neonates have impaired chemotaxis compared to healthy adults and even greater impairment was observed among septic neonates (39). At birth, neutrophil phagocytosis is significantly reduced in both preterm and term neonates as compared to adults; however, this impairment appears to resolve by 3 days after birth (38). This may further explain some of the observed differences in host response to early- and late-onset sepsis, although an additional explanation may be the use of umbilical cord blood (UCB) compared to peripheral blood in this study. UCB may be impacted by administration of antepartum antibiotics as well as the physiological stresses of labor. Healthy preterm neonates have fewer circulating neutrophils compared to term neonates (41) and exhibit a lower percentage of NRBA compared to term neonates and

adults (37). In samples from septic patients, both term and preterm neonates demonstrate diminished NRBA. Neonatal neutrophils additionally fail to form NETs following an hour of *ex vivo* lipopolysaccharide (LPS) stimulation, whereas adult neutrophils readily form NETs which bind and kill bacteria extracellularly (40). However, neonatal neutrophils began to form NETs following 2 h of LPS stimulation and nearly reach adult levels by 3 h of stimulation (42).

FUTURE DIRECTIONS

Successful modulation of the neonatal immune system to reduce the frequency of sepsis, sepsis mortality, and sepsis survivor morbidity would represent substantial advances in the field. Despite multiple attempts to modulate neonatal immunity in an effort to improve sepsis outcomes in neonates, most therapies have been largely unsuccessful to date (43). This is not particularly surprising given the equivalent failures of immune modulation in adult sepsis, which have prompted clinical trials with rapid molecular assessments of immune status and provision of the appropriate immunomodulatory strategy (anti-inflammatory or immune stimulation) (https://clinicaltrials.gov/ct2/show/NCT02576457). Previous immunomodulatory strategies which have been studied in neonates include granulocyte transfusions, administration of GM-CSF, intravenous immunoglobulin, activated protein C, glutamine, pentoxifylline, anti-endotoxin antibodies, probiotics, and breast milk (43) (**Table 1**). With the exceptions of breast milk and bovine lactoferrin, these therapies have shown minimal to no reduction in sepsis incidence, and no intervention is associated with an improvement in sepsis-related mortality (43).

Therapeutics aimed at preventing or treating neonatal sepsis must take into consideration the unique immunological status of the subject. One example of this is the observation that healthy preterm neonates have elevated blood concentrations of IL-18 (63), a proinflammatory member of the IL-1 superfamily. Healthy adults do not manifest high concentrations of circulating IL-18 (64), and their increase in sepsis is markedly lower than the increases seen in neonates (65). Targeting the IL-18/IL-17A axis through the use of neutralizing IL-17 receptor antibodies represents a novel approach to treating neonatal sepsis (59). While IL-18 is known to exert proinflammatory effects such as increased neutrophil phagocytosis and production of reactive oxygen species, paradoxically, IL-18-null (IL-18$^{-/-}$) neonatal mice demonstrated markedly improved survival and reduced bacteremia in comparison to wild-type neonatal mice when challenged with polymicrobial sepsis (59). Unlike human preterm neonates, healthy neonatal mice do not exhibit increased circulating IL-18. When IL-18 was given to septic mice to mimic the human condition, mortality was dramatically increased compared to septic mice alone and was associated with increased bacteremia, intestinal injury, and an increased systemic inflammatory response predominated by IL-17A. The deleterious effects of IL-18 on neonatal sepsis survival were dependent upon IL-1R1 signaling and IL-17A production by gamma delta cells in the intestine and lung (59). Compared to wild-type or isotype control treated mice, transgenic mice lacking IL-17A or wild-type mice that received antibody-mediated receptor blockade of IL-17A through IL-17A receptor or cytokine neutralizing antibodies exhibited markedly reduced mortality to sepsis (59). Currently, two antibodies directed against IL-17A (secukinumab and ixekizumab) have obtained FDA approval for use in other inflammatory diseases, and an additional antibody against IL-17A receptor (brodalumab) is currently awaiting final FDA approval. These therapies may prove beneficial in septic neonates.

The prevention rather than treatment of sepsis is an alternative approach and would be expected to have a significant benefit over the current reactive paradigm. Defined exposure to non-infectious components of bacteria that are recognized

TABLE 1 | Summary of strategies aimed at improving the neonatal immune response.

Strategy	Proposed mechanism	Reference
Granulocyte transfusions	Addition of functional granulocytes	(44)
GM-CSF, G-CSF	Stimulates proliferation, differentiation, and functional activity of myeloid precursors	(45, 46)
Intravenous immunoglobulin	Increase antibody titer and potential antigen–antibody interactions	(47–50)
Activated protein C	Anti-inflammatory and anti-coagulant properties	(51, 52)
Glutamine	Enhance function of immune cells	(53, 54)
Pentoxifylline	Inhibit release of TNFα	(55)
Anti-endotoxin antibodies	Inhibit deleterious effects of endotoxins	(56)
Probiotics	Maintain integrity of the intestinal barrier function	(57)
Breast milk	Provides immunoglobulin A, lactoferrin, oligosaccharides	(58)
Anti-IL17A, anti-IL17A receptor antibodies	Inhibit pathological proinflammatory effects of IL-18	(59)
Toll-like receptor agonists	Augment innate immunity	(15)
Topical emollient	Protect against skin breakdown, prevent pathogens entry	(60)
Lactoferrin	Iron sequestration, disruption of microbial cell membranes	(61)
FFP	Provides humoral immune factors	(62)

GM-CSF, granulocyte-macrophage colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor; FFP, fresh frozen plasma; IL, interleukin; TNFα, tumor necrosis factor alpha.

by the innate immune system is one approach being considered as a means to positively modify neonatal immune responses. Of the adjuvant therapies currently being studied, toll-like receptor (TLR) agonists appear most promising (66). TLRs play a vital role in early recognition of microbial invasion and activation of the innate immunity (67). TLRs are membrane-spanning receptors present on the cell surface and within intracellular vesicles of leukocytes and other non-immune cell populations such as endothelial cells and fibroblasts (68). Stimulation of these receptors occurs through the binding of ligands, specifically, pathogen-associated molecular patterns (PAMPs) (69). PAMPs may include components of either the bacterial or fungal cell wall or membrane such as LPS, peptidoglycan, or flagellin, or intracellular components such as single- or double-stranded RNA or deoxyribonucleic acid. Activation of these receptors induces downstream molecular signaling events which ultimately triggers the production of inflammatory cytokines, type I interferons, chemokines, and antimicrobial peptides (70).

Toll-like receptor agonists, in particular those affecting TLR 4 and TLR 7/8, have been shown to augment innate immunity, magnify but abbreviate the early systemic inflammatory response, reduce bacteremia, and increase survival to polymicrobial sepsis in neonatal murine models (15). Burl and colleagues also demonstrated that *in vitro* stimulation of newborn cord blood with TLR agonists led to significant production of TNFα, IL-6, IL-1β, and IL-10 (71). Interestingly, several first-generation inactivated and attenuated vaccines including rabies, typhoid, and Bacillus Calmette–Guérin (BCG) possess inherent TLR activity (72). Hence, the inclusion of adjuvants in vaccines has provided non-specific heterologous benefits and enhanced immune responses in traditionally poor-responding populations such as neonates. When BCG was administered at birth to newborns in sub-Saharan Africa, there was a 41% reduction in all-cause mortality at 12 months among VLBW neonates, which was attributed to fewer cases of neonatal sepsis, respiratory infections, and fever (73).

CONCLUSION

Neonatal sepsis is prominent cause of morbidity and mortality. A clear understanding of the neonatal response to sepsis represents a critical knowledge gap that greatly limits the opportunity to discover novel diagnostics and therapies to treat and potentially prevent this devastating disease. Evaluation of the transcriptomic response of blood leukocyte populations offers both a global view of the neonatal response to infection and sepsis, and the potential for identification of novel therapeutic opportunities, diagnostic tests, and prognostic markers. Accurate biomarkers would facilitate both targeted treatment, thus avoiding the overuse of empiric antibiotics in non-septic neonates, and an enrichment strategy that facilitates better selection of study participants for future clinical trials.

AUTHOR CONTRIBUTIONS

All the authors contributed extensively to the work presented in this paper. SR, JS, and JM wrote the manuscript. SL, JW, and LM gave conceptual advice and edited the manuscript.

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REVIEW
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Neonatal Meningitis: Overcoming Challenges in Diagnosis, Prognosis, and Treatment with Omics

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Neonatal meningitis is a devastating condition. Prognosis has not improved in decades, despite the advent of improved antimicrobial therapy and heightened index of suspicion among clinicians caring for affected infants. One in ten infants die from meningitis, and up to half of survivors develop significant lifelong complications, including seizures, impaired hearing and vision, and delayed or arrested development of such basic skills as talking and walking. At present, it is not possible to predict which infants will suffer poor outcomes. Early treatment is critical to promote more favorable outcomes, though diagnosis of meningitis in infants is technically challenging, time-intensive, and invasive. Profound neuronal injury has long been described in the setting of neonatal meningitis, as has elevated levels of many pro- and anti-inflammatory cytokines. Mechanisms of the host immune response that drive clearance of the offending organism and underlie brain injury due to meningitis are not well understood, however. In this review, we will discuss challenges in diagnosis, prognosis, and treatment of neonatal meningitis. We will highlight transcriptomic, proteomic, and metabolomic data that contribute to suggested mechanisms of inflammation and brain injury in this setting with a view toward fruitful areas for future investigation.

Keywords: meningitis, neonatology, cytokines, metabolomics, proteomics, transcriptomics

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INTRODUCTION

Meningitis is a life-threatening disease, affecting 0.1–0.4 neonates per 1,000 live births, with a higher incidence in preterm and chronically hospitalized infants (1, 2). Approximately 10% of affected infants die, and 20–50% of survivors develop seizures, cognitive deficiencies, motor abnormalities, and hearing and visual impairments (3). Despite declines in mortality, morbidity has not improved since the 1970s.

Rapid initiation of appropriate broad-spectrum antimicrobial therapy in response to suspected neonatal meningitis is critical to optimize outcomes (4). Empiric therapy often chosen in the setting of suspected early-onset sepsis/meningitis ensures coverage of Group B Streptococcus (GBS), *Listeria monocytogenes*, and Gram-negative organisms, such as *Escherichia coli* (4, 5). Suspected late-onset infections are typically treated with even broader antimicrobial therapy to cover additional organisms in the nosocomial environment, including *Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus*. Antibiotic therapy can only be narrowed if bacterial cultures reveal an offending organism, which hinges on swift, successful performance of a technically challenging, and sometimes risky, lumbar puncture (LP) prior to administration of antibiotics.

At a minimum, recommended therapy for meningitis lasts several weeks and may be longer based on organism or more extensive disease, such as ventriculitis or abscess formation (5). Infants with negative cultures despite a suspicion of meningitis are often managed conservatively and receive a full course of antibiotic therapy. In neonates, long-term antibiotic therapy is associated with emergence of resistant bacteria, superimposed fungal infections, and increased risk of necrotizing enterocolitis (6). Additional risks associated with long-term antibiotic therapy include need for central vascular access and its attendant complications (7). Thus, infants may sustain harm not only from meningitis but also from associated interventions.

Prognosis in the setting of neonatal meningitis is also fraught with difficulty. While outcomes are influenced by time to diagnosis and therapy, prognosis may also relate to virulence of the infecting pathogen. With our current understanding of meningitis, however, it is not possible to predict which infants will die, which infants will survive with disabilities, and which infants will survive with a normal neurodevelopmental outcome. Indeed, many infants still die, and many survivors still sustain lifelong morbidities, despite rapid initiation of appropriate antimicrobial therapy (8, 9).

Several adjuncts to antibiotic therapy have been proposed and tested to improve poor outcomes associated with meningitis. Steroid administration has not been shown to be beneficial as an adjunct to antimicrobial therapy in neonatal bacterial meningitis (10). While there is evidence for improved neurologic and auditory outcomes in pediatric *Hemophilus influenzae* B meningitis following steroid therapy, this pathogen is not a frequent cause of meningitis in neonates (11). The data surrounding benefit of steroids in pediatric or neonatal meningitis secondary to GBS, *E. coli*, *Streptococcus pneumonia*, and *Neisseria meningitidis* is unclear or poor, while long-term risks of exposing neonates to steroids are incompletely understood. Therefore, steroids are not recommended as adjunctive therapy in neonates evaluated for meningitis, unless there is strong suspicion for *H. influenzae* infection based on Gram stain or culture results. Trials of other adjunctive therapies, such as intravenous immunoglobulin and granulocyte-monocyte colony-stimulating factor, have also shown disappointing results.

To enhance outcomes, we must gain deeper insights into the pathophysiology of meningitis to identify diagnostic and prognostic tools and therapies that facilitate bacterial clearance but limit deleterious immune-mediated damage to brain tissue. Herein, we review challenges associated with the rapid and accurate diagnosis of neonatal meningitis. We discuss how a variety of large-scale datasets have extended our understanding of the host response to meningitis. Finally, we integrate the findings into a model to highlight new avenues for basic and translational investigations into the key immunologic pathways active in the setting of neonatal meningitis.

DIAGNOSTIC CHALLENGES IN NEONATAL MENINGITIS

Culture of cerebrospinal fluid (CSF) is the traditional gold standard for diagnosis of bacterial meningitis. However, deciding when to perform LP to obtain and analyze CSF is challenging. Factors complicating this decision include the non-specific signs and symptoms of meningitis in the infant, cardiorespiratory instability that may preclude positioning of an infant for LP, and considerable practice variation (12–15). Meningitis is estimated to occur in approximately 1–2% of suspected cases of sepsis within the first 72 h of life, or early-onset sepsis, though the risk is limited almost entirely to symptomatic infants (16). The American Academy of Pediatrics policy statement on suspected or proven early-onset bacterial sepsis supports performing LP as part of the sepsis evaluation of the neonate with symptoms concerning for early-onset sepsis, but recommends a more limited evaluation in the asymptomatic neonate with sepsis risk factors (17). In contrast to early-onset sepsis, evaluations for late-onset sepsis (after the first 72 h of life) are almost always performed in response to concerning signs and symptoms. Several studies have noted discordance between blood culture and CSF culture results, with negative blood cultures in up to a third of infants with bacterial meningitis, highlighting that this diagnosis could be missed if LP is deferred or not performed (13–15). These findings strongly support obtaining CSF *via* LP prior to antibiotic administration in neonates evaluated for late-onset sepsis. In infants with positive blood cultures, an LP is essential to guide duration of therapy and provide prognostic information.

Interpretation of CSF results is frequently problematic. If LP is delayed, and infants are exposed to empiric broad-spectrum antibiotics, clinical yield of bacterial culture of CSF can be compromised (18–20). In these situations, clinicians rely on interpretation of CSF parameters, such as cell count, glucose, and protein levels to presumptively diagnose meningitis. However, there is considerable overlap in laboratory evaluation of CSF between groups of infected and uninfected infants, leading to difficulty in establishing cutoff levels that possess sufficient sensitivity and specificity for the diagnosis of bacterial meningitis (14, 15). Many other factors influence interpretation of these values in neonates, including gestational age, postnatal age, and trauma sustained during LP causing contamination of CSF with blood (12, 14, 15, 18, 21).

Furthermore, diagnostic markers may differ depending on the gestational maturity of the infant and may confound analyses unless proper controls matched for gestational age are included. Additional studies are warranted to understand how these markers vary during normal development and to better inform how they vary in the context of neonatal meningitis. Indeed, many studies have highlighted differences in the immune system of newborns and infants relative to that of children and adults (22, 23). The premature infant may be additionally immune-compromised compared to full-term infants, due, in part, to deficiencies in innate and adaptive immune system (22). Clinically, these deficiencies manifest as increased risk for coagulase-negative *Staphylococcus* spp., *Staphylococcus aureus*, and *Candida* spp. Of note, however, in an investigation of infants with sepsis, Smith et al. found no significant differences in key genes activated or repressed in the blood of infants of varying gestational ages (24). These data suggest that some key immune pathways activated upon serious bacterial infection may be similar between the preterm and the full-term infant.

PATHOGEN-BASED EVALUATION OF CSF

A variety of approaches have been examined to improve the rapidity and fidelity of diagnosis of neonatal bacterial meningitis over conventional methods. These tests can be categorized broadly as: (1) identification of microbial signatures in CSF and (2) detection of a host response signature pathognomonic of sepsis and meningitis. There is much interest in development of bacterial nucleic acid-based polymerase chain reaction (PCR) assays for detection of common pathogens implicated in sepsis and meningitis (25). These tests have the potential advantage of rapid turnaround time compared to conventional microbiologic culture methods. Further, PCR-based testing has the ability to identify small amounts of nucleic acid signatures from non-viable bacteria, thereby improving diagnostic yield, especially in situations with low bacterial load, such as following antibiotic pretreatment (26). Investigators have studied conventional and real-time PCR methods, and several have employed broad-based bacterial PCR techniques directed against the 16S ribosomal RNA subunit that is conserved across bacterial species. However, in recent years, greater technical success has been noted with multiplex PCRs targeted to several common pathogens implicated in meningitis (27–29). Boriskin et al. reported the use of a focused microarray to detect and distinguish known genomic sequences of 13 viruses causing meningitis (30). Ben and colleagues employed a similar type of array to detect sequences of 20 common bacteria implicated in meningitis (31). Recently, a multiplex PCR assay has been approved by the FDA for detection of 14 pathogens, including *Escherichia coli* K1, *Streptococcus agalactiae*, *Listeria monocytogenes*, and *Haemophilus influenzae* (28, 29). Nonetheless, additional testing is required to determine the reliability of CSF PCR tests when compared with culture results in the management of bacterial meningitis in the clinical setting. To date, bacterial tests of sources other than CSF have shown disappointingly poor accuracy in diagnosis of meningitis (32).

CYTOKINE-BASED EVALUATION OF CSF

The second approach to developing diagnostic tests is based on the premise that serious infections such as meningitis elicit a specific host immune and/or metabolic response that could represent a "host signature" pathognomonic of infection (33–36). Several studies of pediatric and neonatal meningitis have evaluated cytokine levels and other candidate biomarkers in CSF for their diagnostic utility (**Table 1**). In a prospective cohort of infants with suspected bacterial meningitis, Srinivasan et al. noted that interleukin-6 and interleukin-10 possessed the best area under the curve (AUC) in receiver operating characteristic analyses of multiple cytokines [**Table 1** and Ref. (33)]. In a study of 140 subjects with pediatric meningitis, Ye et al. identified interleukin-6 alone and the ratio of CSF/blood interleukin-6 as useful diagnostic markers [**Table 1** and Ref. (37)]. However, not all investigations of interleukin-6 have replicated this success. Mukai and colleagues noted poorer diagnostic accuracy with interleukin-6 compared to tumor necrosis factor-α, and Hsieh's group demonstrated that interleukin-6 had good sensitivity but poorer specificity than interleukin-12 [**Table 1** and Ref. (38, 39)]. Prasad et al. also noted that tumor necrosis factor-α and interleukin-8 provided excellent ability to discriminate bacterial meningitis, and outperformed interleukin-6, in their cohort of 87 pediatric patients [**Table 1** and Ref. (40)]. Ours and an additional group concluded that, while tumor necrosis factor-α had good specificity for bacterial meningitis in infants and children, sensitivity was less promising [**Table 1** and Ref. (41, 42)]. Challenges noted by investigators include the short half-life of many cytokines with only transient elevations in CSF in the setting of bacterial meningitis. The timing of LP in relation to onset of illness, therefore, becomes a crucial factor in interpretation of levels. As noted previously, however, it is often a challenge to obtain LPs early in the course of illness in unstable infants.

Biomarkers, or combinations of biomarkers, that demonstrate not only early but sustained elevations over the course of infection may be better candidates for diagnostic tests. Alterations in damage-associated molecular patterns (DAMPs) that aid in perpetuation of the inflammatory response may provide more stable measurement targets. S100-family proteins are DAMPs associated with innate immune activation (43). S100B showed early promise as a biomarker for meningitis due to its concentration in astrocytes and glia (44, 45). Further investigations, however, revealed that S100B in serum or CSF exhibited suboptimal sensitivity and specificity for bacterial meningitis. The highest, but still inconsistent, levels of S100B in CSF and serum were seen in viral encephalitis or in bacterial meningitis complicated by ventriculitis or obvious parenchymal abnormalities on brain imaging (44, 46). While many candidate cytokine and immunologic markers have shown promise as diagnostic tools in small studies, some have demonstrated conflicting findings across studies (**Table 1**). Thus, such markers have not been accepted into clinical practice as adjunctive tests for diagnosis of meningitis. These data emphasize the need for novel investigations into other classes of potential biomarkers to enhance diagnosis of meningitis beyond our current capability with clinical judgment and classical laboratory parameters.

OMICS TO EXTEND KNOWLEDGE OF BACTERIAL MENINGITIS

Recently, investigators have embarked on exploratory approaches, taking advantage of advanced "omics" technologies to more deeply understand serious bacterial infection in infants, children, and adults (34–36, 47). Omics denotes the comprehensive investigation of any family of biologic molecules, including DNA (genomics), RNA transcripts (transcriptomics), proteins (proteomics), and metabolites (metabolomics), among others. In other words, omics are used to interrogate hundreds to thousands of molecules simultaneously from a body fluid or tissue, promoting generation of pathways or networks of molecules associated with presence or absence of a disease state, such as infection. Omics approaches can provide new insights into diagnosis, pathophysiology, risk stratification and prognostication, and potential therapeutic targets (48). Systems-level investigations into host–pathogen interactions involving many cells types have informed countless translational studies of complex human diseases. Bacterial

TABLE 1 | Studies investigating diagnostic accuracy of cerebrospinal fluid (CSF) cytokines in pediatric and neonatal meningitis.

Reference	Study population	Biomarkers evaluated	Findings
Srinivasan et al. (33)	Overall: 684 patients <6 months Bacterial meningitis: *n* = 11 Not meningitis: *n* = 151 Indeterminate (antibiotic pretreated): *n* = 513	CSF levels of TNF-α, IL-1, IL-6, IL-8, IL-10, IL-12	IL-6 and IL-10 had best area under the curve (AUC) when bacterial meningitis was compared with controls; some indeterminate infants had cytokine patterns similar to infants with bacterial meningitis TNF-α: AUC 0.88 IL-1: AUC 0.86 IL-6: AUC 0.91 IL-8: AUC 0.89 IL-10: AUC 0.91 IL-12: AUC 0.63
Ye et al. (37)	Overall: 814 patients <18 years Bacterial meningitis: *n* = 140 [derivation cohort (DC): *n* = 71; validation cohort (VC): *n* = 69] Healthy controls: *n* = 180 Viral encephalitis: *n* = 182 Epilepsy: *n* = 146 Febrile convulsions: *n* = 166	CSF levels of IL-6, IL-10 CSF/blood ratios of IL-6 and IL-10	Bacterial meningitis versus all others IL-6: AUC: 0.988 (DC); 0.985 (VC) IL-10: AUC 0.949 (DC); 0.938 (VC) CSF/blood IL-6 ratio: 0.995 (DC); 0.993 (VC) CSF/blood IL-10 ratio: 0.924 (DC); 0.912 (VC)
Prasad et al. (40)	Overall: 87 patients <14 years Bacterial meningitis: *n* = 57 Viral meningitis: *n* = 15 Not meningitis: *n* = 15	CSF levels of TNF-α, IL-6, IL-8	Bacterial meningitis versus controls TNF-α: AUC: 1 IL-6: AUC: 0.947 IL-8: AUC: 1 Bacterial meningitis versus viral meningitis TNF-α: AUC: 0.961 IL-6: AUC: 0.853 IL-8: AUC: 0.941
Hsieh et al. (39)	Overall: 95 patients <15 years Bacterial meningitis: *n* = 12 Aseptic meningitis: *n* = 41 Not meningitis: *n* = 42	CSF levels of IL-6, IL-12	IL-6: Sensitivity: 96%, specificity: 51% IL-12: Sensitivity 96%, specificity 75%
Mukai et al. (38)	Overall: 35 patients <12 years Bacterial meningitis: *n* = 6 Aseptic meningitis: *n* = 13 Not meningitis: *n* = 16	CSF levels of TNF-α, IL-6	TNF-α was detected in all bacterial meningitis cases and in 84.6% of the children with aseptic meningitis IL-6 did not enable differentiation between bacterial and aseptic infection
Tang et al. (42)	Overall: 171 specimens from 144 patients <14 years Bacterial meningitis: *n* = 23 Aseptic meningitis: *n* = 26 Not meningitis: *n* = 95	CSF levels of IL-1β, TNF-α	IL-1β: Sensitivity 78%, specificity 96% TNF-α: Sensitivity 74%, Specificity 81%
Dulkerian et al. (41)	Overall: 62 patients <6 months Bacterial meningitis: *n* = 20 Aseptic meningitis: *n* = 22 Not meningitis: *n* = 20	CSF and plasma levels of IL-6, TNF	IL-6 (CSF): Sensitivity 100%, NPV 100% TNF (CSF): Sensitivity 60%, specificity 100%

meningitis is a complex infectious disease amenable to omics analyses to generate new hypotheses into mechanisms underlying inflammation and brain injury. A common obstacle to rigorous mechanistic studies of many human disorders, use of primary human tissue to study meningitis is generally not possible. CSF is an attractive source of information, as it communicates directly or indirectly with brain-resident cells, such as neurons, astrocytes, glia, and the blood–brain barrier. CSF is also in direct contact with infiltrating cells of the immune system recruited to the brain space in the setting of meningitis. Thus, CSF contains hosts of transcripts, proteins, and metabolites that, together, can paint a clearer picture of a disease process whose morbidity and mortality remain unacceptably high despite early recognition, definitive treatment with appropriate antibiotic therapy, and intensive care. Herein, we note advances made with model systems of human meningitis, but we will focus on transcriptomic, proteomic, and metabolomic work performed in human patients with bacterial meningitis.

TRANSCRIPTOMICS

Transcriptomics reveals genome-wide changes in gene expression by individual cells or populations of cells and often forms the backbone for gene-targeted analyses in animal models. Transcriptomics has evolved as an increasingly affordable and fruitful analysis, thanks in large part to several large, well-curated databases capable of organizing results into networks of known pathways. Numerous transcriptomic analyses in model systems of bacterial meningitis have utilized cultured human brain microvascular endothelial cells and cultured primary meningioma cells

(49, 50). When cultured appropriately, such cells can recapitulate the blood–brain barrier *in vitro*. Both systems have been used to understand changes in host gene expression occurring upon contact with bacterial pathogens, such as *N. meningitidis* (51–53).

Animal models of meningitis have also provided great insight into the pathophysiology of neonatal meningitis, dating back to the first *in vivo* model in piglets to show disruption of the blood–brain barrier (54). Currently, rodent models of neonatal meningitis are often used to model human bacterial meningitis (55–58). One study by Coimbra and colleagues used a high-fidelity infant rat model of pneumococcal meningitis to separately profile the transcriptomes of the cortex and hippocampus in the early and late phases of disease (59). This model faithfully replicates much of the pathophysiology found in humans insofar as infant rats suffer similar cortical ischemia with resultant necrosis and hippocampal apoptosis as do human neonates who contract bacterial meningitis (60). Many genes associated type 1 inflammation and toll-like receptor activation, such as interleukin-6, interleukin-18, and STAT1, were increased in both the cortex and hippocampus of infant rats after induction of meningitis (59). Consistent with the distinct mechanisms of injury in the cortex and hippocampus, however, Coimbra et al. found differential regulation of genes associated with toll-like receptor signaling and innate immune activation, including CD14 and tumor necrosis factor-α, in each brain region. Supporting histologic findings, pro-apoptotic transcripts were upregulated selectively in the hippocampus, while increased expression of tropomyosin selectively in the cortex may reflect excess vasoconstriction and ischemia.

Another intriguing set of transcripts found to be upregulated in the cortex and hippocampus of infant rats with meningitis were several associated with complement activation, including C1q, C3, and C4 (59). In addition to a canonical role for complement in bacterial clearance, recent work has highlighted an additional role for complement in crosstalk with astrocytes and microglia to eliminate neuronal synapses (61–63). Indeed, a transcript indicative of activation of astrocytes (glial fibrillary acidic protein, or GFAP) was similarly upregulated in both the cortex and hippocampus of infant rats with meningitis. While clearance of synapses in the setting of bacterial meningitis has not been formally addressed, it is tempting to speculate that this may represent a critical mechanism of brain injury. Altogether, these data highlight the critical importance of animal models to our understanding of bacterial meningitis in human neonates. They also shed light on potential therapeutic targets as we gain greater knowledge of appropriate and maladaptive immune responses to bacterial meningitis.

While the transcriptome of brain tissue or CSF has not been examined in humans with meningitis, profiling of the transcriptome of whole blood in human patients with bacterial meningitis was performed by Lill and colleagues (64). Although the bulk of their study cohort was composed of adult patients, three neonates were included in the analysis: two with GBS meningitis and one with *E. coli* meningitis. Of note, clustering analysis of their data revealed that the neonates with meningitis had distinct whole blood transcriptomic profiles from the adults with meningitis. While adults with meningitis had a distinct whole blood transcriptome compared to adults without infection, the whole blood transcriptome of two out of three neonates most closely resembled that of control adults. This emphasizes the need for infant-specific studies of meningitis, as the neonatal and adult host responses to infection vary substantially (23, 65).

In the setting of bacterial meningitis, the most significantly changed individual and networks of transcripts in the blood were those associated with innate and adaptive immunity (64). Interestingly, type 2 immunity-associated genes, including interleukin-5 receptor, Fc-epsilon receptors, and markers of mast cell activation, were among the most upregulated in the blood of patients with bacterial meningitis relative to controls. These data support findings that type 2 immune responses are induced in humans and animal models with serious bacterial infection (66). In fact, type 2 immune signaling to macrophages drives better outcomes in animal models of sepsis and is associated with reduced severity of disease in humans with sepsis (66, 67). The data also are congruent with work to profile the inflammatory cytokine milieu in CSF of infants and children with bacterial meningitis, described above, showing type 2 cytokines interleukin-4 and interleukin-13 significantly upregulated in the CSF of infants and children with bacterial meningitis compared to controls (33, 68). Future studies will be needed to understand the significance of type 2 immunity in the setting of bacterial meningitis, especially in neonates who often exhibit blunted type 1 immune responses (65).

Notably, the study by Lill and colleagues (64) represents a first step to detect a signature of bacterial meningitis in the blood, outside of the brain space. Such lines of investigation may drive discovery and development of minimally invasive tools to diagnose bacterial meningitis, one day obviating the need for LP.

PROTEOMICS

One caveat of transcriptomics is that some transcripts may not be translated into protein due to posttranscriptional modifications or regulation by non-coding RNA species. Transcriptomics also has limited clinical utility in the intensive care unit, as detection of mRNA transcripts can be expensive and time consuming. Proteomics offers the promise of novel biomarker discovery that may easily translate to clinical applications. Jesse and colleagues (69) applied the first large-scale proteomic approach to the study of bacterial and viral meningitis in adults. Analyzing CSF from adult patients with fluorescent two-dimensional difference gel electrophoresis, the authors aimed to find a protein signature of bacterial meningitis that differentiates it from viral meningitis and from controls. Six proteins were identified as candidate markers of bacterial meningitis in a pilot experiment and were subsequently validated in a larger cohort of patients. Interestingly, GFAP was one such protein marker specific to bacterial meningitis that was also identified by Coimbra et al. as an increased transcript in the setting of bacterial meningitis in infant rats (59). Activation of astrocytes and expression of GFAP can be induced by pro-inflammatory cytokines, such as interleukin-6 (70). Another protein marker of bacterial meningitis identified by Jesse et al. was Prostaglandin-H2 D-isomerase or prostaglandin D synthase (69). Recently, prostaglandin D

synthase was implicated as a marker of potent T helper 2 cells that strongly elaborate type 2 cytokines, including interleukin-4, interleukin-5, and interleukin-13 (71). These data invoke the transcriptomic findings of Lill and colleagues, who showed increased levels of transcripts associated with type 2 immunity in the serum of patients with bacterial meningitis (64). As evidenced by this work, multiple omics modalities can be combined and used to link data across multiple studies and disciplines.

Using similar methodology to that of Jesse et al., Goonetilleke and colleagues performed comparative two-dimensional polyacrylamide gel electrophoresis to characterize the proteome of CSF from adult humans with pneumococcal meningitis (72). The focus of their analysis was to identify markers differentially regulated in survival versus death. Among the proteins relevant to the immune response, complement C3 was decreased in non-survivors, suggesting impaired complement-mediated bacterial clearance in the brain space or, possibly, impaired clearance of injured neurons by astrocytes and microglia (61–63), as discussed above. Another interesting finding was an increase in chitotriosidase in CSF of non-survivors. Chitotriosidase is associated with activation of macrophages, especially those exposed to type 2 inflammatory cytokines (73). As discussed above, there are several pieces of data supporting robust type 2 inflammation in bacterial meningitis, but whether excessive type 2 immune bias in the setting of bacterial meningitis is maladaptive and associated with poor outcomes remains to be investigated. The data of Goonetilleke et al. should be interpreted with some caution, as all patients with meningitis were coinfected with HIV in this study, but the interesting findings begin to address a critical need to predict not only diagnosis but also prognosis of bacterial meningitis. This is especially important in the neonatal population, whose potential disabilities due to meningitis are numerous and would benefit from early subspecialist involvement and intervention.

Cordeiro et al. presented the most recent proteomic data in the setting of meningitis, with a goal to differentiate among control subjects, those with pneumococcal meningitis, those with meningococcal meningitis, and those with enteroviral meningitis (74). The cohort consisted of adults and children, and CSF was analyzed using two-dimensional protein gel electrophoresis. The authors propose an algorithm to predict bacterial meningitis based on elevated apolipoprotein A1, also found to be elevated in bacterial meningitis by Song and colleagues (75), and complement C3, which participates in clearance of encapsulated microbes and in clearance of neuronal synapses (61–63). Once classified as bacterial meningitis, kininogen-1 was identified to discriminate between pneumococcal and meningococcal disease, though kininogen-1 is a pleiotropic effector molecule with possible, but poorly defined, effects on pro-inflammatory cytokine signaling, neurotransmission, and blood–brain barrier function (74). Overall, proteomic approaches to the study of bacterial meningitis are powerful and may lay the groundwork for future translational studies and clinical assays to improve rapidity and fidelity of diagnosis. While pediatric subjects were more represented in the study by Cordeiro and colleagues, there remains an absence of work dedicated to comprehensively addressing the proteome of neonates with bacterial meningitis.

METABOLOMICS

While approaches to simultaneously evaluate multitudes of metabolites have become available only recently, metabolic analysis of CSF is nearly a century old (76). Consistent with presumed cellular stress and high metabolic demand, elevated levels of lactate and decreased levels of glucose were among the first markers to be associated with meningitis. Since the original studies, lactate has been well validated as a highly accurate marker of bacterial meningitis (77). Additional investigations into the CSF metabolome of patients with meningitis have been performed, with a goal of discovering novel biomarkers of disease and uncovering aberrantly regulated pathways contributing to maladaptive inflammation and brain injury. Metabolomics offers an advantage over proteomics and transcriptomics in that metabolites are the final products of active enzymatic reactions in a cell or group of cells. In other words, metabolomics most accurately reflects the true cellular phenotype without having to consider posttranscriptional or posttranslational modifications that may add layers of complexity to transcriptomic or proteomic datasets, respectively. Challenges to pursuing metabolomic work, however, include expense, non-standardized modalities to acquire data, and still-evolving databases with which to compare and contextualize results.

Several studies of CSF in humans with bacterial meningitis employ proton nuclear magnetic resonance (NMR) spectroscopy to study a varied number of metabolites (78–80). Using NMR spectroscopy, Subramanian et al. investigated whether metabolites could distinguish CSF of children with tuberculous meningitis from CSF of children with non-tuberculous bacterial meningitis, viral meningitis, or no meningitis (79). One fascinating finding was detection of the metabolite cyclopropane specifically in CSF from children with tuberculous meningitis (79). Modifications to mycolic acids with cyclopropane in the cell wall of *M. tuberculosis* are critical for virulence (81). This piece of data highlights the potential of metabolomics to identify key pathways relevant to diagnosis and pathophysiology of bacterial meningitis.

In the study by Subramanian et al., other metabolites significantly elevated in CSF of those with bacterial meningitis relative to viral meningitis or controls included urea, creatine, alanine, citrate, pyruvate, acetoacetate, and beta-hydoxybutyrate (79). These data, particularly elevations in acetoacetate and beta-hydroxybutyrate, suggest increased ketosis and an increase in circulating free fatty acids that are subsequently metabolized into ketone bodies. Interestingly, poor outcomes in a cohort of adults with sepsis have been associated with increased levels of fatty acids in the serum (82). Further underscoring the importance of fatty acid oxidation in the immune response to infection, pro-inflammatory responses have been linked to activation of glycolytic pathways, while oxidation of fatty acids and ketosis has been linked to anti-inflammatory pathways in neonates with sepsis (22, 24). Altogether, these data may reflect inappropriately blunted immunity that may predispose to poor outcomes after serious bacterial infection, including sepsis and meningitis.

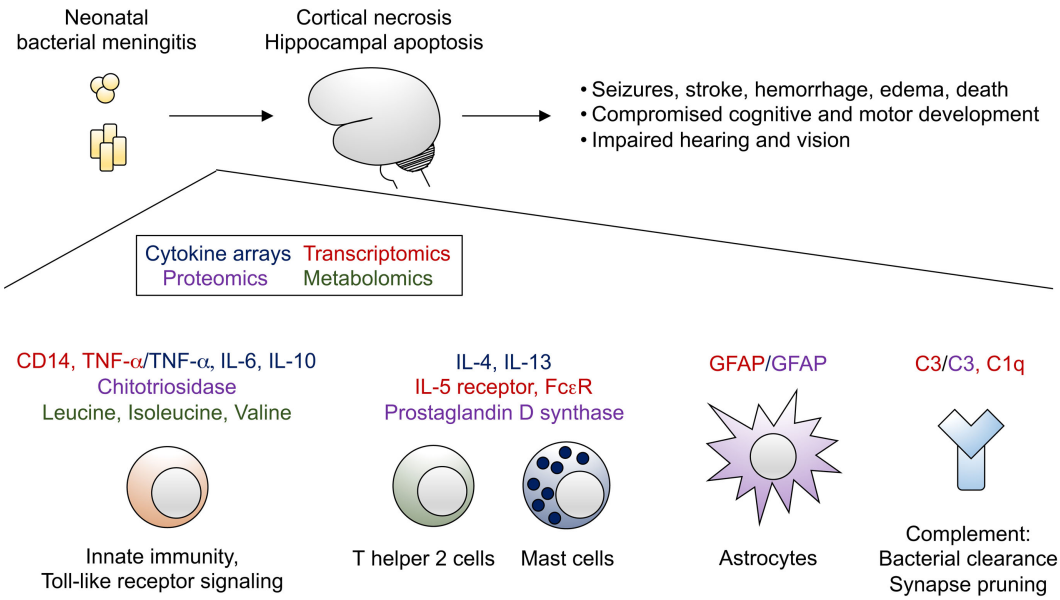

FIGURE 1 | Bacterial meningitis of the neonate leads to profound brain injury, characterized by cortical necrosis and hippocampal apoptosis. Selected transcripts (highlighted in red), cytokines (highlighted in blue), other proteins (highlighted in purple), and metabolites (highlighted in green) identified from omics analyses have been integrated into a proposed model of immune activation in the setting of bacterial meningitis.

Overall, Subramanian et al. quantified twelve metabolites but distinguished non-tuberculous bacterial meningitis from controls with sensitivity and specificity of only 74 and 67%, respectively. When clinical variables were also considered, however, the model's sensitivity and specificity rose to nearly 100%. These data speak to the value of combining multiple modalities in omics research, an approach recently used to create a high-fidelity clinical-metabolomic model of outcomes after bacterial sepsis in adult humans (82).

Coen et al. evaluated the CSF metabolome of mainly adult humans with bacterial, fungal, and viral meningitis, as compared to that of control adults and those with indwelling neurosurgical hardware (80). Of note, the cohort did include two infants with *S. agalactiae* (Group B Strep) meningitis. The group addressed a critical question relevant to the population in the NICU, as a small but significant proportion of infants born extremely prematurely will develop intraventricular hemorrhage and, subsequently, posthemorrhagic hydrocephalus. Using NMR spectroscopy to ascertain metabolic composition of CSF, Coen and colleagues were able to distinguish patients with bacterial meningitis from those with viral meningitis and those with no meningitis but with indwelling hardware. The most "influential" metabolites used to separate experimental groups were glucose and lactate, with additional contribution from beta-hydroxybutyrate, pyruvate, acetate, acetone, isoleucine, leucine, and valine. Of note, branched-chain amino acids, especially valine, have been shown to promote maturation, antigen presentation capability, and cytokine production of innate immune cells (83). The investigators were not able to distinguish CSF infected with Gram-positive organisms from CSF infected with Gram-negative organisms (80), in contrast to the findings of Lill and colleagues, who found unique changes in the plasma transcriptome of patients with bacterial meningitis due to pneumococcus relative to those with meningitis due to other bacterial organisms (64). This is perhaps due to small sample size, though it is feasible that the active metabolic pathways and mechanisms of cellular injury contributing to the CSF metabolome may not vary substantially among different microorganisms.

Although expensive, mass spectrometry-based approaches to metabolomics are alternatives to NMR spectroscopy and are capable of characterizing hundreds of metabolites from body fluids and tissues (84). No studies utilizing mass spectrometry-based metabolomics have been performed in patients, especially infants, with bacterial meningitis. To fill this gap in knowledge and complement known transcriptomic and proteomic data, it will be necessary to more comprehensively investigate the metabolome of CSF in model organisms and in human infants with bacterial meningitis.

CONCLUSION

Despite improvements in neonatal intensive care and timely administration of appropriate broad-spectrum antimicrobial agents, bacterial meningitis remains a major cause of morbidity and mortality in the neonatal period. Our understanding of mechanisms of neonatal brain injury due to meningitis remains limited. Although use of cytokines as biomarkers of meningitis has not achieved sufficient accuracy to be employed in clinical practice, these data complement a wealth of omics data from humans and model organisms with bacterial meningitis that now exist. Efforts to integrate these data sets and assemble pathway maps of genes, proteins, and metabolites altered in the

setting of bacterial meningitis (**Figure 1**) will be of great benefit to advance the field. Subsequent approaches combining omics analyses of primary human tissue (e.g., CSF) with targeted studies in animal models will be required to understand the complex interplay among the numerous infiltrating and brain-resident cell types affected by bacterial meningitis. Such work can drive development of novel, rapid diagnostics and adjunctive therapies that may prevent the devastating, lifelong sequelae of bacterial meningitis in infants.

AUTHOR CONTRIBUTIONS

SG, LS, and MH jointly conceived, wrote, and critically revised the manuscript.

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A Meningococcal Outer Membrane Vesicle Vaccine Incorporating Genetically Attenuated Endotoxin Dissociates Inflammation from Immunogenicity

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Background: Group B *Neisseria meningitidis*, an endotoxin-producing Gram-negative bacterium, causes the highest incidence of group B meningococcus (MenB) disease in the first year of life. The Bexsero vaccine is indicated in Europe from 8 weeks of age. Endotoxin components of outer membrane vesicles (OMVs) or soluble lipopolysaccharide (LPS) represent a potential source of inflammation and residual reactogenicity. The purpose of this study was to compare novel candidate MenB vaccine formulations with licensed vaccines, including Bexsero, using age-specific human *in vitro* culture systems.

Methods: OMVs from wild type- and inactivated *lpxL1* gene mutant-*N. meningitidis* strains were characterized in human neonatal and adult *in vitro* whole blood assays and dendritic cell (DC) arrays. OMVs were benchmarked against licensed vaccines, including Bexsero and whole cell pertussis formulations, with respect to Th-polarizing cytokine and prostaglandin E2 production, as well as cell surface activation markers (HLA-DR, CD86, and CCR7). OMV immunogenicity was assessed in mice.

Results: Δ*lpxL1* native OMVs (nOMVs) demonstrated significantly less cytokine induction in human blood and DCs than Bexsero and most of the other pediatric vaccines (e.g., PedvaxHib, EasyFive, and bacillus Calmette–Guérin) tested. Despite a much lower inflammatory profile *in vitro* than Bexsero, Δ*lpxL1* nOMVs still had moderate DC maturing ability and induced robust anti-*N. meningitidis* antibody responses after murine immunization.

Conclusion: A meningococcal vaccine comprised of attenuated LPS-based OMVs with a limited inflammatory profile *in vitro* induces robust antigen-specific immunogenicity *in vivo*.

Keywords: group B meningococci, outer membrane vesicles, vaccine, newborn, dendritic cells

INTRODUCTION

Human newborns and infants suffer a high frequency of infection compared to older children and adults (1), in part due to distinct early life immunity with impaired host defense (2). Early life immunization is desirable, but vaccine-induced responses of newborns and young infants demonstrate slow initiation, low immunogenicity, and reduced persistence of functional antibodies and cell-mediated responses (2). Although the majority of global immunization schedules are focused on the pediatric age group, development of early life vaccines has been hampered by this distinct immunity and an *ad hoc* approach to developing vaccines, relying on adult-derived results that may inaccurately predict infant responses (3).

Neisseria meningitidis (meningococcus) is a Gram-negative, endotoxin-producing organism that is a normal commensal of the human nasopharynx and is an important cause of invasive bacterial infection in children worldwide (4). Recent immunization programs with capsular-polysaccharide vaccines have dramatically reduced the incidence of serogroup C and A meningococcal disease in North America, Europe, and Africa (5). However, meningitis and septicemia caused by serogroup B meningococci remain a major health concern in young children, as similar capsule-based vaccines cannot be developed against this serogroup (6). Outer membrane vesicle (OMV)-based vaccines have historically been used, with some success, to control outbreaks of disease caused by serogroup B meningococci. OMVs are produced by the blebbing of membranes of clinical-derived live Gram-negative bacteria during *in vitro* growth and are useful vaccine components, as immuno-stimulatory membrane components [lipids, proteins, lipopolysaccharide (LPS), etc.] from meningococci are represented (7). As OMV yield from culture alone is too low for vaccine production, detergent extraction is used to force vesiculation and increase yield and has the added advantage of reducing LPS content to prevent overt reactogenicity. Novartis' 4-component aluminum hydroxide (Alum)-adjuvanted group B meningococcus (MenB) vaccine (4CMenB, Trade name: Bexsero) has been licensed by both the European Medicines Agency and the U.S. Food and Drug Agency and comprised OMVs and three recombinant immunogenic *N. meningitidis* proteins identified by reverse vaccinology (8). Another meningococcal serogroup B vaccine, Trumenba, contains two recombinant proteins with no OMV component, is approved in the U.S. for use in individuals 10 through 25 years of age, but lacks the potentially broader antigen repertoire inherent to OMVs.

The currently licensed meningococcal vaccines have the potential to reduce mortality and morbidity associated with MenB infections, but do have some limitations. Although detergent extraction of OMVs removes the majority of the LPS, the remaining endotoxin, particularly the soluble LPS, may still result in residual reactogenicity, necessitating the use of the Alum to ameliorate excess toxicity (9). Even so, immunization with the OMV-containing Bexsero may correlate with rates of reactogenicity (e.g., fever ≥38°C, tenderness at injection site) as common as 10% in infants less than 1 years of age (10), and severe reactogenicity is reported in some cases (11). Bexsero may also enhance reactogenicity when given together with other vaccines (12), prompting some primary care physicians to prescribe anti-inflammatory agents (13) to prevent potential reactions. Furthermore, as conventional assays for preclinical and release testing of vaccines, such as the rabbit pyrogenicity test and *Limulus* amebocyte lysate (LAL) assay, were developed for vaccines containing no or negligible amounts of endotoxin, predicting the reactogenicity of LPS-containing OMV vaccines is difficult and requires more sophisticated models (14).

To overcome the potential reactogenicity of wild-type (WT) OMV-based vaccines while maintaining the inherent adjuvant activity of vesicles, a vaccine has previously been developed using a *N. meningitidis* strain with genetically attenuated endotoxin (15). This enabled the use of detergent-free manufacturing processes to produce native OMVs (nOMVs) (16). To assess the potential of such OMV vaccines, we reasoned that it would be important to better understand their interactions with human leukocytes, including dendritic cells (DCs). DCs are professional antigen-presenting cells (APCs) that play a vital role in shaping adaptive immunity. DC maturation begins when endogenous or exogenous danger molecules are recognized by pattern recognition receptors [e.g., *Toll*-like receptors (TLRs)] triggering upregulation of costimulatory molecules and production of immune polarizing cytokines (17). Of note, in addition to interactions *via* TLR4, *N. meningitidis* LPS also directly interacts with DCs through the C-type lectin receptor DC-SIGN and modulates their function (18).

In this study, we benchmarked novel candidate MenB vaccine formulations against licensed vaccines, including Bexsero, using physiologically relevant human neonatal and adult whole blood (WBA) and monocyte-derived dendritic cell (MoDC) *in vitro* culture systems, both of which employ age-specific autologous plasma, a rich source of age-specific immunomodulatory molecules (2, 19). We assessed vaccine-induced production of Th-polarizing cytokines, upregulation of surface activation markers (20), and production of potential vaccine reactogenicity biomarkers such as IL-1β and prostaglandin E2 (PGE_2) (3, 21, 22). We found that a meningococcal vaccine comprised of attenuated LPS-based OMVs with a relatively low inflammatory profile toward human leukocytes *in vitro* induced robust antigen-specific immunogenicity *in vivo*. Our observations, may inform translational development of MenB vaccines in high-risk populations, such as newborns and infants.

MATERIALS AND METHODS

Ethics Statement

Peripheral blood samples were collected after written informed consent from healthy adult volunteers with approval from the Ethics Committee of Boston Children's Hospital, Boston, MA, USA (X07-05-0223). Non-identifiable cord blood samples were collected immediately after elective cesarean section delivery (epidural anesthesia) of the placenta. Births to HIV-positive or febrile mothers were excluded. Human experimentation guidelines of the U.S. Department of Health and Human Services, the Brigham and Women's Hospital, Beth Israel Medical Center, and Boston Children's Hospital were observed, with approval

from the Ethics Committee of The Brigham and Women's Hospital, Boston, MA, USA (protocol number 2000-P-000117) and Beth Israel Deaconess Medical Center Boston, MA, USA (2011P-000118). Mice were obtained from Envigo (Indianapolis, IN, USA), and studies were approved by the Janssen Research and Development, LLC Institutional Animal Care and Use Committee.

Human Blood Processing and *In Vitro* Stimulation

Human blood was anti-coagulated with 20 U/ml pyrogen-free sodium heparin (American Pharmaceutical Partners, Inc., Schaumberg, IL, USA). All blood products were kept at room temperature and processed within 4 h from collection. Whole blood (WB) was mixed 1:1 with pre-warmed (37°C) RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) and 180 µl plated in a 96-well U-bottom plate (Becton Dickinson, Franklin Lakes, NJ, USA) containing 20 µl of 10× freshly prepared treatment, as described previously (23).

Human MoDCs

Blood was layered onto Ficoll-Hypaque gradient (GE Healthcare, Waukesha, WI, USA) to collect cord blood mononuclear cells (CBMCs) or peripheral blood mononuclear cells (PBMCs). Monocytes were isolated by positive CD14 selection with magnetic microbeads (Miltenyi Biotec, Auburn, CA, USA). Preparations were routinely >95% pure as assessed by flow cytometry (23–26). Monocytes were cultured in tissue culture dishes at 0.4×10^6 cells/ml in RPMI media containing fresh 10% autologous platelet-poor plasma, supplemented with recombinant human (rh) IL-4 (50 ng/ml) and rh granulocyte-macrophage colony-stimulating factor (rhGM-CSF) (100 ng/ml) (R&D Systems, Minneapolis, MN, USA) with one replenishment of fresh media/cytokines at day 3. After 5–6 days, immature MoDCs were handled, as described previously (23, 25).

Vaccines, TLR Agonists, and Multi-Analyte Assays

Licensed vaccines were commercially obtained. Ultrapure LPS (*Salmonella minnesota* R595, List Biological Laboratories, Campbell, CA, USA) was used at 100 ng/ml. Supernatants were assayed by ELISA for TNF (BD Biosciences, San Jose, CA, USA), IL-1β (eBiosciences, San Diego, CA, USA) and by competitive monoclonal enzyme immunoassay (EIA) for PGE_2 (Cayman Chemical, Ann Arbor, MI, USA). Additionally, assay supernatants were analyzed by magnetic bead multiplex cytokine/chemokine assay (Millipore, Billerica, MA, USA) and analyzed on the Luminex® 100/200™ System employing xPOTENT® software (Luminex, Austin, TX, USA) and Millipore Milliplex Analyst (version 3.5.5.0). PGE_2 concentrations were determined using the analysis tool at www.myassays.com.

Flow Cytometry

Monocyte-derived dendritic cells were treated with blocking agent for 10 min (Sigma-Aldrich), transferred to staining buffer [1× PBS, 0.5% (v/v) human serum albumin] and stained for 30 min at 4°C in the dark (1×10^5 cells/staining) with fluorophore-labeled antibodies [CD80/Fluorescein isothiocyanate/Clone L307.4, CD86/phycoerythrin/Clone 2331, CCR7/V450/Clone 150503, and HLA-DR/PerCP-Cy5.5/Clone L243 (BD Biosciences)]. Cells were then centrifuged ($500 \times g$, 10 min), washed, fixed [1% (v/v) paraformaldehyde], and acquired using a LSRII flow cytometer employing FACSDiva software (BD Biosciences). Data were analyzed using the FlowJo (Tree Star, Inc., Ashland, OR, USA). Typically, 10,000 events per sample were acquired.

Production of OMVs and Characterization

Outer membrane vesicles were provided by Janssen Vaccines and Prevention B.V. Briefly, OMVs were produced from *N. meningitidis* H44/76 (B:P1.7,16;F3-3) WT or derivatives. nOMVs were produced from a H44/76 ΔRΔL (15, 16, 27), resulting in a pentaacylated meningococcal LPS from the *lpxL1* mutant *N. meningitidis* strain, lacking the secondary C12:0 acyl chain at the 2′-position of the lipid A. Detergent-extracted OMVs were produced from the WT strain or the ΔRΔL mutant (28). Adsorption to aluminum hydroxide (Sigma-Aldrich) was performed, as described elsewhere (29). Before use, OMVs were diluted in 10 mM Tris pH 7.4/3% (w/v) sucrose to 50 µg total protein/ml. OMV size was determined by dynamic light scattering (DLS) using the Zetasizer nanoseries (Malvern Nano-ZS, l 1/4 532 nm, Westborough, MA, USA).

In Vivo Immunogenicity

Adult Balb/c mice (6–8 weeks, female, 10/group) were immunized with two doses of OMVs (2.5 µg total protein/dose), two doses of Bexsero [at 2.5 µg total protein/dose (equivalent to 1/10th human dose)], or buffer control, subcutaneously in the scruff of the neck. Doses were administered 4 weeks apart, with terminal bleed taken 2 weeks after the second dose. Serum bactericidal assays (SBAs) were performed, as described previously (30), against the WT H44/76 strain with sera from individual mice using baby rabbit sera (Cedarlane, Burlington, VT, USA) as an exogenous complement source. The H44/76 strain was chosen as our OMV formulations were produced from a *N. meningitidis* H44/76 strain and since H44/76 is used as an immunogenicity indicator strain for Bexsero approval. Reported titers are the reciprocal of the serum dilution giving 50% killing. If 50% killing was not achieved the sample was assigned a titer of 4 (half of the lower limit of detection).

Statistical Analyses and Graphics

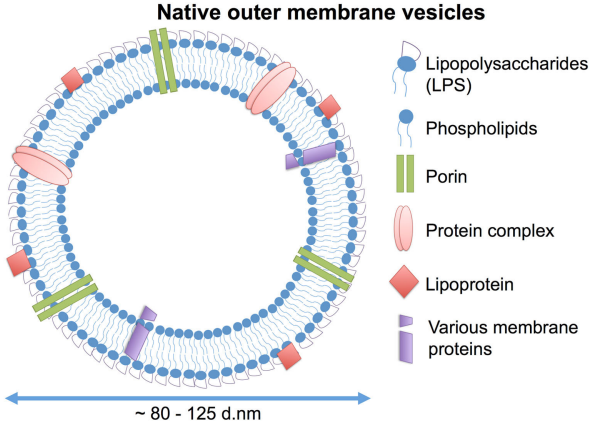
Statistical significance and graphs were generated using Prism v. 5.0b (GraphPad Software, La Jolla, CA, USA) and Microsoft Excel (Microsoft Corporation, Redmond, WA, USA). For data analyzed by normalization to control values, column statistics were conducted using the two-tailed Wilcoxon signed-rank test or unpaired Mann–Whitney test as appropriate. Results were considered significant at $p < 0.05$ and indicated as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{+}p < 0.05$, $^{++}p < 0.01$, and $^{+++}p < 0.001$.

RESULTS

OMVs Incorporating Genetically Attenuated Endotoxin Have Reduced Cytokine Induction Potential in Human Newborn and Adult Blood

Wild-type OMVs were produced from *N. meningitidis* H44/76 (B:P1.7,16;F3-3), while nOMVs (**Figure 1A**), incorporating a genetically attenuated version of endotoxin, were produced from a H44/76 ΔRΔL mutant strain (16, 27). All OMVs were characterized by DLS for particle size (**Figure 1B**; Figure S1 in Supplementary Material), with all formulations falling within a range of 80–120 nm in diameter. Next, we tested the ability of (1) the commercially available Bexsero vaccine, (2) detergent-extracted WT OMV (WT dOMV) (i.e., a Bexsero-like OMV formulation), (3) ΔRΔL mutant nOMV strain (Δ*lpxLI* nOMV) (28), and (4) a Δ*lpxLI* dOMV formulation (**Figure 2A**), to induce titration-dependent cytokine production in human neonatal and adult blood (**Figures 2A,B**). Non-detergent treated WT OMVs containing WT LPS were not tested as these are known to be highly cytotoxic. As indicated by measurement of TNF and IL-1β, both Bexsero and the WT dOMV robustly activated neonatal cord and adult peripheral blood in a titration-dependent manner in all concentrations tested, where levels of these two mediators were significantly increased over baseline ($p < 0.001$). The mutant OMVs also significantly induced production of TNF and IL-1β, but only at the highest concentration in newborn blood ($p < 0.05$) (**Figures 2A,B**; Figure S2 in Supplementary Material). When compared to Bexsero at the highest equivalent volume-to-volume (v/v) dilution tested (1:10), Δ*lpxLI* nOMV demonstrated reduced TNF production in neonatal ($p < 0.001$) and adult ($p < 0.01$) blood. Δ*lpxLI* nOMV were also less effective in inducing IL-1β production in newborn ($p < 0.05$) and WBA ($p < 0.01$). Similar trends were observed when comparing the Δ*lpxLI* nOMV to WT dOMV.

We next broadened our characterization of the ability of WT dOMV, Δ*lpxLI* dOMV, and Δ*lpxLI* nOMV (at v/v 1:1000–1:10) to induce concentration-dependent cytokine production from newborn and WBA using multiplexing assays (**Figures 2C,D**; Figure S3 in Supplementary Material). When compared head-to-head, the Δ*lpxLI* dOMV and Δ*lpxLI* nOMV induced markedly lower cytokine production profiles as compared to the WT dOMV, especially at the lowest concentration tested. The addition of Alum, the most commonly used adjuvant worldwide, and a component of the Bexsero vaccine to the OMV formulations altered the OMV (both wild type and mutant) induced innate cytokine production profiles (Figures S3 and S4 in Supplementary Material). Most notably, Alum reduced the ability of the WT dOMV to induce IFNγ and the chemokine CXCL10 (interferon inducible 10) (Figures S5A,B in Supplementary Material).



B

Characteristics of Outer membrane vesicle formulations studied.

Formulation Name	LPS	Detergent extraction	Ave. Diameter (nm)	PdI Index
WT dOMV	wildtype	Yes	83.27	0.13
Δ lpxLI nOMV	lpxL1	No	99.17	0.14
Δ lpxLI dOMV	lpxL1	Yes	121.27	0.23

OMV: Outer membrane vesicle; LPS: Lipopolysaccharides; lpxL: Lipid A expression/biosynthesis; PdI: Polydispersity; nm: nanometer.

FIGURE 1 | Depiction and characterization of *Neisseria meningitidis* outer membrane vesicles. (A) Outer membrane vesicles (OMVs) are composed of outer membrane lipids, proteins, and lipopolysaccharide and are produced by the blebbing of membranes of live Gram-negative bacteria during *in vitro* and *in vivo* growth [adapted from Sanders et al. (7)]. **(B)** Characterization of each OMV type used in this study, including LPS type, if detergent extraction was used, quantification of average size in nanometers and polydispersity (PdI) index.

Δ*lpxLI* nOMV Mature Human Dendritic Cells without Bexsero-Associated Inflammatory Profile

To further characterize the innate immune effects of OMV formulations, 96-well human MoDC-based arrays were generated after culturing CD14 selected monocytes with IL-4 and GM-CSF in the presence of autologous plasma, a rich source of age-specific soluble immunomodulatory factors (19), as described previously (23, 25). In response to Bexsero, a strong upregulation of CD80, HLA-DR (MHCII), and CCR7 and a modest increase in CD86 were observed in both newborn and adult DCs (**Figures 3A,B**). Both the Δ*lpxLI* nOMV and WT dOMV (with or without Alum) produced slightly reduced DC maturation profiles as compared to the Bexsero. Interestingly, the Alum-adjuvanted Δ*lpxLI* nOMV formulation induced a similar upregulation profile to Bexsero in adult but not newborn MoDCs. Titration-dependent induced MoDC cytokine induction (**Figure 3C**) supported the flow cytometry results and mostly mirrored those observed in WB. When compared head-to-head, the mutant OMV formulations induced lower cytokine production profiles as compared to the WT formulations, especially at the lowest concentration tested. Interestingly, as seen in the WBA, the addition of Alum selectively enhanced WT dOMV-induced IL-1β production from human DCs (**Figure 3D**). Moreover, the addition of Alum to the Δ*lpxLI*

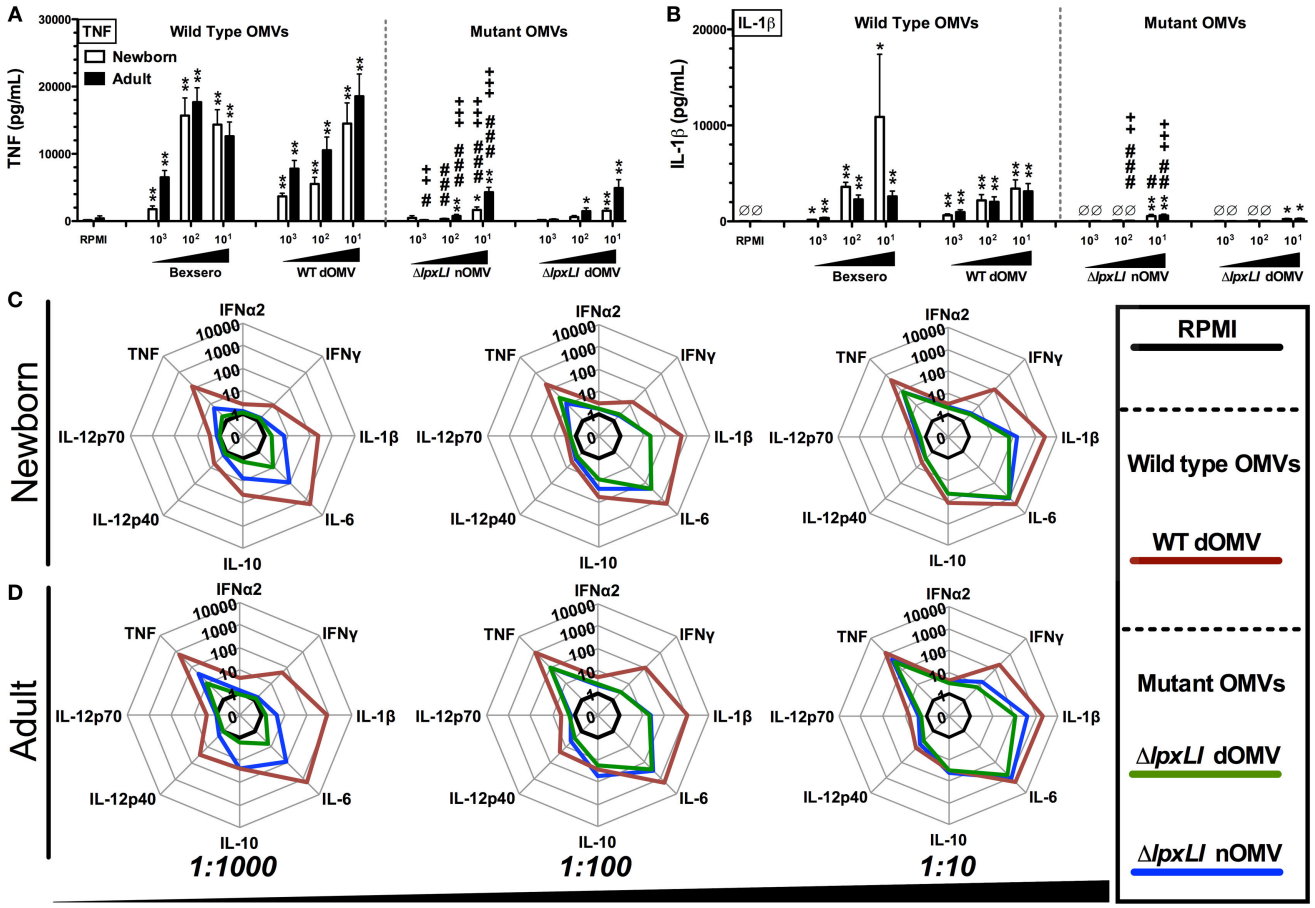


FIGURE 2 | OMVs containing genetically attenuated endotoxin demonstrate relatively low cytokine induction in human newborn and adult blood. Human neonatal and adult blood cultured *in vitro* for 6 h with buffer control (RPMI) or with increasing concentrations of wild type (WT) and mutant OMV formulations (1:1000–10 v/v). Supernatants were collected for ELISA **(A,B)** and multiplex assay [**(C)** newborn, **(D)** adult]. Results represent means ± SEM of $N = 7$. For analyses at individual treatments (e.g., control RPMI vs. Bexero 1:10), unpaired Mann–Whitney test was applied at each concentration, and statistical significances are denoted as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. For comparison of Δ*lpxLI* nOMV to Bexsero, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$. For comparison of Δ*lpxLI* nOMV to WT dOMV, $^{+}p < 0.05$, $^{++}p < 0.01$, and $^{+++}p < 0.001$.

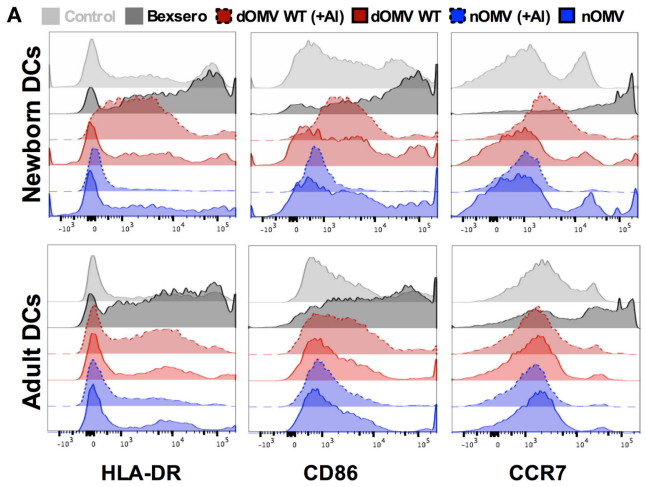
nOMV formulation only induced a slight increase in IL-1β from newborn (**Figure 3D**; Figure S6A in Supplementary Material) and adult DCs (**Figures 3E,F**; Figure S6B in Supplementary Material) at the highest concentration tested (1:10 v/v).

Δ*lpxLI* nOMV Are Less Inflammatory toward Human Leukocytes than the Majority of Pediatric Vaccines

To gain further insight into the inflammatory potential of the Δ*lpxLI* nOMV formulation, we next benchmarked it against a number of conventionally licensed pediatric vaccines (Table S1 in Supplementary Material) in both WB and MoDC assays. We have previously demonstrated that several of these licensed vaccines, such as the EasyFive (DTwP-HepB-Hib), induce distinct reactogenicity biomarker profiles *in vitro* (31, 32). When tested at equivalent v/v treatment concentrations, Δ*lpxLI* nOMV conversely induced a lower cytokine response for most innate cytokines (**Figures 4A,B**) and chemokines (Figure S7 in Supplementary Material) tested, grouping closer to pediatric vaccines such as PCV13 and HBV, than the more inflammatory (Alum + TLR agonist)-containing pediatric vaccines (i.e., PedvaxHIB and EasyFive).

Next, immature newborn (**Figure 5A**) and adult DCs (**Figure 5B**) were assessed for vaccine-induced production of PGE_2, a molecule whose *in vitro* production has been correlated with reactogenicity *in vivo* (31–33). Of note, newborn DCs demonstrated significantly reduced Δ*lpxLI* nOMV-mediated PGE_2 responses as compared to both Bexsero ($p < 0.01$) and WT dOMV (with Alum) ($p < 0.05$) (**Figure 5A**). A similar pattern was observed for adult DCs, but with significance only observed between Bexsero and Δ*lpxLI* nOMV ($p < 0.01$) (**Figure 5B**).

Newborn (**Figure 5C**) and adult DCs (**Figure 5D**) responses to Δ*lpxLI* nOMV were also benchmarked against conventional licensed pediatric vaccines (at 1:10 v/v, Tables S1 and S2 and Figure S8 in Supplementary Material) in the MoDC array. Here,



B

Newborn MoDC	CD80	CD86	CCR7	MHCII
RPMI	1.00	1.00	1.00	1.00
Bexsero	3.72	2.21	7.63	6.02
WT dOMV + Alum	1.32	1.12	2.08	3.36
WT dOMV	1.31	3.06	2.20	1.19
ΔlpxLI nOMV + Alum	1.21	1.10	2.22	2.31
ΔlpxLI nOMV	2.34	1.27	2.12	1.17

Adult MoDC	CD80	CD86	CCR7	MHCII
RPMI	1.00	1.00	1.00	1.00
Bexsero	4.83	2.29	5.81	19.44
WT dOMV + Alum	1.15	1.40	1.99	3.77
WT dOMV	1.29	1.47	1.45	2.36
ΔlpxLI nOMV + Alum	4.01	3.62	7.78	6.78
ΔlpxLI nOMV	1.40	1.84	2.07	3.71

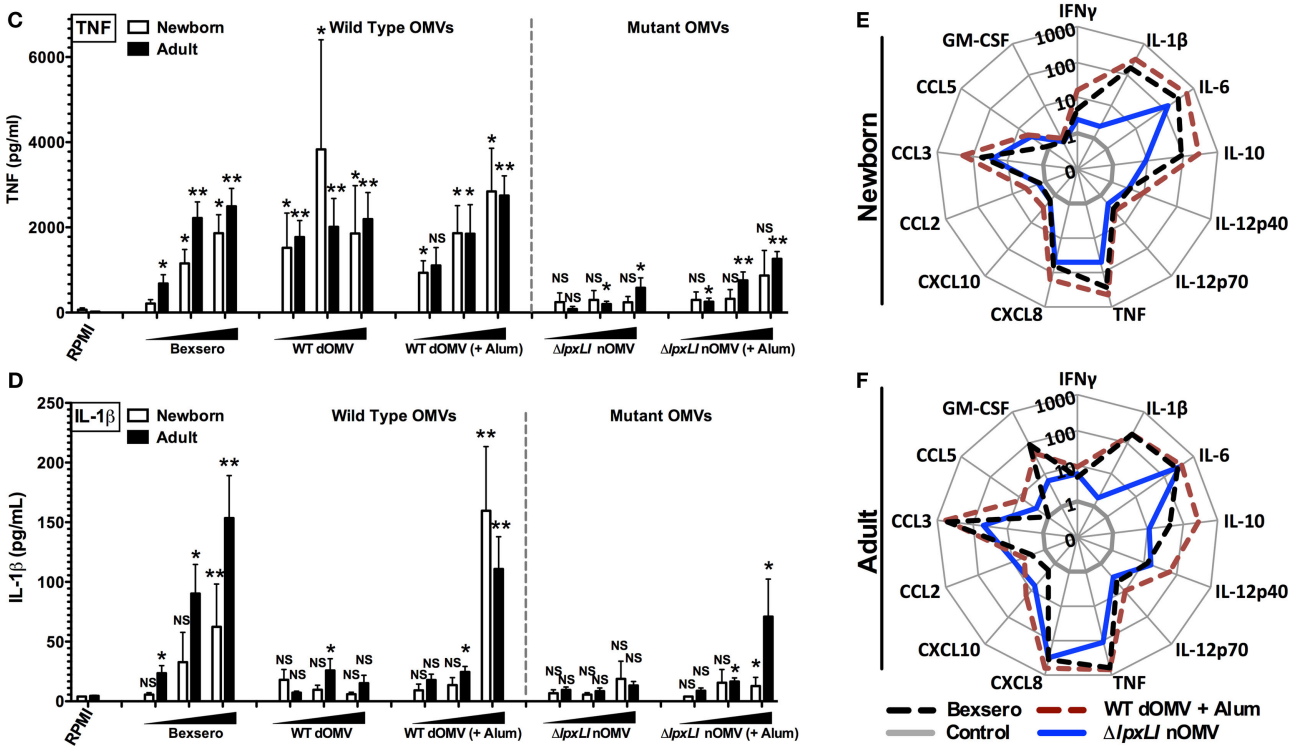


FIGURE 3 | *ΔlpxLI* nOMV mature human dendritic cells without the high *in vitro* inflammatory profile induced by Bexsero. **(A)** Human newborn and adult MoDCs were cultured in 10% autologous plasma (v/v) and stimulated for 24 h with OMV vaccines (each at 1:10 v/v). Surface expression of costimulatory molecules and HLA was determined by flow cytometry. **(B)** Average MFI of newborn and adult MoDCs. **(C,D)** MoDCs cultured 24 h with increasing concentrations of OMV vaccines (each at 1:1000, 1:100, and 1:10 v/v). **(E,F)** Multiplex cytokine production from treated DCs. Mean ± SEM, $n = 5–6$. For analyses at individual treatments (e.g., control RPMI vs. Bexsero), unpaired Mann–Whitney test was applied at each concentration, and statistical significances are denoted as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, and not significant (NS).

we focused on the correlation of both PGE_2 and IL-1β production, as co-production of both in human monocytic assays may predict rabbit pyrogenicity (i.e., fever) *in vivo* (33). Overall, a common trend was observed. The bacillus Calmette–Guérin (BCG) vaccine, Bexsero and WT dOMV consistently induced the highest production of both PGE_2 and IL-1β. Δ*lpxLI* nOMV conversely induced a lower MoDC PGE_2/IL-1β profile than WT OMV-based vaccines, suggesting similarity to low reactogenicity pediatric vaccines such as PCV13 and HBV (**Figures 5C,D**).

Δ*lpxLI* nOMV Dissociates Inflammation from Immunogenicity

Having characterized the relatively low reactogenicity potential of Δ*lpxLI* nOMV toward human leukocytes *in vitro*, we next

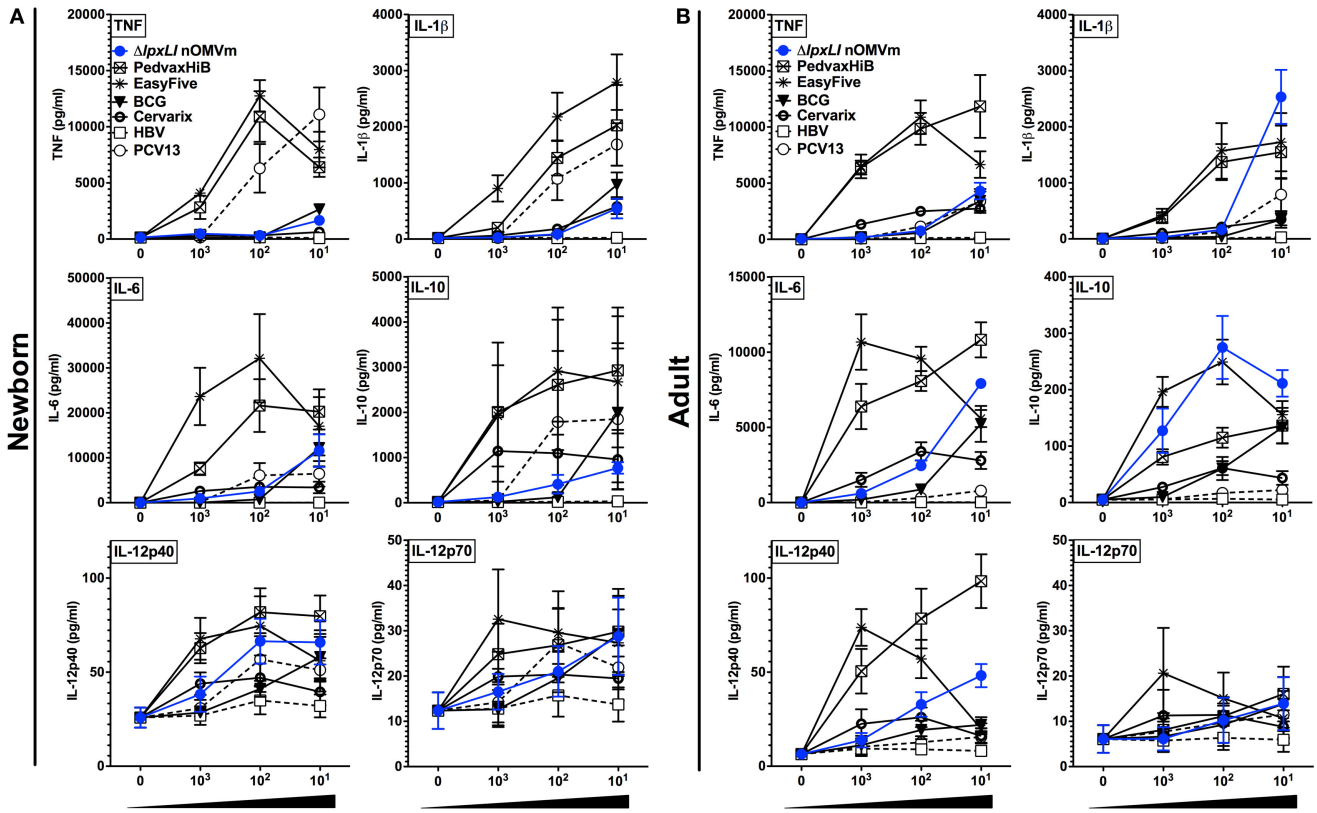


FIGURE 4 | Δ*lpxLI* nOMV are less inflammatory *in vitro* than licensed combination vaccines. Human **(A)** neonatal and **(B)** adult blood cultured *in vitro* for 6 h with buffer control (RPMI) or with increasing concentrations of WT and Δ*lpxLI* OMV formulations (each at 1:1000, 1:100, and 1:10 v/v) as well as the vaccines PedvaxHib, EasyFive, BCG, Cervarix, PCV13, and HBV (1:1000–10 v/v). Results for TNF, IL-1β, IL-6, IL-10, IL-12p40, and IL-12p70 are shown and represent means ± SEM of $N = 7$.

assessed the ability of Δ*lpxLI* nOMV to induce immunogenicity in mice *in vivo*. Four cohorts of 10 mice each (**Figure 6**) were immunized subcutaneously with Tris/Sucrose (buffer control), Δ*lpxLI* nOMV or WT dOMV (2.5 μg total protein/dose, dOMVs formulated with Alum), or a 1/10th dose of Bexsero (equivalent to 2.5 μg dOMV). Two doses were administered 4 weeks apart, with blood collected 2 weeks after the second dose to obtain serum for SBA. The buffer control alone failed to induce meningococcal bactericidal antibody responses. In marked contrast, Bexsero ($p < 0.05$), WT dOMV ($p < 0.01$), and Δ*lpxLI* nOMV ($p < 0.01$) all induced robust and significant antibody responses as compared to baseline (**Figure 6**). Remarkably, even though Δ*lpxLI* nOMVs demonstrated reduced inflammation potential toward human leukocytes *in vitro*, there was no significant difference in immunogenicity as compared to either Bexsero or WT dOMV.

DISCUSSION

The majority of global immunization schedules are pediatric, with particular focus on newborns and young infants, yet most vaccine discovery programs do not rationally design vaccine formulations for use in humans in early life. Indeed, even when employed, *in vitro* modeling of human responses often occurs in late stage vaccine development. Such current pre-clinical approaches may contribute to vaccine formulations inducing sub-optimal responses in the very young (3). To more completely evaluate an OMV-based *N. meningitidis* vaccine, we took a rational vaccine design approach, in which we combined a relevant *in vivo* model and age-specific human *in vitro* culture systems that together may better model OMV-induced innate immunomodulatory capacity and immunogenicity potential. Overall, when compared to the WT detergent-extracted OMV (WT dOMV), mutant detergent-extracted OMV (Δ*lpxLI* dOMV), and multiple licensed vaccines with respect to innate signaling toward human newborn and adult leukocytes *in vitro*, a prototype Δ*lpxLI* nOMV vaccine generated a pattern of response suggestive of low reactogenicity potential while still demonstrating robust immunogenicity in mice *in vivo*. Demonstration of non-inferior *in vivo* immunogenicity of the Δ*lpxLI* nOMV vaccine, as compared to Bexsero, is noteworthy, especially as the *in vitro* DC maturation profiles are divergent. Such a result may indicate that the inclusion of native endotoxin molecules into traditionally designed OMV-based vaccines may not be as essential for immunogenicity as often assumed.

A key concern regarding modern vaccine development is reactogenicity (33), the propensity of a formulation to cause acute inflammatory events either locally, such as erythema or tenderness

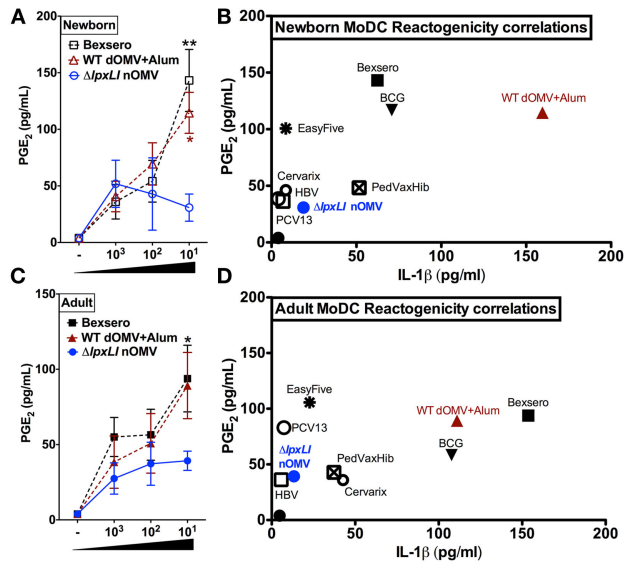


FIGURE 5 | Δ*lpxLI* nOMV-induced lower MoDC-PGE_2 and -IL-1β than Alum-adjuvanted WT OMV and the licensed Bexsero vaccine. Human newborn and adult MoDCs were cultured in 10% autologous plasma (v/v) and stimulated for 24 h with increasing concentrations of OMV vaccines (each at 1:1000, 1:100, and 1:10 v/v). **(A)** Newborn and **(C)** adult PGE_2 production, as measured by ELISA. Potential reactogenicity biomarker correlation of PGE_2 with IL-1β for newborn **(B)** and adult **(D)** MoDCs after treatment with wild WT and mutant OMV formulations (at 1:10 v/v) as well as the vaccines PedvaxHib, EasyFive, BCG, Cervarix, PCV13, and HBV (at 1:10 v/v). Mean ± SEM, $n = 5–6$. PGE_2 levels are depicted with untreated basal levels subtracted. For analyses at individual treatments (e.g., Bexsero 1:10 vs. Δ*lpxLI* nOMV 1:10), Wilcoxon test was applied at each concentration and statistical significances are denoted as follows: $^*p < 0.05$ and $^{**}p < 0.01$.

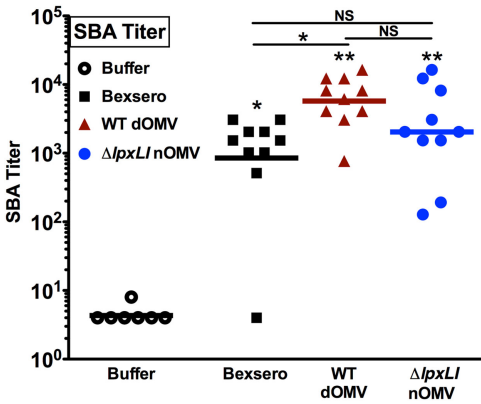


FIGURE 6 | Δ*lpxLI* nOMV induce robust immunogenicity *in vivo*. Mouse functional antibodies levels were determined by serum bactericidal assays (SBAs) after subcutaneous immunization with Δ*lpxLI* nOMV or WT dOMV (2.5 μg total protein/dose) formulations, as well as the vaccines Bexsero (1/10th human dose) or buffer control. Two doses were administered 4 weeks apart, with terminal bleed taken 2 weeks after the second dose. Mutant OMV demonstrated enhanced immunogenicity over buffer control treatment, and equivalent induction of functional antibodies to the WT formulations, $n = 10$. Horizontal line indicates geometric mean. Wilcoxon test was applied between buffer and each OMV formulation, or between OMV formulations as indicated. Statistical significance is denoted as follows: $^*p < 0.05$, $^{**}p < 0.01$, and not significant (NS).

at the injection site, or systemically, such as fever. In this context, *in vitro* assays that provide potential reactogenicity biomarker data may be highly advantageous in de-risking formulation selection for *in vivo* use (21). By employing an *in vitro* human DC array, we benchmarked the immunomodulatory abilities of an Δ*lpxLI* nOMV-based vaccine against several licensed pediatric vaccines. DCs are logical targets for such *in vitro* studies as they efficiently process antigens for induction of immunity against pathogens and their components (34). The few published studies that assessed the activating effects of OMV on human leukocytes *in vitro* (35–40) evaluated fixed concentrations with limited cytokine measurements. One study evaluated concentration-dependent OMV responses in human WB and PBMC assays, solely from adult donors (39). Accordingly, our study is the first to comprehensively investigate (a) the ontogeny of OMV-induced immune responses and (b) the responses of human DCs to OMVs as benchmarked against licensed vaccines. Δ*lpxLI* nOMV induced cytokine responses significantly lower than those induced by either Bexsero or licensed vaccines (i.e., PedvaxHIB and EasyFive). As demonstrated by the stronger induction of costimulatory molecule expression by the Δ*lpxLI* nOMV with Alum in adult but not newborn MoDCs, the dramatically enhanced human DC IL-1β production in response to Alum-adjuvanted WT dOMV, as well as our prior studies demonstrating distinct ontological effects of Alum on *in vitro* DC IL-1β production (24), it is reasonable to suggest that our age-specific *in vitro* systems may be useful tools with respect to characterizing candidate vaccine formulations with or without Alum.

Our study has multiple strengths including (a) robust age-specific human *in vitro* modeling, (b) extensive benchmarking to multiple licensed vaccines, and (c) confirmation of immunogenicity *in vivo*. That said, our study also has some limitations including (a) the unavoidable use of different species for *in vitro* (human) and *in vivo* (mouse) studies, (b) that *in vitro* systems, no matter how carefully developed, are imperfect models for responses *in vivo*, (c) the lack of full concordance in the antigens profile found in Bexsero- which contains added recombinant protein- and our Δ*lpxLI* nOMV formulations that do not, and (d) the lack of non-detergent treated- WT strain-derived OMVs that contain WT LPS. While such a formulation was initially not evaluated as part of our current study due to the known cytotoxic effects in humans, based on our results, such WT OMVs may provide a benchmark in future studies employing human *in vitro* systems. Of note, our experiences to date with *in vitro* benchmarking suggest that our *in vitro* studies provide insights relevant *in vivo* (2). Additionally, studies evaluating OMV vaccine-based reactogenicity *in vivo* using similar strategies to ours in mice (41) and humans (42) support these conclusions.

Several aspects of our results will prompt future investigations. For example, nucleotide-binding oligomerization domain (NOD)-like receptor-mediated IL-1β production is greater in human newborn cord than adult peripheral blood (43, 44) and slowly decreases to adult levels over the first years of life (45). A similar phenomenon for basal PGE_2 levels in WB has been

observed (32). Therefore, further study of the IL-1β/PGE_2 axis as a predictive marker of vaccine reactogenicity may need to take ontogeny into account. Recent use of proteomics (secretomics) has profiled hundreds of proteins released by human monocytes when stimulated *in vitro* with Alum or TLR agonists, several of which have been validated upon study of licensed adjuvanted vaccines, providing opportunities for further refinement of future adjuvanticity and reactogenicity biomarkers (31). Finally, the evaluation of different pentaacyl lipid A mutants (46) and comparison of their modified agonist properties using age-specific human WB and DC assays should also considered.

The persistently high global burden of meningococcal disease in the very young infants and adolescents provides a compelling rationale for developing additional safe and effective early life vaccines (47). Overall, two key aspects of our study deserve particular emphasis: (a) human *in vitro* systems model age-specific biomarker responses that may correspond to reactogenicity and immunogenicity as benchmarked to licensed vaccines and (b) these in *vitro* systems may enable systematic comparison of formulations with and without candidate adjuvants early in the design process to inform translational development. Overall, if further validated, such an approach of *in vitro* assay system-informed age-specific vaccine development may open new paths to more precise vaccine development for distinct vulnerable populations.

AUTHOR CONTRIBUTIONS

DD, GD, and OL designed the study. DD, WC, SJ, SB, IB, CP, and SH conducted the *in vitro* experiments. HS, SA, and JF produced OMV vaccines and conducted the *in vivo* experiments. DD wrote the manuscript. OL provided overall mentorship and assisted in writing the manuscript. HS, WC, SJ, SB, IB, CP, SH, SA, JF, and GD contributed to helpful discussions and the careful approval of the final manuscript. All the authors have given final approval for the version submitted for publication.

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SUPPLEMENTARY MATERIAL

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Immunization of Newborn Mice Accelerates the Architectural Maturation of Lymph Nodes, But AID-Dependent IgG Responses Are Still Delayed Compared to the Adult

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Lymph nodes (LNs) have evolved to maximize antigen (Ag) collection and presentation as well as lymphocyte proliferation and differentiation—processes that are spatially regulated by stromal cell subsets, including fibroblastic reticular cells (FRCs) and follicular dendritic cells (FDCs). Here, we showed that naïve neonatal mice have poorly organized LNs with few B and T cells and undetectable FDCs, whereas adult LNs have numerous B cells and large FDC networks. Interestingly, immunization on the day of birth accelerated B cell accumulation and T cell recruitment into follicles as well as FDC maturation and FRC organization in neonatal LNs. However, compared to adults, the formation of germinal centers was both delayed and reduced following immunization of neonatal mice. Although immunized neonates poorly expressed activation-induced cytidine deaminase (AID), they were able to produce Ag-specific IgGs, but with lower titers than adults. Interestingly, the Ag-specific IgM response in neonates was similar to that in adults. These results suggest that despite an accelerated structural maturation of LNs in neonates following vaccination, the B cell response is still delayed and reduced in its ability to isotype switch most likely due to poor AID expression. Of note, naïve pups born to Ag-immunized mothers had high titers of Ag-specific IgGs from day 0 (at birth). These transferred antibodies confirm a mother-derived coverage to neonates for Ags to which mothers (and most likely neonates) are exposed, thus protecting the neonates while they produce their own antibodies. Finally, the type of Ag used in this study and the results obtained also indicate that T cell help would be operating at this stage of life. Thus, neonatal immune system might not be intrinsically immature but rather evolutionary adapted to cope with Ags at birth.

Keywords: neonates, immunization at birth, FDCs, FRCs, germinal centers, class-switched antibodies

Abbreviations: AID, activation-induced cytidine deaminase; ASC, antibody-secreting cell; CSR, class switch recombination; CR, complement receptor; DNP-KLH, dinitrophenyl hapten conjugated to keyhole limpet hemocyanin; DLN, draining lymph node; EF, extrafollicular; FcγR, Fc-gamma receptor; FRC, fibroblastic reticular cell; FDC, follicular dendritic cell; GC, germinal center; IC, immune complex; LT, lymphotoxin; PNA, peanut agglutinin; SLO, secondary lymphoid organ; SHM, somatic hypermutation; SCS, subcapsular sinus; Tfh, follicular helper T cells.

INTRODUCTION

Neonates are often thought to be immunologically immature (1, 2), in part due to anti-inflammatory dendritic cells (3, 4) and immature lymphocytes (4–6). However, secondary lymphoid organs (SLOs), which bring antigen (Ag)-presenting cells into contact with their cognate lymphocytes and provide niches for the differentiation of lymphocytes, are also incompletely developed in neonates. The architecture of SLOs is supported by non-hematopoietic stromal cells (7), including fibroblastic reticular cells (FRCs) and follicular dendritic cells (FDC) (7, 8). Nevertheless, it is not clear how these cells influence the architecture of neonatal SLOs or whether early exposure to Ag affects the maturation of these cells (7, 8).

In adults, the FRC network extends from the subcapsular sinus (SCS) of the lymph node (LN) through the entire T-cell zone and is contiguous with high endothelial venules (HEVs) (9). FRCs maintain lymphocyte homeostasis *via* the production of IL-7 (10–12) and direct leukocyte traffic *via* chemokine secretion (13–15). FRCs also form a conduit system through which the LNs can collect small molecules (12). Importantly, FRCs provide strength and flexibility to LNs and allow them to be restructured following inflammation, thereby providing space for the influx or proliferation of lymphocytes following antigenic exposure (16). In contrast to FRCs, FDCs are found exclusively in the B cell follicle, where they support B cell homeostasis, maintain the follicular architecture, and promote robust humoral immune responses (13, 17–19). FDCs express complement receptors (CRs)-1 and -2 and can be induced to express Fc-gamma receptor (FcγR) IIb (17, 20, 21), which are important for their retention of immune complexes (ICs). FDCs also release ICs in the form of iccosomes (22), so that B cells can acquire Ag and present it to follicular helper T cells (Tfh). FDCs also provide costimulatory signals that enhance B cell proliferation and antibody (Ab) production (23).

An essential step for primary B cell responses is the germinal center (GC) reaction, which is a complex microenvironment that supports B cell clonal expansion and affinity maturation in response to T-cell-dependent Ags. GCs are critically influenced by the establishment of a functional FDC network capable not only of retaining Ag–Ab complexes through complement- and Fc-receptors but also of promoting the survival of GC B cells (24–26). FDCs are prominent in the light zone of GCs, where they facilitate B cell selection by displaying Ags (17, 21).

During the GC reaction, cognate interactions between Tfh cells and GC B cells are critical for the follicular T cells to provide the necessary signals for GC B cell survival and/or differentiation. CD40-ligand (CD40L) and IL-4 are among the crucial molecules of the T cell help to B cells and require close cell–cell interactions. It is established that Tfh cells are needed to maintain and to regulate GC B cell differentiation into Ab-secreting cells (ASCs) and memory B cells (27). ASCs and memory B cells provide both immediate as well as long-term protection against re-infections (28–30). Importantly, immunoglobulin (Ig) class switching (CSR) and somatic hypermutation (SHM) of Ig V regions both occur in the GC (23). These activities are dependent on the enzyme activation-induced cytidine deaminase (AID), which is a protein specifically expressed in GC B cells (31, 32). As a result, this enzyme is very important for successful Ab responses (33, 34) and can be used as a marker of T-dependent B cell activation.

Given the importance of GCs, stromal cell populations and the expression of AID in the generation of primary Ab responses, we examined these structures and the cell types, as well as AID and the Ab production in the context of immune responses in newborn mice upon early immunization at birth. We showed that mice on the day of birth have poorly organized LNs with few B cells or FDCs. However, we found that immunization at birth accelerated the accumulation of both B cells and Thy-1+ T cells inside follicles, and promoted FDC maturation and FRC organization in neonates. Nevertheless, the GC response was still delayed and reduced in neonates as compared to that in adults. Importantly, relatively few B cells in neonatal LNs expressed AID and as a result, they had fewer IgG-ASCs and lower IgG titers than adults did. Interestingly, the Ag-specific IgM response in neonates was similar to that in adults. These results suggest that despite an accelerated structural maturation of LNs in neonates following vaccination, the B cell response is still reduced in its ability to isotype switch.

ANIMALS AND METHODS

Animals and Immunizations

Adult and female pregnant C57BL/6 mice were obtained from the animal facilities (UPEAL) of the Center for Advanced Research (CINVESTAV-IPN). This was arranged to ensure that by keeping the animals under discreet careful observation we could use the pups immediately at birth, which was considered day 0 in our studies. Mice were immunized with 100 μg of dinitrophenyl-conjugated keyhole limpet hemocyanin (DNP-KLH, Calbiochem), in combination with the adjuvant, Titermax Gold (Sigma, 1:10 v/v), *via* a single subcutaneous injection in the right inguinal region. Immunized pups were kept with mothers until weaning at 3 weeks. Serum was collected from anesthetized mice by cardiac puncture. Mice inoculated with sterile pyrogen-free saline solution (SS) were used as controls.

For the maternal transfer experiments, female C57BL/6 mice (6–8 weeks old) were immunized with DNP-KLH or SS and the day of immunization these female mice were mated. Fourteen days post-first-immunization, pregnant mice were boosted with another dose of DNP-KLH. Serum was collected from pups and mothers at various times after birth. Anesthetized mice were bled by cardiac puncture. To have enough sera, at least three litters of pups were used for each time point of the experimental design. All experiments were performed in accordance with the institutional guidelines for animal care and experimentation (UPEAL-CINVESTAV-IPN). The protocol and procedures employed were reviewed and approved by the UPEAL-CINVESTAV Ethics Review Committee.

Antibodies and Lectins

Immunohistochemistry on frozen sections of draining LNs was performed using Abs against B220 (RA3-6B2), MFG-E8

(FDC-M1) and Thy-1 (G7) from Pharmingen, ER-TR7 against FRCs (ER-TR7) from Serotec, and AID (mAID-2) from eBioscience. Horseradish peroxidase (HRP)-conjugated goat anti-rat IgG (Thermo Scientific 31470), biotinylated anti-rat IgG (Vector BA-4001), and HRP-conjugated streptavidin (Pierce 21124) were used as secondary reagents. The lectin, peanut agglutinin (PNA) was obtained from Vector Labs.

Immunohistochemistry

At necropsy, the inguinal draining LN (DLN) was excised from each animal, immediately frozen in Tissue-Tek OCT compound (Sakura) and cut in 3-μm cryosections. Serial cryostat sections were prepared from each specimen, fixed in pure cold acetone for 20 min, air dried, and stored at −20°C until staining, which usually occurred within 3 days. Sections were used to detect B cell follicles (B220), T cells (Thy-1) and then mature FDCs (FDC-M1), or FRCs (ER-TR7), PNA-positive GC B cells, and AID-positive cells (mAID-2) *in situ*. The specimens were obtained from at least three mice at each time point. For single staining, sections were incubated for 1 h with 9% H_2O_2 to block endogenous peroxidase at room temperature (RT). Before specific Ab staining, non-specific binding sites were blocked with Tris-buffered saline (TBS) 1× containing 1% human serum and 1% bovine serum albumin. For immunolabeling, we incubated the sections for 1 h with optimal concentrations of monoclonal Abs (mAb). After washing with TBS 1×, the sections were incubated with HRP-conjugated rabbit anti-rat IgG for 1 h at RT, washed and then stained with a solution of 3,3′-diaminobenzidine (DAB; Sigma) and H_2O_2 in TBS 1× to determine the peroxidase activity, and then washed with TBS 1×. For double immunolabeling, the slides were treated again with H_2O_2 to quench the specifically bound HRP and then peroxidase activity was visualized with SG substrate (Vector) which produces a blue-gray colored reaction. Results were recorded using Image-Pro PLUS software for the Olympus BX51 system microscope. Pictures were analyzed by ImageJ 1.47v (National Institutes of Health, USA).

Flow Cytometry Analysis and Assessment of Antibody-Secreting Cells (ASC)

Draining lymph nodes were suspended in sterile phosphate-buffered saline and then pooled to have enough tissues and cells. Cell suspensions of DLNs from adults and from neonates were first pre-treated with purified rat anti-mouse CD16/CD32 mAb (2.4G2 BD Pharmingen Cat No. 553141) to block FcγRs II/III and then stained for 30 min on ice with the following fluorochrome-conjugated Abs: rat anti-mouse CD19-APC (1D3 BD Pharmingen Cat No. 550992), rat anti-mouse CD138-PE (281-2 BD Pharmingen Cat No. 553714), and rat anti-mouse Gr-1-PercP (RB6-8C5 BD Pharmingen Cat No. 552093). Cells were then washed, re-suspended in Cytofix/Cytoperm Kit (BD Pharmingen Cat No. 54714) on ice for 10 min, and washed with 1× Perm Wash Buffer 1:10 (BD™ Phosflow Cat No. 557885). Cells were then intracellular stained with goat anti-mouse IgG-FITC (Jackson Labs Cat No. 115-095-205). Cells were acquired on a CyAn™ ADP Analyzer. Data were analyzed with FlowJo software v7.6.5 (TreeStar).

ELISA

The 96-well EIA/RIA Immunoplates (Costar 3590, Cambridge, MA, USA) were coated with 5 μg/ml of DNP-KLH at 4°C overnight in a moist chamber. After washing and blocking, serial dilutions of serum samples were incubated at 4°C overnight in a moist chamber. After further washes, HRP-conjugated goat anti-mouse IgG (Bio Rad 170-6516) was added for 2 h at 37°C. The reaction was visualized by the addition of 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) substrate (Sigma-Aldrich A1888). Absorbance was measured at 405 nm in a Sunrise Tecan microplate reader, software Magellan v.3.0 (Switzerland). Relative Ab titers were calculated after plotting the optical density of each well against the serum dilution and were derived from the linear portion of the resulting curves.

Statistical Analysis

Values are expressed as median ± the interquartile range. Differences between mean values were analyzed for statistical significance using the GraphPad Prism 5 Software (La Jolla, CA, USA), using the Mann–Whitney *U* test. In the case of multiple comparisons, Kruskal–Wallis test with the Dunn's multiple comparisons were used. *P* values less than 0.05 were considered statistically significant ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$).

RESULTS

Immunization at Birth Accelerates the Maturation of B Cell Follicles and FDCs

To determine the effect of immunization on LN architecture, we immunized neonatal mice (day of birth) and adults (6 weeks old) with either SS or DNP-KLH with Titermax Gold adjuvant and evaluated B cell follicles and FDCs by immunohistochemistry. As expected (35–37), we found well-formed B cell follicles and extensive FDC networks in adult mice, even before immunization (**Figure 1A**). Without immunization, the number of follicles per section remained steady (**Figure 1E**), and the follicular area expanded slightly over the next 4 weeks (**Figures 1A,F**). However, following immunization, the follicular area expanded dramatically especially in the first time points post-inoculation assessed (**Figures 1B,F**) and slightly more follicles were observed (**Figure 1E**). Nonetheless, both follicular size and number returned to baseline by day 28 after immunization (**Figures 1E,F**).

Regarding FDCs, while few of them were detected at different times in the control group (SS), Ag immunized adult animals showed a more prominent FDC mesh. Because in adult mice the FDCs were already forming dense networks inside B cell follicles, it was not possible to count individual cells in these animals. Accordingly, FDCs network was bigger at days 7, 14, and 21 in Ag immunized animals than in control groups (**Figures 1A,B**). By day 28, however, there were no apparent differences for both, the B cell follicles and the FDCs networks between Ag immunized and SS inoculated (control) adult mice. Thus, by day 28, the *in situ* cellular response generated to the Ag appeared to decrease to pre-immunization basal stages in adult mice.

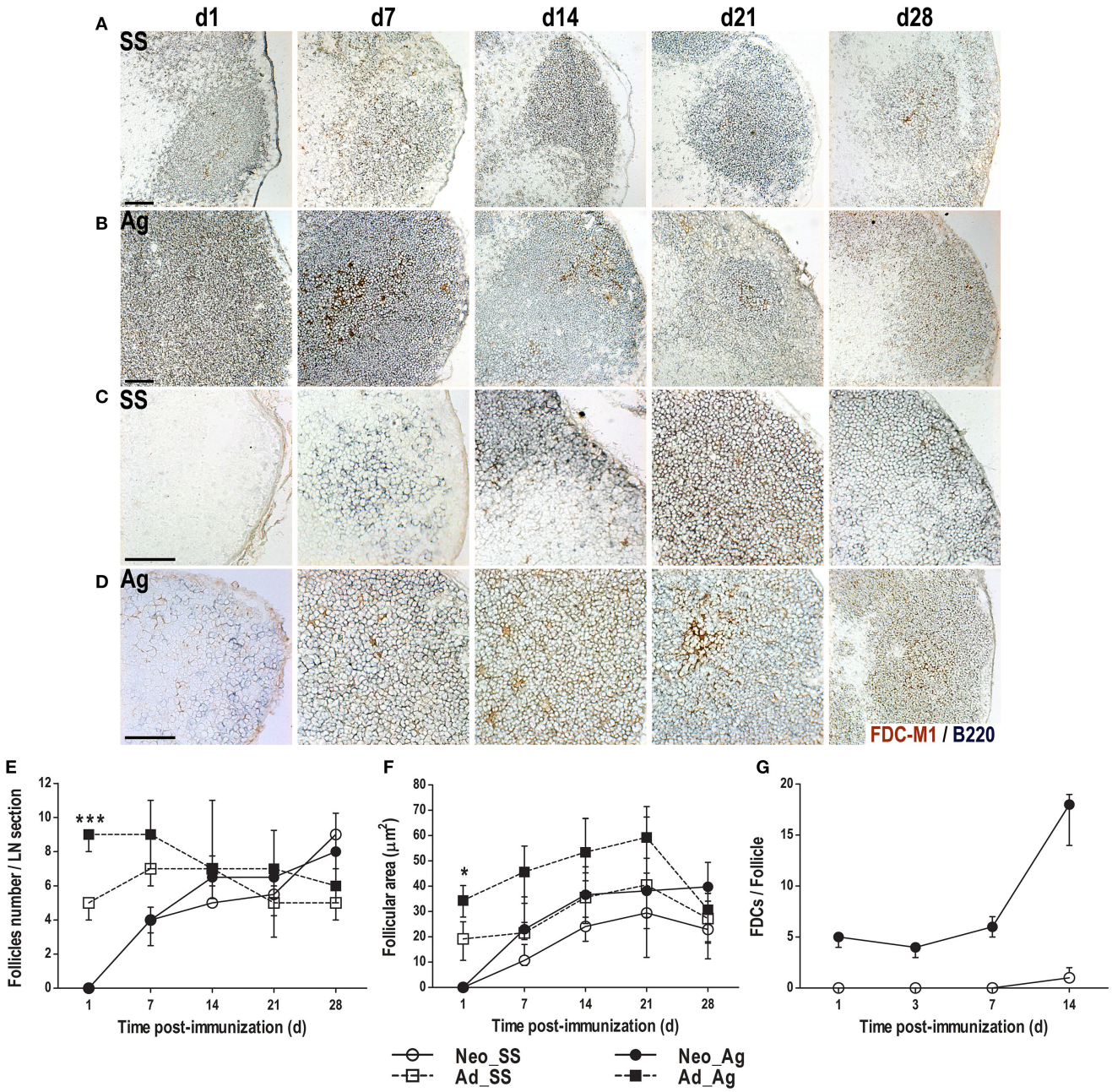

FIGURE 1 | Immunization at birth accelerates the formation of B cell follicles and appearance of follicular dendritic cell (FDC) networks. Adult **(A,B)** or neonatal **(C,D)** mice were inoculated with sterile saline solution (SS) or DNP-KLH (Ag) and the draining lymph nodes were examined by immunohistochemistry on days 1, 7, 14, 21, and 28 after inoculating. Cryosections were probed with antibodies to FDCs (FDC-M1, brown) and B cells (B220, blue). The number of B cell follicles in each lymph node (LN) section **(E)** was counted and the area of B cell follicles in each section **(F)** was calculated. The number of FDCs **(G)** inside B cell follicles was determined in neonates during the first 14 days post-inoculation. Scale bar 100 μm. Data represent the median ± interquartile range from three to nine mice per group at each time point and were analyzed with Kruskal–Wallis test. Mice immunized as adults versus neonates immunized at birth. $^{*}P < 0.05$, $^{***}P < 0.001$.

In control LNs from neonatal mice, we observed very few scattered B cells on day 1, with no apparent follicular organization or FDCs (**Figure 1C**). We already found B cell follicles in LNs of control mice on day 7, but only minimal evidence of FDCs (**Figure 1C**). However, both B cell follicles and FDCs were clearly evident on days 14, 21, and 28, even without immunization. At these times, the number of follicles and their size resembled those of adult LNs (**Figures 1E,F**).

In contrast, immunization of newborns triggered the accumulation of B cells forming follicles-like below the SCS as early as

1 day later (**Figure 1D**). Likewise, FDCs were also observed at this time following immunization. In these animals, DLNs by day 7 exhibited the typical B cell follicles with FDCs inside, as seen in adult animals (**Figures 1A,B**). At this time, FDCs in immunized newborns were found in clusters and were more apparent than in control SS neonates. Thus, in immunized neonates, it was possible to quantify individual FDCs since day 1 until day 14 post-immunization (however, by day 21, FDCs were in a crowded cell mesh making very difficult to count them). The number of FDCs inside B cells follicles was bigger in immunized neonates than in the control animals during this period (**Figure 1G**). Bigger follicular areas and more prominent FDCs were maintained in the Ag-inoculated neonates in the following days (days 21 and 28) of the experimental protocol (**Figures 1C–F**). Thus, early experimental Ag exposure somehow accelerated the adult-like organization in neonates immunized at birth (day 0).

As shown in newborns, the quantity of follicles increased rapidly being very similar between the two groups (Ag-immunized and SS control, with an average of four follicles per section/node by day 7); reaching adult-like levels by day 14 (**Figure 1E**). In adults, the follicle number increased after 1 day post Ag immunization, declining to basal levels by day 21. Since in both the adults and the neonates experimentally immunized, the follicles were apparently bigger in size than in the control SS animals (**Figures 1A–D**); we measured the follicular areas at each time point in the different groups (**Figure 1F**). In adult mice, the follicular area was almost double in size 1 day after immunization than that of the SS control group. This ratio continued until day 21, declining after and being similar in both groups by day 28. In the newborns immunized at birth, the follicular areas at day 7 also had at least doubled the size of follicles from the control SS neonates. Bigger follicles in Ag immunized newborns continued by days 14 and 21. By day 28, the follicular areas were comparable between the two groups of newborns. It is worth mentioning that after day 7 post-immunization, follicular areas in neonates were rather similar to that of adults.

Immunization at Birth Triggers the Rapid Reorganization of the FRC Network

Fibroblastic reticular cells are another important type of stromal cells needed for the efficient induction of adaptive immune responses (7, 8, 10, 13–15, 38, 39). To test whether FRC networks were expanded following immunization in adults and neonates, we probed tissue sections using the ER-TR7 Ab. As expected in LNs from control adult mice, we found FRCs in the T cell area and to a lesser extent in the B cell follicle and particularly at the T:B border surrounding the B cell follicles (**Figure 2A**). FRCs also delineated large blood vessels that were likely HEVs, both in control and in immunized mice. Nevertheless, this FRCs

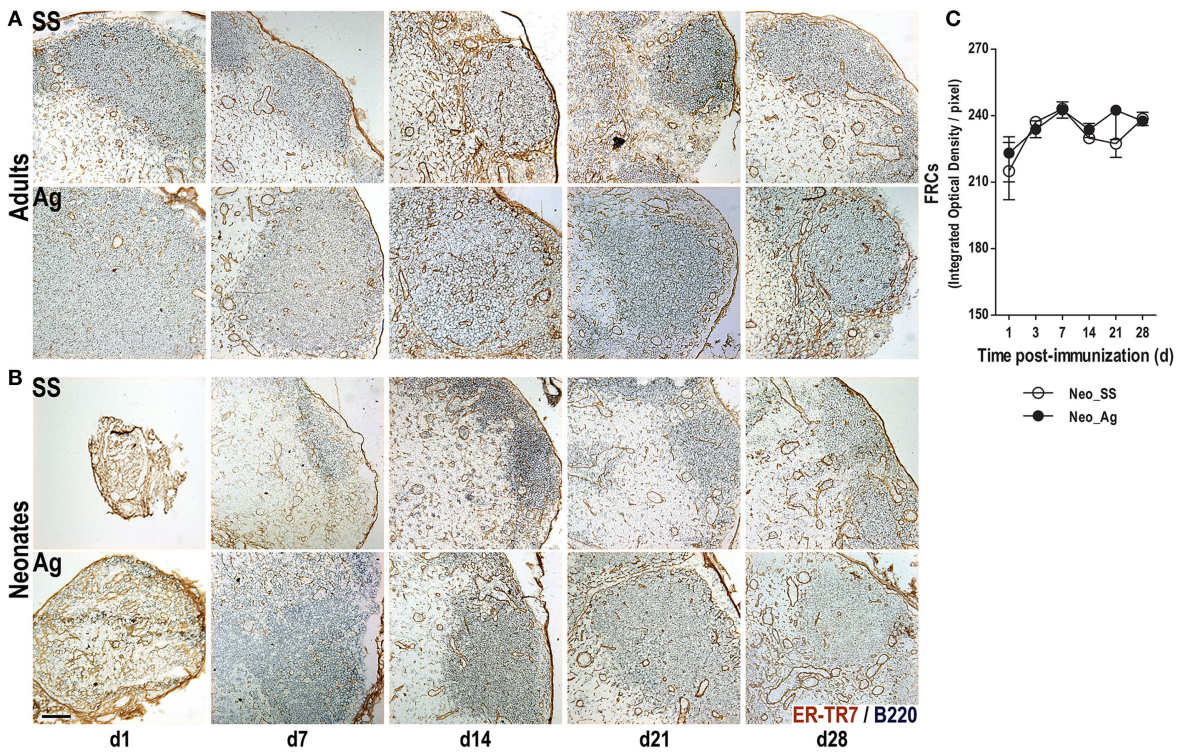

FIGURE 2 | Immunization at birth expands the fibroblastic reticular cell (FRC) networks. Adult **(A)** or neonatal **(B)** mice were inoculated with sterile saline solution (SS) or DNP-KLH (Ag), and the draining lymph nodes were examined by immunohistochemistry on days 1, 7, 14, 21, and 28 after inoculation. Cryosections were probed with Abs for B lymphocytes (B220, blue) and FRCs (ER-TR7, brown). The integrated optical density of the DAB color (FRCs color quantity) by pixels was determined in control and in immunized neonates **(C)**. Scale bar, 100 μm. Data shown are pooled from three independent experiments and represent the median ± interquartile range.

conduit system in adults appears better structured 1 week after experimental immunization (**Figure 2A**).

Interestingly, we observed robust staining for FRCs in neonatal LNs, even before immunization (**Figure 2B**). In fact, at day 1, the FRC network extended through the entire LN without interruption, probably because the B cell follicles are not yet formed. The FRCs network in the control neonates appeared (and was semi-organized) by day 7, whereas in immunized neonates, this network is clearly seen as early as 1 day after immunization. We tried to quantify the FRC networks in neonates by measuring the integrated optical density (IOD) of the brown color (produced by DAB) in neonates. Although the IOD between controls and Ag-immunized animals was rather similar, FRC networks were better structured in the Ag-inoculated neonates than in non-immunized ones (**Figure 2C**). Thus, Ag immunization at birth seemed to accelerate in the neonates the adult-like organization of FRCs too.

Immunization at Birth Promotes the GC Reaction

The preceding data suggested that immunization at birth accelerated the architectural organization of LNs. To test whether the accelerated architectural maturation promotes a more efficient immune response, we assessed the capacity of B cells to undergo GC reactions. To accomplish this goal, we probed tissue sections with the lectin PNA. As expected, adult mice that received SS did not have PNA+ B cells at any time consistent with the absence of GCs in these mice (**Figure 3A**). However, immunized adult mice generated large PNA+ B cell clusters starting on day 7, a pattern that continued on day 14, and waned by days 21 and 28 (**Figure 3A**). Similarly, we did not observe any PNA+ B cell clusters in neonatal mice that received SS. However, following immunization, neonatal mice did develop small clusters of PNA+ B cells by day 14 (**Figure 3B**), which were still seen by day 21, but were difficult to detect by day 28. While PNA+ B cell clusters were absent in control SS neonates, they occupied about 2% from the total LN area in neonates at day 21 post-immunization (**Figure 3C**). These data suggest that neonatal mice are capable of generating a GC response following immunization at birth, but that GC formation is delayed and importantly attenuated compared to adults.

We next tested whether B cells expressed AID, the enzyme responsible for both CSR and SHM, two key molecular mechanisms that occur within GCs (33, 34). In immunized adults, we found well-defined clusters of AID+ cells on days 14 and 21 post-immunization (**Figure 4A**); however, AID+ cells were never observed in the control SS adult animals (not shown). In neonates immunized at birth, we observed AID+ cells at days 14 and 21 post-immunization (**Figures 4B,C**), although these cells were relatively rare and were observed scattered about rather than in clusters that resembled GCs. Again, we did not observe AID

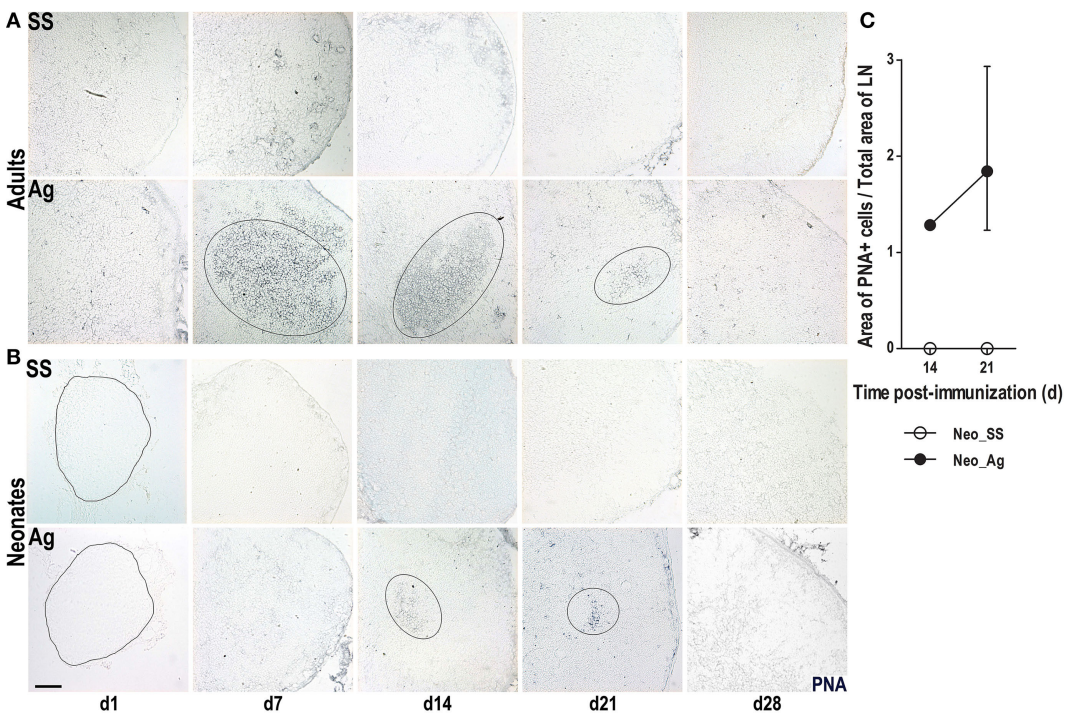

FIGURE 3 | Germinal center responses are delayed in neonatal lymph nodes (LNs). Adult **(A)** or neonatal **(B)** mice were inoculated with sterile saline solution (SS) or DNP-KLH (Ag) and the draining lymph nodes were examined by immunohistochemistry on days 1, 7, 14, 21, and 28 after inoculating. Cryosections were probed with the lectin peanut agglutinin (PNA, blue). Germinal centers (GCs) are delimited by elliptical lines. The outline of LNs at day 1 in neonates is indicated by dashed lines. **(C)** The GC area of control versus immunized neonates is represented as percentage of the Area of PNA+ cells from the total lymph node (LN) area on days 14 and 21. Scale bar, 100 μm. Data shown are pooled from three independent experiments and represent the median ± interquartile range.

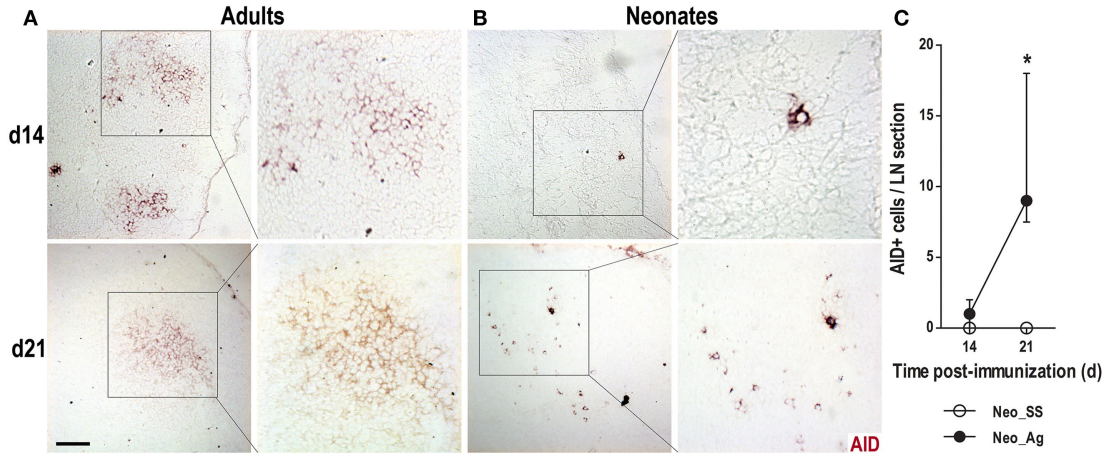

FIGURE 4 | Neonate lymph nodes (LNs) show few activation-induced cytidine deaminase (AID)+ cells after immunization. Adult **(A)** or neonatal **(B)** mice were immunized with DNP-KLH (Ag), and the draining lymph nodes were examined by immunohistochemistry on days 14 and 21 after inoculating. Cryosections were probed with an antibody against AID (red). **(C)** The AID+ cells per LN cryosection in control and immunized neonates are shown on days 14 and 21. Insets show enlargements of indicated areas of AID+ cells. Scale bar, 100 μm. Data represent the median ± interquartile range from three to nine mice per group at each time point and were analyzed with Mann–Whitney *U* test. Control neonates versus neonates immunized at birth. $^*P < 0.05$.

expression in newborns treated with SS (not shown). Altogether, these data suggest that newborns are capable of generating GC reactions, including AID expression, following immunization. However, this response seems to be delayed and very much reduced compared to that in adults.

Immunization at Birth Prompts the Rapid Appearance of T Cells Inside B Cell Follicles

As GC reactions develop in response to T-cell-dependent Ags and because GCs were observed in neonates by day 14 post-immunization (14 days after birth), we searched for T cells inside follicles in the preceding days. A requisite for T cells to interact with follicular B cells is the recruitment of T cells into B cell follicles. Thus, we probed tissue sections with Thy-1 Ab to quantify the Thy-1+ T cells inside (B220+) B cell follicles at days 1, 3, 7, and 14 post-immunization. Similarly to B cells, in control LNs from neonatal mice, we observed very few scattered T cells on day 1, these T cells increased over time, some were inside B cell follicles (**Figure 5A**). By contrast, in immunized neonates, Thy-1+ cells inside B cell follicles were readily detected from 1 day post-immunization (**Figure 5B**). In the following days (3, 7, and 14) increasing numbers of these T cells inside B follicles were found in the Ag-inoculated neonates (**Figures 5B,C**). Thus, experimental Ag exposure at birth somehow accelerated the recruitment of T cells into B cell follicles.

Immunization at Birth Elicits IgG-Secreting Cells and Ag-Specific IgGs

As we found that neonates immunized at birth underwent GC reactions and also that T cells were present within follicles, we next wanted to know whether these neonates produce class-switched Abs. Therefore, using flow cytometry, we first enumerated total IgG-secreting cells in the LNs after immunization. We found that IgG-ASC numbers in immunized adults were already high by day 7, reached a peak by day 14, and declined by days 21 and 28 (**Figure 6A**). Interestingly, in neonates immunized at birth, IgG-ASC numbers also peaked at day 14 and fell by day 21 (**Figure 6A**). Although the IgG-secreting cell numbers in immunized neonates were much lower than those in adults, they followed a very similar kinetic to that of immunized adult mice.

We also measured Ag-specific serum IgGs in both adults and neonates at 7, 14, 21, and 28 days post-immunization (**Figure 6B**). In adult animals, we found that the titers of Ag-specific IgGs were already very high 1 week after immunization and increased steadily on days 14, 21, and 28. By contrast, in neonates immunized at birth (day 0), the titers of Ag-specific IgGs were low for the first 2 weeks, but they clearly increased by day 21 and were still increasing by day 28. Nevertheless, the titers of Ag-specific IgGs were generally about 10-fold higher in adults than in neonates, consistent with the size of GCs and numbers of AID-expressing B cells.

We also examined Ag-specific IgMs. As expected, we found that adults produced high titers of Ag-specific IgMs by day 7 post-immunization and that the titers declined by day 14 (**Figure 6C**). The kinetics of Ag-specific IgGs and IgMs in adults were as expected, whereas the circulating IgGs increased, the IgM Ab response decreased over time. However, in neonates immunized at birth, the kinetic of Ag-specific IgMs was similar to that of Ag-specific IgGs (**Figures 6B,C**). Of note, the Ag-specific IgM response in neonates was similar to that in adults.

Ag-Naïve Pups Born to Immunized Mothers Have High Titers of Ag-Specific IgGs

Although neonatal mice were capable of generating class-switched IgGs and IgG-ASCs following immunization, this

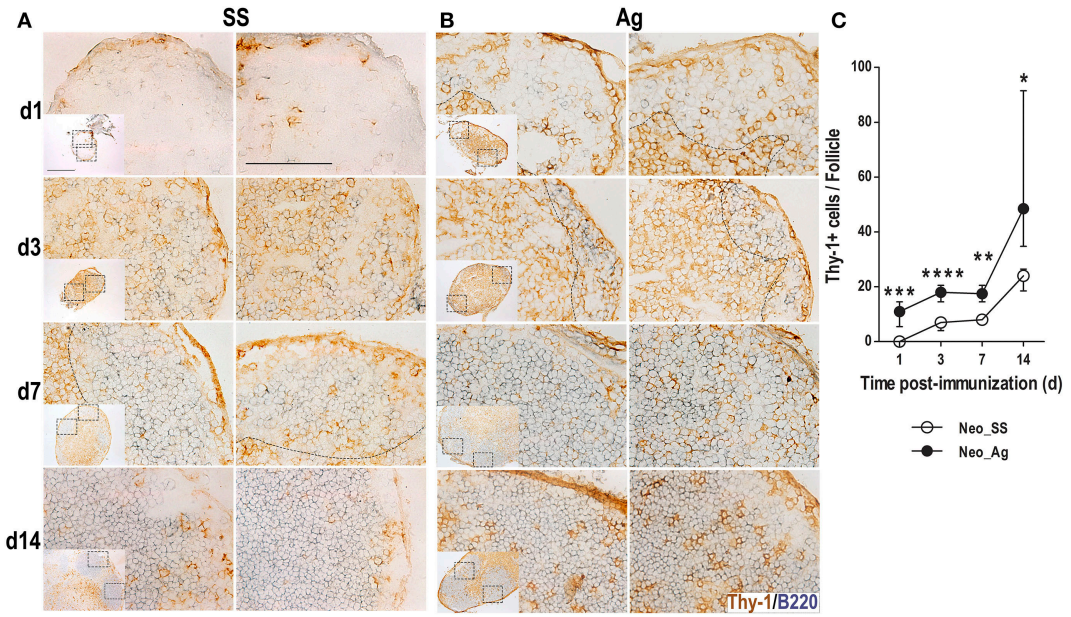

FIGURE 5 | **Immunization at birth accelerates the recruitment of T cells inside B cell follicles**. Neonatal mice were inoculated with sterile [saline solution (SS) **(A)**] or DNP-KLH [Ag **(B)**], and the draining lymph nodes were examined by immunohistochemistry on days 1, 3, 7, and 14 after inoculation. Cryosections were probed with Abs for B lymphocytes (B220, blue) and T cells (Thy-1, brown). Two B cell follicles (scale bar 100 μm) are shown from each lymph node (insets, scale bar 300 μm). The number of (Thy-1+) T cells inside B cell follicles **(C)** from neonates was determined during the first 14 days post-inoculation. Data represent the median ± interquartile range and were analyzed with Mann–Whitney U test. Control neonates versus neonates immunized at birth. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

response was somewhat delayed compared to that in adults, suggesting that humoral immunity might be limited immediately after birth. Since it is known that maternal Abs could be passively transferred to neonates (40, 41), we investigated how newborns will cope with new foreign Ags to which the mother has been exposed in the surroundings, and most likely newborns will be too immediately after birth. Therefore, we tested whether at birth neonates will show Abs specific for the Ag that we have previously used to immunize the mothers. Thus, adult female mice were immunized with either SS or DNP-KLH and the day of immunization these female mice were mated. Once pregnant, mice were boosted with another dose of DNP-KLH 14 days after their first immunization and serum was obtained at various times after birth. We found that neonates born to immune mothers had high titers of Ag-specific serum IgGs on the day of birth. Titers of these Abs were maintained high during 3, 7, and 14 days after birth, peaking at day 7; however, the presence of these Ag-specific IgGs in non-immunized neonates declined by day 21 (**Figure 7**). Interestingly, the decrease of the passively transferred Abs by day 21 concurs with the time that neonates reach high titers of their own Ag-specific Abs (**Figures 6B,C**).

DISCUSSION

Early upon birth some tissues are still ongoing modifications to attain what is deemed the proper adult functioning. Although the immune system and SLOs are most likely no exception to this, the published research is contradictory (1, 2, 5, 42, 43). How the LNs in neonates are organized very early after birth and whether Ag plays a role in this process are not well known (16, 44).

We show in this work that immunization on the day of birth accelerated the FRC organization in neonatal LNs. FRCs, as part of the stromal cell supporting the LN architecture, organize the compartmentalization for B and T cells and direct the traffic of both, leukocytes and incoming Ags (13–15). Despite the importance of FRCs, there are very few publications about their organization in early postnatal stages (35). It has been shown that during the first week of life FRCs change dramatically and that by day 3, they are around the follicular area. During LN colonization with mature lymphocytes, B cells accumulate and generate follicles and the networks formed by the FRCs undergo progressive remodeling. These data are consistent with ours regarding "basal" conditions (i.e., in absence of experimental stimulus), as the distribution of FRCs 24 h after birth is shown throughout the LN, even in the subcapsular area, while B cells were sporadically observed. In the LNs from neonates immunized at birth and analyzed 24 h later, we already observed the accumulation of B cells in the subcapsular region, which were forming a sort of follicles. Interestingly, we found that the FRCs organization at day 7 in neonates that were immunized at birth is similar to that reported in immunized adults. It is neither clear whether B cells *per se* and/or the FDCs development are involved in this remodeling phenomenon nor which soluble and/or surface molecules might be taking part. Several concurrent mechanisms may lead to the progressive remodeling of this reticular conduit system (44). Progressive reorganization of FRCs networks from developing

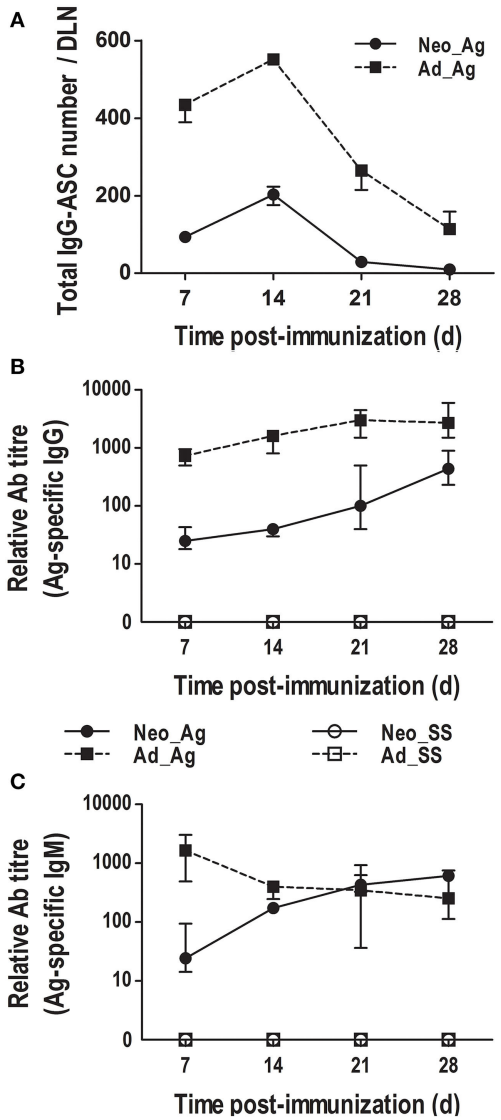

FIGURE 6 | Class-switched antibody (Ab) production in neonates immunized on the day of birth. Adult or neonatal mice were inoculated with sterile saline (SS) or DNP-KLH (Ag). **(A)** The number of IgG-secreting cells (IgG-ASCs) was quantified on days 7, 14, 21, and 28 in draining lymph nodes (DLNs) using flow cytometry. **(B)** The titers of Ag-specific IgGs in serum were determined at days 7, 14, 21, and 28 by ELISA. **(C)** The titers of Ag-specific IgMs in serum were determined by ELISA. Data represent the median ± interquartile range from three independent experiments with three to nine mice per group at each time point.

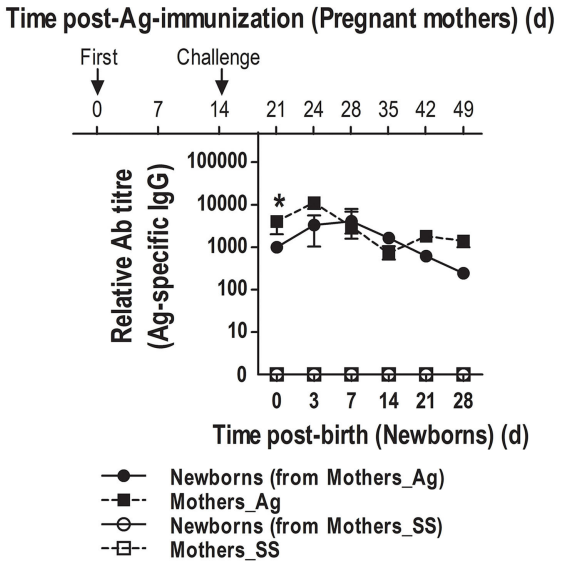

FIGURE 7 | Naïve neonates born to antigen (Ag)-immunized mothers show at birth Ag-specific IgGs. Female mice were inoculated with sterile saline (SS) or DNP-KLH (Ag) and mated. Serum was obtained from the mothers and naïve pups at the indicated times and Ag-specific IgGs were measured by ELISA. Data represent the median ± interquartile range and were analyzed with Mann–Whitney *U* test. Immunized mothers versus naïve neonates born to immunized mothers. *$P < 0.05$.

follicles may result from the loss of their integrity, facilitating the expansion of lymphoid organs and the restructuring reported in adults when facing antigenic or pro-inflammatory stimulation (10, 11).

On the other hand, despite the fact that FDCs represent the stromal cell compartment inside follicles, maintaining B cell homeostasis and organizing B cell responses in LNs (including the production of high affinity Abs), research on FDCs in early neonatal stages is very scant. Some reports described the absence of FDCs in 1-week-old neonates. However, when these mice were immunized and analyzed 1 week later (i.e., at 14 days of age), FDCs were then observed (35, 43). It thus seems that the presence of FDCs in very early neonates has been previously unrecognized. Certainly, we detected few FDCs as early as 24 h after experimental Ag immunization at birth, though not exactly within B cell clusters but rather at the side. This might not be unexpected since it has been reported that FDCs precursors are located immediately below the LN SCS, at the edge of follicles, and upon pro-inflammatory stimuli, they proliferate differentiating into FDCs (8, 45). In our protocol, the early antigenic challenge at birth might have provided the stimulation required to induce the appearance of FDCs (or FDCs precursors) at the edge of B cell follicles-like 24 h after Ag inoculation. In the non-immunized newborns, FDCs were observed until 14 days of life but not intrafollicular, just below the SCS where marginal reticular cells, their putative precursors, can be found. Thus, the behavior of these neonatal FDCs is consistent with the one described for FDCs in adults (17, 46). It is likely that this FDCs network might also provide positive co-signals for B-cell activation and differentiation at these early stages, but this requires much further research.

Regarding B cell development, at basal conditions in non-immunized neonates, we could see B cell follicles in the cortical area from day 7 after birth, which is consistent with previous reports (35, 47). This initial organization (even in the apparent absence of FDCs) could be due to the secretion of CXCL13 by the marginal reticular cells or by the presence of the lymphoid tissue inducers, with which the B cells establish a positive feedback for

continuous secretion of CXCL13. CXCL13 has a dual role in the follicular compartmentalization of B cells: mediating B cell attraction and inducing increased LTα1β2 expression on the recruited cells. LTα1β2 then engages LTβRs on non-hematopoietic stromal cells, promoting differentiation/maturation of FDCs and leading to increased expression of CXCL13 to initiate the LN reorganization (48). Our data indicate that the antigenic stimulus prompts the functional reorganization of lymphoid tissues accelerating both the appearance and the structuring of cellular subcompartments as early as 24 h after stimulation.

Therefore, we decided to analyze the humoral response in the mice immunized at birth. Pihlgren et al. defined the neonatal humoral response as delayed, as they observed that ASCs reached their peak by day 14 whereas in adults peaked at day 10 after antigenic stimulus (43). Our work revealed that the humoral adaptive immune response (IgG) of the neonates immunized at birth was of a similar kinetic for ASCs than that of adults but of much lower magnitude. Moreover, Ag-specific IgGs were observed by day 7 in neonates immunized at birth, and PNA+ B cell clusters *in situ* were seen by day 14 post-Ag stimulation. Although PNA+ cell clusters have been described in mice immunized at 7 days of age and assessed 10 days later, i.e., at 17 days of age (49); we found these clusters at day 14 of age in neonates immunized just at birth. To this respect, the *in situ* GC response assessed by means of PNA labeling in neonates would be delayed (about 7 days) compared with that in adults. The presence of Ag-specific Abs by day 7, before the peak of the ASCs (day 14) could be because after BCR engagement, partially activated B cells can migrate to the T:B border and proliferate and differentiate there, creating clusters of proliferating B cells and ASCs (some even might undergo isotype switching). This is the extrafollicular (EF) response and those ASCs may last a few days as short-lived ASCs (50). On the other hand, some of these activated B cells might in turn migrate from the T:B border into follicles where they continue proliferating, forming a nascent GC. As a result, the long-lived ASCs from GCs maintain an increased Ab concentration. These data suggest that the Ab kinetic observed in the immunized neonates could be due first to an EF response, followed by GC reactions. Importantly, parallel to the decreasing of total ASCs within the DLNs from both adults and neonates, the systemic levels of Ag-specific IgGs were clearly augmented and were still increasing by day 28, revealing that the B cell response to Ags (including Ig class switching) can be mounted rather early after immunization at birth. Of note, this Ab response in neonates is also of lower magnitude than that in adults, but whether it is completely efficient needs further examination.

Since T cell help to B cells is critical for GC formation and for the generation of ASCs and memory B cells, and we found that neonates immunized at birth are able to induce the formation of GCs, we searched for T cells within the follicles. Although we did not perform a precise fine characterization of follicular T cells, our data show that the appearance of Thy-1+ T cells inside B cell follicles is accelerated upon very early immunization, compared to neonates in basal conditions. It has been reported that in early life the follicular T cells do not complete their differentiation into fully activated GC Tfh cells and thus fail to optimally support GC B cell differentiation and survival, compared to Tfh from adults. Whether this failure is due to the limited capacity of Tfh cells to localize in GCs is uncertain. In addition to this, the proportion of Tfh cells expressing Foxp3 seems to be significantly higher in neonates, suggesting a preferential induction of T follicular regulatory cells (49, 51). However, our *in vivo* data altogether suggest that these cells would be already operating at this early stage of life, revealed by the increasing numbers of T cells inside B cell follicles, the induction of GCs, and by the increase of ASCs and of Ag-specific IgGs over time.

A report of Ag-specific IgMs in neonates experimentally immunized 1 week after birth only mentioned (without showing the data) "the presence of certain quantities but minimal and transient IgMs" (42). However, we found that in neonates immunized at birth, Ag-specific IgMs and IgGs increased very similarly over time. In the case of adults, IgM decreased post-immunization concomitantly with the increase of IgGs, as previously characterized (52). In contrast to Barrios et al. (42), the differences with our work might be due to both the Ag and the time of inoculation we used. Regarding this, we wanted to test the capacity of the neonate immune system to an Ag to which they would be exposed precisely in similar quantities/conditions than adults, as it would be likely happening in the first days of extrauterine life. As B cell populations colonize peripheral SLOs in neonates, maybe a similar kinetic between the production of IgMs and IgGs might be expected. It suggests that B cells that generate IgM- and IgG-ASCs would be involved in the response at approximately the same time in these neonates. The regulation of IgMs and IgGs very early after birth has been poorly explored (53) and more studies are clearly needed. In adults, it is described that IgMs might act directly on B cells to regulate the magnitude of the IgG response (53–55). Due to the ability of IgM–Ag complexes to bind complement and activate the complement cascade, these might activate B cells by binding to CRs, providing important costimulatory signals. Another possibility is that Ag-free, pentameric IgM may engage Ag on Ag-bearing BCRs on B cells, triggering them (54). IgM would thus enhance the signals provided to B cells *via* the BCR (54, 56, 57). Something similar could be happening in neonates, enhancing the adaptive IgG response.

Furthermore, as the Ag-specific IgM response was similar between neonates and adults by day 14 post-immunization, it is conceivable that the EF reaction in neonates might be generating bigger quantities of IgM-ASCs compared to IgG-ASCs, as reported in adults (50). To this respect, the start of the IgG response would be delayed in neonates, whereas the IgM response would reach adult-like titers faster. Nonetheless, regarding Ag-trapping it is worth remembering that, as IgM is pentameric and IgG bivalent, it is conceivable that less quantities of IgM might compensate for the low IgG response during the first days after birth.

As mentioned, the enzyme AID acts in Ag-stimulated B cells allowing SHM and CSR. We found AID *in situ* by days 14 and 21 in the LNs of immunized neonates, AID+ cell clusters were fewer than in adults and seen in the cortical area where B cell follicles are present and where SHM and CSR could be happening. It has been shown in adult mice that during the immune response to a model Ag, specific B cells in LNs get initially activated at the follicle borders and differentiate into short-lived ASCs in EF foci (58, 59), which could be observed also in immunized newborns. Since

AID-triggered CSR can also be induced during EF plasmablast differentiation (60), the presence of AID in newborns immunized at birth could represent the development of EF reactions. Besides the presence of GC reactions, the GC-independent CSR may represent an ancient pathway of AID induction that evolved before proper GCs appeared in evolution, as seen in lower vertebrates that develop ASCs and undergo CSR without GCs (60, 61). Of note, these neonates immunized at birth are capable of undergoing CSR since (a) we observed the presence of AID *in situ* after Ag stimulation and (b) we identified class-switched IgG Abs both by means of total IgG-ASCs in DLNs and of Ag-specific IgGs in circulation. Presumably, these neonates would be doing SHM too, but this needs to be demonstrated. Thus, compared to adults, the B cell response in neonates would be somewhat diminished in its ability to isotype switch, most likely due to poor AID expression. To the best of our knowledge, AID expression has not been previously shown in DLNs from neonates after Ag stimulation at birth.

As high titers of Ag-specific IgGs were not seen until day 21 post-immunization, we investigated whether neonates would be coping with totally new Ags immediately after birth through passively transferred maternal Abs, as it has been reported (40, 41). We found that the levels of Ag-specific Abs from both the mother and the naïve neonates were highly correlated. These passively transferred Abs confirmed a mother-derived coverage to neonates against Ags to which mothers, and most likely neonates, are exposed. This would ensure the protection of newborns while they structure the microarchitecture and subcompartmentalization within SLOs to efficiently mount their own responses.

Finally, besides the finding that rather early antigenic exposure (at birth) prompts the neonate immune system to engage in efficient immune responses, the type of Ag we used (62, 63) and the results we obtained are both indicative of efficient T cell help to B cells operating already at these early stages, although we did not examine this directly in this paper.

Thus, neonatal immune system might not be intrinsically immature but rather adapted for early postnatal life. Our results suggest that some responses of the neonatal immune system differ from immune responses observed in later life stages. However, it seems that evolutionary adaptations have provided these newborns with some elements to cope with Ags at birth.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: RM-F, JY-P, and LF-R. Performed the experiments and the acquisition of data: RM-F, JY-P, EM-J, IG-H, and JC-A. Analyzed the data, interpretation, and discussion; wrote and revised the paper: RM-F, JY-P, AS-S, TR, and LF-R. Final approval of the version to be published: LF-R.

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Antifungal Immunological Defenses in Newborns

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Newborns are prone to fungal infections, largely due to *Candida* species. The immunological basis for this vulnerability is not yet fully understood. However, useful insights can be gained from the knowledge of the maturation of immune pathways during ontogeny, particularly when placed in context with how rare genetic mutations in humans predispose to fungal diseases. In this article, we review these most current data on immune functions in human newborns, highlighting pathways most relevant to the response to *Candida*. While discussing these data, we propose a framework of why deficiencies in these pathways make newborns particularly vulnerable to this opportunistic pathogen.

Keywords: neonate, immunology, fungus, *Candida*, humans, infection, prematurity

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INTRODUCTION

Fungi are present everywhere in the environment, including in water, on solid surfaces, on our skin, and gastrointestinal tract. Taxonomists estimate the existence of 1.5 to over 5 million fungal species, although only a small minority (<300 species) causes diseases in humans (1). Despite their ubiquitous presence, fungi rarely become invasive in healthy adults due to multiple levels of immune defenses. In contrast, fungal infections are common in newborns and can be particularly invasive in those born very prematurely (2, 3).

A number of studies have investigated the functional characteristics of newborn immune cells [reviewed in Ref. (4, 5)]. The immune system is composed of two main arms involved in the recognition of fungi. Developmental changes in some of the main immune pathways involved in responses against Candida are illustrated in **Figure 1**. During gestation, innate immune cells are skewed toward anti-inflammatory responses. Adaptive immune cells also lack immunological memory from prior exposure to antigens and are skewed toward a T helper 2 (Th2) effector profile. These changes are essential to prevent allogeneic maternal rejection and during the establishment of tolerance toward self-antigens. Moreover, the expansion and maturation of immune cells is incomplete in infants born very prematurely, which further increases their vulnerability to infections (4, 5). These functional limitations are also affected by pregnancy complications, which can be linked to a premature birth (6, 7).

While our knowledge of the maturation of immune pathways in human newborns has greatly progressed recently, few of these studies have focused on fungi as model organisms. Therefore, our understanding of the immunological basis for the increased susceptibility of the neonatal immune system to fungi remains limited. Nonetheless, insights can be gained from rare genetic mutations predisposing to localized or invasive *Candida* infections in humans. These data have been recently covered by other experts (8, 9). The clinical presentation, risk factors, and treatment of neonatal *Candida* infections have also been reviewed recently (10, 11). This review discusses recent data

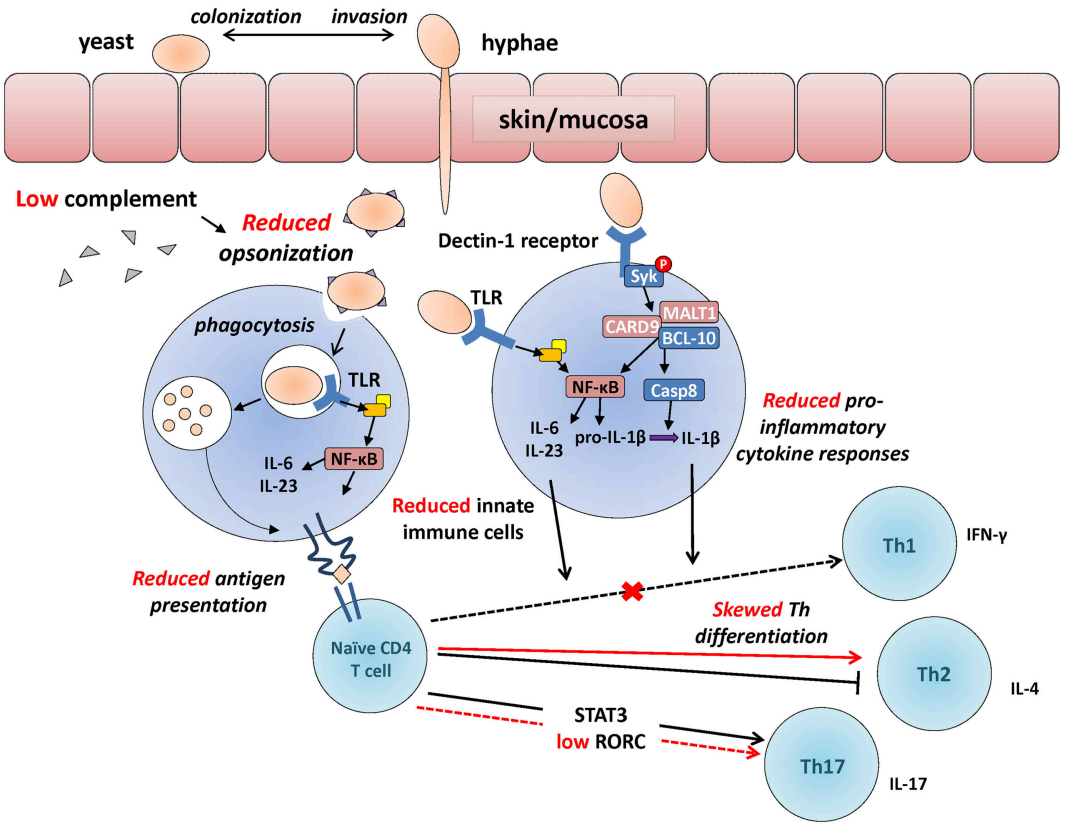

FIGURE 1 | Developmental changes in the immunological response to *Candida*. Developmental differences in some of the main immunological events involved in the recognition of *Candida*, between adults (in black) and neonates (in red, cross also indicates a reduction in neonates compared to adults). *Candida* colonizes the skin or mucosa as yeast but often invades in hyphae form allowing to penetrate through epithelial barriers more efficiently. Upon innate immune detection of *Candida* through pattern recognition receptors [e.g., toll-like receptors (TLRs); Dectin-1 receptor], microorganisms get opsonized (reduced in preterm neonates), facilitating phagocytosis and resulting in the production of pro-inflammatory cytokines (also reduced in neonates) through Syk and NFκB-mediated intracellular signaling. Internalized *Candida* antigens are presented (reduced in neonates) to naïve CD4 T cells, resulting in their differentiation into Th1/Th17 effector cells (skewed toward T helper 2 in neonates). Whereas deficiencies in innate functions (e.g., MALT-1/CARD9) can lead to invasive candidemia, selective deficiencies in adaptive functions (e.g., IL-17 responses) most often lead to chronic mucocutaneous infections.

underlying the immunological basis for newborns' increased susceptibility to *Candida* infections.

NEONATAL *CANDIDA* INFECTIONS

In newborns, *Candida* is responsible for the common oral thrush and rash in skin folds and in the diaper area. Before the advent of modern sanitary measures and topical antifungal treatments, infants died from dehydration due to severe oral mucocandidiasis (12). Nowadays, invasive infections are rare with the exception of infants born very premature, those who require prolonged indwelling medical devices, or in cases of a primary immunodeficiency (8, 13).

Once invasion occurs, the mortality from *Candida* infections in newborns is high, and so is the associated morbidity: up to two-thirds of those who survive will suffer long-term impairments (14). Similarly, fungemia due to other genera such as *Malassezia* (15), Aspergillosis (16), and Zygomycosis (17) also carry a high mortality, though these infections are more rare. Dermatophytes infrequently cause skin infections in young infants.

At birth, neonates generally have a low fungal burden (18–20); however, colonization occurs in a majority of neonates through both vertical (mother-to-child) and horizontal (nosocomial) transmission (20–28). Most invasive *Candida* infections occur between the second and sixth week postnatal age (29, 30) owing to the timing of colonization. *Candida albicans* is the most frequently isolated *Candida* species, but other species, particularly *Candida parapsilosis*, but also *Candida tropicalis*, *Candida glaberata*, and *Candida kruzei* are becoming more prevalent (11, 31–33). Interestingly, major variations have been reported in the incidence and species distribution of *Candida* infection among neonatal intensive care units across the world (3, 34, 35). For example, in North America and Europe, invasive disease almost exclusively occurs in infants of birth weight less than 1,000 g (2, 11, 36, 37), whereas up to 15% of infants born below 33 weeks in neonatal center in Shanghai were diagnosed with a systemic fungal infection (38). These variations may be due to racial differences in immune phenotypes, although this has not been formally examined in the context of *Candida* infections. On the

other hand, these differences in epidemiology are more likely due to geographical variations in infection control measures and in the use of broad spectrum antibiotics.

INNATE IMMUNE RESPONSES

The innate immune system is the first-line of immune defenses responsible for signaling the presence of microorganisms and riding our body from an invasion through opsonization (i.e., targeted labeling), cell-to-organism killing, and phagocytosis. The epithelial layers (skin and mucosa) are the first line of defense of the innate immune system against a fungal invasion (39, 40). Highly premature infants lack *vernix caseosa*, which is a natural substance composed of antimicrobial sebum, covering the skin of term newborns (41, 42). This lack of *vernix caseosa* may increase fungal invasion by affecting the balance between the infant's bacterial and fungal flora (42). However, this contention, at this point, remains speculative and requires further study.

Antimicrobial Peptides

Antimicrobial peptides are a major component of innate immune defenses. These peptides generally show reduced levels in those born prematurely [reviewed in Ref. (43)]. Levels of α-defensin have been correlated with the presence of mannan in bronchoalveolar lavage fluids from preterm neonates, indicating a role in controlling fungal growth at mucosal surfaces (44). Complement proteins are another major component, consisting of at least 20 interdependent components that are deposited on the surface of pathogens, resulting in phagocytosis *via* opsonization, killing *via* pore-formation, and activation of inflammatory cytokine responses. In mice, deficiencies in complement proteins, particularly C3, result in an inability to clear infections due to *C. albicans* and *Candida glabrata* (45). Human C5a also appears important for induction of inflammatory cytokine responses to *C. albicans* (46). In one case, a child with a hereditary C3 deficiency was unable to opsonize this microorganism and normal function was restored with C3 supplementation (47); deficiencies in factor H and factor I have also been shown to negatively affect killing of *C. albicans* (48). Production of complement proteins is detectable early on in the fetus during ontogeny (49) and increases in a gestational age-dependent manner until the term of gestation and even after birth (50, 51). Therefore, it is possible that relative complement deficiencies in newborns may increase susceptibility to invasive *Candida* infections, but to a relatively minor extent.

Phagocytes

Innate immune cells such as neutrophils, macrophages/monocytes, and dendritic cells play important roles in preventing a fungal invasion [reviewed in Ref. (52)]. In mice, depletion of neutrophils increased susceptibility to cutaneous *Candida* infections (53) and also increased the risk of lethal invasion following experimental mucosal damage (54). Humans with genetic defects that impair neutrophil functions, such as the autosomal recessive myeloperoxidase deficiency, are at greatly increased risk of systemic Candidiasis, suggesting an important role for neutrophils and other phagocytes (55). Neutrophil extracellular traps (NETs) facilitate killing of *C. albicans*, although their functional importance against this pathogen is debated (55–57). NET formation upon *Candida* exposure is operational in newborns and, therefore, a neonatal deficiency in NET does not explain their susceptibility to infections (58). On the other hand, neutropenia due to a central, bone marrow cause, severely predisposes to systemic candidiasis in adults (59). In the fetus, bone marrow production of hematopoietic cells rapidly increases after the 20th week of gestation (4, 5). Consequently, lower neutrophil and monocyte cell counts are observed in extremely low gestation preterm infants, which may play a more important role in increasing the risk of systemic infections in these infants (60, 61). Interestingly, neutropenia is often not observed during *Candida* sepsis in preterm neonates, in contrast to Gram-negative bacteria, which may indicate a more limited role for these cells once invasive infection has occurred (62).

In addition to a relatively limited neutrophil cell count, some studies have reported reduced neutrophil function in very preterm neonates. In a whole blood assay, reduced migration and phagocytosis of *C. guillermondii* was observed in very low birth weight (<1,500 g) infants compared to term neonates and adults (63). However, a recent study reported no difference in phagocytosis and oxidative burst between age groups (64). In general, phagocytosis functions are relatively preserved in very preterm neonates (65–67). These differences in findings may be due to differences in the assay or strain of *Candida* that have been used between these studies. More functional *in vitro* studies are required using *Candida* in order to help resolve these findings.

Monocytes/macrophages also appear to play an important role in preventing a *Candida* invasion based on mouse studies (52). Monocytes rely primarily on non-opsonic phagocytosis *via* Dectin-1 and Dectin-2 (68). To the best of our knowledge, the response of macrophages (or monocytes) to *Candida* has not been studied in infants born prematurely.

Pathogen Recognition

The lipid bilayer of *Candida* is surrounded by chitin, an inner cell wall component made of polysaccharides (β,1-3 glucan, β,1-6 glucan) and an outer cell wall composed of N-linked glycoproteins coated with mannan polymers (69). *C. albicans* can transform between yeasts and hyphae based on the environmental conditions (70). These two forms have different virulence and elicit different immune responses due to structural changes in their cell wall (70, 71). Immune cells recognize the presence of pathogens through innate receptors called pattern recognition receptors (PRRs). PRR can be free circulating in body fluids (e.g., pentraxin, collectins, or ficollins) or cell associated. Cell-associated PRRs include toll-like receptor (TLR), C-type lectin receptors (CLRs), and the intracellular (cytoplasmic) NOD-like receptors (NLRs) and RIG-I-like receptors (RIRs). Several PRR are involved in the immune recognition of *Candida*, including TLR2, TLR4, TLR6, and CLRs and in the recognition of *Candida* DNA (e.g., TLR3 and TLR9) (72–74). Recognition of fungi by multiple PRRs triggers a cascade of immune activation events including the production of cytokines, reactive oxygen species, and the activation of phagocytosis. These multiple levels of immune recognition enhance immune protection in healthy individuals.

In newborns, PRR functionality develops early in the third trimester of gestation, beginning with endosomal/cytoplasmic PRR around 20–24 weeks, followed by extracellular PRR until about 33 weeks of gestation when the PRR functionality compares to that of full-term infants (7, 75, 76) [reviewed in Ref. (4, 5)]. These maturational changes are likely to play an important role in preterm infants' increased vulnerability to infections. Indeed, cytokine responses (TNF-α, IL-6, IL-1β, and IFN-γ) are decreased and skewed toward an anti-inflammatory phenotype early in gestation (5). Reduced cytokine responses have been linked to reduced downstream signaling, in part due to decreased expression of the main MyD88 signaling molecule, as well as a gestational age-dependent reduction in phosphorylation of p38 and ERK1/2 (65, 77–81). The *S*-type lectin receptor Galectin-3 is expressed on neutrophils, monocytes, macrophages, endothelial cells, and epithelial cells, can be secreted, and confers protection in *Candida* infection leaving galectin-3-deficient mice more susceptible to *Candida* infection (82, 83). However, conflicting results have been published regarding whether Galectin-3 levels are higher (84) or lower (83) in cord vs. adult blood and whether the levels increase (85), decrease (86), or remain constant (83) with decreasing prematurity.

Dectin-1 Receptor

Dectin-1 is a CLR and main extracellular PRR mediating the recognition of β-glucan in the *Candida* cell wall. Reduced Dectin-1 receptor function naturally occurs in ~1% of the general population due to a genetic polymorphism that introduces a stop-codon in the *CLEC7A* gene encoding this receptor. Humans with this polymorphism may display a marginally increased susceptibility to cutaneous fungal infections (87). However, these infections are generally mild due to a high degree of functional redundancy with other PRRs such as Dectin-2 (88, 89). Upon recognition of β-glucan at the surface of *Candida*, a phagocytic synapse containing Dectin-1, active Src, and Syk kinases is formed (90). The intracellular signaling molecule Syk becomes phosphorylated, resulting in the cytosolic colocalization of the signalosome complex composed of CARD9, MALT1, and Bcl-10 (see **Figure 1**). Assembly of this protein complex leads to two main sequences of events: (1) nuclear translocation of the transcription factor and main inflammatory regulator NF-κB, which then leads to induction of pro-inflammatory cytokine gene transcription (91) and (2) activation of the caspase-8 enzyme, which cleaves pro-IL-1β into its mature, secreted IL-1β form. Because of the central importance of the signalosome complex in antifungal immune defenses, a deficiency in CARD9 or MALT1 results in a marked increased risk for invasive fungal infections in humans (8, 92, 93). The function of Dectin-1 signaling has not been studied in premature newborns, requiring further studies to understand how this pathway may increase their susceptibility to fungal infections.

ADAPTIVE IMMUNE RESPONSES

Adaptive immune responses, mediated through dendritic cells, B and T lymphocytes, are essential to limit a *Candida* invasion. Following penetration of *C. albicans* through epithelial surfaces, dendritic cells become activated through PRR, resulting in their uptake and presentation of antigen fragments to CD4 T lymphocytes (also called "helper lymphocytes"). CD4 T cells producing the cytokine interleukin-17 (termed Th17 cells) are particularly important for controlling the proliferation of *Candida*, as evidenced by increased chronic mucocutaneous candidiasis in humans with genetic mutations in cytokines (e.g., IL-17A, IL-17F), receptors (e.g., IL-12β1R), or transcription factors (e.g., RORC, GATA2, STAT1, APS-1, and ACT1) along these pathways (94). In newborns, T cells are largely naïve and display reduced activity against microbial antigens as they have not been exposed during gestation (95). Moreover, neonatal CD4 T cells are intrinsically less able to differentiate into Th17 cells due to reduced expression of the transcription factor RORC (96). Adults with genetic mutations impairing RORC or STAT3 function have increased susceptibility to chronic mucocutaneous candidiasis due to diminished Th17 function (97, 98). STAT3 phosphorylation occurs in neonatal T cells although whether reduced expression may limit Th17 differentiation in this age group is unclear (99). Neonatal T lymphocytes also have a reduced ability to differentiate into interferon-γ-producing CD4 lymphocytes (5), which play a role protecting against fungi, through the activation of other cellular immune components (e.g., phagocytes) (9).

The role of innate immune cells in promoting the development of Th17 responses has been studied in newborns. In term newborns, antigen-presenting cells produce high levels of Th17-polarizing cytokines (i.e., IL-1β and IL-23) (100). However, the production of these cytokines and antigen presentation are profoundly reduced in dendritic cells and monocytes of preterm infants below 29 weeks of gestation (7, 101), which may further contribute to their susceptibility for invasive fungal infections. Other T lymphocyte subsets such as γδ T cells develop early during fetal life and are able to produce interleukin-17, naturally, in an innate-like manner in the absence of effector differentiation (102). In mice, these cells are an important source of interleukin-17 (103). However, their functional role in preventing fungal invasions in premature newborns remains to be determined.

LIMITATIONS OF *IN VITRO* STUDIES

An important limitation of studies investigating fungal immune responses by human primary immune cells *in vitro* is that this situation may not reflect the complex life cycle of this microorganism during an infection *in vivo*. For example, heat-killed *C. albicans*, which is commonly used as a model in *in vitro* assays, exposes more β-glucan on its surface than live yeast (104). Dectin-1 specifically recognizes β-glucan structures in the cell wall of yeast, but not hyphae forms of *C. albicans*, where the β-glucan is less accessible to immune cells. As such, filamentous growth of *C. albicans* is not recognizable by Dectin-1, resulting in deficiency of ROS production and a reduction in Th17 differentiation of T cells (104, 105). However, *Candida* hyphae, but not yeast, induce a strong immune response in macrophages (71). Hyphae can specifically activate the NLRP3 inflammasome, which is important for production of IL-1β (106). Moreover, these changes can be strain specific (107). These limitations may significantly restrain interpretation of data.

Likewise, experimental conditions influence the interaction between immune cells and *Candida*. Sasse and colleagues showed that neutrophils can phagocytose yeast *Candida* in a suspension (3D-setting) but fail to phagocytose opaque cells on a surface, 2D-setting (i.e., glass slide) (108). Moreover, it has been suggested that yeast are important for colonization and hyphae are responsible for invasion and that the switching between the two forms itself is responsible for activation of the immune system [reviewed in Ref. (109, 110)]. Unfortunately, this is not accounted for in most *in vitro* studies as live fungal pathogens are rarely used. To mitigate these problems, animal models have been developed (111) [reviewed in Ref. (112)]. However, it is important to remember that mice are not a natural host for *Candida* and that considerable differences in immune functions across species warrant validation in humans (113).

ENHANCING NEONATAL ANTIFUNGAL IMMUNE DEFENSES

Basic science research findings need to be translated into clinical practice. Systemic antifungal drugs reduce the incidence of colonization and invasive fungal infections (114). However, the applicability of these approaches is somewhat limited by concerns of increasing antimicrobial resistance (115). Also, the microbial flora of preterm infants differs considerably from adults, or term infants, suggesting a role for a bacterial dysbiosis in promoting Candidemia in preterm neonates (116). Indeed, one study showed high fungal diversity in stool samples from very low-birth weight infants (117). In light of these findings, altering the gastrointestinal flora of preterm infants through the use of probiotics may represent a more viable approach to reduce the risk of invasive infections in the neonatal intensive care unit (118). Reciprocally, a better understanding of the immune response to *Candida* in newborns could help design vaccine interventions (119).

More research is required to understand how immune responses can be modulated specifically in the very preterm infant. Innate immune training using ultra-low exposure to β-glucan enhances responses to *Candida in vitro* (120). In support of the application of this concept to newborns, TLR and Dectin-1 co-stimulation induced strong Th1-polarizing conditions in neonatal dendritic cells *in vitro* (121). However, without a clear knowledge of whether these pathways are functional in premature neonates, the applicability of this strategy in preventing systemic infection in the youngest age group of neonates remains speculative. Research in this area has been traditionally hard to pursue due to obvious ethical and logistical factors (4, 122). Indeed, blood volumes are extremely limited in these small infants even using non-invasive sources such as the placenta. The challenge of enrolling a large enough number of premature neonates into clinical trials is also a major limitation (3). In the absence of interventional studies, basic science research remains crucial to lay the foundation for more evidence-based medicine in our approach to neonatal fungal infections.

AUTHOR CONTRIBUTIONS

CM, BK, and PL conceived, wrote, and edited this manuscript.

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METHODS
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Investigation of *OCH1* in the Virulence of *Candida parapsilosis* Using a New Neonatal Mouse Model

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Candida parapsilosis is an opportunistic human fungal pathogen that poses a serious threat to low birth weight neonates, particularly at intensive care units. In premature infants, the distinct immune responses to *Candida* infections are not well understood. Although several *in vivo* models exist to study systemic candidiasis, only a few are available to investigate dissemination in newborns. In addition, the majority of related studies apply intraperitoneal infection rather than intravenous inoculation of murine infants that may be less efficient when studying systemic invasion. In this study, we describe a novel and conveniently applicable intravenous neonatal mouse model to monitor systemic *C. parapsilosis* infection. Using the currently developed model, we aimed to analyze the pathogenic properties of different *C. parapsilosis* strains. We infected 2 days-old BALB/c mouse pups via the external facial vein with different doses of *C. parapsilosis* strains. Homogenous dissemination of yeast cells was found in the spleen, kidney, liver and brain of infected newborn mice. Colonization of harvested organs was also confirmed by histological examinations. Fungal burdens in newborn mice showed a difference for two isolates of *C. parapsilosis*. *C. parapsilosis* CLIB infection resulted in higher colonization of the spleen, kidney and liver of neonatal mice compared to the *C. parapsilosis* GA1 strain at day 2 after the infection. In a comprehensive study with the adult mice infection, we also presented the attenuated virulence of a *C. parapsilosis* cell wall mutant (*OCH1*) in this model. Significantly less *och1*Δ/Δ null mutant cells were recovered from the spleen, kidney and liver of newborn mice compared to the wild type strain. When investigating the cytokine response of neonatal mice to *C. parapsilosis* infection, we found elevated TNFα, KC, and IL-1β expression levels in all organs examined when compared to the uninfected control. Furthermore, all three measured cytokines showed a significantly elevated expression when newborn mice were infected with *och1*Δ/Δ cells compared to the wild type strain. This result further supported the inclusion of *OCH1* in *C. parapsilosis* pathogenicity. To our current knowledge, this is the first study that uses a mice neonatal intravenous infection model to investigate *C. parapsilosis* infection.

Keywords: *Candida parapsilosis*, neonatal, mouse model, intravenous infection, cytokines

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INTRODUCTION

Candida species are common agents causing invasive diseases in neonates (Stoll et al., 2002; Benjamin et al., 2006; Chow et al., 2012). Although the incidence of invasive fungal infections among premature infants in neonatal intensive care units (NICUs) has decreased during the past decade (Aliaga et al., 2014; Kelly et al., 2015), candidiasis and infection-related death is still of major concern (Le et al., 2013).

Previously, epidemiological reports have demonstrated the difference in the range of infections caused by *Candida* species (Dotis et al., 2012; Juyal et al., 2013; Jordan et al., 2014). While *Candida albicans* is the leading cause of invasive candidiasis in general, children under the age of 2 are at greater risk of infections caused by *C. parapsilosis,* an emerging non-*albicans Candida* species (Dotis et al., 2012; Juyal et al., 2013; Jordan et al., 2014).

Neonatal animal injection methods have been in use for more than 30 years with the purpose of modeling newborn diseases (Billingham and Silvers, 1961; Pope et al., 1979; Domer, 1988; Chen et al., 1989; Rodewald et al., 1992; Tang et al., 1996; Genovese et al., 1999; Venkatesh et al., 2007; Trofa et al., 2011; Tsai et al., 2011). These models proved to be excellent for studying *Escherichia coli*- (Cox and Taylor, 1990), *Pseudomonas aeruginosa*- (Tang et al., 1996), *Listeria monocytogenes*- (Chen et al., 1989; Genovese et al., 1999), and *Streptococcus*- (Rodewald et al., 1992) caused sepsis in neonate mice and analyzing the virulence factors of these pathogens or the possible treatment of invasive diseases. Also, the neonate animal models have proven to be efficient to study newborn-related *Candida* infections (Pope et al., 1979; Domer, 1988; Venkatesh et al., 2007; Trofa et al., 2011; Tsai et al., 2011). Early studies laid foundations to establish systemic candidiasis in newborn mice by gastric inoculation (Pope et al., 1979; Domer, 1988). Using-2-day old mouse pups, the intraperitoneal route of injection was used to demonstrate the disseminated infection and assessing virulence properties of the different *C. albicans* strains, including comparisons of survival proportions or the extent of organ colonization (Tsai et al., 2011). Trofa et al. (2011) provided the first report of *C. albicans* and *C. parapsilosis* infection in a neonatal rat model. This research also demonstrated the crucial role of the secreted lipases in the virulence of these species. The study concluded that preterm rodents show higher susceptibility to *Candida* infections and confirmed the utility of neonatal animal models to characterize *C. parapsilosis* pathogenesis.

In newborn mice, gastric or intraperitoneal inoculation has previously been carried out as a route of infection to model systemic candidiasis (Pope et al., 1979; Domer, 1988; Venkatesh et al., 2007; Tsai et al., 2011). However, these models still have several limitations. The colony counts have shown considerable variation between the already mentioned inoculation methods. Gastric infection of infant mice led to rapid transmission of *C. albicans* cells from the gut to the liver, and less frequently to other organs such as the kidneys and spleen (Pope et al., 1979; Domer, 1988). Following peritoneal injection, strong spleen attachment occurred, either by direct contact with the organ or through lymphatic channels (Tsai et al., 2011).

Systemic infection models in neonatal mice are valuable tools for studying severe newborn diseases (Vizler et al., 1993; Kienstra et al., 2007; Glascock et al., 2011). In this study, we introduce a new intravenous neonatal mouse model of *C. parapsilosis* infection, which could help to understand the complications in the response of the premature immune system to the systemic fungal invasions.

MATERIALS AND METHODS

Ethics Statement

All animal experiments were performed by national (1998. XXVIII; 40/2013) and European (2010/63/EU) animal ethics guidelines. The experimental protocols were approved by the Animal Experimentation and Ethics Committee of the Biological Research Centre of the Hungarian Academy of Sciences and the Hungarian National Animal Experimentation and Ethics Board (clearance number: XVI./03521/2011.) with the University of Szeged granted permission XII./00455/2011 and XVI./3652/2016 to work with mice.

Candida Strains

Candida parapsilosis GA1 (SZMC 8110) (Gacser et al., 2005), CLIB 214 (SZMC 1560) (Laffey and Butler, 2005) wild-type strains and *C. parapsilosis och1*Δ/Δ (Perez-Garcia et al., 2016) and *C. parapsilosis och1*Δ/Δ+*OCH1* (Perez-Garcia et al., 2016) were used in this study. *Candida* cells were grown overnight at 30°C in liquid YPD medium (1% yeast extract, 2% peptone, and 2% glucose). Before experiments, cells were harvested by centrifugation, washed twice with PBS (phosphate-buffered saline; 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4; pH 7.4) and counted using a hemocytometer.

C. parapsilosis Intravenous Infection

Timed pregnant BALB/c mice were obtained in the specific pathogen-free animal facility of the Biological Research Center (BRC, Szeged, Hungary, XVI./2015.). Mice were monitored to determine the date of the nativity. They received commercial mouse food pellets and water *ad libitum*. On a post-partum day 2, mouse pups were weighed (weight of pups was around 2,2-2,7 g depending on the number of pups in the same litter) and randomized within cages prior the injection with 1×10^7 or $2x10^7$ yeast cells/20 μl of *C. parapsilosis* strains or with sterile PBS (control). Briefly, the injection of newborn mice was performed with a 1 ml insulin syringe with 30 G X 8 mm needle via the external facial vein of newborn mice. Bubbles in the suspension were eliminated to prevent potentially lethal air emboli. The vein was visualized by transilluminating the head with a light source placed on the opposite side. After injection, pups were examined daily. At the indicated time-points, pups were sacrificed via decapitation for the analysis of fungal burden, cytokine response and histology. Adult mice data were previously published (Perez-Garcia et al., 2016). 10–12 weeks-old male BALB/c mice were injected via lateral tail vein with 2×10^7 yeast cells/100 μl of *C. parapsilosis* strains or

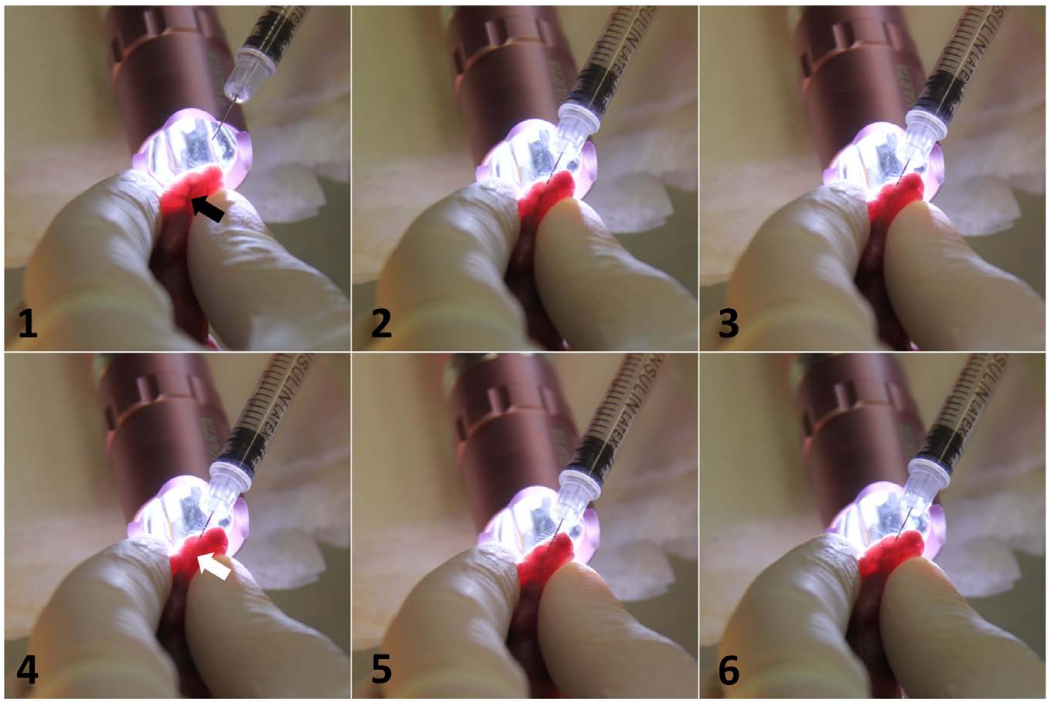

FIGURE 1 | Intravenous infection of *C. parapsilosis* via injecting external facial vein of newborn mice. Transillumination of the head optimized the visualization of the blood vessel. Black arrow shows the target vein (1). After the insertion of the needle, the slow clearance of the vein from the blood is visible upon the expelling of the *Candida* suspension (2,3). White arrow shows the blanching of the vessel from blood (4). After the injection, the needle was withdrawn, and the blood returned to the vascular space (5,6).

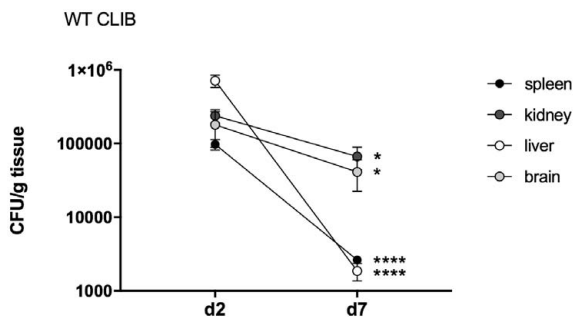

FIGURE 2 | Fungal burden in neonatal mice after wild-type *C. parapsilosis* strain infection. CFU in spleen, kidney, liver and brain in newborn mice after 2 and 7 days of post-infection. At least $n = 4$ mice/group/experiment were infected with the 1×10^7/20 μl of *C. parapsilosis* CLIB 214 (WT CLIB) wild-type strain. Results (mean ± SEM) are pooled data from two independent experiments. Not significant (ns), $^*p < 0.05$ and $^{****}p < 0.0001$ when the CFUs were compared between the two-time points of the analysis as determined by Mann–Whitney *U* test.

with sterile PBS (control). They were terminated at the same time-points as pups.

Fungal Burden

For the colony counts, kidneys, spleens, livers, and brains were collected, weighed, and homogenized in sterile PBS (in the case of the kidney, in sterile PBS containing one Complete Protease Inhibitor Tablet (Santa Cruz Biotechnology Lot.: J1012) per 50 ml). Homogenates were used for determination of fungal burdens by colony counting after plating serial dilutions on YPD agar plates per tissue. The CFUs were counted after 48 h of incubation at 30°C and expressed as CFU/g tissue. (No colonies were recovered from samples from mice challenged with PBS alone.) The remaining homogenates were centrifuged at 3000 rpm, 4°C, for 15 min, and the supernatants were stored at –20°C until cytokine measurement.

Cytokine Measurement

TNFα, IL-1β, IL-10, and KC levels were determined by commercial ELISA kits (R&D Systems) according to the manufacturer's instructions. Cytokine levels were measured from kidney and liver of *C. parapsilosis*-infected (1×10^7 yeast cells/20 μl) and PBS control mice after 2 and 7 days of infection. The concentration of each cytokine was determined in units of pg/ml, and then recalculated as pg/g tissue in the sample.

Histology

Whole spleens, kidneys, livers and brains of PBS control and *C. parapsilosis*-infected (2×10^7 yeast cells/20 μl) mice were fixed and kept in 4% buffered formalin until processed for histology. Fixed organs were sectioned and stained with periodic acid-Schiff (PAS) using conventional staining methods. Tissue sections were analyzed with a BX51 OLYMPUS or Zeiss Imager Z1 microscope.

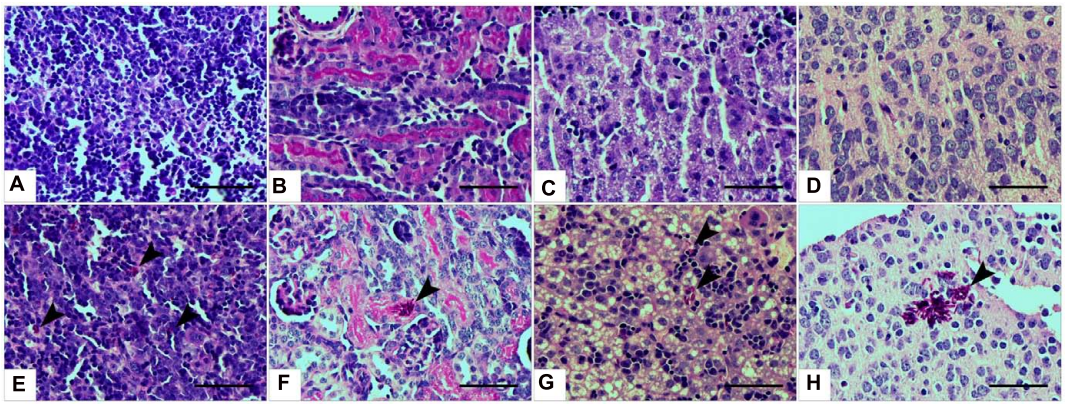

FIGURE 3 | Histopathology during systemic *C. parapsilosis* infection in neonate mice. Sections were stained with periodic acid-Schiff (PAS) stain. **(A)** Spleen, **(B)** kidney, **(C)** liver, and **(D)** brain show the organs from PBS-injected mice. Fungal cells (indicated by arrowheads) are present in the organs of mice infected with the 2×10^7/20 μl *C. parapsilosis* CLIB 214 (WT CLIB) (**E,** spleen, **F,** kidney, **G,** liver, **H,** brain) wild-type strain. All observations were performed at day 2 post-infection. Scale bar represents 100 μm.

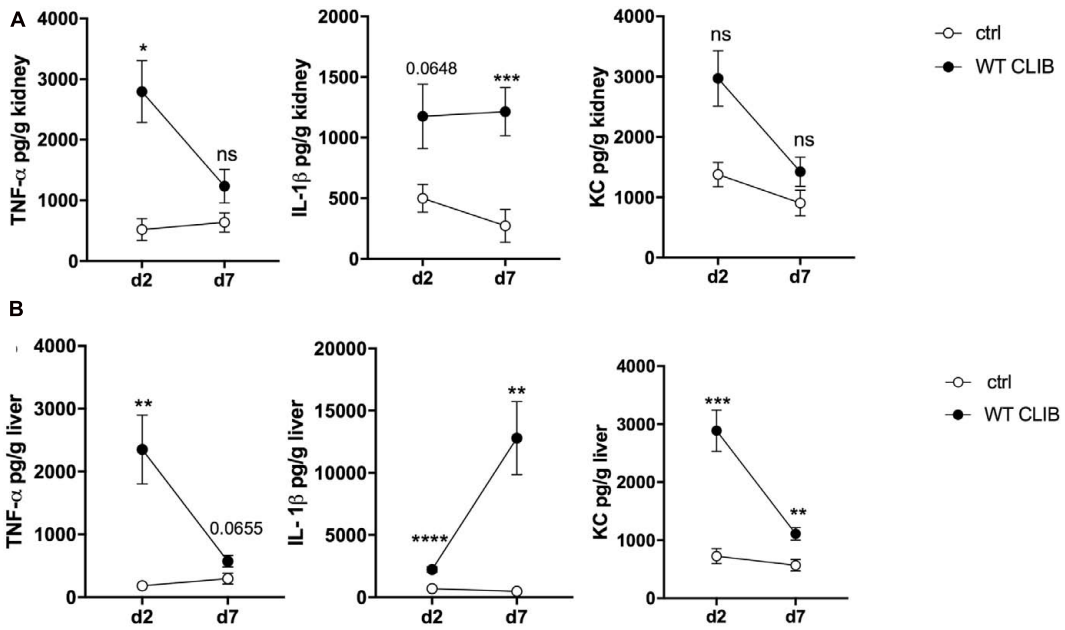

FIGURE 4 | *In vivo* cytokine response in neonate mice after wild-type *C. parapsilosis* infection. TNFα, IL-1β, and KC level in the kidney **(A)** and liver **(B)** after 2 and 7 day of post-infection. Newborn mice were infected with the dose of 1×10^7/20 μl *C. parapsilosis* CLIB 214 (WT CLIB) strain or with 20 μl of PBS (control group, ctrl). Results (mean ± SEM) are pooled data from at least two independent experiments (at least 4 mice/group/experiment). Not significant (ns), $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$ when it was compared to the control group and determined by unpaired *t*-test.

Statistical Analysis

Statistical analysis was performed using the GraphPad Prism 7 software. Differences in fungal burden were determined using the Mann–Whitney *U*-test. Unpaired *t*-test was used for analysis of cytokine measurements. Differences between groups were considered significant at *p*-values of <0.05. The majority of the experiments were performed at least twice and at least 4 mice/group/time point/experiment. Data are presented as means with standard errors of the means (SEM).

RESULTS

Characterization of the Intravenous *C. parapsilosis* Infection of Newborn Mice

In this research, we aimed to develop a new neonatal mouse model of invasive *C. parapsilosis* infection, using intravenous infection through the external facial vein. This method was originally developed for the adoptive transfer of hemopoietic

cells (Billingham and Silvers, 1961). **Figure 1** shows the method of intravenous infection. Following transillumination of the pups' head to aid the visualization of the vascular anatomy, the injection of newborn mice was performed via the external facial vein. BALB/c mouse pups were injected on post-partum day 2 with PBS (control) or wild-type *C. parapsilosis* (WT CLIB) (1×10^7 yeast cells/20 μl).

To monitor the course of the infection in this neonatal model, we included an early (day 2 post-infection) and a late (day 7 post-infection) time point examination. Fungal burdens were detectable in the examined organs (kidney, spleen, liver, brain) on day 2, indicating homogenous dissemination of *C. parapsilosis* cells. The highest number of CFUs was detected in the liver on day 2. Therefore, the injection via the external facial vein was not associated with higher fungal attachment to the brain compared to other organs. Following the course of the infection, decrease of the colony counts was detectable to day 7. On this day, the kidney showed the highest number of yeast cells, followed by the fungal burden in the brain, while the highest clearance was observable in the spleen and the liver (**Figure 2**).

Histopathological examinations also indicated the presence of *C. parapsilosis* yeast cells in all the examined tissues at day 2 post-infection, when the 2×10^7 inoculation dose was used, showing the widespread colonization of the organs via the blood vessels (**Figures 3E–H**). However, no fungal cells were detected in the harvested organs during histology analysis at day 7 post-infection (2×10^7), or at either time points in mice infected with 1×10^7 *C. parapsilosis* cells (data not shown). Single cells of WT CLIB penetrated the different sites of the spleen (**Figure 3E**) and liver (**Figure 3G**). Colonization of the brain (**Figure 3H**) and the small blood vessels of the kidney was visible at day 2 post-infection (**Figure 3F**). The presence of fungal cells in the organs of the WT CLIB-infected mice was not associated with lesions or specific signs of immune cell infiltration. Of note, PBS-injected mice from the same litter showed no evidence of fungal cells or immune cell infiltrates in the examined organs (**Figures 3A–D**).

We also assessed the *in vivo* cytokine response in the colonized neonatal organs after 1×10^7 dose of WT CLIB infection. TNFα, IL-1β, IL-10, and KC chemokine levels were measured from tissue homogenates of kidneys and liver, using enzyme-linked immunosorbent assay (ELISA). We could not detect these cytokines in spleen and brain homogenates. In the kidney, elevated TNFα level was found at day 2 post-infection stimulated by WT CLIB, and the quantity of this cytokine decreased at the later time point (**Figure 4A**). IL-1β was also induced at day 2 post-infection and its level remained elevated at day 7. Similar to TNFα, the secretion of KC was induced at day 2 post-infection, and a decrease in KC production was observed at day 7.

In the liver, high amount of TNFα was triggered by the WT CLIB at day 2 after infection. Similar to what was measured in the kidney, a decrease in the level of this cytokine was found at the later time point. At day 7 post-infection, an increase in the IL-β level was measured in this organ after WT CLIB injection. In comparison with the PBS control group, a rise in KC level was measured at day 2 after inoculation. Also, a reduction in the KC level was found at day 7 (**Figure 4B**). No significant IL-10 secretion was assessed from the kidney and liver samples after stimulation with WT CLIB (data not shown). The first set of analyses confirmed the applicability of this injection method in modeling disseminated candidiasis in neonatal mice.

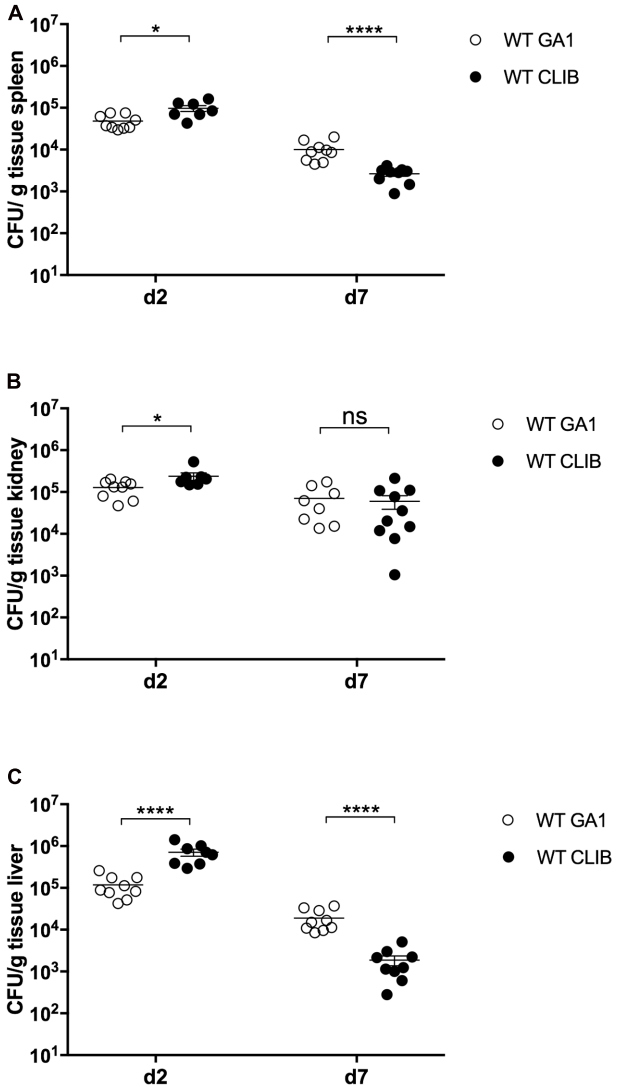

FIGURE 5 | Fungal burden in neonatal mice after wild-type GA1 and CLIB *C. parapsilosis* strains infection. CFU in spleen **(A)**, kidney **(B)**, and liver **(C)** in newborn mice after 2 and 7 day post-infection. n = 4–5 mice/group/experiment were infected with 1×10^7/20 μl of *C. parapsilosis* GA1 (WT GA1) and *C. parapsilosis* CLIB 214 (WT CLIB) wild-type strains. Results (mean ± SEM) are pooled data from two independent experiments. Not significant (ns), $^*p < 0.05$ and $^{****}p < 0.0001$ as determined by Mann–Whitney *U* test. Republished from Perez-Garcia et al. (2016).

The Utility of the Newborn Mice Model to Investigate the Virulence Properties of *C. parapsilosis*

We have previously reported that the GA1 and CLIB wild type *C. parapsilosis* strains elicit distinct host responses during *in vitro* experiments with murine and human macrophages

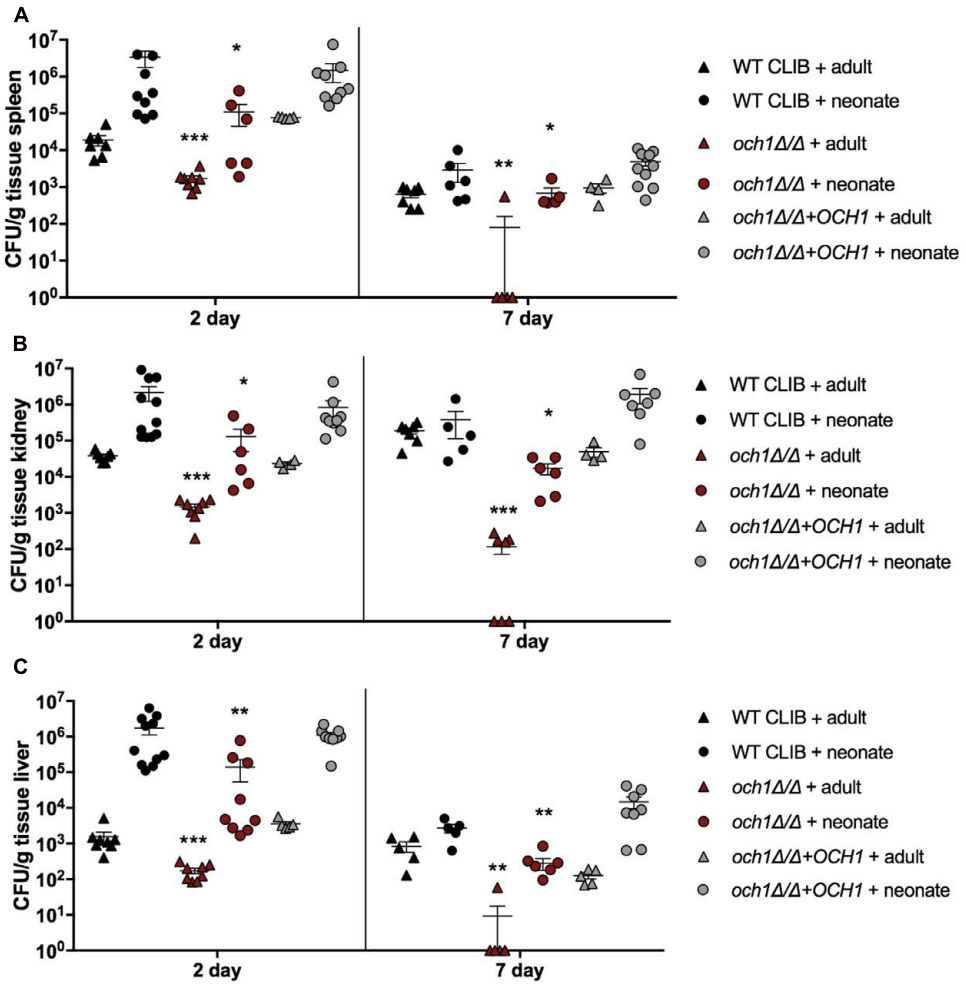

FIGURE 6 | Comparison of fungal burden in the adult and neonate mice after the WT CLIB, *och1*Δ/Δ, and *och1*Δ/Δ+*OCH1* challenge. Newborn ($\times 10^7$/20 μl) and adult (2×10^7/100 μl) mice were infected with the *C. parapsilosis* CLIB 214 (WT CLIB), the cell wall mutant (*och1*Δ/Δ) and the reintegrated (*och1*Δ/Δ+*OCH1*) strain. CFUs in spleen **(A)**, kidney **(B)**, and liver **(C)** at day 2 and 7 post-infection are shown. Results (mean ± SEM) are pooled data from two independent experiments (n = 5 adult mice/group/experiment, neonates at least 4 mice/group/experiment). Not significant (ns), $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ when compared to the corresponding WT CLIB infected mice group as determined by Mann–Whitney U-test.

(Toth et al., 2015). Following the inoculation of newborn mice with these strains (infection dose 1×10^7), differences between the two strains were detectable in terms of fungal burden (**Figure 5**). At day 2 post-infection, the WT CLIB strain showed notably higher fungal loads in the spleen, kidney and especially in the liver, when compared to the WT GA1 strain. In contrast, 7 days after the infection, newborn mice showed higher ability to clear the WT CLIB strain from spleen and liver. However, no difference was found in the colony counts in the kidney.

Next, we wanted to examine the neonatal immune response to a cell wall mutant *C. parapsilosis* strain (*och1*Δ/Δ) that was previously constructed in our laboratory (Perez-Garcia et al., 2016). This mutant lacks the *N*-linked mannan outer chain in the cell wall, and it has been shown to have a significantly attenuated virulence in a BALB/c mouse model of systemic infection (Perez-Garcia et al., 2016). We analyzed the fungal burden in newborn and adult mice after injection with the 2×10^7 dose of *och1*Δ/Δ mutant or the wild-type WT CLIB or the reintegrated strain (*och1*Δ/Δ+*OCH1*). The virulence differences between the wild-type and the mutant strains were evident from the significantly decreased burdens in the spleen, kidney and liver in both adults and pups challenged with the *och1*Δ/Δ strain at 2 as well as at 7 day post-infection (**Figure 6**). Neonatal mice infected with the same dose (2×10^7) of *C. parapsilosis* cells displayed similar trend in organ colonization in comparison with adult mice, as it was previously published (Perez-Garcia et al., 2016). However, while *och1*Δ/Δ-infected adult mice almost completely cleared the infection by day 7, fungal burdens in newborn mice were still high at this time point. Infection with the lower dose (1×10^7) similarly showed the attenuated virulence of the cell wall mutant strain with the significantly decreased colony counts of the *och1*Δ/Δ in the spleen, kidney and liver compared to the WT CLIB. Interestingly, the fungal burdens in the brain of newborn mice were similar

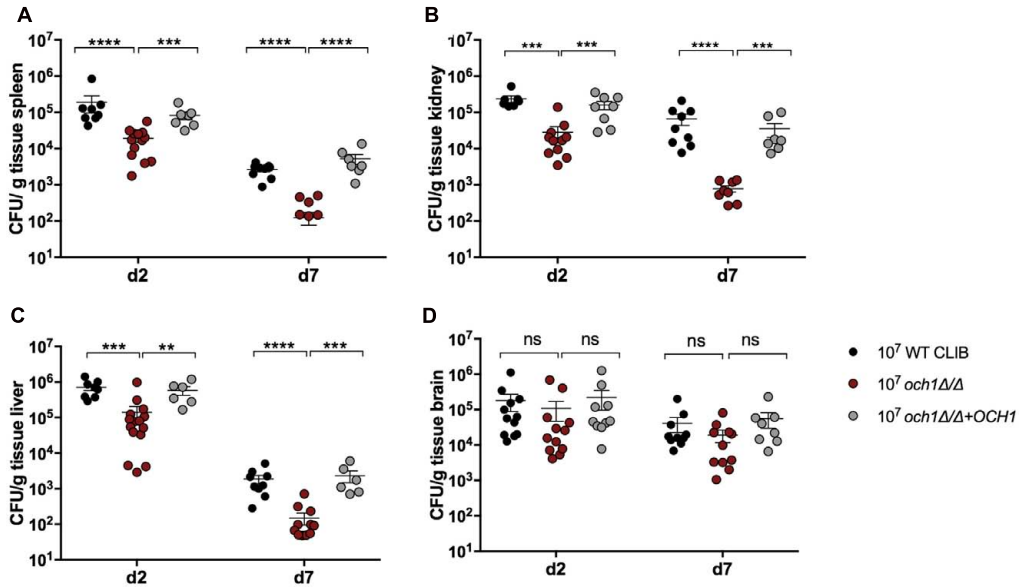

FIGURE 7 | Comparison of fungal burden in neonate mice after the 1×10^7 dose of WT CLIB, *och1*Δ/Δ and *och1*Δ/Δ+*OCH1* infection. Newborn (1×10^7/20 μl) mice were infected with the *C. parapsilosis* CLIB 214 (WT CLIB), the cell wall mutant (*och1*Δ/Δ) and the reintegrated (*och1*Δ/Δ+*OCH1*) strain. CFUs in spleen **(A)**, kidney **(B)**, liver **(C)**, and brain **(D)** at day 2 and 7 post-infection are shown. Results (mean ± SEM) are pooled data from two independent experiments ($n = 4$ mice/group/experiment). Not significant (ns), $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ when compared to the WT CLIB infected mice group as determined by Mann–Whitney *U*-test.

following WT CLIB, *och1*Δ/Δ, or *och1*Δ/Δ+*OCH1* challenge (**Figure 7**).

Histopathological examinations indicated the presence of *och1*Δ/Δ, *och1*Δ/Δ+*OCH1* and wild-type *C. parapsilosis* yeast cells in the brain, kidney, liver and spleen of newborn mice at day 2 post-infection, when $2x10^7$ inoculum dose was used (**Figure 8**). The cell morphology of the strains were consistent with our previously published research (Perez-Garcia et al., 2016). The *och1*Δ/Δ cells were not able to develop pseudohyphae, and the rounded shape of the null mutant cells was distinguishable from the elongated cell form of WT CLIB or the *och1*Δ/Δ+*OCH1* strains during the histological analysis of the tissues. Single cells were spread in the spleen and liver of the three *C. parapsilosis* strains (**Figures 8B–D,J–L**). In the kidney, colonization by the fungal cells was observed around the small blood vessels (**Figures 8F–H**). Colonies were present in the small foci of the brain tissue (**Figures 8N–P**). However, the hematogenous spread of the mutant and the control strains was found in the tissues, specific signs of cellular response were generally not visible in the organs of the pups (**Figure 8**).

According to the ELISA measurements, the lack of the *N*-linked mannan component in the *C. parapsilosis* cell wall resulted in altered cytokine response in newborn mice (infection dose 1×10^7). In the kidney, *och1*Δ/Δ triggered significantly higher TNFα production compared to the wild-type and the reintegrated strain at the two time points of examination (**Figure 9A**). IL-1β and KC secretion was markedly increased in *och1*Δ/Δ-challenged newborn mice at day 7 post-infection compared to the WT CLIB, and the *och1*Δ/Δ+*OCH1*-challenged mouse groups. No significant differences were detected in the level of TNFα and KC in the liver of WT CLIB-, *och1*Δ/Δ-, and *och1*Δ/Δ+*OCH1*-challenged mice. However, the *och1*Δ/Δ strain induced significantly less IL-1β in the liver at day 7 post-infection compared to WT CLIB (**Figure 9B**). No difference was detectable in the levels of IL-10 from the kidney and liver of the different mouse groups (data not shown).

In conclusion, the neonatal mouse model proved to be suitable for the study of the virulence of different *C. parapsilosis* strains and compare the systemic *C. parapsilosis* infection in adult and newborn mice. In addition, our results further support the importance of the cell wall *N*-linked mannan in the pathogenesis of *C. parapsilosis*.

DISCUSSION

Mouse models have been widely used to study human diseases. The increased susceptibility of newborn mice and rat pups to systemic *C. albicans* infection has previously been reported (Pope et al., 1979; Domer, 1988; Trofa et al., 2011; Tsai et al., 2011). First studies demonstrated that gastrointestinal colonization and systemic disease could be achieved by gastric inoculation with *C. albicans* in a neonatal mouse model (Pope et al., 1979; Domer, 1988). In these studies, 5 to 6-day old mouse pups were infected and monitored for survival. Furthermore, tissue burden showed the colonization of spleen, kidney, liver and lung by *C. albicans* strains. Therefore, these early studies demonstrated that infant mice lend itself to study the host response against *Candida* infections. In 2011, new mouse model was developed by Tsai et al. (2011). In that model, mouse pups were infected

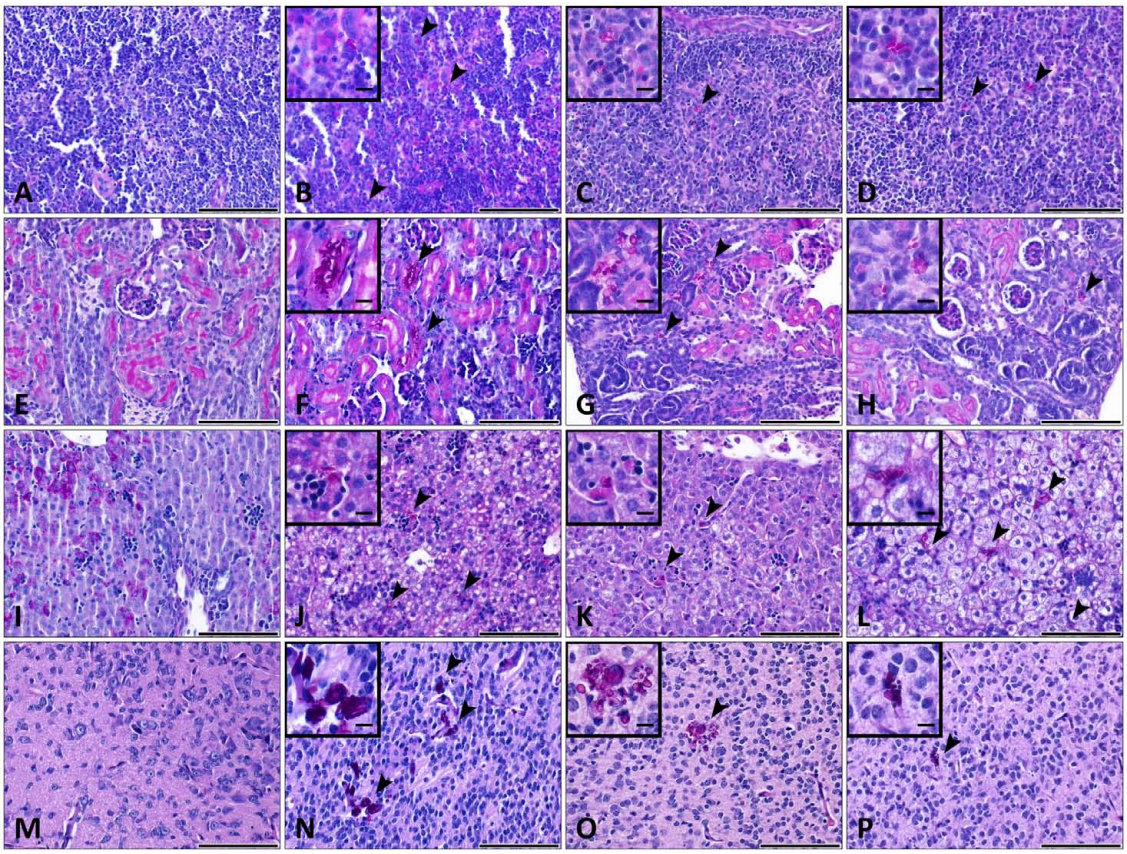

FIGURE 8 | Histopathology during systemic WT CLIB, *och1*Δ/Δ, and *och1*Δ/Δ+*OCH1 C. parapsilosis* infection in neonate mice. Sections were stained with periodic acid-Schiff (PAS) stain. Fungal cells (indicated by arrowheads) are evident in the organs of mice infected with the *C. parapsilosis* CLIB 214 (WT CLIB) (**B** spleen, **F** kidney, **J** liver, **N** brain), the *och1*Δ/Δ (**C** spleen, **G** kidney, **K** liver, **O** brain) and the *och1*Δ/Δ+*OCH1* (**D** spleen, **H** kidney, **L** liver, **P** brain) strain. (**A** spleen, **E** kidney, **I** liver, **M** brain) Control organs from PBS-injected mice. All observations of the infected organs were performed at day 2 of 2 × 10^7/20 μl dose of infection. Scale bar represents 100 μm.

with *C. albicans* by intraperitoneal route on post-partum day 2. Similarly, survival, fungal burden and histopathology were analyzed. They showed that intraperitoneal injection of *C. albicans* induced mortality in a dose-dependent fashion and led to disseminated disease as colonization of the spleen, kidney, liver, and lung were detected. Although this infection method resulted in the dissemination of the fungal elements to different organs, CFU counts were highly variable. Another neonatal animal model was described by Trofa et al. (2011), where 2–3 day old rat pups were infected with *C. albicans* and *C. parapsilosis* via intragastric, intraperitoneal and intravenous routes. In that study, colony counts demonstrated that lipase mutants *Candida* strains displayed lower virulence in the neonatal rats. In that experiment, also, *C. parapsilosis* infection resulted in overall lower fungal burden compared to *C. albicans*. Further investigation showed that the neonatal rat model proved to be suitable for the testing of antifungal prophylaxis with fluconazole, supported by significantly enhanced survival and weight gain after coinfection with *C. albicans* and *S. epidermidis* (Venkatesh et al., 2007). Taken together, these studies confirmed that neonatal rodent models are useful for studying *Candida* pathogenesis that may be specific to an immature host. Despite the importance of *C. parapsilosis* in neonatal infections, study of the *in vivo* immune response against *C. parapsilosis* has been hindered by the lack of appropriate animal models. The mouse intravenous challenge is well investigated and reproducible model to define *Candida* virulence (Arendrup et al., 2002). Therefore, here, we characterized a newborn mouse model of intravenous *C. parapsilosis* infection. The first part of our results revealed the utility of facial external vein injection as a novel method to induce systemic *Candida* infection in newborn mice. We observed widespread dissemination of the yeast cells in the spleen, kidney, liver and brain at day 2 and 7 post-infection during CFU determination and histopathological analysis. The benefit of the intravenous injection used in our study is the consistency in fungal burden measurements in the kidney, liver and spleen of newborn mice, indicating effective colonization of these organs. The fact that the route of the temporal vein injection was not associated with higher yeast attachment to the brain further supported the reliability of this model to characterize disseminated candidiasis in neonatal mice. Furthermore, ELISA measurements demonstrated that

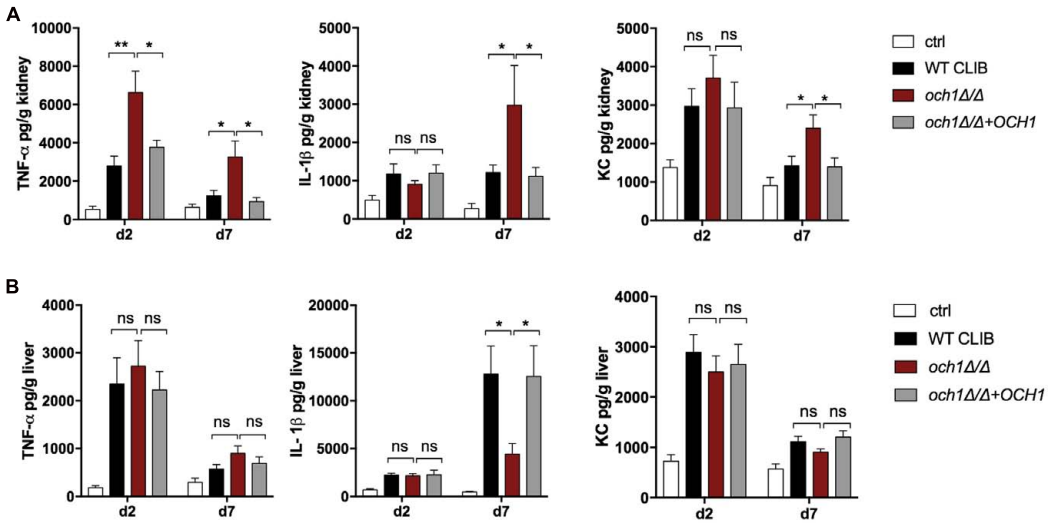

FIGURE 9 | *In vivo* cytokine response in neonate mice after WT CLIB, *och1*Δ/Δ, and *och1*Δ/Δ+*OCH1 C. parapsilosis* infection. TNFα, IL-1β, and KC levels in the kidney **(A)** and the liver **(B)** after day 2 and 7 post-infection. Newborn mice were infected with the lower dose of 1×10^7/20 μl with *C. parapsilosis* CLIB 214 (WT CLIB), the cell wall mutant (*och1*Δ/Δ), the reintegrated (*och1*Δ/Δ+*OCH1*) strains or with 20μl of PBS (control group, ctrl). Results (mean ± SEM) are pooled data from at least two independent experiments (at least 4 mice/group/experiment). Not significant (ns), $*p < 0.05$, $**p < 0.01$, when it was compared to the wild-type *C. parapsilosis* infected mice group determined by unpaired *t*-test.

C. parapsilosis infection induced increased TNFα, IL-1β, and KC secretion in the kidney and liver in newborn mice. Therefore, the route of the injection and the features of this neonatal mouse model grant the opportunity to compare the susceptibility of neonate mice to the different *C. parapsilosis* strains and analyze the induced immune response in the adult and the neonatal settings during systemic infections.

In this research, we tested the utility of the neonatal candidiasis model to analyze the pathogenic properties of the different *C. parapsilosis* strains. Previously, it has been demonstrated that different isolates of this species trigger various host cell responses (Toth et al., 2015). It has been shown that macrophage migration is significantly enhanced toward WT CLIB than toward the WT GA1 strain. Furthermore, both murine macrophages and human PBMC-DMs require more time to engulf WT CLIB cells than WT GA1 cells. The altered cellular immune responses have been suggested to originate from potential differences in the cell wall structure or composition and fungal signaling molecules released by the two strains (Toth et al., 2015). In the present study, fungal burden results reflected the differences in the virulence properties of the two isolates. Compared to WT GA1, WT CLIB infection led to higher colonization of spleen, kidney, and liver at the early time point of infection. However, clearance by the neonatal host was more effective against the WT CLIB strain, as the WT GA1 showed higher tissue burden in the spleen and liver at 7 day after the infection. According to the study of Trofa et al. (2011), *C. parapsilosis* GA1 strain inoculation of rat pups did not induce mortality and the intragastric and intraperitoneal inoculation with 1×10^7 cells of this pathogen led to complete clearance by the used host model organism on day 6. Similarly, during our experiments, infection of neonatal mice with the two *C. parapsilosis* isolates was not associated with mortality and the colonization showed decreased fungal burdens overtime. Therefore, these results contribute to a deeper insight into *C. parapsilosis* infection in the neonatal animal settings.

The *N*-linked mannosylation has recently been shown to play a role in the virulence of *C. parapsilosis* (Perez-Garcia et al., 2016). Disruption of *C. parapsilosis OCH1* results in morphological changes and decreased fungal load in the mouse model of systemic candidiasis. Our data support these results by the significantly reduced fungal burdens in the spleen, kidney and liver of newborn mice after even the lower dose (1×10^7) or the higher dose (2×10^7) of the *och1*Δ/Δ stimulus compared to the wild-type strain. No significant difference was detected in the colony counts in the brain between the wild-type and the cell wall mutant strain infected groups. *In vitro* studies with *C. albicans* demonstrated the invasion of brain endothelial cells by trans-cellular migration and the role of fungal invasins, Als3 and Ssa1 mediated trafficking to the brain (Jong et al., 2001; Liu et al., 2011). Therefore, our results indicate that the *N*-mannan component in the cell wall structure may not affect the mechanism of how *C. parapsilosis* enters and colonizes the brain of mice. Future research could target the investigations of how other *Candida spp*. invade brain tissue from the circulation.

During the histopathological analysis, yeast cells of the less virulent cell wall mutant, as well as the wild-type strain were present in the organs without specific damage of the tissues or visible cellular changes after the 2×10^7 dose of the infection. This phenomenon likely relates to the route of infection and to the inoculation dose, and may also be influenced by the early developmental stage of the animal, as no evidence of yeast cells were detected at the lower dose of the infection.

Perez-Garcia et al. (2016) have shown that *C. parapsilosis N*- and *O*-linked mannans play different roles in host response than *C. albicans* mannans. *C. albicans* cells lacking *N*-linked mannosyl residues induced lower levels of inflammatory cytokines in human mononuclear cells and significantly decreased TNFα and IL-6 in kidneys in infected mice (Netea et al., 2006). In contrast, IL-6, IL-10, TNFα, and IL-1β production by human PBMCs was found to be higher following incubation with *och1*Δ/Δ compared to the wild-type *C. parapsilosis* strain (Perez-Garcia et al., 2016). Here, we found increased TNFα, IL-1β, and KC secretion in the kidney of *och1*Δ/Δ-injected neonate mice, which result correlates with the previous findings in human PBMCs (Perez-Garcia et al., 2016). The liver showed slightly elevated TNFα as well, but we found decreased IL-1β and KC level after the cell wall mutant infection. Preceding research with glycosylation mutant *C. albicans* strains reported the varying chemokine or cytokine responses between tissues (spleen and kidney) from infected mice (Castillo et al., 2011). Thus, despite the mentioned differences between the kidney and liver in our research, cytokine measurements stimulated by the *och1*Δ/Δ correlates with the significantly decreased fungal burden. As the neonatal host showed less susceptibility against the cell wall mutant strain, the higher KC chemokine and TNFα could explain the higher activation of the immune cells, which contributed to the more effective clearance of the *och1*Δ/Δ. Taken together, our model has proven useful for studying the role of the cell wall elements during the host-pathogen interactions and our findings further strengthened the importance of *N*-linked mannosylation in the virulence of *C. parapsilosis*.

CONCLUSION

In our results confirm that injection via the external facial vein is a reliable and efficient method to induce disseminated *Candida* infection in newborn mouse. Therefore, for the future studies this novel model will be an adequate tool to compare the host responses of adult and newborn mice and will be useful for the analysis the pathogenic features of the different *Candida* strains in the neonatal host system. This model may also allow for testing of novel therapeutic strategies against systemic neonatal candidiasis.

AUTHOR CONTRIBUTIONS

AG, HM-M, and KC contributed to the conception of the study, HM-M, CVA, LT, CVI, and KC designed the study. KC, MV, RT, LT, and AM carried out experiments, AG, EZ, and KC analyzed the data. AG, AT, and KC wrote the main manuscript text, EZ, MV, and KC prepared manuscript figures. All authors reviewed the manuscript, contributed to the discussion and approved the final version.

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Candida parapsilosis Protects Premature Intestinal Epithelial Cells from Invasion and Damage by *Candida albicans*

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Candida is a leading cause of late-onset sepsis in premature infants and is thought to invade the host *via* immature or damaged epithelial barriers. We previously showed that the hyphal form of *Candida albicans* invades and causes damage to premature intestinal epithelial cells (pIECs), whereas the non-hyphal *Candida parapsilosis*, also a fungal pathogen of neonates, has less invasion and damage abilities. In this study, we investigated the potential for *C. parapsilosis* to modulate pathogenic interactions of *C. albicans* with the premature intestine. While a mixed infection with two fungal pathogens may be expected to result in additive or synergistic damage to pIECs, we instead found that *C. parapsilosis* was able to protect pIECs from invasion and damage by *C. albicans*. *C. albicans*-induced pIEC damage was reduced to a similar extent by multiple different *C. parapsilosis* strains, but strains differed in their ability to inhibit *C. albicans* invasion of pIECs, with the inhibitory activity correlating with their adhesiveness for *C. albicans* and epithelial cells. *C. parapsilosis* cell-free culture fractions were also able to significantly reduce *C. albicans* adhesion and damage to pIECs. Furthermore, coadministration of *C. parapsilosis* cell-free fractions with *C. albicans* was associated with decreased infection and mortality in zebrafish. These results indicate that *C. parapsilosis* is able to reduce invasion, damage, and virulence functions of *C. albicans*. Additionally, the results with cellular and cell-free fractions of yeast cultures suggest that inhibition of pathogenic interactions between *C. albicans* and host cells by *C. parapsilosis* occurs *via* secreted molecules as well as by physical contact with the *C. parapsilosis* cell surface. We propose that non-invasive commensals can be used to inhibit virulence features of pathogens and deserve further study as a non-pharmacological strategy to protect the fragile epithelial barriers of premature infants.

Keywords: *Candida albicans*, *Candida parapsilosis*, fungal pathogenesis, premature infant, intestinal epithelium, zebrafish model system

INTRODUCTION

Candida albicans and *Candida parapsilosis* are the leading causes of invasive fungal disease in premature infants (1), with the intestinal tract being an important site for *Candida* invasion (2–4). For example, life-threatening gastrointestinal tract diseases that occur in premature infants such as necrotizing enterocolitis and spontaneous intestinal perforation are highly associated with concurrent diagnoses of invasive candidiasis. *C. albicans* and *C. parapsilosis*, along with other fungi, are prevalent commensals of the intestinal tract of infants (5–7), with high amounts of *Candida* colonization within the intestine being correlated with an increased risk for invasive disease (8). Administration of prophylactic doses of fluconazole to infants has been shown to decrease *Candida* colonization of the intestinal tract as well as the incidence of invasive candidiasis in premature infants (9). Concern remains, however, regarding off-target effects of antimicrobial agents, in particular, the impact on the developing intestinal microbiome and longer-term health (10).

Candida albicans is capable of forming three primary morphologies: ovoid yeast cells, chains of elongated yeast cells known as pseudohyphae, and extremely elongated filamentous cells known as true hyphae. Most *Candida* species, including *C. parapsilosis*, exist as yeast or pseudohyphae; only *C. albicans*, *Candida tropicalis*, and *C. dubliniensis* have been observed to form true hyphae (11). The ability to undergo hyphal morphogenesis is associated with the ability of *C. albicans* to invade and damage various human epithelial and endothelial tissues (12, 13). In particular, our laboratory has shown that *C. albicans* hyphae, but not yeast forms, cause significant invasion and damage of premature intestinal epithelial cells (pIECs) (14, 15). Other *Candida* species that do not form hyphae, including *C. parapsilosis*, are essentially inert with respect to this epithelial cell line (14).

Mixed infections that include more than one pathogen often have additive or synergistic effects on pathogenesis or virulence features as compared to infection with a single microbe. With respect to *C. albicans* infections, coinfection of vaginal epithelial cells with *C. albicans* and *C. glabrata* has been observed to result in increased epithelial cell injury as compared to infection with either single species (16). In addition, intra-abdominal infection of *C. albicans* along with *Staphylococcus aureus* results in 100% mortality in mice, whereas the mono-microbial infections are avirulent (17). Some microbes, on the other hand, have been observed to have activities that confer protection from pathogenic features of *C. albicans* infections. For example, *Pseudomonas aeruginosa* produces phenazines that inhibit the formation of *C. albicans* biofilms *in vitro* (18), and probiotic bacteria of the *Bifidobacterium* and *Lactobacillus* genera appear to reduce *Candida* colonization in extremely premature infants, although their efficacy in reducing fungal sepsis has not yet been conclusively shown (19). Collectively, these observations support the idea that the particular behavior of a microbe toward commensalism versus pathogenesis can be influenced by the relative activity of neighboring microbes.

In this study, we tested the hypothesis that *C. parapsilosis*, the second leading cause of fungal sepsis in premature infants, modulates *C. albicans*-induced damage and invasion of pIECs *in vitro*, as well as *in vivo* using a zebrafish model of candidiasis. Together, our results add mechanistic insight into pathogenic interactions between *C. albicans* and the premature intestine and how these interactions might be prevented.

MATERIALS AND METHODS

Fungal Growth Conditions and Preparation of Cell and Cell-Free Fractions for Assays

Yeast strains (**Table 1**) were propagated and maintained as described previously (20). Strains were recovered from 15% glycerol stocks stored at −80°C by plating onto Yeast Peptone Dextrose agar and incubating at 30°C overnight. Individual colonies were then suspended and grown in synthetic dextrose complete medium containing 2% glucose at 30°C overnight prior to assays being performed. Cell concentrations were determined microscopically using a hemacytometer. To obtain cell-free culture fractions, yeast cells were grown as described above, sub-cultured into H4 tissue culture medium at a concentration of 2×10^6 cells/mL and grown at 30°C for 12 h. Yeast cells were pelleted by centrifugation at 13,000 rpm for 3 min. The supernatants were removed carefully using a pipet, so as not to disturb the cell pellet. Supernatants were visualized microscopically using 60× magnification in multiple random fields to ensure that no yeast cells were present.

pIEC Culture and Maintenance

Primary premature human enterocytes (cell line H4) were cultivated and maintained using H4 growth medium and conditions as previously described (23).

Epithelial Cell Damage (Cytotoxicity) Assay

Premature intestinal epithelial cell damage was assessed as previously described (14, 24). Briefly, pIECs were cultured at a concentration of 2×10^4 cells/well and grown to approximately 80% confluence in a 96-well flat-bottomed tissue culture plate (BD Biosciences, San Jose, CA, USA). The pIECs were incubated with yeast (1×10^6 *C. albicans* and/or 2×10^6 *C. parapsilosis*, both in ~10 μL volume), *C. albicans* (1×10^6 cells) with addition of *C. parapsilosis* cell-free culture fraction from 2×10^6 cells, or *C. parapsilosis* cell-free culture fraction alone. After 8 h of incubation, the amount of lactate dehydrogenase released from injured epithelial cells was measured using the Cyto-Tox-96® assay

TABLE 1 | Strains used in this study.

Strain	Description/genotype	Source
Ca SC5314	*Candida albicans* laboratory strain	(21)
Cp 4175	*Candida parapsilosis* clinical isolate, neonate, cardiac mass	(14)
Ca A022b	*C. albicans* clinical isolate, neonate, pleural fluid	(14)
Cp LOW	*C. parapsilosis* JMB72	(22)
Cp HIGH	*C. parapsilosis* JMB77	(22)
Ca GFP	*C. albicans* SC5314 *ENO1:GFP-NAT1/ENO1*	J. Berman (U of MN)

(Promega, Madison, WI, USA) per the manufacturer's instructions. The amount of cell damage is represented as a percent of the control group for each comparison and the reported results are the averages of three independent experiments, each performed in triplicate. Statistical analyses were performed as follows. For two group comparisons, Student's *t*-test (pairing the data by day) was used. For three or more group comparisons, a blocked ANOVA was employed to account for day-to-day variation in pIEC passage number followed by *post hoc* separation of means using Tukey's HSD.

Invasion Assay

Candida albicans penetration of pIECs was determined as described previously (14, 15, 24). In general, the yeast morphologic form is non-invasive whereas the hyphal form is capable of penetration with respect to H4 pIECs (14, 15). Hyphal cells were determined to be invading or not invading pIECs after 3 h of infection with *C. albicans*, in the presence or absence of *C. parapsilosis* or *C. parapsilosis* supernatant, using an immunocytochemical method. Briefly, non-invading fungal cells are labeled fluorescently *via* the Alexa 568 fluorophore, whereas invading fungal cells are inaccessible to the primary antibody and are not labeled. Approximately 30 fungal cells were analyzed for each experimental condition and day. Statistical analysis was performed using Student's *t*-test, pairing the data by day to account for pIEC passage number.

Adhesion Assay

Fungal cell adhesion to pIECs was determined as described previously (25). Briefly, pIECs were inoculated into 96-well tissue culture microplates at a density of 2×10^4 cells/well in a final volume of 100 µL and incubated overnight. The medium was aspirated, and pIECs were infected with 100 µL of 1×10^6 *C. albicans* cells suspended in either H4 media or *C. parapsilosis* cell-free fractions and incubated for 3 h at 37°C with 5% CO_2. Following incubation, the wells were washed with phosphate-buffered saline (PBS), the cells were fixed with 4% *p*-formaldehyde, and the yeast cells were stained with 0.5% crystal violet. The optical densities at 600 nm were determined using a spectrophotometer, and the results were expressed as the percent *C. albicans* adhesion inhibition as compared to 100% adhesion of *C. albicans* alone to pIECs. Results are reported as the average of three independent experiments, each with six replicates. Statistical analysis was performed using Student's *t*-test, pairing the data by day to account for pIEC passage number.

Zebrafish Growth, Maintenance, and Infection

All animal studies were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All animals were treated in a humane manner according to guidelines of the University of Maine IACUC as detailed in protocol number A2015-11-03. The UMaine IACUC/Ethics Committee approved this protocol. Animals were euthanized by tricaine overdose. Infected animals were monitored twice daily for signs of infection and morbid animals were euthanized. Wild-type AB zebrafish were maintained as described previously (26). Zebrafish larvae were grown in E3 medium (5.0 mM NaCl, 0.17 mM KCl, 0.33 mMCaCl, 0.33 mM MgSO4, 2 mM HEPES, pH 7) plus 0.3 µg/mL methylene blue for the first 6 h post-fertilization, then switched to E3 supplemented with 10 µg/mL 1-phenyl-2-thiourea to suppress pigmentation.

The *C. albicans* CAF2.1-dTom-NATr strain was used for all experiments in zebrafish (26–28) and was grown and prepared for infections as described previously (26). Overnight cultures were washed three times in calcium- and magnesium-free PBS and yeast cell concentrations were determined microscopically using a hemocytometer. Cell suspensions were adjusted to a concentration of 5×10^7 cells/mL in 5% polyvinylpirrolidone dissolved in H4 media alone or fungal culture supernatants (described above in Section "Fungal Growth Conditions and Preparation of Cell and Cell-Free Fractions for Assays").

Zebrafish larvae were infected with yeast by injection into the swimbladder at 4 days post-fertilization (dpf) as previously described (29). Infected fish were individually screened at 2 h post-infection (hpi) on a Zeiss Axiovision Vivatome microscope. Mock-infected and infected fish were divided randomly into two cohorts. One cohort of 10 fish was held for 4 days, with counting and removal of deceased fish each day. The second cohort of fish was rescreened at 24 hpi and infection parameters (swimbladder deflation and epithelial breaching) were recorded. Statistical analysis was performed on GraphPad Prism version 7.00 for Mac (GraphPad Software, La Jolla, CA, USA, www.graphpad.com). Survival curves were analyzed using a log-rank (Mantel–Cox) test. Fisher's exact test, with Bonferroni correction for multiple tests, was used to detect differences in infection parameters among groups.

RESULTS

pIEC Damage and Invasion by *C. albicans* Is Reduced by *C. parapsilosis*

To determine the extent to which *C. parapsilosis* is able to modify host cell injury induced by *C. albicans* infection, pIEC damage was measured in the presence and absence of *C. parapsilosis*. Incubation of a mix of *C. albicans* and *C. parapsilosis* cultures (1:2 ratio) with pIECs caused significantly less damage as compared to infection with *C. albicans* alone (**Figure 1A**). By itself, *C. parapsilosis* strain 4175 does not damage pIECs (**Figure 1A**), consistent with the results of our previous study (14). *C. parapsilosis* had the same effect of decreasing pIEC damage caused by two different *C. albicans* strains (**Figure 1A**), a common laboratory strain (SC5314) and a clinical isolate (A022b), both previously shown to significantly damage pIECs (14).

During incubation of pIECs with *C. albicans*, yeast cells form elongated hyphae that start to penetrate the host cells after ~3 h; *C. parapsilosis* cells, which do not form hyphae, exhibit minimal invasion of pIECs (14). In a mixed infection of pIECs using *C. parapsilosis* and *C. albicans*, we found a reduction in the number of *C. albicans* hyphae able to penetrate pIECs as compared

to infection with *C. albicans* alone (**Figure 1B**). The efficiency of *C. albicans* hyphal morphogenesis in the presence or absence of *C. parapsilosis* did not differ, thus the reduction in invasion was not due to differences in hyphal formation by *C. albicans* (data not shown). We also observed that *C. parapsilosis* cells tended to localize along the surface of *C. albicans* hyphae, raising the possibility that physical interactions between the fungal species were inhibiting *C. albicans* from interacting with and injuring host cells.

C. parapsilosis Cell-Free Culture Fraction Reduces *C. albicans*-Induced pIEC Damage

To determine if the *C. parapsilosis* activity that reduces pIEC damage and/or invasion by *C. albicans* is contained in the cell-free culture fraction, *C. parapsilosis* culture supernatants were used to resuspend *C. albicans* cells prior to infection of pIECs. Supernatants were harvested from *C. parapsilosis* cultures grown to the same density (2×10^6 cells/mL) as those used in the invasion and damage assays (**Figure 1**) to ensure consistent quantities of supernatant components between experiments. For pIEC invasion by *C. albicans* hyphae, addition of *C. parapsilosis* supernatants had no apparent effect, either in reducing or enhancing invasion (data not shown). For pIEC damage, *C. albicans* cells resuspended in *C. parapsilosis* supernatants had significantly reduced ability to damage pIECs, although not as much as when *C. parapsilosis* cells were also present (~20% reduction for supernatants alone; ~50% reduction for supernatants along with cells) (**Figure 2**). Control *C. albicans* cells that were resuspended in conditioned medium (incubated overnight at 37°C) that was not exposed to *C. parapsilosis* were able to damage pIECs to the same extent as observed previously for unconditioned media (data not shown). These results suggest that *C. parapsilosis* cells secrete a factor that interferes with the ability of *C. albicans* to damage pIECs and that *C. parapsilosis* cells themselves also contribute to reducing damage to pIECs. Of note, *C. parapsilosis* supernatants had no effect on *C. albicans* growth or ability to form hyphae (data not shown). Thus, its function in reducing *C. albicans* damage of pIECs appears to occur independently of these two virulence factors.

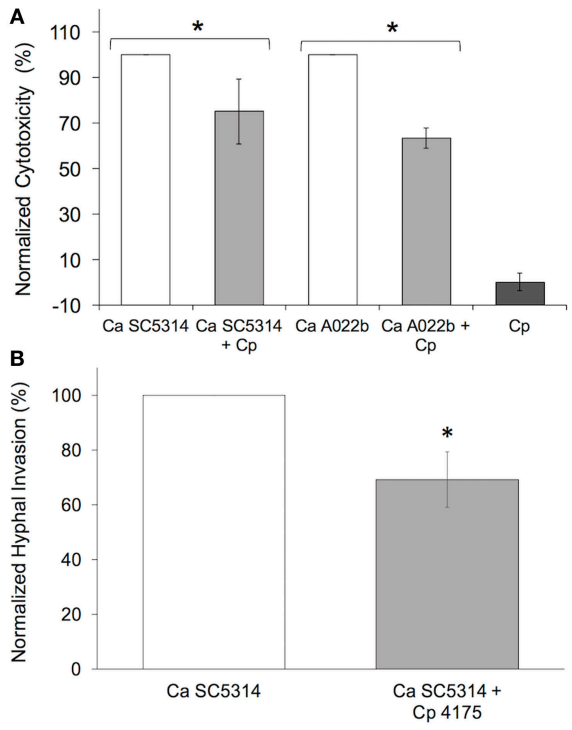

FIGURE 1 | *Candida parapsilosis* reduces premature intestinal epithelial cell damage (A) and invasion (B) by *C. albicans* strains. **(A)** Cell damage (lactate dehydrogenase amount) is plotted as a percentage of that caused by *Candida albicans* (Ca) strains (SC5314 and A022b) alone ("normalized cytotoxicity"). Cp, *C. parapsilosis* strain 4175. **(B)** *C. albicans* hyphal invasion in the presence of *C. parapsilosis* (Cp 4175) is plotted as a percentage of that observed for *C. albicans* (Ca SC5314) alone. Data shown are the average of three individual experiments. For both panels **(A,B)**, an asterisk (*) indicates a statistically significant difference with $p \leq 0.05$. Error bars represent SEM.

C. parapsilosis Reduces the Ability of *C. albicans* to Adhere to pIECs

Fungal adhesion to host cells is thought to be an early step in the pathogenesis of *C. albicans* infections (30, 31). To determine how the *C. parapsilosis* cell-free fraction affects *C. albicans* adhesion to pIECs, we again used supernatants harvested from *C. parapsilosis* cultures as described above. Addition of *C. parapsilosis* supernatants caused an ~15% reduction in the number of *C. albicans* cells able to adhere to pIECs ($p < 0.05$). Thus, the decreased pIEC damage from addition of *C. parapsilosis* supernatants correlates with reduced *C. albicans* adhesion to pIECs.

Most adhesion assays, including the one employed by us as described above, utilize a cell staining method to quantify yeast

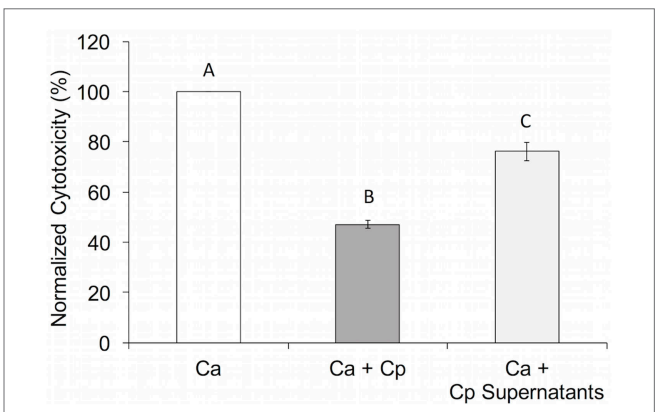

FIGURE 2 | *Candida parapsilosis* cell-free culture fraction reduces *Candida albicans*-induced premature intestinal epithelial cell damage. Cell damage (lactate dehydrogenase amount) is plotted as a percentage of that caused by *C. albicans* (Ca SC5314) alone ("normalized cytotoxicity"). Data shown are the average of three individual experiments. Letters are used to indicate statistical significance, where letters differ, $p < 0.05$. Error bars represent SEM.

cells spectrophotometrically. These methods cannot distinguish cells of different yeast species from each other, thus we are not able to determine the effect of *C. parapsilosis* cells on the adhesion of *C. albicans* to pIECs. As an alternative, we explored the extent to which adhesiveness of *C. parapsilosis* cells contributes to reducing pIEC damage caused by *C. albicans* by comparing two *C. parapsilosis* strains that differ with respect to adhesion to human FaDu epithelial cells (22). By themselves, both of these *C. parapsilosis* strains caused a similar, low amount of damage to pIECs that did not differ from that observed with *C. parapsilosis* strain 4175 (**Figure 3A**). Both strains were able to reduce *C. albicans*-induced pIEC damage (similar to strain 4175) and did not differ with respect to their specific activities. Thus, adhesiveness to FaDu cells does not correlate with differences in the ability of *C. parapsilosis* to inhibit pIEC damage by *C. albicans*. By contrast, pIEC invasion by *C. albicans* hyphae was reduced by coincubation with *C. parapsilosis* strain 4175 (**Figure 1B**) and the FaDu high-adhesion strain (**Figure 3B**), but not with the low-adhesion strain (**Figure 3B**). Furthermore, both strain 4175 and the FaDu high-adhesion strain were observed to localize along *C. albicans* hyphae whereas the FaDu low-adhesion strain was not (**Figure 4**). Thus, adhesiveness of *C. parapsilosis* strains correlates with their abilities to interact with *C. albicans* hyphae and to reduce *C. albicans* invasion of pIECs.

C. parapsilosis Supernatants Protect Zebrafish from Infection by *C. albicans*

The zebrafish swimbladder is a mucosal organ that is used for buoyancy and is both physiologically and ontologically most closely related to the mammalian lung (**Figure 5A**) (32–34). To test the protective ability of *C. parapsilosis in vivo*, zebrafish were infected in the swimbladder with *C. albicans* yeast suspended in *C. parapsilosis* supernatant, *C. albicans* supernatant, or unconditioned medium. This infection model recapitulates a number of aspects of *in vitro C. albicans*–epithelial interactions, permits mucosal infection in the context of a vertebrate immune system and allows for high inoculum doses that cause infection without immunosuppression (28, 29). Therefore, this optically transparent disease model is more complex than *in vitro* challenge of epithelial

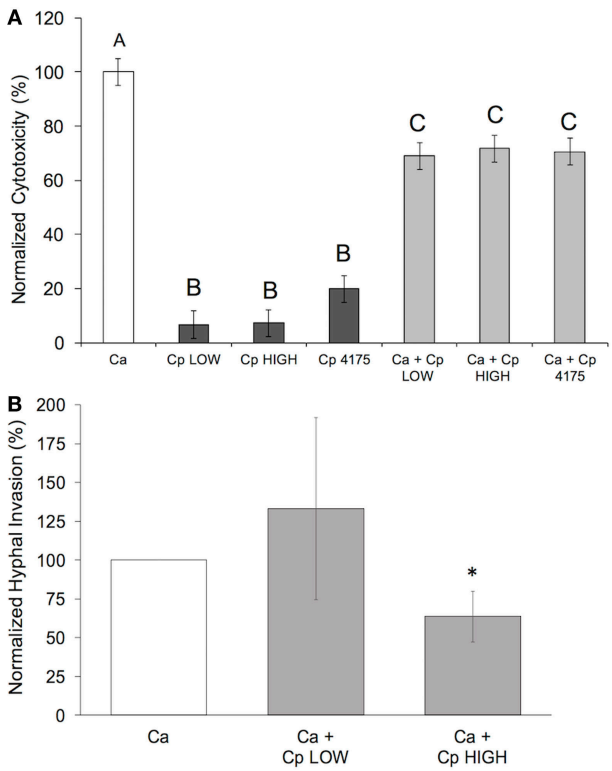

FIGURE 3 | Adhesiveness of *Candida parapsilosis* strains correlates with ability to reduce *Candida albicans* invasion, but not damage, of pIECs. **(A)** Cell damage (lactate dehydrogenase amount) is plotted as a percentage of that caused by *C. albicans* (Ca SC5314) alone ("normalized cytotoxicity"). Data shown are the average of three individual experiments. Letters are used to indicate statistical significance, where letters differ, $p < 0.05$. Error bars represent SEM. **(B)** *C. albicans* hyphal invasion in the presence of *C. parapsilosis* (Cp) strains is plotted as a percentage of that observed for *C. albicans* (Ca SC5314) alone. Data shown are the average of three individual experiments. An asterisk (*) indicates a statistically significant difference with $p \leq 0.05$. Error bars represent SEM.

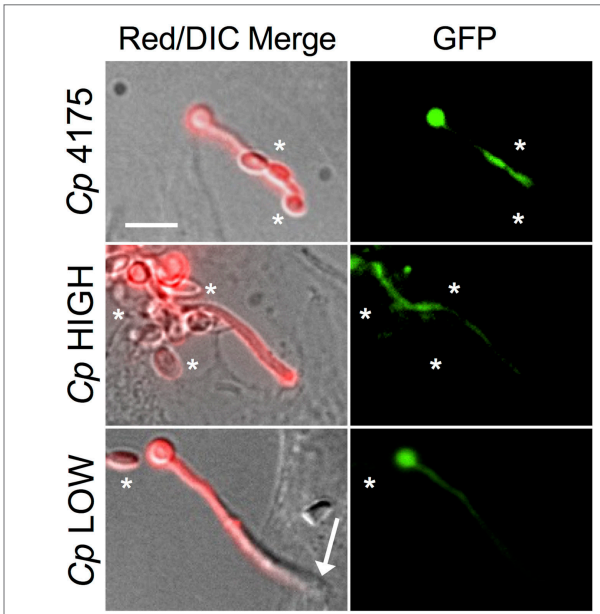

FIGURE 4 | *Candida albicans* and *Candida parapsilosis* interactions on premature intestinal epithelial cells (pIECs). Representative photomicrographs of *C. albicans* (*Ca* GFP) coincubated with *C. parapsilosis* (*Cp*) strains on pIECs. A *C. albicans* strain expressing GFP was used to differentiate between *C. albicans* (red and green fluorescence) and *C. parapsilosis* (red fluorescence, no green fluorescence, see asterisks in GFP panels) yeast cells. Alexa 568 conjugated to phalloidin was used to stain fungal cells with red fluoroscence, as previously described (14). A penetrating *C. albicans* hypha lacks red fluorescent signal (*Cp* LOW, Red/DIC Merge panel, arrow). Non-penetrating *Candida* cells exhibit red fluorescent signal. *Cp* 4175 and *Cp* HIGH cells make contacts with *C. albicans* hyphae (top and center merge panels, asterisks), while *Cp* LOW cells do not (bottom merge panel, asterisk). Scale bar, 10 μm.

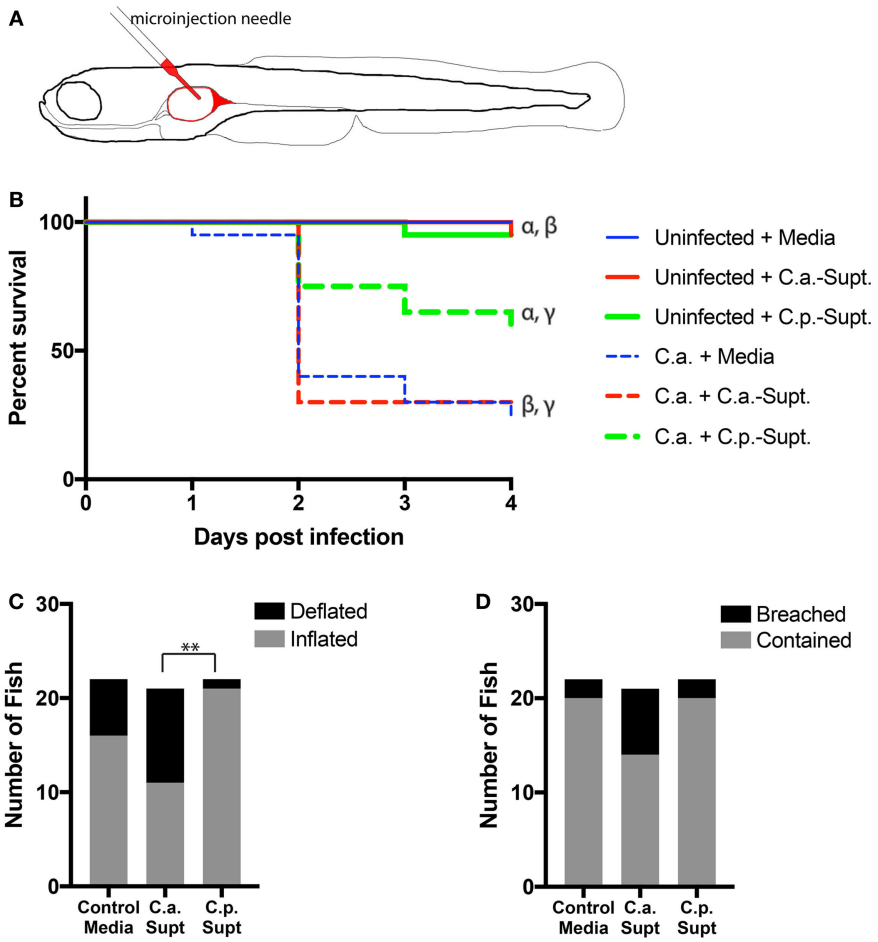

FIGURE 5 | *Candida parapsilosis* cell-free culture fraction protects zebrafish from infection by *Candida albicans*. **(A)** Schematic of infection model. The larval zebrafish swimbladder offers a transparent vertebrate mucosal infection model that is amenable to non-invasive imaging of both the host and the pathogen. **(B–D)** Zebrafish at 4 days post-fertilization with inflated swimbladders were infected in their swimbladders by glass needle injection with *C. albicans* yeast cells (C.a.) suspended in control (H4) media or in supernatants from *C. parapsilosis* (C.p. Supt) or *C. albicans* (C.a. Supt.) cultures. **(B)** Relative survival of fish infected with *C. albicans* with or without *Candida* supernatants. All *C. albicans*-infected fish cohorts are significantly different from their respective controls. *C. parapsilosis* supernatants significantly reduce the mortality of a *C. albicans* infection (denoted by γ). Matching Greek letters label individual comparisons: α, $p < 0.01$; β, $p < 0.0001$; γ, $p < 0.05$. Survival data are pooled from two independent experiments, $n = 20$ per group. All pair-wise comparisons were made with the Mantel–Cox test. **(C,D)** Fish from the experiment in **(B)** were viewed by fluorescence microscopy at 24 h post-infection and scored for two indicators of infection, swimbladder deflation [**(C)** $^{**}p < 0.01$] and breaching of epithelial barrier **(D)**. Examples of these phenotypes are shown in Figure S1 in Supplementary Material. Data were pooled from two independent experiments and analyzed by Fisher's exact test with Bonferroni correction (Control media, $n = 22$, *C.a.* supernatant, $n = 21$, *C.p.* supernatant, $n = 22$).

cells but is not as complex as the mouse intestinal colonization model, which is reliant on antibiotic treatment for colonization and requires both physical disturbance and immunosuppression to yield lethal infection (35). Cell-free fractions of *C. albicans* and *C. parapsilosis* cultures, as well as medium alone, had no effect on zebrafish viability. When *C. albicans* was suspended in *C. parapsilosis* supernatant, mortality was significantly reduced (**Figure 5B**) as compared to *C. albicans* suspended in its own supernatant or fresh unconditioned medium. The effect of *C. parapsilosis* cells along with cell-free fractions on zebrafish viability could not be tested due to the number of yeast in the mixed inoculum being beyond the physical constraints of microinjection into the zebrafish swimbladder.

Deflation of the swimbladder and breaching of the swimbladder epithelium by hyphae are visual hallmarks of *C. albicans* infection in zebrafish (29). Nearly half of the fish infected with *C. albicans* suspended in *C. albicans* supernatant experienced swimbladder deflation at 24 hpi. By contrast, significantly fewer fish had deflated swimbladders when *C. albicans* was suspended in *C. parapsilosis* supernatants (**Figure 5C**). In addition, there was a trend toward more breaching of the swimbladder epithelium by *C. albicans* hyphae with addition of *C. albicans* supernatant than for *C. parapsilosis* supernatant or control media, although this difference did not reach statistical significance (**Figure 5D**). Together, these results indicate that *C. parapsilosis* supernatants protect zebrafish from the effects of *C. albicans* infection.

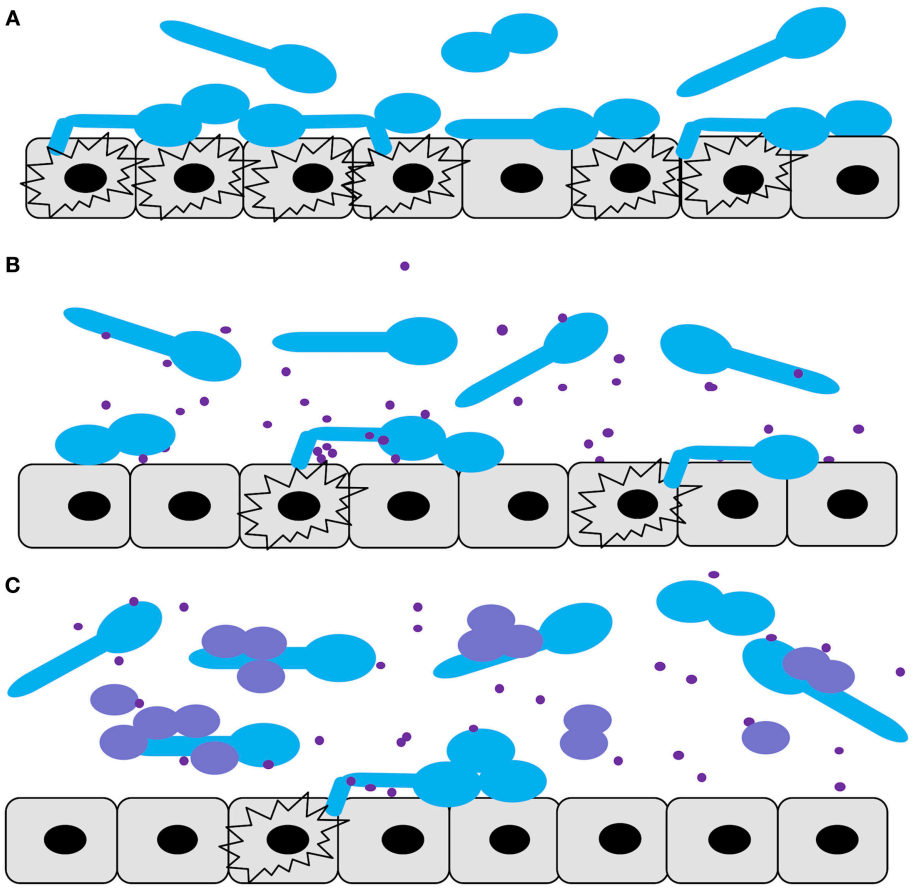

FIGURE 6 | Diagram summarizing the *Candida*–premature intestinal epithelial cell (pIEC) interactions described in this study. Infection models: **(A)** *Candida albicans* alone, **(B)** *C. albicans* along with *Candida parapsilosis* cell-free culture fraction, **(C)** mix of *C. albicans* and *C. parapsilosis* cultures. For all panels: *C. albicans* yeast and hyphal cells, light blue; *C. parapsilosis* cells, purple; *C. parapsilosis* cell-free culture factors, purple dots; pIECs, gray; damaged pIECs, black explosion outlines.

DISCUSSION

Three epithelial cell infection models were compared in this study (**Figure 6**): *C. albicans* alone (**Figure 6A**), *C. albicans* with the addition *C. parapsilosis* cell-free fractions (supernatants) (**Figure 6B**), and a mix of *C. albicans* and *C. parapsilosis* cultures (**Figure 6C**). Comparison of *C. parapsilosis* cellular versus cell-free culture fractions allowed us to study the relative contributions of each and gain mechanistic insight with respect to pathogenic interactions of *C. albicans* with pIECs. The amount of *Candida* cells that we used for infection of pIECs (~10^5 cells/mL) is within the range of colonization levels that have been reported in a few studies of fecal fungal colonization of infants and children (~10^1–10^8 cells/mL), with the higher levels being more typical for subjects with concurrent diarrhea (5, 36, 37). Thus, we think that our infection models are within a physiologically relevant range. Although infection with two fungal pathogens may be expected to cause more host cell damage, we found that *C. parapsilosis* reduces pathogenic interactions of *C. albicans* with host cells. This is consistent with the idea that pathogens can behave as commensals if they coexist with other microbes that are capable of modulating their virulence functions. Conversely, it is also possible that pathogens could exhibit heightened virulence functions depending on the neighboring microflora. It is notable that the zebrafish swimbladder is not sterile, so the conservation of a protective effect in this mucosal model suggests that this work has relevance *in vivo*. As is true for all studies with infection models, the effects on virulence that we observe must be tested and corroborated in further animal and, eventually, human studies before being brought to the clinic.

Candida parapsilosis supernatants alone were able to reduce *C. albicans* adhesion to, and damage of, pIECS (**Figure 6B**), and occurred independent of effects on *C. albicans* growth or hyphal formation. It seems reasonable that *C. albicans* would need to establish and maintain close contact with pIECs in order to damage them *via* physical penetration and/or *via* targeted delivery of membrane-degrading enzymes such as secreted aspartyl proteinases (SAPs) or candidalysin (38–40). The result that *C. parapsilosis* supernatants reduce pIEC damage by *C. albicans*, then, may be related to their ability to reduce *C. albicans* contacts with host cells, potentially *via* competitive blockade of hydrophobic contacts or fungal adhesin–host receptor interactions (41).

In addition, or alternatively, *C. parapsilosis* supernatants may contain a factor that inhibits *C. albicans* secreted enzymes (e.g., SAPs) that degrade pIEC membrane components or that alter the biology of the pIECs (e.g., downregulate a receptor) such that they are less susceptible to adhesion and damage. Addition of *C. parapsilosis* cells along with their supernatants had a greater protective effect against *C. albicans*-induced pIEC damage than with the addition of supernatants alone (**Figure 6C**). The result that there is an additive effect of *C. parapsilosis* cell and cell-free fractions suggests that each of them is acting *via* different inhibition mechanisms.

It seems likely that fungal-host cell contact would also be important for *C. albicans* to successfully invade pIECs. In support of this mechanistic step, when *C. parapsilosis* cells plus supernatants were coincubated with *C. albicans*, only the *C. parapsilosis* strains that were seen to localize along *C. albicans* hyphae were able to reduce *C. albicans* invasion of pIECs. *C. parapsilosis* supernatants by themselves also reduce the ability of *C. albicans* cells to adhere to pIECs; however, they appeared to have no effect on *C. albicans* invasion of pIECs. Importantly, the invasion assay employed here only analyzes the invasion efficiency of those fungal cells that are already in contact with pIECs (in the same microscopic plane of view). No conclusion can be drawn, then, about the connection between adhesion and invasion using this particular assay. We think it likely that, in the presence of *C. parapsilosis* supernatant, more *C. albicans* cells never made contact with pIECs and were washed away during the invasion assay. Consistent with a protective effect of *C. parapsilosis* supernatants in inhibiting *C. albicans* invasion of epithelia, although not statistically significant, cell-free fractions tended to reduce the ability of *C. albicans* to penetrate the epithelial lining of zebrafish swimbladders (**Figure 5D**). Thus, our collective data suggest that *C. albicans* invasion and injury of pIECs can be prevented by both *C. parapsilosis* cell and cell-free fractions (**Figures 6B,C**), either partially or completely, by inhibiting *C. albicans* adhesion to the host cell.

Two mechanisms have been described for *C. albicans* invasion of adult intestinal epithelial cells: active, physical penetration by hyphae, and endocytosis induced by protein–protein interactions (12). For pIECs, our laboratory has observed that the primary mechanism of invasion is penetration by hyphae; only live, actively elongating hyphae are capable of penetrating pIECs (Sara Gonia and Cheryl A. Gale, unpublished data). The identity of the specific factors and processes that mediate hyphal invasion of pIECs remain unknown, but fungal adhesion, physical forces, and targeted secretion of host membrane-degrading enzymes (e.g., proteinases, lipases, candidalysin) from the hyphal tip are likely to be involved as these features have been shown to facilitate invasion of other human cell lines (31, 38).

Cell-free fractions from the yeast *Saccharomyces boulardii* also appear to have a protective effect with respect to pathogenic interactions of *C. albicans* with human intestinal epithelial cells. *S. boulardii* supernatants reduce adhesion of *C. albicans* to adult Caco-2 cells and this is associated with decreased IL-8 cytokine release by enterocytes (25). *Saccharomyces* species are typically regarded as non-pathogenic for the majority of individuals, except in rare cases of severe immunosuppression (42). *C. parapsilosis*, on the other hand, is regarded as a pathogen, particularly in infants (1, 43). Thus, its role in protection against *C. albicans*-induced mortality in zebrafish, and injury and invasion of epithelia *in vitro*, seems somewhat counter-intuitive. Of note, although the same *C. parapsilosis* clone has been isolated from both the intestinal tract and blood of premature infants with fungal sepsis (4), *C. parapsilosis* consistently causes minimal to no damage of pIECs [**Figures 1** and **5**; (14)]. These observations suggest that *C. parapsilosis* gains entry to the host *via* alternative sites [e.g., intravascular catheters and endothelium (44)] and support the idea that pathogenesis mechanisms employed by *Candida* species are host site- and species-specific (12).

In summary, *C. parapsilosis* reduces *C. albicans*-induced injury and invasion of pIECs and these effects are at least partially due to the ability of both secreted and cellular *C. parapsilosis* factors to reduce *C. albicans* physical interactions with host cells. Further studies are needed to understand the molecules on the surface of *C. parapsilosis* as well as the secreted factors that inhibit *C. albicans* adhesion to, and injury of, pIECs. Potential cell-surface candidates include homologs of *C. albicans* Als proteins and other adhesins (45, 46). For secreted factors, identification of molecules in supernatant fractions that confer inhibitory activities will provide additional mechanistic insight. Ultimately, a more complete understanding of microbe–microbe and host–microbe interactions that are associated with protection from disease may lead to the development of therapeutic strategies that promote intestinal health in at-risk patient populations.

AUTHOR CONTRIBUTIONS

SG designed and performed experiments, analyzed data, and wrote the manuscript draft. LA designed and performed experiments, analyzed data, assisted in manuscript writing, and revised the manuscript. MS, MA, and EF performed experiments, analyzed data, and revised the manuscript. JB assisted in development of the project, data analysis, and writing and revision of the manuscript. RW assisted in development of the project, designing of experimental approach, data analysis, and manuscript writing, and revised the manuscript. CG conceived the project, assisted in experimental design, data analysis, and writing of the manuscript, and revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fped.2017.00054/full#supplementary-material.

VIDEO S1 | Animated Z-stack of Figure S1A in Supplementary Material. Z-stack of a representative fish with partial swimbladder deflation and epithelial invasion. In fish like this representative, the swimbladder retains its air bubble (black oval). *Candida albicans* hyphae (purple) fill the distended space behind the air bubble and grow along the epithelial lining of the swimbladder. Filaments can be seen penetrating the epithelial barrier and invading the tissue under and behind the swimbladder. The animation begins near the midline of the fish and moves outward to the skin. Interslice interval = 5 μm. Scale bar = 150 μm.

VIDEO S2 | Animated Z-stack of Figure S1B in Supplementary Material. Z-stack of a representative fish with complete swimbladder deflation and extensive invasion of epithelium. In fish like this representative, the swimbladder becomes completely deflated during infection. Here, many *Candida albicans* hyphae have breached the swimbladder epithelium, have penetrated the tissues surrounding the swimbladder, and are even growing through the skin. The animation begins near the midline of the fish and moves outward to the skin. Interslice interval = 5 μm. Scale bar = 150 μm.

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