REDUCING NEONATAL INFECTIOUS MORBIDITY AND MORTALITY: JOINING UP OUR THINKING

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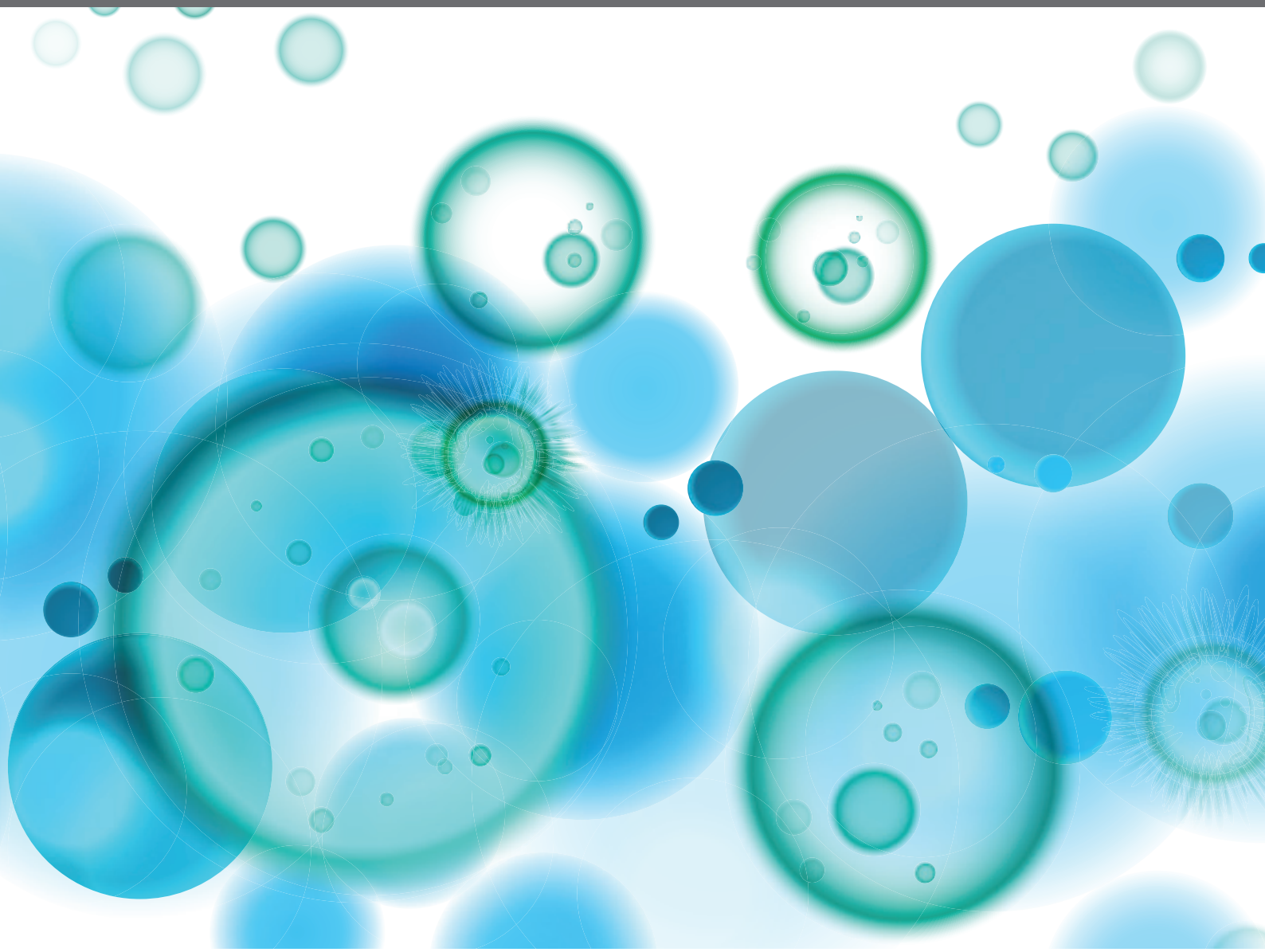

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REDUCING NEONATAL INFECTIOUS MORBIDITY AND MORTALITY: JOINING UP OUR THINKING

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Table of Contents

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HYPOTHESIS AND THEORY
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Outgrowing the Immaturity Myth: The Cost of Defending From Neonatal Infectious Disease

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Newborns suffer high rates of mortality due to infectious disease—this has been generally regarded to be the result of an "immature" immune system with a diminished disease-fighting capacity. However, the immaturity dogma fails to explain (i) greater pro-inflammatory responses than adults *in vivo* and (ii) the ability of neonates to survive a significantly higher blood pathogen burden than of adults. To reconcile the apparent contradiction of clinical susceptibility to disease and the host immune response findings when contrasting newborn to adult, it will be essential to capture the entirety of available host-defense strategies at the newborn's disposal. Adults focus heavily on the disease resistance approach: pathogen reduction and elimination. Newborn hyperactive innate immunity, sensitivity to immunopathology, and the energetic requirements of growth and development (immune and energy costs), however, preclude them from having an adult-like resistance response. Instead, newborns also may avail themselves of disease tolerance (minimizing immunopathology without reducing pathogen load), as a disease tolerance approach provides a counterbalance to the dangers of a heightened innate immunity and has lower-associated immune costs. Further, disease tolerance allows for the establishment of a commensal bacterial community without mounting an unnecessarily dangerous immune resistance response. Since disease tolerance has its own associated costs (immune suppression leading to unchecked pathogen proliferation), it is the maintenance of homeostasis between disease tolerance and disease resistance that is critical to safe and effective defense against infections in early life. This paradigm is consistent with nearly all of the existing evidence.

Keywords: neonate, infection, defense, tolerance, sepsis

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INTRODUCTION

The world has seen under-five mortality greatly reduced over the last two decades but this progress has least benefited those in the first 28 days of life—the neonatal period—which now accounts for nearly half of all under-five deaths (1). Infectious disease is one of the most common causes of newborn death, accounting for more than a third of all neonatal mortality (1). Unfortunately, the underlying reasons for this are not clear, preventing a rational approach to preventing newborn death across the globe. Unquestionably, newborns are much more susceptible to infection causing clinical disease (2–5). Also clear is that the neonatal immune system is very different than that of adults (6, 7). Many immunomodulatory approaches to improving outcome in neonatal infectious disease

have been unsuccessful (8, 9), which necessitates a careful reexamination of our assumptions and beliefs regarding the nature of neonatal immune responses. The current dogmatic view, namely that the neonatal immune system is immature and therefore deficient to resist infection as compared to that of an adult (10), is inadequate as it does not capture the existing body of evidence (11). We recently reviewed the molecular mechanisms guiding the ontogeny of immune response from birth throughout infancy, emphasizing that newborns harbor an immune phenotype that is a match to the unique environmental pressures and challenges in the first days of life (11, 12). Balancing disease tolerance and resistance, while a challenge throughout the entire life span, is also unique for newborns (12). We here place the existing evidence in a larger framework to expand on this concept of host defense as a balance between disease tolerance and resistance to help guide the search for actionable answers.

NEONATAL HOST DEFENSE FROM INFECTIOUS DISEASE: THE COMPLETE PICTURE

Host defense to infection can broadly be divided into three different, not mutually exclusive, categories: disease avoidance, disease resistance, and disease tolerance (13). A more detailed exploration into the finer details of these defense strategies has been previously been outlined by Medzhitov et al. in their 2012 *Science* paper titled "Disease Tolerance as a Defense Strategy" (13). Here, each strategy is briefly summarized and connected explicitly to the neonatal immune response, which has been found time and time again to be distinct from the adult immune response (4, 11, 14–17):

(i) An avoidance strategy reduces the risk of infection by preventing exposure to infectious agents. Human avoidance of rotten meat consumption through an olfactory response to the metabolites produced by bacteria breaking down tissue is an example of avoidance (13). Limitations in both newborn mobility and exclusive breastfeeding can prevent potentially harmful exposure and represent an example of an avoidance strategy in early life (18). However, there are obvious physiological, physical as well as social and cultural limitations to this strategy; there is an unfortunate inevitability to some degree of pathogen exposure.

When avoidance has failed and infection has been established, the (ii) disease resistance approach aims to reduce pathogen burden and has traditionally been considered the primary *modus operandi* of the immune system (and thus the focus of most prophylactic or therapeutic interventions). However, unleashing antimicrobial immune responses can also cause collateral damage (13). In fact, much of what is clinically recognized as signs and symptoms of infection relates to this immune pathology (19). For example, a recent comparison of sepsis models showed that lipopolysaccharide (LPS) treatment in mice "induced a very similar course of inflammation" as infection (20). Given that LPS has no intrinsic virulence, the pathology of LPS challenge must result from host response, and thus similarities between LPS-induced sepsis and, e.g., polymicrobial sepsis (21) implicate host-mediated immune pathology as a key agent of disease. This is further evidenced by murine studies showing that knocking out anti-inflammatory cytokine production during infection is associated with worse outcomes without impacting bacterial clearance or viral replication (22, 23). Importantly, the newborn is particularly susceptible to this host-mediated immune pathology (e.g., intraperitoneal LPS challenge at 10 mg/kg resulted in 100% mortality in neonatal mice and 0% in adults) (11, 24). It is therefore not surprising that evolution has selected for a higher threshold that needs to be overcome in early life before a full-fledged immune response can be unleashed (12). This leaves the newborn with a conundrum; a disease avoidance approach has clear limitations [indiscriminately avoiding bacterial colonization is not only impossible, but would be harmful as the first few days of life are extremely important for establishing a healthy and diverse community of commensal enteric bacteria (25)], while a disease resistance approach carries substantial risk for immune-mediated damage (11, 13, 24, 26).

Newborns thus likely also rely on employing the third strategy of host defense, disease tolerance. (iii) Disease tolerance reduces potential harm to the host without reducing pathogen burden, generally by minimizing the level of immunopathology that results from a resistance response (13). This strategy is understood to be widely employed by plants (27) but the notion that animals (and humans) may rely on a disease tolerance defense as well has only recently begun to be considered (13). It is important to note that disease tolerance is different from the concept of adaptive immune tolerance: the former is a broad, categorical term for a defense strategy of coping with infection, and the latter is the immunological phenomenon of immune unresponsiveness to specific antigens. To our knowledge, the concept of disease tolerance as a defense strategy in early life has never been experimentally examined. However, existing evidence, while not proof, is at least consistent with its existence. Lastly, the host microbiota has increasingly been recognized as key to host defense, impacting all aspects of from avoidance (colonization resistance) to immune development; however, its role and relation to disease tolerance is significant and unexplored, as the tolerance to a range of microbial commensals is essential for a healthy human host (28).

THE CASE FOR HIGHER DISEASE TOLERANCE IN EARLY LIFE

A disease tolerant vs. intolerant phenotype would be expected to display a lower morbidity/mortality relative to a same pathogen load, and/or a higher pathogen load at a similar mortality level (13). While many suspected cases of bacterial sepsis in both neonates and adults are not confirmed by a positive blood culture (29, 30), within culture-positive cases, neonates have consistently been found to exhibit much higher circulating bacterial loads than adults (31). Despite expected variability depending on the pathogen involved, studies generally report bacterial counts in adults (with an active bacterial infection) to be somewhere in the range of 1–30 CFU/ml blood (31–34), while in neonates, the more commonly detected range lies between 50 and 500 CFU/ml blood with one-third of infected newborns harboring bacterial counts in excess of 1,000 CFU/ml (31, 35). Furthermore, while 50% of

adult culture-positive cases harbor <1 CFU/ml blood (considered a "low" bacterial load), 78% of culture-proven newborn sepsis cases reported >5 CFU/ml of blood, and <50 CFU/ml blood was considered to be low for neonates (31). Most of these studies were not set up to compare newborn vs. adult bacterial loads in sepsis but rather were framed in the context of describing how much blood would be needed in order to confidently determine a culture-positive or a -negative state, thus do not directly address this comparison (34). However, this relationship also holds true in more controlled animal models, where much higher bacterial counts are consistently found in the blood as well as visceral organs of septic neonatal vs. adult mice challenged with the same pathogen (36).

Perhaps, a higher bacterial load in infected newborns does not seem surprising at first glance—after all, neonates are more susceptible to suffer from infection, and higher bacterial loads would seem to be entirely in line with this observation. However, this simple concept begins to unravel when age-specific mortality statistics are taken into consideration. While bacterial load correlates with outcome across all ages, there are log-fold differences in the scale of circulating bacteria which neonates are able to survive in comparison to adults. Studies have shown 100% mortality in adult patients with greater than 100 CFU/ml blood (37) and 84% mortality when the bacterial load was greater than 5 CFU/ml blood (38). By contrast, a cohort of neonates with sepsis suffered 73% mortality when bacterial loads were greater than 1,000 CFU/ml blood and 37% when less than 1,000 CFU/ml blood (35). This particular study describes the "low bacterial count with 37% survival" group as those with bacterial loads between 5 and 49 colonies per ml of blood—an amount that would be considered extremely high and lethal in adult patients (31). As stated above, the most recent studies tend not to report the magnitude of bacterial burden in human patients with sepsis, but simply whether they were culture positive or negative; this precludes a full assessment of the relationship between bacterial load and mortality across the age groups. However, many animal models using CFU/ml blood as an outcome validate the observation that neonates are able to survive much higher circulating bacterial loads than adults (36, 39). Note that this is not to suggest that newborns are able to survive higher levels of bacterial exposure than adults (in fact, the opposite is true, as detailed below), rather that neonates are able to survive levels of bacteremia that adults cannot.

THE BALANCE OF DISEASE RESISTANCE VS. IMMUNOPATHOLOGY

Many studies have described deficiencies in the neonatal innate immune system that could be responsible for the decreased ability to clear invasive pathogens. For example, kinetics of pathogen clearance in animal models of neonatal infection show that neonates take longer to clear invasive bacteria than their adult counterparts (36, 39). A recent study comparing methicillin-resistant *Staphylococcus aureus* infection in neonatal and adult mice attributed a delayed clearance in neonates to inefficient phagocytosis and a limited neutrophil recruitment to the site of infection. Specifically, in neonates, neutrophil production dropped off despite the continued presence of bacteria, whereas in adult animals, a diminishing neutrophil production corresponded with bacterial clearance. Other studies have implicated impaired neutrophil recruitment as a potential explanation for the increased susceptibility to infection in early life (40, 41). Furthermore, while neonates have higher basal levels of circulating phagocytic cells than adults, they are generally considered to be less efficient phagocytes (40, 42–45). For example, *in vitro* neonatal monocytes and neutrophils in whole blood cultures have been shown to have an impaired phagocytic ability of *Escherichia coli* and *S. aureus* when compared to adults (44). However, other groups that found similarly reduced phagocytosis of *S. aureus* by newborn polymorphonuclear leukocytes (PMNs) also found that the exposure of neonatal PMNs to adult plasma resulted in adult levels of bactericidal activity and hydrogen peroxide production (against *S. aureus*) (17, 46). Similarly, phagocytosis of group B *Streptococci* and *E. coli* by adult and neonatal purified monocytes had similar phagocytic activity between the different age groups (45, 47). This brief excursion into the literature of just one aspect of host defense immediately highlights that the ability of newborn immune cells to fight infection is a purposeful response and not simply a state "deficient as compared to the adult."

Just as *in vitro* comparisons of neonatal and adult phagocytic cells have contributed to the theory that neonatal susceptibility to infection is a result of "immaturity," so has the evidence accrued which describes diminished *in vitro* pro-inflammatory responses when comparing neonatal and adult cells (7, 10, 48, 49). However, animal models of neonatal sepsis using a variety of pathogens (both bacterial or viral) or TLR agonists have found neonates to generate an inflammatory response equal to or greater than that of adults (1, 24, 39, 50–52). Furthermore, exogenous supplementations of pro-inflammatory cytokines have been shown to greatly increase mortality in a polymicrobial model of sepsis in neonatal mice (53, 54). This increased mortality of neonatal sepsis does not relate to a decreased bacterial clearance, as neonatal mice also suffer a much greater mortality than adults when challenged with purified TLR agonists in the absence of an infection (24, 26). This has led to the realization that the inflammatory response itself is considered to be largely responsible for the higher mortality of infected newborns vs. adults (53, 54).

Given this higher risk of the newborn vs. adult to suffer from the immune response to an infection (or TLR agonist), newborns would benefit from mechanisms that would reduce the risk to unleash a harmful antimicrobial immune response. The molecular mechanisms related to this have recently begun to be deciphered and highlight a direct connection to disease tolerance. An *E. coli* model of neonatal sepsis found that neonatal TRIF$^{-/-}$ mice suffered a higher mortality than WT or MyD88$^{-/-}$ strains with the opposite being true in young adults (55). Neonatal prioritization of TRIF-dependent pathway activation when exposed to TLR agonists was then linked to a strong induction of type 1 interferon regulatory responses, as opposed to the adult MyD88-dependent pro-inflammatory response. A molecular explanation for these age-dependent differences in defense strategy has recently been identified as the endogenous, heterodimeric complex of TLR4 ligands S100A8/A9: high levels of S100A8/A9 shift TLR signaling from MyD88- to TRIF-dependent pathways. S100A8/A9

alarmins are also known to be massively released at birth. This alarmin release is entirely incongruous with the "immune immaturity" paradigm as it represents a purposeful shift away from MyD88 pathway activation, the preferred adult pathway. If neonatal death was driven by a simple lack of adult-like features, one would expect that any external shift toward a more adult-like immune response would lead to better outcome. But the opposite is in fact the case, as *S100a9*$^{-/-}$ neonatal mice suffer much higher mortality than their WT counterparts when infected, implying the alarmin release at birth; i.e., the subsequent shift away from an adult-like response is an important and necessary step to successfully mount a defense against an early-life infection (11, 56). The age-dependent production of S100A8/A9 thus represents an example of disease tolerance unique to neonates that has developed to avoid immunopathology from an MyD88-driven pro-inflammatory response at the potential cost of rapid bacterial clearance.

This emphasis on the TRIF-dependent response is entirely incongruous with the "immune immaturity" paradigm. While this is true for pathogens that signal through TLR4, other mechanisms of disease tolerance to, e.g., Gram-positive infections still need to be identified. For example, there are several other mechanisms in place in early life that commonly are described as immune suppressive, with the notion that these are remnants of the mechanisms that allow semiallogeneic mismatch *in utero* without rejection of maternal cells by the fetus (12). However, these mechanisms persist far beyond the immediate perinatal period and thus likely have other benefits in postnatal life, such as increasing disease tolerance by reducing immune-mediated pathology ("immune cost") even if it comes at the cost of an increased bacterial burden (13).

THE BALANCE OF DISEASE RESISTANCE VS. DISEASE TOLERANCE

The benefit of disease tolerance as a host-defense strategy depends on the capacity for virulence of a given invasive agent. If the only pathogen ever encountered by a host organism secreted virulence factors that inflicted mortality in 100% of cases, there would be intense pressure to improve resistance and no pressure to improve tolerance. More relevant to humans is the opposite case; there are myriads of bacteria that rarely cause mortality and provide both direct and indirect fitness advantages to the host. This creates a situation where disease tolerance is a viable defense strategy, but to a finite degree. Even very low virulence organisms, if left totally unchecked, would cause disease. To prevent disease from occurring upon the transition from the semi-sterile environment *in utero* into the microbe-rich *ex utero* world, disease tolerance (immunosuppression preventing immunopathology) and disease resistance (inflammatory/antimicrobial responses preventing virulence) must maintain a state of homeostasis for optimal host defense. Without active suppression of inflammatory innate signaling, the initial influx of microbes from the birth process could prompt an enormous, potentially lethal inflammatory response; even if this inflammatory response did not result in mortality, there would be serious short- and long-term health ramifications as a result of inadequate bacterial diversity in the gut (25, 57). If there was no disease resistance, opportunistic colonizers would inevitably reach the blood stream and cause disease (**Figure 1**). Since adults (a) are less sensitive to immunopathology caused by inflammation, (b) have already established an enteric microbiome, and (c) are not hindered by the energetic requirements of development and environmental change (see below); the benefits of disease resistance (keeping pathogens out) outweigh the costs of disease tolerance (letting pathogens in).

THE COST OF HOST DEFENSE

Any form of host response (or lack thereof) to an invasive agent must be weighed in terms of the potential for self-inflicted damage, or immunopathology. The resultant immunopathological impact of any given response can range from negligible (i.e., mild fever) to fatal (i.e., septic shock), and thus the immunopathology associated with an immune response has been described as the "immune cost" of a response (58). The three principle host-defense strategies of avoidance, disease tolerance, and disease resistance can be ordered in terms of increasing immune cost, i.e., immune pathology: avoidance has a very low cost, resistance a very high cost, and tolerance lies somewhere in between (13). In addition to the cost of damage from an immune response, however, there is also an associated "energetic cost" which describes the amount of energy required to deploy a given strategy. Ordering the strategies by energetic cost indicates the same order as that of immune cost—disease avoidance very low (primarily behavioral, little to no regulation), disease resistance very high (58) (massive, highly regulated cell mobilizations across the body), and disease tolerance in the middle (some regulatory maintenance to avoid resistance and tissue healing). Both types of costs, immune and energetic, are particularly important to consider when discussing infections in neonates, as newborns are (a) particularly sensitive and prone to immunopathology (24) and (b) in the midst of a rapid growth and development phase which demands a high energy input to be maintained (59), i.e., neonates are unable to "pay" the costs of a full resistance response (**Figure 2**). Avoidance has failed by definition when discussing an already established active infection, which leaves disease tolerance as the primary defense strategy for newborns to cope with an invasive agent. This comprehensive, holistic point of view takes into account aspects of immunity (i.e., energy balance) which fall beyond the narrowly defined immune system and is best captured with the phrase "host fitness cost." The concept of host fitness cost helps better explain some seemingly paradoxical observations in neonatal immunity and can inform interventions moving forward.

THE ROLE OF THE MICROBIOME IN NEONATAL HOST DEFENSE

In the last decade, a vast body of research has emerged, implicating the microbiome as a critical mediator of neonatal immune development (2, 6, 28, 60–62). Dysbiosis during the neonatal period has been associated with necrotizing enterocolitis, and both early- and late-onset sepsis (60, 63–65). Given the potential

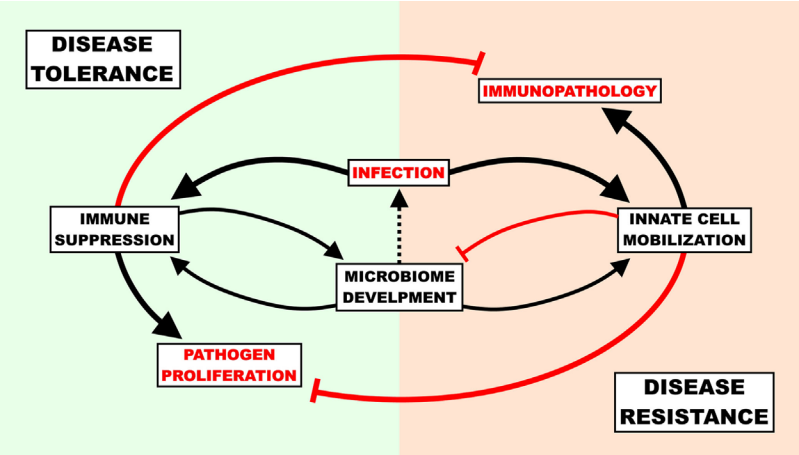

FIGURE 1 | The cost of host-defense strategies in newborn infection. The immune response must be suppressed to a degree in order to allow healthy commensal colonization of the gut, though unchecked suppression can result in gut "leakiness" and lead to infection. Upon infection, newborns must balance the potential self-inflicted harm associated with the pro-inflammatory/antimicrobial response (immunopathology) with the dangers of unencumbered pathogen proliferation and ensuing virulence. A disease tolerance strategy reduces immunopathology and supports microbiome development at the cost of pathogen load, while a disease resistance strategy reduces pathogen load at the cost of microbiome development and immunopathology.

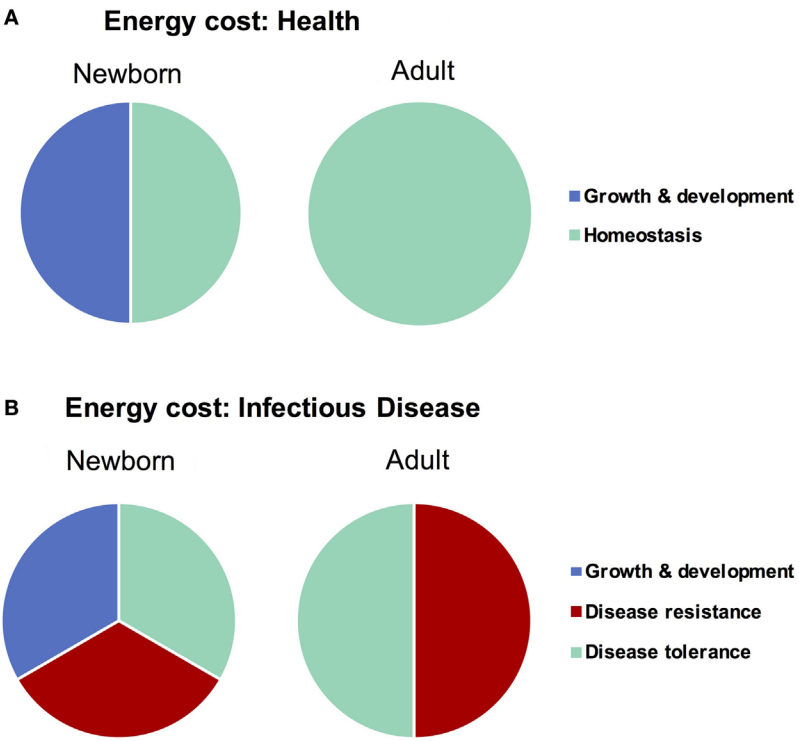

FIGURE 2 | Difference in energy demands of the newborn and adult as it relates to infectious disease. Newborns must devote a large amount of energy toward growth and development which adults are able to spend on maintaining homeostasis. When healthy **(A)**, these differences in energetic demands may not be important, though when fighting infectious disease **(B)**, the newborn is unable to expend the resources required to employ a strategy of disease resistance and must therefore rely more heavily on disease tolerance.

costs of impaired microbiome development, the neonatal immune system seems to have developed specific mechanisms to ensure "safe colonization" of the interphase between external and internal environments. Some of these mechanisms are reviewed below. The active immunosuppressive portion of the neonatal immune response may not only serve to minimize the damage associated

with immunopathology but also ensure that the neonatal gut can be colonized with a large and diverse array of commensal bacteria. The interplay and dependence on commensal bacteria begins immediately with colonization, as transcriptomic analysis of germ-free mice exposed to common commensal bacteria showed the most prominent changes in genes associated with toll-like receptor (TLR) and type 1 interferon (IFN1) signaling pathways, which, as evidenced by the aforementioned importance of the TRIF-dependent signaling, are crucial in early life (66).

Newborns heavily depend on avoidance to prevent infection. In the context of microbial colonization, they rely on their mothers to introduce them to the right organisms and promote their growth, meanwhile avoiding unwanted colonizers from outcompeting the beneficial ones. While maternal influence on prepartum colonization is still being debated (67), postpartum colonization has been shown to be largely derived from the vagina during birth (68). In newborns sampled during the first week of life alongside their mothers, the majority of taxa detected in their stools were also detected in stool samples taken from their mothers at the same time (69). While newborns are colonized by bacterial families such as *Bacteroides* and *Clostridia* acquired from their mothers, the composition of their microbiota is still different from their mothers with *Escherichia*/*Shigella*, *Bifidobacterium*, *Streptococcus*, and *Enterococcus* occupying roughly half the space of the entire intestinal microbiome in newborns but only about 10% in their mothers [the exception is *Bacteroides*, which feature prominently in both (69)]. While life will eventually expose an individual to a multitude of different foods and diverse microbial environments, newborns subsist solely on breast milk and have no environmental exposure in their control. Thus, microbes found in human milk or other maternal sources that utilize human milk oligosaccharides dominate initial colonization (70, 71) and in turn provide resistance to colonization by potential pathogens (72) and other forms of immune support (28). Consequences of less-controlled exposure are suggested by the detriment of deviation of exclusive breastfeeding practices; excusive breastfeeding in low- and middle-income countries (and perhaps high income) is associated with a substantial reduction in newborn disease and mortality (18).

Avoiding inflammatory or deleterious responses to commensal microbes is important throughout the life span of colonized hosts. Strategies put in place by the newborn are appropriate for the environmental pressures and physiological requirements of this unique early-life period. Adults depend on a thick mucous layer packed with antimicrobial peptides and dimerized IgA alongside trained innate and adaptive mucosal responses to prevent microbial translocation into host tissues (73). Since the development of these defenses first requires stimulation by the microbiota, newborns must employ a different repertoire of tolerance strategies prior to the introduction of solid food. For example, newborn intestinal epithelial cells (IECs) produce a micro-RNA molecule that targets IRAK-1, a necessary signal transducer of inflammatory TLR signaling for degradation and thus reduces inflammation caused by commensal stimulation of intestinal TLRs (74). However, this mechanism requires continuous TLR4 stimulation for its maintenance and is absent in pups delivered by C-section. Also, murine IECs produce antimicrobial peptide CRAMP only prior to Paneth cell development and show some efficacy against *Listeria* infection (75). In fact, weaning appears to be a massive transitory period for IEC regulation. Transcriptional regulator Blimp1 is active in the newborn intestine and ceases to be expressed upon weaning; its deletion results in an adult-like intestinal architecture at birth and with it a substantial early-life mortality in animal models (76). A more comprehensive evaluation of intestinal transcriptional regulation showed a more global postweaning shift in rodents with an increase in IL-1/TLR signaling post weaning that was lost in MyD88/TRIF$^{-/-}$ mice, showing that the intestinal immune environment is very sensitive to changes in early-life transitions (77) and is likely guided by the changing microbial and nutritional environment.

Newborn colonizers also play an instrumental role in preventing immune hyperresponsiveness within and outside the mucosal immune system. Widely studied commensal *Bacteroides fragilis* promotes an anti-inflammatory environment by inhibiting the recruitment of invariant NKT cells to the gut and lung mucosa, leaving mice less susceptible to inflammatory disease later in life (78). dsRNA from lactic acid bacteria (LAB) preferentially promotes IFN-β expression in mucosal dendritic cells to concentrations that predominantly drive their anti-inflammatory effects in adult rodents (79). Since LAB are prominent colonizers of the newborn gut, it is likely that they perform similar functions during this period—although that mechanism still needs to be investigated. An influx of highly activated regulatory T cells into the neonatal skin has been linked with tolerance to commensal skin bacteria, an event that was not replicated when the same experiment was performed in adult animals. Selective inhibition of these specific Tregs completely prevented tolerance to commensal bacteria colonization later in life (80).

There is evidence in adult animal models that microbiome-derived products can reduce disease pathology, i.e., increase disease tolerance. Recently, the clostridia-derived metabolite desaminotyrosine (DAT) was shown to promote type 1 IFN signaling in lung dendritic cells, resulting in a less damaging response to influenza challenge and an increased animal survival, while the viral burden in DAT-treated mice remained unchanged (81). Newborns are thought to be at risk for "inside-out" infections, where the pathogen escapes mucosal compartments, supported by the identification of the same strain of bacteria from septic newborns in their feces (82). The microbiome is not only instrumental in excluding potential pathogens, but by promoting an anti-inflammatory environment, it likely also plays a role in reducing the potential harm from responses to inflammatory microbes. For example, newborn mice given probiotic strains of *Lactobacillus* were rescued from death caused by *Citrobacter rodentium* infection (a mouse model of enteropathogenic *E. coli*) *via* a mechanism involving the recruitment of Tregs to the colon (83). A second group was administered *L. acidophilus* alongside a prebiotic to newborn mice prior to challenge with *C. rodentium* in young adulthood, a finding that treated mice had an enhanced IL-10 and a diminished NF-kB response to infection, in addition to a faster recovery from disease (84).

Taken together, host-commensal bacteria crosstalk in newborns is highly dependent on maternal care for both original inoculation and continued support through breastfeeding and

controlled environmental exposure. This complete dependence is unique to the newborn period, highlighted by both the devastating consequences of suboptimal breastfeeding practices and the reworking of intestinal and microbial architecture once solid foods are introduced. During this time, commensals selected to thrive in newborns promote disease tolerance by boosting anti-inflammatory immune responses and disease avoidance by excluding potentially pathogenic organisms from coming into contact with the epithelium, thus preventing infection of the mucosal and systemic sites.

TRANSLATIONAL IMPACT

Successful immune defense relies on a balance between disease resistance and disease tolerance strategies to bring the host back to homeostasis. The ideal intervention is one that would hasten restoration of homeostasis or enable the system to deal with an extreme imbalance in either direction for longer periods. A promising approach that fits this requirement is to work in the realm of innate immune memory or trained immunity—the concept that an initial infection or an exposure to a pathogen can provoke an enhanced innate immune response when the organism is re-exposed or exposed to a different pathogen (85) Unlike many traditional interventions, prophylactic or treatment approaches reliant on trained immunity are not dependent on shifting the response only toward resistance. Numerous examples of successful interventions reliant on innate immune memory have been described in animal models and human clinical trials (85). Various TLR agonists have, for example, shown to protect nonspecifically against mortality from a polymicrobial challenge 24 h later in neonatal mice (41). A similar model used a *Listeria monocytogenes* challenge and found TLR agonists to be protective as well. Cord blood monocytes stimulated with endotoxin showed an enhanced activity both 7 and 14 days later. Moreover, a retrospective analysis revealed correlation between histological chorioamnionitis (a condition prompting an inflammatory response) exposure and a reduction in late-onset neonatal sepsis (85). Most impressively, probiotics in newborns have been shown to be very powerful in reducing both necrotizing enterocolitis (86), a devastating disease characterized by colonization with proteobacteria and excessive inflammation (87), and most recently, sepsis and respiratory disease when administered within days of birth (88). Finally, certain live vaccines (particularly Bacille Calmette–Guérin) have been shown to reduce all-cause neonatal mortality, presumably through nonspecific protection against unrelated pathogens in the first month of life (89).

SUMMARY

The paradigm that neonates are more susceptible to infectious disease than adults is well known, well documented, yet poorly understood. The high susceptibility and mortality figures have largely been attributed to "immune immaturity," a vague concept that is predicated on findings of weaker antimicrobial responses of newborns than those of adults. Here, we posit that an increased susceptibility to infection in neonates is not a result of immaturity but rather one of immunosuppressions, which is in part an active defense strategy termed disease tolerance. This is supported by the finding that neonates can survive significantly higher bacterial loads than adults during active infection. This observation is consistent across many studies, yet still is oft ignored and cannot be adequately explained by the immaturity paradigm. Employing a defense strategy of disease tolerance during infection rather than disease resistance confers some advantages but is more likely a virtue of necessity. Compared to the adult-like disease resistance strategy, disease tolerance is (a) less energetically intensive (critical during a period of rapid development), (b) less likely to incur serious damage associated with bacterial clearance (neonates are more sensitive to immunopathology than adults and seem to have a heightened innate immune response), and (c) less likely to interfere with the development of the gut microbiome (overactive resistance pathways could result in a dangerous inflammatory response and interfere with colonization). Maintaining homeostasis between disease resistance and disease tolerance is a critical outcome of fighting and preventing infection. Fortunately, interventions, which work within these constraints, have been identified and promise to finally usher in the desperately needed reduction of global newborn mortality rates.

AUTHOR CONTRIBUTIONS

DH, NA, and TK conceived of the presented ideas. DH wrote the manuscript with assistance from RB-O, NH, and TK. All authors provided critical feedback and editing through the writing process.

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REVIEW
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Neonatal Immunization: Rationale, Current State, and Future Prospects

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Infections take their greatest toll in early life necessitating robust approaches to protect the very young. Here, we review the rationale, current state, and future research directions for one such approach: neonatal immunization. Challenges to neonatal immunization include natural concern about safety as well as a distinct neonatal immune system that is generally polarized against Th1 responses to many stimuli such that some vaccines that are effective in adults are not in newborns. Nevertheless, neonatal immunization could result in high-population penetration as birth is a reliable point of healthcare contact, and offers an opportunity for early protection of the young, including preterm newborns who are deficient in maternal antibodies. Despite distinct immunity and reduced responses to some vaccines, several vaccines have proven safe and effective at birth. While some vaccines such as polysaccharide vaccines have little effectiveness at birth, hepatitis B vaccine can prime at birth and requires multiple doses to achieve protection, whereas the live-attenuated Bacille Calmette–Guérin (BCG), may offer single shot protection, potentially in part *via* heterologous ("non-specific") beneficial effects. Additional vaccines have been studied at birth including those directed against pertussis, pneumococcus, *Haemophilus influenza* type B and rotavirus providing important lessons. Current areas of research in neonatal vaccinology include characterization of early life immune ontogeny, heterogeneity in and heterologous effects of BCG vaccine formulations, applying systems biology and systems serology, *in vitro* platforms that model age-specific human immunity and discovery and development of novel age-specific adjuvantation systems. These approaches may inform, de-risk, and accelerate development of novel vaccines for use in early life. Key stakeholders, including the general public, should be engaged in assessing the opportunities and challenges inherent to neonatal immunization.

Keywords: neonatal, vaccine, protection, trained immunity, novel adjuvants

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INTRODUCTION

Despite the success of the Millenium Development Goal era from 2000 to 2015, during which the under five mortality rate was reduced by 53%, ~ 2 million infants under 6 months die annually due to infections (1). Of the 5.9 million children under 5 years of age who died in 2015, 45% were in the first month of life (2). Many of these deaths are attributed to vaccine preventable illnesses, occurring before protection is afforded by routine immunization given as part of the expanded program of immunization (EPI). Although this commences at 6–8 weeks of age, the first dose does not provide

immediate protection and multiple doses are required, leading to vulnerability in the first 6 months of life. In an effort to reduce the under 5-year old mortality rate further, to ≤25/1,000 live births by the end of 2030, a number of strategies are being explored and implemented as part of the sustainable development goal-3. These include maternal immunization, which, although it shows great promise for a number of pathogens, including pertussis and influenza, is limited by safety and ethical concerns, and is of limited value for the ~2.6 million infants born preterm, prior to maternal antibody (Ab) transfer (3). The reality, that we rely on immunization occurring early in life, coupled with recent advances in our understanding of neonatal immune responses (4–6), has led to renewed interest in neonatal immunization as a promising and effective strategy, to reduce morbidity and mortality in young infants. Thus, the topic of early life immunity, and in particular neonatal immunization, is one of tremendous public health relevance.

Great strides in vaccine development over the last century have resulted in a number of effective vaccines being given in early life, but only Bacille Calmette–Guérin (BCG), hepatitis B (HBV), and polio vaccine [oral polio vaccine (OPV); or inactivated polio vaccine (IPV)] have been routinely recommended at birth. For some pathogens, including pertussis and tuberculosis (TB), better vaccines are needed, while for others such as human immunodeficiency virus (HIV) and respiratory syncytial virus (RSV), efficacious vaccines have yet to be developed and licensed for any age group. Among the approaches to improving protection against infection in early life, neonatal immunization is ripe for further research and development. Herein, we review the rationale for neonatal immunization and highlight essential research areas, including the study of immune ontogeny and the development of vaccines optimized for early life administration.

Rationale for Use of Vaccines in the Neonatal Period

The neonatal period is defined as the first 28 days of life. For the purpose of this review, we define neonatal vaccines as those given "at birth" or within the first 28 days of life. Of note, EPI vaccines are licensed to be given within the first few weeks of life, and in reality, the "birth dose" is given across a range of time in the first month of life, a variability that to our knowledge has not been systematically studied with respect to relative vaccine efficacy. In contrast, we define infant vaccines as those given *after* the first 28 days of life. In countries following the EPI schedule, after the neonatal doses of BCG, HBV, and polio vaccines, the next EPI schedule dose is typically given between 6 and 8 weeks of life. As with any vaccine approach, development of neonatal vaccines must take into account potential limitations, including: (a) need to establish safety, (b) lack of effectiveness of some vaccines in early life, (c) challenges of a translational path that typically starts with formulations optimized for adults, rather than generating formulations that are optimal for the young, and (d) potential blunting of neonatal Ab responses after maternal immunization. Nevertheless, the rationale for neonatal immunization is robust and includes: (a) the heavy burden of early life infection; (b) that birth is a practical point of healthcare contact, and pairing immunization with birth may lead to health benefits for both mothers and newborns; (c) immunization at birth may provide earlier protection than existing immunization schedules; (d) the likely benefit of protection to babies born preterm for whom maternal Ab transfer is limited, with an increased risk of serious infections throughout childhood (7); and (e) emerging evidence that the heterologous benefit of the live-attenuated BCG vaccine and other live vaccines may be greatest in early life (8).

LESSONS FROM IMMUNE ONTOGENY

Neonatal immunization occurs in a backdrop of distinct early life immunity. Recent reviews have highlighted that both cellular and soluble aspects of the immune system are distinct at birth (9, 10). Neonatal immunity must not only defend the newborn against a potential onslaught of potential pathogens, but also mediate the acquisition of a colonizing microbiome over the first hours and days of life. In this context, neonatal immune responses are apparently designed to avoid excessive inflammation with a generally reduced production of pro-inflammatory and Th1-polarizing cytokines to microbial components/pattern recognition receptors (PRR) agonists. Age-specific composition of soluble and cellular factors shape neonatal immunity. The distinct composition of human newborn cord blood plasma includes soluble mediators such as maternal Abs, high levels of immunosuppressive adenosine, and low levels of complement, important for triggering adaptive immune responses (11). Accordingly, modeling age-specific immunity *in vitro* should take into account distinct composition of age-specific autologous plasma, rather than, for example, fetal bovine serum (9). Distinct cellular immunity in the newborn includes reduced Th1 but robust anti-inflammatory IL-10 responses of antigen-presenting cells to stimulation by PRR agonists, high frequency of naïve- and regulatory-T cells and CD71+ erythroid precursors that may limit, for example, responses to pertussis immunization (10, 12, 13). Nevertheless, neonatal immunity is capable of mounting antigen-specific effector responses, as demonstrated by BCG-specific IFNγ production following vaccination at birth (14). Overall, detailed study and modeling of age-specific human immunity may help inform development of vaccine formulations, with or without adjuvants as needed, that may trigger a protective immune response in early life.

PROOF OF CONCEPT: ROUTINE NEONATAL VACCINES

Bacille Calmette–Guérin

Bacille Calmette–Guérin is a live-attenuated strain of *Mycobacterium bovis*. Given in areas with high-endemic TB to prevent disseminated TB in infancy, BCG is the most commonly given vaccine with ~4 billion doses administered to date. Although it has been administered for nearly 100 years, several key issues regarding BCG have emerged, including: (a) lack of a clear correlate of protection (CoP); (b) marked heterogeneity

between licensed BCG formulations (15); and (c) growing evidence that BCG has heterologous ("non-specific") beneficial effects, particularly when administered in newborns (16). It is hypothesized that these non-specific benefits may protect against unrelated infections, supporting the use in neonates, beyond any protection against TB infection or disease. Furthermore, association studies suggest that early immunization with BCG-containing regimens may protect against leukemia, allergy, and childhood diabetes among others, possibly *via* heterologous trained immunity (17–19).

A CoP is an immune measure that corresponds to vaccine-induced protection from disease (20). Despite substantial efforts to characterize classic adaptive immunity, including multiple studies of polyfunctional CD4 T cells, a clear CoP for BCG has yet to be established (21). Indeed, increasing evidence that BCG induces trained immunity—i.e., enhanced subsequent innate responses to a range of stimuli (8)—raises the possibility that these innate immune enhancing effects may not only underlie heterologous (non-specific) benefits of BCG vaccine, but may also contribute to, or conceivably be, the major factor in the so called "specific" effect of BCG, i.e., protection against early life TB.

A critical issue with respect to BCG immunization is marked variability between vaccine formulations produced in different production facilities, with the result that "BCG" is not a single entity. After its original manufacture in the Pasteur Institute (Paris, France) in 1921, BCG was shipped to 20 different international sites where the vaccine was repeatedly subcultured under different conditions. This has resulted in diverse licensed BCG formulations that are distinct both by content of live mycobacteria as well as genetic composition. These strains have been shown to have differing immune responses and furthermore, the clinical relevance of this has been illustrated in comparative studies, which suggest, for example, that BCG-Denmark and BCG-Japan may have greater benefit in reducing TB disease than BCG-Russia (15, 22).

Much remains to be learned regarding mechanisms underlying BCG-induced protection. As a complex vaccine comprised of live-attenuated mycobacterium, BCG engages the innate immune system *via* PRRs. Analogy to *M. tuberculosis* as well as direct human *in vitro* studies suggests that BCG may activate *via* multiple toll-like receptors (TLRs) including TLRs-2, -4, -7, -8, and -9 as well as C-type lectin receptors and NLRs (23). Studies of BCG-immunized adults demonstrate a re-programing of monocyte precursors such that the higher expression of PRRs and greater reactivity to stimuli such as TLR agonists. This innate immune enhancing effect of BCG is reminiscent of the effect of administration of TLR agonists to neonatal mice that enhanced innate immune responses, including cytokine induction and phagocyte recruitment, and improved bacterial clearance and survival in a model of neonatal sepsis (24). Such innate immune enhancing effects of prior stimulation have been termed "trained immunity" reflecting an adaptive arm of innate immunity that is noted in plants, insects, and mammals (25). That BCG heterologous (non-specific) benefits are greater in early life (26) suggests ontogeny of underlying immune mechanisms, potentially including trained immunity (27).

Hepatitis B Vaccine

Hepatitis B vaccine is an alum-adjuvanted vaccine containing hepatitis B surface antigen (HBsAg). The alum-adjuvanted HBV is given within the EPI (**Table 1**) and also in Australia, Europe, and United States, where a birth dose is recommended (28). With respect to innate immune activation, while the Alum adjuvant present in HBV may engage the inflammasome, HBsAg also interacts with CD14 to activate dendritic cells (29). Importantly, there is a measurable CoP for HBV, namely the titer of anti-HBsAg Abs. Although this CoP has been defined, as with many vaccines, it is not fully understood how HBV vaccine induces protective immunity, as a newborn dose is protective, despite only ~30–50% of newborns responding with "protective" titers after a single dose. This observation suggests that additional mechanisms, including cell-mediated immunity (CMI), may contribute to protection (30). Ab and T cell responses to HBV given to infants are distinct from those of adults, in that infants produced markedly higher serum anti-hepatitis B surface (HBs) Ab titers in one study, and low-Ab levels were associated with lower HBs Ag-specific IFNγ responses and a more Th2-polarized memory response to HBsAg

TABLE 1 | Immunizations given at different ages.

	Vaccines licensed	Vaccines tested	Future vaccine targets
Pregnancy	• aPertussis • Tetanus • Influenza	• (RSV) • GBS	• Group B *Streptococcus* • HIV • Malaria
Birth	• OPV • HepB • BCG	• DTaP • Hib • PCV • Malaria (e.g., RTS,S/ASO1/2) • Recombinant BCG vaccines (e.g., VPM1002) • HIV (phase I/IIa) • Rotavirus	• RSV • *Salmonella* • ETEC • ncHI • Malaria
Infant doses Age 2–4 months	• DTaP and DTwP • IPV • Hib • HepB • PCV • MenB • MenC • Rotavirus	• Malaria (e.g., RTS, S/ASO1/2, and Spf66) • Recombinant BCG vaccines (e.g., VPM1002) • Novel TB candidates (e.g., MVA85A) • HIV (phase I/IIa)	• RSV • Men ACWY • *Salmonella* • ETEC • ncHI • Malaria
Infant doses Age 12–13 months	• Hib • PCV • MMR • MenB • MenC • Varicella	• LAIV	

RSV (respiratory syncytial virus), Hib (haemophilus influenzae B), BCG (Bacille Calmette–Guerin), OPV (oral polio vaccine), IPV (inactivated polio vaccine), HepB (hepatitis B), DTaP (diphtheria, tetanus, acellular pertussis), DTwP (diphtheria, tetanus, whole cell pertussis), PCV (pneumococcal conjugate vaccine), HIV (human immunodeficiency virus), ETEC (enterotoxic Escherichia coli), ncHI (non-encapsulated Haemophilus influenzae), Men B,C, ACWY (meningococcal B,C, ACWY), MMR (measles, mumps, rubella), TB (tuberculosis), LAIV (live-attenuated influenza virus). ( ) indicates Trial in progress.

at 1 year (30). Genetic factors, low-birth weight and low-Apgar scores were risk factors for poor HBV response in a study of twins in China (31). Haplotype analysis of Gambian infants suggested that *CDC42*, *IL19*, and *IL1R1* genes associated with peak anti-HBsAg Ab level (32). Much remains to be learned regarding how HBV protects in early life.

Polio

A birth dose of OPV has been recommended by the World Health Organization since 1984. It is hypothesized that a birth dose of OPV may induce mucosal protection prior to colonization or infection with enteric organisms which may interfere with the immune response to doses given later in life. Data on seroconversion following this individual dose of trivalent OPV (tOPV) vary greatly, from 10 to 15% in India to 76% in South Africa, however, the positive impact on levels of neutralizing Abs and seroconversion rates on completion of the routine immunization schedule are undisputed (33). A systematic review of 5,257 infants given tOPV at birth, found that the percentage of newborns who seroconverted at 8 weeks ranged between 6 and 42% for poliovirus type 1, 2 and 63% for type 2, and 1 and 35% for type 3 (34). In addition, there were four studies of IPV in newborns with a seroconversion rate of 8–100% for serotype 1, 15–100% for serotype 2, and 15–94% for serotype 3, measured at 4–6 weeks of life. No serious adverse events related to OPV or IPV doses at birth were reported in these studies, including no cases of acute flaccid paralysis. Some groups have advocated a shift to using IPV because (a) tOPV has been associated with rare cases of vaccine-associated paralytic poliomyelitis (~2–4 cases/million), (b) concerns about the use of live vaccines in immunocompromised individuals, including those with HIV infection, and (c) potential risk of strain reversion. Of note, however, some studies have suggested that similarly to BCG vaccine, a birth dose of live OPV may induce heterologous ("non-specific") beneficial effects (35). Further research is warranted prior to replacing OPV with IPV (36).

CLINICAL STUDIES OF OTHER VACCINES AT BIRTH

Pertussis

Initial studies of the role of a birth dose of whole cell pertussis vaccine demonstrated low-Ab titers at 4 months, although there were no randomized studies at that time comparing a birth dose with a dose at 6–8 weeks of age (37). Further studies of neonatal whole cell pertussis immunization were deterred by the suggestion in 1965 that immunization with the whole cell pertussis vaccine combined with diphtheria and tetanus toxoids (DTwP) within 24 h of birth may introduce "immune paralysis" (38). Twenty years later, comparison studies of a birth dose of DTP with routine immunization at 2 months demonstrated significantly lower titers to pertussis toxin (PT) at 9 months of age, and an inverse correlation between cord Ab titers and infant responses (39). Safety concerns about the whole cell vaccine led to a switch to acellular pertussis vaccines (aP, or DTaP) in the 1990s. Initial neonatal studies of aP vaccines, both with and without diphtheria and tetanus antigens, were promising (40, 41), but a conflicting later study showed poorer responses (42). Reports of bystander interference resulting in lower *Haemophilus influenza* type B (Hib) vaccine and HBV responses were concerning (41). A pilot study of the GSK monovalent aP vaccine at birth and 4 weeks demonstrated significantly higher IgG Ab against pertussis antigens at 2 months of age, without reducing subsequent pertussis Ab responses. A larger study of doses at birth and 6 weeks, including influence of maternal immunization, on Ab responses up to 5 years of age is ongoing (43, 44). Follow-up of children from the initial pilot study to 4 years of age demonstrated higher cytokine responses to pertussis antigen stimulation in those who received a birth dose compared with controls at 2 years of age (44). These observations were similar to those in a long-term follow-up study of children vaccinated at birth which showed increasing CMI, as measured by lymphoproliferative capacity, compared with controls (45). As the role of maternal immunization with pertussis becomes more established, it is crucial to include the effect of maternal interference in studies, as even pre-pregnancy immunization may influence later-born infant responses (46). Englund et al. demonstrated a lack of maternal Ab interference on infant immunizations given at 2 months—that is, the PT Ab response to DTaP, unlike DTwP, was not affected by pre-existing Ab to PT (47). Whether this observation also holds true for birth doses requires future study. Given current concerns of waning immunity to aP (48), novel pertussis vaccine formulations, potentially including developing pertussis vaccines that are safe and effective in newborns, are needed to induce robust and durable immunity against this pathogen.

Haemophilus Influenza Type B

The role of a birth dose of Hib vaccine was explored by a number of groups following the initial success of its introduction into the EPI. Three different conjugate vaccines tested {HIB polysaccharide conjugated to tetanus toxoid, Hib polysaccharide conjugated to a genetically modified diphtheria toxin (HbOC), and Hib polysaccharide conjugated to a *Neisseria meningitidis* outer membrane protein [HbOMP; subsequently noted to be a TLR2 agonist (49)]} all resulted in significantly higher PRP Ab levels at 2 or 4 months compared with controls, suggesting neonatal priming was possible (50, 51). However, these higher Ab levels did not persist for HbOC and declined more rapidly than controls for HbOMP, leading to concerns about waning protection. Further studies have not been undertaken, as epidemiological studies have demonstrated a protective effect of herd immunity on early life burden of invasive Hib disease, presumed due to reduction of asymptomatic nasopharyngeal carriage of Hib among vaccines (52, 53). Conjugate vaccines, through a T cell-dependent immune response, result in very high-protective Ab responses in infants of all ages, which results in reduction of carriage. Furthermore, the licensed 10 valent pneumococcal conjugate vaccine (PCV10; PHid-CV), uses a *Haemophilus* outer membrane protein (protein D) as its carrier protein, and immunization with this reduces the incidence of all invasive *Haemophilus* spp. disease, including non-typable or non-encapsulated *Haemophilus influenzae* (ncHI). This may be of particular importance as invasive disease due to ncHI is commonest in the first month of life (54, 55), so the

potential role of the PHid-CV pneumococcal vaccine or a specific ncHI vaccine in neonates should be studied.

Pneumococcal Conjugate Vaccine

To date, only two published trials have assessed effects of a neonatal dose of the seven valent PCV7 (56, 57). In Kenya, 300 neonates were randomized to receive PCV7 at birth, 10 and 14 weeks or at 6, 10, and 14 weeks (EPI schedule). The safety of giving vaccine at birth was an important endpoint in the study and the researchers saw no significant difference in safety events between the two groups. Serotype-specific IgG binding was measured following the completion of the primary infant schedule at 18 weeks of age. The proportion of infants who had an Ab concentration above the accepted protective threshold of >0.35 μg/mL was similar between the two groups. When a higher threshold (>1.0 μg/mL) was used, proportions above 1 for serotypes 4, 18 C, 19 F were lower in the neonatal group. Geometric mean concentrations of IgG for four serotypes (4, 9V, 18 C, 19 F) were lower in the neonatal group compared with the EPI group at 18 weeks of age. In contrast, the mean avidity indices were significantly higher in the neonatal group for three of the four serotypes tested (4, 6B, 19 F) (57). In both groups, maternal IgG measured in cord blood inversely correlated with the GMC at 18 weeks of age with high-serotype specific cord blood levels associated with lower responses to vaccine. At 9 months of age, 5 months following the third PCV dose, there was no difference in the percentage of infants with an Ab concentration above either the 0.35 or 1.0 μg/mL thresholds. The GMC of serotype 4 specific IgG remained lower and serotype 19 F avidity index remained higher in the neonatal group. Responses to a booster dose of PCV given at 9 months of age and measured 2 weeks later were comparable between the two groups suggesting that there is no tolerance induced by the neonatal dose. Carriage was measured at 18 and 36 weeks in this study with no significant differences detected between the groups.

In a trial undertaken in Papua New Guinea (PNG), 318 infants were randomized to receive either PCV7 at birth, 1 and 2 months, PCV7 at 1–3 months, or no PCV7 (56). Local reactogenicity rates were generally low although higher rates were seen in the infant than the neonatal group. There were no differences in the illness episodes or serious adverse events. At 2 months of age, serotype-specific GMCs were significantly higher in the neonatal group than in the infant group for four of the seven serotypes in PCV7 (4, 9V, 18 C, 19 F). At this point, the neonatal group had received two doses of PCV compared with one dose in the infant group. By 4 months of age, following three doses of the vaccine, GMCs were significantly higher for all serotypes in the infant group than in the neonatal group although 2 months had elapsed since the neonatal groups third dose, a gap that was only 1 month for the infant group. Comparable responses were seen following a pneumococcal polysaccharide vaccine administered at 9 months of age and responses in both PCV7 primed groups were significantly greater than responses in those who were not primed. Nasopharyngeal swabs were collected at ages 1–4 weeks and 3, 9, 18 months, and middle ear discharge if present. The prevalence of pneumococcal carriage was 22% at 1 week of age, rising to 80% by age 3 months and remained >70% thereafter (58). There were no significant differences in PCV7 serotype carriage between PCV recipients and controls at any age (22 vs. 31% at 9 months, $p = 0.2$). At age 9 months, the prevalence of non-PCV7 serotype carriage was 17% higher in PCV7 recipients (48%) than in controls (25%, $p = 0.02$). The authors attributed the limited impact of neonatal or accelerated infant PCV7 schedules on vaccine serotype carriage to the early onset of dense carriage of a broad range of pneumococcal serotypes.

A prior report from the same PNG study examined whether a neonatal PCV7 dose might induce immune tolerance (59). In a comprehensive immuno-phenotypic analysis at 9 months of age, no differences in the quantity or quality of vaccine-specific T cell memory responses (including responses to CRM197, tetanus toxoid, and HBsAg) were found between the neonatal and infant vaccination groups. Hospitalization rates in the first month of life did not differ between children vaccinated with PCV at birth or not. Reviewing the data outlined in these two studies demonstrates that neonatal immunization with PCV7 is safe and not associated with immunologic tolerance (56, 58).

CURRENT RESEARCH ON EARLY LIFE IMMUNIZATION

Enhancing Current Vaccines

One approach to developing enhanced neonatal vaccines focuses on improving existing vaccines such as the live "self-adjuvanted" BCG vaccine. For example, the BCG-derivative VPM1002 expresses listeriolysin from *Listeria monocytogenes* designed to enhance MHC-I responses (60). In a phase II open label study comparison with conventional BCG-SSI in South African newborns ($n = 48$), VPM1002 demonstrated safety and immunogenicity with an increased proportion of CD8+ IL-17+ cells at 6 months post-vaccine. The authors speculate that although the significance of such cells is unknown, it is possible that they could contribute to more robust protection against TB and that larger studies are needed to assess this possibility.

Development of Adjuvants for Early Life Immunization

Another approach to enhancing vaccine responses in infants with "age-appropriate" immunity is the addition of adjuvantation systems to enhance vaccine immunogenicity and efficacy. PRR agonists such as mono-phosphoryl lipid A that activates TLR4, have been employed as vaccine adjuvants but the translational path for this approach must take into account that responses to PRR stimulation vary markedly with the age of a given individual (6). In developing adjuvant systems optimized to early life, there may be lessons to learn from live-attenuated vaccines currently in use. Examples from human *in vitro* studies using the BCG vaccine demonstrate this, including TLR2-mediated activation of neonatal NK cells to produce IFNγ; TLR9-mediated activation of pDCs for IFNγ; TLR2- and IFN-mediated activation of conventional DCs IL-12 p70 production and subsequent CD4+ T-cell Th1 polarization (61). Along these lines, TLR7/8 adjuvant-containing nanoparticles mimic effects of BCG on human neonatal monocyte-derived DCs *in vitro* and induce anti-mycobacterial T cells

in humanized TLR8 mice *in vivo* (62). Addition of a TLR7/8 agonist adjuvant to PCV dramatically accelerated and enhanced responses to a birth dose, inducing pneumococcal-specific Ab titers far exceeding the CoP after a single dose (63). As with any vaccine development, safety will be front and center, and in this context, it is worth noting that the most commonly given neonatal vaccine, BCG, activates multiple PRRs including TLRs and is safely given to newborns across the globe. Although this does not necessarily imply that any TLR-stimulating approach would be safe, it does provide an important proof of concept that in certain settings and contexts, PRR activation including TLR-stimulation can be safe and effective approach to vaccine adjuvantation in human newborns.

Mucosal Vaccine Development

In designing new vaccines to be given in the neonatal period, consideration must be given to ideal characteristics (64). Mucosal vaccines, with their potential for needle-free delivery, are very attractive. A number of the pathogens causing severe disease in early infancy are mucosally transmitted [rotavirus, RSV, polio, non-typable *Salmonella* spp., enterotoxic *Escherichia coli* (ETEC)] and the positive experience with OPV provides proof of concept of mucosal vaccines in this age group. Mucosal vaccines could be administered by a number of routes (oral, nasal, conjunctival, rectal, and vaginal) with nasal and oral being the most practical and studied to date. Adjuvanted mucosally administered vaccines stimulate multiple types of immune responses, including secretory IgA Abs which prevent adhesion and invasion of pathogens, serum IgG neutralizing Abs, and a wide array of cell-mediated T cell responses (65). Of note, determining the correlate of immune protection for each vaccine is a challenge. The focus has historically been on neutralizing IgG antibodies, but increasingly, the role of antigen-specific IgA in serum and stool has been explored (66) with need for further standardization of these assays. A number of factors may influence the efficacy and immunogenicity of oral vaccines, particularly in early life, including the presence of pre-existing Abs, malnutrition, enteropathy, micronutrient deficiencies, and breast feeding, factors being studied in the context of OPV and rotavirus vaccines (67). Breast milk contains an array of protective molecules including specific Abs, oligosaccharides, glycoproteins, and receptor analogs, likely to prevent both pathogenic- and vaccine-strains of microorganisms binding to the intestinal wall. A study of Rotarix in infants comparing withholding or not withholding breastfeeding around the time of vaccine administration did not demonstrate an increase in anti-rotavirus IgA seroconversion (68). To the extent that these factors may limit responses to mucosal vaccines, neonates, who are unlikely to yet have malnutrition, enteropathy, and co-infections, may be more responsive to oral vaccines.

A number of oral vaccines with varying immunogenic and protective efficacies have been licensed including those directed against typhoid, rotavirus, polio, and cholera. Other targets include non-typable Salmonella, ETEC, Shigella, and adenovirus (64). Few to date have been studied in newborns, although diarrheal illness accounts for many of the deaths in infants <6 months of age, with rotavirus identified as a leading cause of dehydrating gastroenteritis, associated with ~28% of diarrheal deaths (69). Two live-attenuated oral rotavirus vaccines, the pentavalent human-bovine rotavirus vaccine, RotaTeq, given as three oral doses a month apart; and monovalent human rotavirus vaccine Rotarix, given as two doses a month apart; were licensed in 2006. Protection against severe disease in high- and middle income countries is excellent for both (80–95% efficacy). However, unfortunately, these rotavirus vaccines have been less efficacious in low-income regions where the need is far greater such as sub-Saharan Africa (46% efficacy) and in Southern Asia (50%) (70). Although indirect benefits of rotavirus vaccine, such as those realized *via* herd immunity and protection, have been described in high- and middle income countries, this has not been firmly established in low-income countries to date (71). Factors including breastmilk Abs, concurrent infections, or environmental enteropathy may interfere with the efficacy of the oral rotavirus vaccine (67). Administration of a birth dose could potentially ameliorate these concerns, as well as provide protection in the vulnerable gap when the most severe disease occurs, between birth and the protective response induced by a first dose given at 6 weeks of age (72). Furthermore, the risk of intussusception as a complication of rotavirus vaccine appears to follow an age-related pattern, supporting a neonatal schedule over an infant schedule for this vaccine (73). A phase IIa study of a monovalent human rotavirus vaccine RV3-BB, including a neonatal dose, demonstrated a rotavirus IgA response rate of 11% with stool excretion of 13% after one dose (74). Overall immunogenicity following the non-neonatal schedule at 8, 15, and 24 weeks was 50% after two doses and 74% after 3. A nested study within the trial examined the relationship between rotavirus-specific IgA in cord blood, colostrum and breast milk and infant serum IgA response and stool excretion and found no evidence of an association (75). Although these initial immunogenicity results are disappointing, with low uptake following the neonatal dose, neither RotaTeq nor Rotarix have been tested in the neonatal period such that further studies of rotavirus vaccines in the neonatal period are warranted.

A nasally delivered live-attenuated influenza vaccine (LAIV) has been effective in infants in protection from flu, but has not to date been tested in neonates. Infants between 6 and 12 months experienced relatively high rates of hospitalization in an RCT comparing inactivated vaccine and LAIV and consequently, LAIV has only been recommended in infants greater than 12 months of age (76). There is currently no available influenza vaccine for infants younger than 6 months, however, maternal immunization provides passive protection (77) and in a murine model, there was no evidence of maternal interference (78).

As a leading cause of morbidity and mortality in the neonatal and early infancy period, RSV is an ideal target for a mucosal vaccine (79). The RSV fusion (F) surface glycoprotein has been considered as one of the two major protective antigens for eliciting neutralizing Abs; a humanized monoclonal Ab specific to the F protein (Palivizumab), administered monthly to vulnerable infants during the RSV season, is efficacious in preventing severe disease, but not infection (80). Low levels of

nasal RSV-specific IgA are a risk factor for RSV disease in adults (81), but as yet, although animal models have demonstrated neutralizing Abs in response to RSV vaccine candidates, specific IgA production has not been shown (82, 83). Animal models of live-attenuated vaccines provide grounds for optimism (84) and phase I studies of virus-vectored vaccines in adults have been promising (85), but virus-vectored RSV vaccines have not yet been trialed in infants. Development of vaccines based on the RSV pre-fusion protein, an antigenic target to which protective neutralizing Abs in human sera are directed (82), offers fresh avenues for RSV vaccine development with much to be learned regarding the potential of this antigen when administered in early life.

Systems Biology

Systems biology approaches have been applied to adults and older infants but thus far not to newborns (86). This deficiency is to soon change with the award of a NIH Human Immunology Project Consortium grant to employ systems vaccinology to study HBV-induced molecular signatures in relation to CoP—i.e., anti-HBsAg Ab responses. Led by the Expanded Program on Immunization Consortium, an affiliation of Boston Children's Hospital, Medical Research Council (UK)-Gambia, and the University of British Columbia, focused on application of systems biology to early life vaccinology, this study will leverage transcriptomics and proteomics to provide fresh insights into HBV-induced protection in newborns. Among the novel systems biology approaches is "systems serology" that offers potential for much more precise understanding on the impact of vaccines at different ages measuring all aspects of the response, Ab avidity, titer, specific, and non-specific responses (87). This approach promises to provide deeper insight into the vaccine-induced humoral immune responses of distinct populations such as human newborns.

In Vitro Modeling to Accelerate and De-Risk Early Life Vaccine Development

Vaccine development is inherently costly, slow, and unfortunately beset by multiple failures. Current paradigms of vaccine development tend to presume that all populations will respond similarly to a given formulation and do not take species-specificity, genetic background, and age-specificity into account. Accordingly, many vaccine formulations fail or are less effective in vulnerable subpopulations such as the very young or elderly. In this context, human *in vitro* platforms that model age-specific vaccine-induced innate and adaptive immune responses as benchmarked to licensed vaccines offer the possibility of accelerating and de-risking vaccine development (9, 88). Indeed age-specific human *in vitro* platforms such as newborn whole blood assays, dendritic cell arrays, and microphysiologic tissue constructs (89) have been successfully used to define novel biomarkers of vaccine adjuvanticity (90) and identify TLR7/8 agonists as adjuvants active in early life (63).

Overall these technical advances offer powerful new opportunities to inform, de-risk, and accelerate novel vaccine development for use in early life.

INTEGRATION INTO CURRENT PROGRAM: CHALLENGES AND STRATEGIES

Neonatal immunization carries tremendous potential but further expansion or enhancement of this approach will require both deeper mechanistic insight into how vaccines protect in early life as well as integration into the existing framework of public health, including maternal immunization programs. In response to the concerns of multiple professionals in the field of maternal immunization, the Brighton Collaboration was formed in 2000, followed by the formation of the Global Alignment of Immunization safety Assessment in pregnancy (GAIA) to establish safety and efficacy standards in this area (91). A collection of case definitions and guidelines for data collection, analysis, and presentation of safety data in vaccine trials, relevant for neonates and infants, were published by global experts, largely in the context of maternal immunization, but these are also relevant and of value for monitoring safety of neonatal immunization studies (92). Whilst these definitions and guidelines can be amended for the use in neonatal immunization studies, the formation of an independent group is warranted to establish the framework for safe and efficacious neonatal immunization studies.

In 2010, to identify key topics and research gaps in the field and foster collaboration among investigators focusing on vaccinology and immune ontogeny, a workshop was organized by NIH (NIAID; Division of Allergy, Immunology, and Transplantation) and cosponsored by the Bill and Melinda Gates Foundation (93). Given recent technical and conceptual advances, and their potential to vastly transform the area of early life immunization, further workshops on optimizing early life immunization, including those individuals involved in regulation and safety assessments are warranted. The study populations for any ongoing vaccine trials should include not just healthy term infants, but a number of distinct populations, including preterm infants and those with immunodeficiencies, in particular HIV-exposed infected and uninfected [but not unaffected (94)], to ensure protection of all infants, especially the most vulnerable. Thoughtful design of studies, both of maternal and neonatal vaccines, will be essential to understand mechanisms underlying vaccine–vaccine interactions, including interference. It is essential that whilst adopting the most advanced systems-based approaches, the data are standardized to allow comparison of sample sets from the same or different sites. Ongoing reassessment of infant immunization schedules will allow the development of more effective neonatal vaccine schedules.

CONCLUSION

Overall, neonatal immunization is a common practice across the globe, yet much can be done to optimize its beneficial impact. Taking advantage of pivotal opportunities to enhance this approach will require engagement with stakeholders, including government, funding agencies, and the general public, on: (a) the need for greater precision in our understanding

of how current neonatal vaccines protect, the potential impact of the exact timing of administration in the neonatal period (i.e., first 28 days of life) and of vaccine–vaccine interactions, (b) assessing how maternal and neonatal immunization can be best integrated, and (c) leveraging modern tools including systems biology and human *in vitro* modeling to study the impact of immune ontogeny on vaccine responses thereby informing development of novel vaccines for use in early life against pathogens for which currently vaccines are inadequate (e.g., pertussis, TB, and influenza) or do not yet exist (e.g., RSV, HIV).

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EW and OL outlined the initial manuscript drafts; EW, OL, PM, and DB all contributed to the final manuscript.

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Current Challenges and Achievements in Maternal Immunization Research

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Maternal immunization has the potential to significantly improve maternal and child health worldwide by reducing maternal and infant morbidity and mortality associated with disease caused by pathogens that are particularly relevant in the perinatal period and in early life, and for which no alternative effective preventive strategies exist. Research on all aspects related to vaccines for administration during pregnancy is ongoing with support of multiple stakeholders and global participation. Substantial progress has been made, and the availability of new vaccines licensed exclusively for use in pregnant women to protect their infants has become an achievable goal. This review provides an update of the current challenges and achievements in maternal immunization research, focusing on recent milestones that advance the field and the prospects to make maternal immunization a feasible and accessible strategy to improve global health.

Keywords: maternal immunization, ethics, research, pregnancy, inclusion, regulatory, vaccination

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INTRODUCTION

The goal of maternal immunization is to boost maternal levels of specific antibodies to provide the newborn and young infant with sufficient IgG antibody concentrations at birth to protect them against infections occurring during a period of increased vulnerability, until they are able to adequately respond to their own active immunizations or infectious challenges. Newborns and young infants are at greatest risk of morbidity and mortality from infectious diseases, and they depend of maternal antibodies to resist these infections in early life. Maternal antibodies can be optimized during pregnancy given that pregnant women have intact humoral immune responses to vaccines and adequately produce antibodies, which can be efficiently transferred to the fetus through an active receptor-mediated transport system in the placenta. Higher concentrations of antibody at birth result in protection from infection and disease, or in delayed onset and decreased severity of various infectious diseases in the newborn. Examples of this concept include passive maternal antibody protection against tetanus, pertussis, respiratory syncytial virus (RSV), influenza virus, and group B streptococcus (GBS) infections, among others.

Research on maternal immunization is not new; as vaccines were developed, their administration to pregnant mothers to protect them and/or their infants was considered and evaluated, including protection against small pox with vaccinia vaccine in the late 1800s, whole cell pertussis vaccine (DTP) in the 1940s, influenza vaccine after the 1950s pandemics, and tetanus toxoid vaccine to prevent maternal and neonatal tetanus worldwide since the 1960s. Despite the success of the Maternal–Neonatal Tetanus Elimination program of the World Health Organization (WHO) (http://www.who.int/immunization/diseases/MNTE_initiative/en/), there was a paucity of active research on maternal immunization for several years in the twentieth century, in part due to concerns

with the safety of administering any drug or biologic to women during pregnancy, particularly after the experience of the drug thalidomide in the 1960s, which was associated with severe limb and other deformities in infants born to women who took this unlicensed medication in the US to treat hyperemesis gravidarum.

KEY ISSUES ON MATERNAL IMMUNIZATION RESEARCH

The potential impact of maternal immunization as a public strategy to prevent disease in mothers and infants is well recognized. Yet, there are no vaccines currently approved or licensed specifically for use in pregnant women. Licensed vaccines that are recommended for non-pregnant adults may be administered to pregnant women based on need and a risk:benefit assessment. When the risk of exposure and disease from a vaccine preventable infection is high for a mother and/or her fetus, and an effective vaccine is available, the benefit of the vaccine protection is greater than any potential theoretical risk from the vaccine, which is in turn considered to be lower than the risk of acquiring the infection and disease the vaccine can prevent. Licensed vaccines that have not been formally evaluated in or approved for pregnant women are therefore recommended for administration during pregnancy by the WHO and the US Centers for Disease Control and Prevention (CDC), as well as local organizations in many countries (1, 2) (**Table 1**). These recommendations have evolved over time, and they differ in that the current WHO recommendations do not specifically recommend pertussis vaccination during pregnancy, except when there is a known high burden of disease, as implemented in several countries such as Canada and Australia; while CDC and other Public Health programs such as in the UK, recommend routine vaccination of all pregnant women with the tetanus, diphtheria, and reduced acellular pertussis antigen content (Tdap) vaccine for all women, at every pregnancy. The specific timing of administration of this vaccine is also variable in different countries. Similarly, while tetanus vaccination is recommended for all pregnancies by WHO, most industrialized countries in Europe and North America, where pediatric vaccination coverage is high and the risk of tetanus infection at birth is negligible, do not routinely recommend tetanus vaccine administration during pregnancy. It is only given now because of the use of Tdap. Finally, influenza vaccination during pregnancy is considered an essential element of prenatal care in the US, and pregnant women have one of the highest influenza vaccination coverage rates in this country. However, while pregnant women are not excluded from influenza vaccination, routine administration is not the standard in most countries.

TABLE 1 | Recommended vaccines for maternal immunization [World Health Organization (WHO)].

Generally recommended	Recommended for disease prevention in specific situations	Contraindicated
Tetanus (TT, Td)	Cholera	BCG
	Yellow fever	Measles
Influenza inactivated[a]	Meningitis A (meningococcal)	Mumps
Acellular pertussis vaccine (Tdap) only in areas of burden[b]	Hepatitis A, B, and E,	Rubella
	Japanese encephalitis	Varicella
	Polio (OPV and IPV)	Live typhoid T21a
	Rabies	Live influenza

[a]*Influenza vaccine is recommended by WHO for administration in pregnant women in regions where influenza vaccine programs are already in place. Influenza vaccination is recommended as part of routine antenatal care in the US and several countries in Latin America.*

[b]*Tdap is routinely recommended for pregnant women in the US, the UK, some provinces of high burden in Europe, Canada, and Australia, as well as several countries in Latin America.*

Given that currently licensed vaccines are not specifically indicated for pregnant women, there might be reluctance by some providers and government agencies worldwide to recommend routine vaccination in this population. However, the US Federal Drug Administration (FDA) addresses this concern by approving labeling clearly stating in that licensed vaccines that are recommended for pregnant women (such as influenza and Tdap) are NOT contraindicated for use in pregnant women, and specific considerations regarding safety of use during pregnancy are addressed in the pregnancy subsection of the FDA approved labeling (3). Furthermore, the safety of these vaccines continues to be monitored through post-licensure surveillance mechanisms, such as pregnancy registries and large passive and active adverse event reporting and surveillance systems (4).

Ensuring and evaluating the safety of vaccines administered to pregnant women is a key component of any maternal immunization program or recommendation. This is particularly true now that new vaccines that can benefit pregnant women and their infants are being developed, such as vaccines to protect against GBS and RSV. An important issue is the need for harmonization of standard definitions of key safety outcomes after maternal vaccination and of a systematic approach to the assessment of safety throughout the life cycle of a vaccine, but particularly after implementation as large number of pregnant women are vaccinated. It is critical to consider the inherent risks associated with pregnancy itself, and to clearly understand the background rate of these risks in specific populations. Furthermore, to evaluate the impact of maternal immunization as a public health strategy to impact the burden of morbidity and mortality associated with the infection it prevents, it is necessary to establish baseline rates of these outcomes to demonstrate the efficacy and benefit of the vaccines in both mothers and infants. Finally, the ethical and regulatory aspects surrounding the inclusion of pregnant women as research subjects also influence the progress of the development of vaccines for maternal immunization.

RECENT MILESTONES IN MATERNAL IMMUNIZATION RESEARCH

Substantial progress has occurred in maternal immunization research (**Table 2**). Maternal immunization research has been supported by National Institutes of Health in the US for decades, spanning basic science, clinical, epidemiological, and translational research (5). Studies of relevant pathogens, including GBS, *Haemophilus influenzae* type b, *Streptococcus pneumoniae*, and tetanus were conducted during the 1980s and 1990s; studies of

TABLE 2 | Milestones in the development of vaccines for maternal immunization.

Time period	Milestones
1940s	• Studies of whole cell pertussis vaccine (DTPw) in pregnant women to protect infants in the US (8)
1960s	• Influenza vaccines recommended for pregnant women, considered a high risk group for influenza complications after the 1957 pandemic (8) • Maternal immunization with tetanus toxoid demonstrated to prevent neonatal tetanus in clinical study in Papua New Guinea (9)
1970s	• Tetanus toxoid added to World Health Organization (WHO) Expanded Program on Immunization (10)
1980s	• Maternal–Neonatal Tetanus Elimination program goal set by the WHO (10) • Phase I/II studies of vaccines in pregnant women and various studies related to maternal immunization supported by NIH are initiated in the US (5)
1990s	• Phase I/II studies of vaccines in pregnant women and various studies related to maternal immunization supported by NIH are ongoing in the US (5) • Influenza vaccine is routinely recommended for pregnant women in the US, regardless of underlying medical conditions (11)
2000s	• NIH clinical studies of vaccines in pregnancy continue (5) • Brighton Collaboration is formed (12) • WHO supports influenza vaccine recommendations in pregnancy (13) • Study in Bangladesh demonstrates efficacy of influenza vaccine given to pregnant women in protecting mothers and infants against laboratory confirmed influenza illness (14) • The Bill and Melinda Gates Foundation supports 3 large studies of influenza maternal immunization in Nepal, Mali, and South Africa (15–17) • MenAfrivac program in the African meningitis belt does not exclude pregnant women from receiving the meningococcal A vaccine (18) • The 2009 influenza pandemic results in prioritization of maternal immunization research in the US and worldwide (19)
2010 to date	• Publications of NIH guidance on Maternal Immunization Research and Toxicity Tables for pregnant women (7) • GAIA is formed in response to call from WHO to work toward harmonization of the assessment of safety of vaccines in pregnancy (20) • The WHO's Strategic Advisory Group of Experts recommends influenza vaccination of pregnant women in countries were influenza vaccines are routinely administered (21) • Given the reemergence of pertussis and infant mortality, maternal immunization with Tdap is recommended in the US and the UK in 2012 and subsequently other countries (22, 23) • Safety and effectiveness data from the UK and the US continue to support the administration of Tdap for pregnant women (24–26) • Research and health regulations support the inclusion of pregnant women in research (27–33) • Multiple studies of vaccines for pregnant women are being conducted globally with the support of various stakeholders, including vaccines for the prevention of respiratory syncytial virus and group B streptococcus (19, 34–38)

pertussis and RSV were prioritized from the 1990s to the first decade of the twenty-first century, while studies of seasonal and pandemic influenza vaccine studies have been conducted continuously for 40 years. Experimental and licensed vaccines for these pathogens were evaluated in phase I/II clinical trials in pregnant women under contract with various public and academic institutions in the US. Furthermore, these programs promoted research related to maternal immunization from vaccine antigen identification to the development of pertinent laboratory assays and reference materials, as well as animal models and developmental toxicity studies, and epidemiology and safety studies. In 2013, guidance documents on research, protocol design, and assessment of safety of vaccines during pregnancy were developed (6, 7). Other guidance documents have since been published, providing a framework for the study of vaccines and other biologics in pregnant women.

In 2008, a pivotal study conducted in Bangladesh was published (14). This study demonstrated for the first time that maternal vaccination with influenza vaccine can protect mothers and their infants from laboratory confirmed influenza illness, with an efficacy in preventing infant influenza of 63%, similar to that achieved with active immunization. This study led to the support to three large studies of influenza vaccination of pregnant women by the Bill and Melinda Gates Foundation, conducted in Nepal, Mali, and South Africa. These seminal studies have now been completed, contributing significantly to the knowledge of the benefits and safety of influenza vaccination of mothers and infants, including HIV infected women, and providing critical information to guide decisions and policies surrounding maternal immunization (15–17). One important contribution of these trials was the determination of the relatively limited duration of protection of infants provided by maternally derived antibody, which decreased substantially after the second month of life (39). The 2009–2010 influenza pandemic was another critical event that resulted in the subsequent prioritization of maternal immunization research in the US and worldwide. The number of clinical trials and publications on the topic of maternal immunization has increased substantially since the pandemic. Importantly, the knowledge gained in aspects related to safety, immunogenicity, and implementation of influenza vaccines for pregnant women has resulted in more advances in this field than ever. An example of this was the acquisition of data on the safety and effectiveness of adjuvanted influenza vaccines in pregnant women (40). In general, there is a need for more immunogenic vaccines for use in all populations, including pregnant women, to improve effectiveness and further reduce the impact of influenza.

In 2012, prompted by evidence of reemergence of pertussis disease and associated infant mortality, maternal immunization with Tdap was recommended in the US and the UK as the most immediate and direct intervention to decrease pertussis in the first few months of life (22). Several other countries with high burden of pertussis disease in the Americas, Europe, and Australia also adopted this recommendation. Importantly, research on maternal immunization with Tdap flourished, filling critical gaps of information, such as understanding the optimal timing for maternal vaccination in the second trimester of gestation to achieve higher antibody concentrations in infants at birth, and better and longer duration of protection in the first few months of life until active immunization with pertussis containing vaccines is achieved (41). Another relevant concept associated with the

utilization of Tdap vaccine in pregnancy is the potential blunting of infant immune responses to active immunization when high concentrations of maternal antibodies are present. This has been observed and documented for various antigens in the pertussis vaccines, including pertussis toxin, filamentous hemagglutinin, and pertactin, but relatively lower concentrations of vaccine-specific antibodies in infants after primary vaccination have not been associated with increased incidence or severity of pertussis disease in infants of vaccinated mothers, and preservation of priming and memory immune responses has been documented (42–45). Furthermore, the safety and effectiveness of the Tdap maternal immunization program have been demonstrated in the US and the UK, supporting continuation of this intervention in these countries (23–26). Similar programs are in place now in Latin America and other countries and regions with high burden of pertussis disease.

Currently, several studies are ongoing assessing various aspects of the use of licensed vaccines such as influenza and pertussis in pregnant women, as well focusing on the development of new vaccines specifically designed for administration during pregnancy, for the protection of infants against RSV and GBS in early life. Numerous RSV and GBS vaccines are in various phases of development, from preclinical to clinical trials, supported by multiple stakeholders from industry to private and public organizations (34–36). One RSV vaccine is currently in phase III of clinical development, promising, if successful, to be the first vaccine developed and licensed for specific use in pregnancy. Achieving this milestone has the potential to positively impact and change the landscape and practical applicability of infant disease prevention through maternal immunization. In addition to research focused on basic placental biology and immunology, understanding the role of passive and breast milk antibodies in infant protection and responses to natural infection and active immunization, and determining how to optimize maternal intervention to improve its safety and efficacy, other aspects that require further study include those related to acceptance, feasibility, and logistics of implementation of maternal immunization in different settings and populations. Furthermore, aspects related to education of mothers and providers, utilization, communications, and long-term surveillance and assessment of vaccine safety are paramount for the success of maternal immunization as a public health strategy to improve maternal and child globally. The field of maternal immunization research is therefore open, active, and rich.

PROGRESS IN THE REGULATORY ASPECTS RELATED TO MATERNAL IMMUNIZATION AND RESEARCH

The perception of risk of any intervention during pregnancy has evolved over time. Before the demonstration that the use of thalidomide during pregnancy was associated with birth defects, there were relatively little restrictions to what pregnant women were exposed to (46). This tragic association resulted in a shift toward strict restrictions of what pregnant women could be exposed to, including medications and vaccines, and the exclusion of pregnant women from research. However, there has been a culture change in recent years, driven by the need to develop effective immunization strategies and understanding that pregnant women and their infants can actually benefit from participating in clinical research. Their participation in clinical trials of vaccines and therapeutics ultimately will reduce any potential harm of these products, by generating useful information that is specifically relevant to pregnancy, and avoiding exclusion of women from receipt of potentially beneficial interventions available to the rest of the population. Having access to the benefits of participating in research and the results of this research will promote and improve maternal, fetal, and infant health. Clinical studies in pregnant women are carefully designed to minimize the risks of the intervention, particularly the risk to the fetus, and to balance the risk of participating in research with the risk of not having a potentially beneficial intervention available for mothers and infants.

Several recent milestones have been reached in the regulatory aspects of the assessment of vaccines for use in pregnancy (27). It is clear that for both novel vaccines, as well as for currently licensed vaccines not previously evaluated in pregnant women, regulatory agencies approval for use during pregnancy would result in inclusion of specific information in the product label that would facilitate the acceptance and use of the vaccine by health-care providers and the public in general. One important step toward facilitating the utilization of vaccines in pregnancy is the recent update to the US FRA pregnancy and lactation labeling rule, whereby product label pregnancy risk categories designated with letters as A, B, C, D, and X that were difficult to put into practice have been replaced with a narrative descriptions of the risks of using the vaccine during pregnancy, as informed by any source of information, including both observational and prospective studies (28). In 2015, vaccine manufacturers sought guidance from the Vaccines and Related Biological Products Advisory Committee of the FDA to work toward the development of vaccines for maternal immunization. In their fall meeting, the determination was made that the regulatory approval process of vaccines indicated for maternal immunization to prevent infant disease would be guided by regulations outlined in Title 21 of the Code of Federal Regulations and standards set forth in applicable documents such as the ICH guidelines and FDA guidance documents (29). The groups agreed that the path to development and licensure of a vaccine for pregnant women would be product specific and designed to support the indication being sought. Key aspects to consider would include the use of serologic endpoints as markers of passive protection in the infants, the evaluation of duration of immunity and immune interference with childhood vaccines, and the duration and type of safety follow-up. Importantly, the committee considered that observational studies could be used as an approach to confirm the effectiveness of already licensed vaccines that are recommended for use in pregnancy in the US.

Progress has also been made in regulations that further expand the options for pregnant women to be included in research. The updated "Common Rule," which is the set of federal

regulations for the ethical conduct of human subject research in the US, clearly delineates that pregnant women or fetuses may be involved in research if several conditions are met, including the prior conduct, when scientifically appropriate, of preclinical studies, including studies on pregnant animals (such as reproductive toxicology studies), and clinical studies, including studies on non-pregnant women (30). The document also delineates the risk categories for research based on the prospect of benefit for the women or the fetus, indicating that the risk of the research needs to be balanced with the prospect for benefit for the women OR the fetus, or if there is no such prospect of benefit, the risk to the fetus should be not greater than minimal when the purpose of the research is the development of important biomedical knowledge which cannot be obtained by any other means. The pregnant mother is given the right to provide consent for herself and for her baby, unless the prospect of direct benefit is solely to the fetus, then the consent of the pregnant mother and the father should be obtained, with exceptions allowed based on specific situations that would prevent the father from signing. These provisions help guide the Institutional Review Boards in their decision making regarding the participation of pregnant women in research.

Other advances relate to the change in classification of pregnant women from being considered a "vulnerable" population for research, to no longer being considered "vulnerable." This challenge for maternal immunization was addressed by the National Vaccine Advisory Committee to the Department of Health and Human Services, who also recommended the prioritization of maternal immunization as a public health strategy, and the investment in the development of vaccines for pregnant women (31). Globally, the 2017 updated International Guidelines for Health-Related Research Involving Humans of the Council for International Organizations of Medical Sciences, in collaboration with the WHO, also conclude that women must be included in health-related research, unless a good scientific reason justifies their exclusion, and that women should provide informed consent for themselves (32). Finally, the 21st Century Cures Act, as law enacted by the US Congress in December 2016 and designed to help accelerate medical product development and faster access to patients to innovations, established a task force on research specific to pregnant women and lactating women, to provide advice and guidance to the Secretary of HHS, to address gaps in knowledge and research regarding safe and effective therapies for pregnant and lactating women, and authorized substantial funds for this task (33). A key provision of this law was the inclusion of vaccines administered during pregnancy in the Vaccine Injury Compensation Program, thereby providing coverage for claims of potential vaccination adverse effects on the fetus and the mother, for providers who administer vaccines to pregnant women. Specifically, the law states that "…both a woman who received a covered vaccine while pregnant and any child who was *in utero* at the time such woman received the vaccine shall be considered persons to whom the covered vaccine was administered and persons who received the covered vaccine." This provision is a tremendous step toward the improvement of acceptance, confidence, and coverage of maternal immunization in the US.

EFFORTS IN HARMONIZATION IN THE ASSESSMENT OF SAFETY OF MATERNAL VACCINES

In addition to the work of the NIH and investigators involved in maternal immunization research, one of the organizations that provided early contributions toward the goal of developing a consensus and harmonized assessment of the safety of vaccines during pregnancy is the Brighton Collaboration. This independent and non-profit partnership was formed in the year 2000 as a voluntary international group seeking to facilitate the development, evaluation, and dissemination of high quality information about the safety of human vaccines. The group stated by developing a common language and standardized research methods to improve the accuracy and consistency of vaccine risk assessment. In 2014, stemming from a call by WHO, and with support from the Bill and Melinda Gates Foundation, the GAIA (Global Alignment on Immunization Safety Assessment in pregnancy) consortium was formed, with the goal to develop a globally concerted approach to actively monitor the safety of vaccines and immunization programs in pregnancy (20). The GAIA group utilizes the format of the Brighton Collaboration to assess safety outcomes in mothers and infants after maternal vaccination, determining the level of certainty in the assessment of the event, to ensure uniformity and comparability in different settings. In addition to pertinent clinical case definitions, the GAIA consortium also published guidelines and tools for the assessment of safety of vaccines in maternal immunization clinical trials (47, 48). These guidelines were supported by the Global Advisory Committee on Vaccine Safety of the WHO (21), and various clinical case definitions are undergoing evaluation and validation as they are utilized in various settings from retrospective, to observational and prospective clinical trials worldwide.

CONCLUSION

Maternal immunization has the potential to significantly improve maternal and child health worldwide by reducing maternal and infant morbidity and mortality associated with disease caused by pathogens that are particularly relevant in the perinatal period and in early life, and for which no alternative effective preventive strategies exist. Active research encompassing all aspects related to vaccines for administration during pregnancy is underway, with support of multiple stakeholders and global participation. Substantial progress has been made, and the availability of new vaccines licensed for use in pregnant women is an achievable goal. While many challenges remain to be addressed, the achievements in maternal immunization research to date have advanced the field and the prospects to make maternal immunization a feasible and accessible strategy to improve global health.

AUTHOR CONTRIBUTIONS

FM was responsible for designing and writing this article.

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REVIEW
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The Effect of Human Immunodeficiency Virus and Cytomegalovirus Infection on Infant Responses to Vaccines: A Review

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The success of prevention of mother to child transmission programs over the last two decades has led to an increasing number of infants who are exposed to human immunodeficiency virus (HIV), but who are not themselves infected (HIV-exposed, uninfected infants). Although the morbidity and mortality among HIV-exposed, uninfected infants is considerably lower than that among HIV-infected infants, they may remain at increased risk of infections in the first 2 years of life compared with their HIV-unexposed peers, especially in the absence of breastfeeding. There is some evidence of immunological differences in HIV-exposed, uninfected infants, which could play a role in susceptibility to infection. Cytomegalovirus (CMV) may contribute to the increased immune activation observed in HIV-exposed, uninfected infants. Infants born to HIV-infected women are at increased risk of congenital CMV infection, as well as early acquisition of postnatal CMV infection. In infants with HIV infection, CMV co-infection in early life is associated with higher morbidity and mortality. This review considers how HIV infection, HIV exposure, and CMV infection affect infant responses to vaccination, and explores possible immunological and other explanations for these findings. HIV-infected infants have lower vaccine-induced antibody concentrations following tetanus, diphtheria, pertussis, hepatitis B, and pneumococcal vaccination, although the clinical relevance of this difference is not known. Despite lower concentrations of maternal-specific antibody at birth, HIV-exposed, uninfected infants respond to vaccination at least as well as their HIV-unexposed uninfected peers. CMV infection leads to an increase in activation and differentiation of the whole T-cell population, but there is limited data on the effects of CMV infection on infant vaccine responses. In light of growing evidence of poor clinical outcomes associated with CMV infection in HIV-exposed, uninfected infants, further studies are particularly important in this group. A clearer understanding of the mechanisms by which maternal viral infections influence the developing infant immune system is critical to the success of maternal and infant vaccination strategies.

Keywords: human immunodeficiency virus, cytomegalovirus, vaccines, immune responses, infant

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INTRODUCTION

Immunization is essential to global strategies to reduce infant mortality, especially in low-resource settings where infectious morbidity and mortality remain high (1, 2). In regions of the world where the burden of infectious diseases is high, even a small reduction in vaccine efficacy might have important clinical implications for young infants. Factors affecting infant vaccine responses can be divided into those relating to the infant, mother and environment; an important and potentially modifiable maternal factor is antenatal viral infections. To date, studies of the effects of maternal antenatal viral infections on infant vaccine responses have focused on two important viral infections: human immunodeficiency virus (HIV) and cytomegalovirus (CMV).

In 2015, there were 1.4 million pregnant women living with HIV (3), and an estimated 24% of those did not receive antiretroviral therapy (ART) for prevention of mother-to-child transmission (PMTCT) (4). In infants born with HIV infection, early commencement of combination ART significantly reduces mortality (5, 6) and is associated with increased magnitude and quality of infant antibody responses to vaccines (7–10), but there is some evidence of reduced humoral responses to vaccines even in those children starting ART at 6–8 weeks of age, compared with HIV-unexposed infants (9).

The expansion of PMTCT programs in the last two decades, and continued high numbers of HIV-infected women who become pregnant, have led to an increase in the number of HIV-exposed, uninfected infants, who now represent up to 30% of births in some parts of Southern Africa (11). Compared with HIV-unexposed infants, HIV-exposed, uninfected infants are at increased risk of hospitalization, pneumonia and mortality at 6 and 24 months in some settings, although the risk is reduced when they are breastfed and when infected children and their mothers receive ART (12). Increasing evidence shows immunological differences in HIV-exposed, uninfected infants, who have changes in T-cell populations, lower CD4 counts and increased T-cell differentiation by 10 weeks of age compared with HIV-unexposed infants (13). While these immunological differences may play a part in the increased morbidity which has been observed, factors such as socioeconomic status, maternal health, breastfeeding duration, exposure to ART, and exposure to co-infections are also likely to be important.

Cytomegalovirus is the most common congenital infection worldwide, affecting up to 1.2% of live births in developing countries. Although CMV infection is thought to lead to immune senescence and a poor response to the influenza vaccine in the elderly (14), it is not fully understood how CMV might affect infant responses to vaccines. Congenital CMV infection leads to changes in the infant T-cell population as a whole and is associated with increased morbidity in HIV-infected and HIV-exposed, uninfected infants. In HIV-infected infants who are not receiving ART, those with congenital CMV infection have an increased rate of HIV disease progression (15, 16). HIV-exposed infants may be at higher risk of congenital CMV than HIV-unexposed infants (17, 18). In HIV-exposed, uninfected infants, CMV infection may contribute to immune activation (11, 19, 20) and increase the risk of postnatal HIV infection (21, 22).

This review summarizes the evidence for alterations in infant vaccine responses associated with exposure to maternal HIV and CMV infection, and explores possible immunological and other explanations for these findings.

METHODS

A literature search of English language publications was performed using Medline. Key search terms included: maternal, fetal, neonate, HIV, CMV, vaccination, and immunization. The full search strategy is detailed in Table S1 in Supplementary Material. A formal systematic review was not undertaken; however, we sought to carry out a comprehensive review of the published literature. We screened titles and abstracts, selecting all articles with outcomes that included infant immune responses to routine Expanded Program on Immunizations vaccinations following antenatal exposure to either maternal HIV or CMV infection, or both.

Studies of HIV-infected infants in which the majority of participants received ART were selected when possible, for three main reasons: (1) ART is associated with increased magnitude and quality of infant antibody responses to vaccines (7–10); (2) studies in which infants do not receive ART are prone to survival bias because there is often a high mortality rate in the HIV-infected group (7, 9, 23); and (3) this review is intended to be relevant to current and future practice, looking toward universal ART in HIV-infected children. Since 2015, the World Health Organization guidelines have recommended that all children infected with HIV receive ART. The proportion of children with HIV receiving ART worldwide was 49% in 2015, and ART coverage in children and adults with HIV has been increasing year on year (24). Studies of vaccine responses in HIV-infected infants were excluded if they did not include a HIV-unexposed control group. Papers including a comparison between vaccine responses in HIV-exposed, uninfected infants with HIV-unexposed, uninfected infants were included. This review aimed to review the contemporary literature; there were no studies comparing vaccine responses of HIV-infected infants treated with ART and HIV-exposed, uninfected infants, therefore this comparison is not made in this review.

VACCINE RESPONSES IN HIV-INFECTED INFANTS

Humoral Responses

The published literature shows some heterogeneity in the differences in concentrations of specific immunoglobulin (Ig) G and proportion of infants protected following primary and booster doses of routine vaccines in HIV-infected infants receiving ART compared with HIV-unexposed infants (**Table 1**).

Following primary immunization, HIV-infected infants aged 20 weeks had significantly lower specific antibody concentrations to tetanus, diphtheria, pertussis and hepatitis B surface antigen (HBSAg), than HIV-unexposed infants in a South African study (9). However, the clinical significance of this is unclear, as high proportions (92–100%) of infants from both groups had

TABLE 1 | Effect of HIV infection on infant responses to vaccines.

	Vaccine	Age at vaccination	Age at blood sampling	HIV-infected infants on ART, *n*	HIV-unexposed infants, *n*	Main findings in HIV-infected compared with HIV-unexposed infants
South Africa (7)	PCV	7, 11, and 15 weeks	20 weeks	172	125	• No significant difference in % protected or concentration of specific IgG • Opsonophagocytic assay: higher concentration of antibody needed for 50% killing activity for 2/3 serotypes (antibody (95% CI) for 50% killing 24 (21–27) vs 19 (16–23), $p = 0.045$ for serotype 19F; 4 (3–5) vs 2 (2–3), $p = 0.007$ for serotype 23F)
South Africa (8)	PCV	7, 11, and 15 weeks	7, 11, 15, and 20 weeks	205	119	• Pre-vaccination, lower concentration of specific IgG for 3/7 serotypes (mean GMC 0.14 vs 0.22, $p \leq 0.004$) and lower % protected for 4/7 serotypes (mean 30 vs 45%, $p \leq 0.008$) • Following dose 1, significantly lower specific IgG concentrations for 3/7 serotypes (mean 0.42 vs 0.60, $p \leq 0.016$) and lower % protected (mean 34 vs 49%, $p \leq 0.007$) for 4/7 serotypes • No difference after dose 2
South Africa (9)	DTwP-Hib, HBV	6, 10, and 14 weeks	7 and 20 weeks	172	114	• Pre-vaccination, lower concentration of antibody to tetanus (GMC 0.086 vs 0.421 IU/ml N/n^m, $p < 0.001$), HBsAg (GMC 5.81 vs 7.74 mIU/ml N/n, $p = 0.01$), and pertussis (GMC 17.67 vs 40.65 IU/ml, $p < 0.001$) • Post-vaccination antibody concentration lower for tetanus (GMC 0.405 vs 0.952 IU/ml N/n^m, $p < 0.001$), diphtheria (GMC 0.200 vs 0.272 IU/ml N/n, $p = 0.001$), HBsAg (GMC 924.87 vs 2,521.03 mIU/ml N/n, $p < 0.001$), and pertussis (GMC 40.48 vs 61.24 IU/ml, $p < 0.001$), but no difference in % protected
South Africa (10)	Measles	9 and 15.5 months	2.5, 4, 15.5, 16, and 24 months	182	115	• Pre-vaccination, significantly lower % protected (41.8 vs 65.2%) • At 24 months, no significant difference in antibody concentration or % protected
Zambia (21)	OPV	0, 6, 10, and 14 weeks, 12 months	18 months	17	397	• Lower antibody titer (log_2 antibody titer 4.71 (SD 2.97) vs 8.15 (SD 2.09), $p < 0.01$) • Lower proportion had protective antibody levels (64.5 vs 98.4%, $p < 0.01$)

PCV, pneumococcal conjugate vaccine; DTwP-Hib, diphtheria, tetanus, whole cell pertussis, Hib; OPV, oral polio vaccine; % protected, proportion protected; CI, confidence interval, Ig, immunoglobulin; HBSAg, hepatitis B surface antigen; ART, antiretroviral therapy; HIV, human immunodeficiency virus.

concentrations of antibody to tetanus, diphtheria, and HBsAg that are deemed protective (9).

Pneumococcal conjugate vaccine (PCV) antibody concentrations were lower in HIV-infected infants after the first and second PCV doses (8), but after the third dose the antibody concentration and proportion of infants protected were similar in HIV-infected and HIV-unexposed infants (7). However, an opsonophagocytic assay showed that antibody against two out of three PCV serotypes tested in HIV-infected infants receiving ART had 26–50% lower killing activity than that of HIV-unexposed infants. This suggests that HIV-infected infants may not mount as effective an antibody response against pneumococcal disease as HIV-uninfected infants, despite producing a similar concentration of antibody following vaccination (7). A UK study of older children (mean age 12.8 years, range 1–17.4 years) showed that a lower proportion of HIV-infected individuals were protected against three of 13 PCV serotypes, compared with HIV-unexposed children and adults. This was despite an equal or larger proportion of HIV-infected children having previously received the 7-valent or polysaccharide pneumococcal vaccines (25).

In a Zambian cohort in which mothers received short-course intrapartum nevirapine to prevent mother-to-child transmission during labor and delivery, but infants did not receive ART, HIV-infected infants who received oral polio vaccine (OPV) had 42% lower neutralizing antibody responses at 18 months ($p < 0.01$), and a lower proportion had protective antibody levels than HIV-unexposed infants (64.5 vs 98.4%, $p < 0.01$) (26).

Following measles vaccination at age 9 months, there was no significant difference in measles-specific IgG levels at 24 months in HIV-infected infants compared with HIV-unexposed infants in a study from South Africa, and no difference in the proportion of infants with protective antibody levels (10).

Human immunodeficiency virus-infected newborns are potentially more susceptible to vaccine-preventable diseases for a longer period than HIV-unexposed infants (8, 10). As well as having lower responses to the first two doses of vaccine (as seen with PCV and measles), compared with HIV-unexposed infants, HIV-infected infants had lower pre-vaccination concentrations of antibody to three of seven PCV serotypes (8), tetanus (GMC 0.086 vs 0.421, $p < 0.001$), HBsAg (GMC 5.81 vs 7.74, $p = 0.01$), and pertussis (GMC 17.67 vs 40.65, $p < 0.001$) (9, 10). Similarly, before vaccination a lower proportion of HIV-infected than HIV-unexposed infants had protective levels of antibody to PCV (for four serotypes, mean 30 vs 45% protected, $p \leq 0.008$) and measles (9, 10).

Cellular Responses

In a study in South Africa, in which mothers received PMTCT and infected infants (diagnosed at 6 weeks) were not breastfed and did not receive ART, T-cell responses to BCG in HIV-infected infants were compared with those in HIV-unexposed infants (27).

After BCG vaccine on day 1 of life, HIV-infected infants had severely impaired T-cell responses at 3 months, and by 9–12 months the response was almost absent (27). Both the magnitude of the CD4 and CD8 T-cell responses, and the polyfunctionality of the CD4 response were markedly reduced (27). Secreted cytokines interferon (IFN)-γ and interleukin-2 were also present in significantly lower concentrations in HIV-infected than HIV-unexposed infants at 3 months, although tumor necrosis factor (TNF)-α concentration was not significantly different (27).

Mechanisms of Altered Vaccine Responses in HIV-Infected Infants

Both maternal and infant factors are likely to be involved in the observed differences in antibody and cellular responses to vaccines seen in HIV-infected compared with HIV-unexposed infants (8). In HIV-infected infants, lower CD4 count may impair the mechanisms leading to induction and maintenance of immunological memory to vaccine antigens (26). The observation that specific antibody concentrations are lower in HIV-infected infants before vaccination may suggest reduced transfer of antibody across the placenta (8, 10). Both reduced antibody concentration and impaired placental function in HIV-infected mothers may contribute to this (28, 29).

There are inherent difficulties in comparing vaccine responses in HIV-infected with HIV-unexposed infant populations, as the two groups are likely to differ in duration of breastfeeding, exposure to ART, socioeconomic status, exposure to co-infections, nutritional status, and survival. In a multivariable analysis of factors associated with response to OPV (primary course and booster at age 12 months) at 18 months of age, increasing breastfeeding duration was associated with increasing poliovirus antibody level (26). In this study, median breastfeeding duration was 6 months in HIV-infected mother-infant pairs compared with 15 months in HIV-uninfected pairs ($p < 0.01$). Differences in breastfeeding duration in HIV-infected and unexposed groups were not stated in the other studies described earlier, although in four of the studies, infants were co-enrolled in the CHER trial in South Africa, a randomized controlled trial evaluating antiretroviral treatment strategies, in which only 14% of infants were breastfed (30). Short duration or refraining from breastfeeding in low and middle-income settings, including for HIV-infected infants, is associated with increased infectious morbidity, stunting, and wasting (31–33).

A recent review of the effects of maternal nutritional status on infant vaccine responses concluded that maternal macro- and micronutrient deficiency during pregnancy is likely to impair infant responses to vaccines, even in the presence of nutrient supplementation (34).

Increased exposure to opportunistic infections through breastfeeding (for example, CMV infection) or close contact with HIV-infected mothers who may have co-infection may affect immune responses in HIV-infected infants. In HIV and CMV co-infection, infants have accelerated HIV progression, increased mortality, growth delay and cognitive impairment, compared with HIV-infected infants without CMV (35). In Malawi, breastmilk CMV load had a stronger negative association with infant growth than breastmilk HIV load (36).

In summary, HIV-infected infants receiving ART may have impaired ability to mount quantitatively and qualitatively adequate antibody responses to vaccines compared with HIV-unexposed infants. The clinical effect of impaired vaccine responses on morbidity from vaccine-preventable disease is not known. Shorter breastfeeding duration, poorer nutritional status and increased exposure to co-infections in HIV-infected infants may be contributing factors, and their impact requires further investigation to fully understand the mechanisms underlying the changes in vaccine responses in HIV-infected infants. CMV infection is almost ubiquitous in low-resource settings, and its clinical effects on infants with HIV suggest it is having an important effect on the immune system, and could be an important modifiable factor in reducing morbidity and mortality of HIV-infected infants.

VACCINE RESPONSES IN HIV-EXPOSED, UNINFECTED INFANTS

Humoral Responses

Detailed studies in HIV-exposed, uninfected infants have demonstrated differing patterns of antibody responses to vaccines compared with HIV-unexposed infants (**Table 2**), and revealed likely underlying mechanisms.

After three doses of pertussis-containing vaccine, antibody levels in HIV-exposed, uninfected infants were two to seven times higher than in unexposed infants (9, 29, 37). However, following the booster dose at 18 months, the proportion of children with protective antibody levels was non-significantly higher in HIV-exposed, uninfected infants than in HIV-unexposed children (37).

Following the first one to two doses of Hib vaccine, concentration of specific antibody was 12 times higher at 16 weeks in HIV-exposed, uninfected than unexposed infants (29), but there was no significant difference when infants had received all three doses (9, 29, 37, 38). Similarly, tetanus antibody concentration was higher after one to two doses, but not significantly different at 4, 5, or 6 months following three doses (9, 29, 37). Six months after the fourth (booster) dose, at 24 months, the antibody concentration was significantly higher in the HIV-exposed, uninfected children than in the HIV-unexposed children ($p < 0.05$) (37). One study found that at 7 months tetanus antibody concentration was significantly lower in HIV-exposed, uninfected infants than unexposed infants, but neither study found any significant difference in the proportion of infants protected (37, 39).

In studies in South Africa, HIV-exposed, uninfected infants who received all three doses of PCV had significantly higher antibody concentrations at 16 and 20 weeks (7, 29), although opsonophagocytic activity was reduced compared with unexposed infants for 1 out of 3 serotypes (7).

The increased antibody responses to the initial doses of Hib, tetanus, and other vaccines can be explained by reduced interference from maternally derived antibody in HIV-exposed, uninfected infants. Before vaccination, HIV-exposed, uninfected

TABLE 2 | HIV-exposed, uninfected infant responses to vaccines.

	Vaccine	Age at vaccination	Age at blood sampling	HIV-exposed uninfected infants, *n*	HIV-unexposed infants, *n*	Findings in HIV-exposed uninfected compared with HIV-unexposed infants
South Africa (7)	PCV	7, 11, and 15 weeks	20 weeks	120	125	• Overall no difference in specific IgG concentration or % protected after third dose (median 99 vs 98% protected)
South Africa (8)	PCV	7, 11, and 15 weeks	7, 11, 15, and 20 weeks	124	119	• Pre-vaccination: for 7/7 serotypes, significantly lower specific IgG concentration (median GMC 0.12 vs 0.21, $p \leq 0.006$) and % protected (median 18 vs 32%, $p \leq 0.005$) • Post-dose 1: significantly lower specific IgG concentration (4/7 serotypes, median GMC 0.26 vs 0.53, $p \leq 0.003$) and % protected (5/7 serotypes, median 35 vs 49%, $p \leq 0.019$) • Post-dose 2: overall no significant difference in GMT or % protected
South Africa (9)	DTwP-HibCV, HBV	6, 10, and 14 weeks	7 and 20 weeks	120	114	• Pre-vaccination: lower antibody concentration against tetanus (GMC 0.219 vs 0.421 IU/ml N/n^m, $p = 0.001$); higher antibody concentration and % protected against diphtheria (61 vs 29% protected, $p < 0.001$) and HBsAg (81 vs 50% protected, $p < 0.001$) • Post-vaccination: lower antibody concentration against HBsAg (GMC 2,019.28 vs 2,521.03, $p = 0.041$) but mean 99.2% protected. Higher antibody concentration (GMC 261.30 vs 134.34, $p < 0.001$) and % achieving fourfold increase (76.7 vs 39.1%, $p < 0.001$) in response to pertussis
South Africa (10)	Measles	9 and 15.5 months	2.5, 4, 15.5, 16, and 24 months	116	115	• Pre-vaccination: no significant difference in GMT or % protected at 2.5 months • Before booster (15.5 months): significantly higher antibody concentration (GMT 3,009 vs 2,212, $p = 0.008$) but no difference in % protected • After booster: significantly lower antibody concentration at 16 months (GMT 2,532 vs 3,124, $p = 0.015$) and 24 months (GMT 1,773 vs 2,248, $p = 0.004$), and lower % protected at 24 months (79.6 vs 94.3%, $p = 0.002$)
Malawi (13)	BCG, OPV	Birth	10 weeks	13	21	• No difference in anti-*M. tb* and anti-polio IgG
Zambia (21)	OPV	0, 6, 10, and 14 weeks, 12 months	18 months	133	397	• Significantly lower antibody titer (difference in $\log_2$ antibody titer −0.62, 95% CI −1.04; −0.21, $p < 0.01$)
South Africa (25)	DTP-Hib or DTaP-IPV/Hib, HBV, PCV	6, 10, and 14 weeks	Birth, 16 weeks	38	55	• Pre-vaccination: significantly lower antibody concentrations to Hib, pertussis, pneumococcus, and tetanus; lower % protected against Hib (17 vs 52%, $p < 0.001$), pertussis (24 vs 57%, $p = 0.001$), tetanus (43 vs 74%, $p = 0.002$), and hepatitis B (21 vs 54%, $p = 0.01$) • Post-vaccination: following 1–2 doses, higher antibody concentration against Hib (6.46 vs 0.52 mg/L, $p = 0.02$), pertussis (81.16 vs 11.6 FDA IU/mL, $p < 0.001$), pneumococcus and tetanus (1.86 vs 0.50 IU/mL, $p = 0.01$). Following 3 doses, higher antibody concentration against pertussis (270.1 vs 91.7 FDA U/mL, $p = 0.006$) and pneumococcus (47.32 vs 14.77 mg/L, $p = 0.001$) • Greater fold increase in antibody level against Hib (21.15 vs 2.97, $p = 0.007$) and pertussis (9.51 vs 2.16, $p = 0.007$) • Infant:maternal antibody ratio (proxy for placental transfer of antibody) lower by 23% for Hib, 40% for pertussis, and 27% for tetanus in HIV-infected compared with HIV-uninfected mothers
South Africa (33)	DTP, Hib, HBV, measles	6, 10, and 14 weeks; DTP booster 18 months; measles 9, 18 months	0.5, 1.5, 3, 6, 12, 18, and 24 months	27	28	• Pre-vaccination, significantly lower antibody levels against tetanus ($p < 0.025$) and higher against hepatitis B ($p < 0.025$) • DTP, Hib, and HBV: after 2 doses, no difference in antibody levels or % protected. After 3 doses: higher antibody level and % protected against pertussis. At 24 months higher antibody level against tetanus • *Measles*: no differences between groups
Denmark (34)	Hib	3, 5, and 12 months	15 months	19	7	• No difference in antibody concentration

(*Continued*)

TABLE 2 | Continued

	Vaccine	Age at vaccination	Age at blood sampling	HIV-exposed uninfected infants, *n*	HIV-unexposed infants, *n*	Findings in HIV-exposed uninfected compared with HIV-unexposed infants
Brazil (35)	HBV, DTP/Hib	HBV: 0, 1, and 6 months DTP/Hib: 2, 4, and 6 months	7 months	53	112	• HBV: more non-responders (6.7 vs 3.6%, χ^2 10.93, df = 1) and more very good responders (64.4 vs 38.8%, non-significant) among HIV-exposed infants • Tetanus: significantly lower antibody titer against tetanus (GMT 1.520 vs 2.712, $p = 0.013$), but 100% of infants were protected • Diphtheria: no significant differences between groups

M. tb, Mycobacterium tuberculosis; OPV, oral polio vaccine; PCV, pneumococcal conjugate vaccine; DTwP-Hib, diphtheria, tetanus, whole cell pertussis, Hib; HBV, hepatitis B vaccine; DTaP-IPV/Hib, diphtheria, tetanus toxoid, and acellular pertussis combined with inactivated polio vaccine and Hib; HBsAg, hepatitis B surface antigen; % protected, proportion protected; GMC, geometric mean concentration; GMT, geometric mean titer; HIV, human immunodeficiency virus; Ig, immunoglobulin; CI, confidence interval.

infants consistently had lower antibody concentrations against PCV (seven serotypes), pertussis, Hib, and tetanus in a number of studies (8, 9, 29, 37). For each of these specific antibodies, there was significantly reduced placental transfer, with reductions of 15–40% in the ratio of maternal antibody to infant antibody concentrations at birth (29). Individual infants with lower antibody levels at birth had larger antibody responses at 16 weeks, and HIV-exposed, uninfected infants had a significantly larger fold increase than unexposed infants following vaccination against PCV, pertussis, and Hib (29).

Two South African studies have compared measles vaccine responses in HIV-exposed, uninfected, and unexposed infants. One study found antibody concentrations were 36% higher at 16 months, but after the booster dose, both the antibody titer and proportion of infants protected were lower at 24 months (79.6 vs 94.3% protected, $p = 0.002$) (10). The other study found the opposite; however, in this study antibody responses were lower in all groups, especially HIV-unexposed infants, for whom only 50% had antibody concentrations associated with protection at 2 years (37). Pre-vaccination levels of measles antibody did not differ significantly in HIV-exposed and unexposed infants in either study (10, 37).

Antibody responses to hepatitis B vaccine in HIV-exposed, uninfected infants were heterogeneous, with higher proportions of both non-responders (6.7 vs 3.6%, χ^2 10.93, df = 1) and very good responders (64.4 vs 38.4%, non-significant) at 7 months, compared with HIV-unexposed infants in Brazil (39). At time points between 3 and 24 months, no significant differences were found in the overall proportion of infants with protective antibody levels in HIV-exposed, uninfected, and unexposed groups in Brazil and South Africa (9, 29, 37, 39). Before vaccination, the proportion of infants with protective antibody levels was higher in HIV-exposed than unexposed infants in two studies from South Africa and lower in one study in the same country (9, 29, 37).

In two studies of responses to diphtheria vaccine, antibody responses in HIV-exposed, uninfected, and HIV-unexposed infants did not differ, with more than 98% protected following the primary course (9, 39). Pre-vaccination anti-diphtheria toxin antibody levels were significantly higher in HIV-exposed, uninfected infants (GMC 0.136 vs 0.078, $p < 0.001$) (9). There were no differences found in IgG concentrations against OPV at 10 weeks (13). The response to OPV at 18 months was lower in HIV-exposed uninfected infants than unexposed infants, but this was no longer significant after adjusting for breastfeeding duration (26).

In summary, typically vaccines for which the antibody concentration is lower before vaccination result in higher concentrations after vaccination. This is true for only the first one to two doses of Hib and tetanus vaccines, but persists to the end of the course of PCV and pertussis. For all four vaccines there is reduced maternal trans-placental transfer of antibody (29). There are less clear trends for measles and hepatitis B vaccines, which may be more dependent on population transmission and prevalence. HIV exposure did not appear to have any effect on antibody responses to diphtheria or OPV. Overall, HIV-exposed, uninfected infants respond at least as well to vaccines as their unexposed peers.

Cellular Responses

Human immunodeficiency virus-exposed, uninfected infants produce strong T-cell responses to BCG vaccine. In South Africa, BCG-specific CD4 and CD8 T-cell proliferation increased significantly after vaccination in HIV-exposed, uninfected, and unexposed infants at 14 weeks (40). In another study, all 94 HIV-exposed, uninfected infants formed a scar (41). T-cell proliferation and cytokine secretion were not affected by maternal HIV infection or *Mycobacterium tuberculosis* (*M. tb*) sensitization at time points between 6 weeks and 12 months (13, 27, 40, 42, 43).

Differences in the frequencies of specific T-cell subpopulations have been found between HIV-exposed, uninfected, and unexposed infants before and after BCG vaccination (40, 42). In HIV-exposed and uninfected infants, the CD4 and CD8 T-cell response at 14 weeks was less polyfunctional, indicating a less effective response (42). However, this may simply reflect immaturity, as infants were vaccinated within 3 days after birth, and another study in which the infants were vaccinated at 6 weeks found very little difference in T-cell subpopulations at 16 weeks, compared with HIV-unexposed infants (40).

At birth, no differences in BCG-specific T-cell proliferation or functionality are seen between HIV-exposed, uninfected, and unexposed infants (40). However, there are differences in the frequencies of some T-cell subsets, some of which correlate between mother-infant pairs, with the strongest associations between HIV-infected, *M. tb* sensitized mothers, and their infants (40). Secretion of TNF-α and IFN-γ in response to BCG antigens was increased at birth in HIV-exposed uninfected infants compared

with HIV-unexposed infants, but only when their mothers had evidence of latent tuberculosis infection (40). These findings support the idea that HIV-exposed uninfected infants are able to mount just as robust a response to BCG vaccine as unexposed infants, but that the immune system may be primed by antenatal exposure to maternal HIV and tuberculosis infection (40).

Two studies have investigated the cellular response to other vaccine antigens in HIV-exposed, uninfected infants compared with unexposed infants. In response to pertussis vaccine, one study found no significant differences in T-cell proliferation at 14 weeks in HIV-exposed, uninfected, and unexposed infants, but HIV-exposed infants showed reduced polyfunctionality in CD4 and CD8 responses (42). Similarly, tetanus vaccine-specific T-cell responses showed no differences at 3 months, but at 12 months HIV-exposed uninfected infants had reduced polyfunctionality, and a lower proportion of effector memory T-cells compared with HIV-uninfected infants (43).

A similar pattern was seen in response to stimulation with staphylococcal enterotoxin B (SEB) in one study, even after adjusting for differences in birthweight, breastfeeding, and gestational age (42). However, another study found that cytokine production and polyfunctionality were increased overall at 3 months but reduced at 12 months (43).

Mechanisms of Altered Vaccine Responses in HIV-Exposed, Uninfected Infants

Human immunodeficiency virus-exposed, uninfected infants are exposed to antenatal factors that might affect both their antibody and T-cell responses to vaccines. There is compelling evidence that in mothers with HIV infection, less IgG is transferred across the placenta than in HIV-uninfected mothers, resulting in lower pre-vaccination levels of IgG specific to several vaccines (8–10, 29, 37). Results from analysis adjusting for maternal age, gravidity, and socioeconomic status show that maternal HIV infection is associated with the concentration of specific IgG following Hib, pertussis, PCV, and tetanus vaccines in exposed, uninfected infants (26, 29). In this study, mothers received ART during and after pregnancy, infants received zidovudine for the first month after birth, and no HIV-exposed, uninfected infants were exclusively breastfed. This finding is likely to be a result of lower vaccine-specific antibody levels in HIV-infected mothers, which correlate with CD4 count (29), and placental dysfunction resulting in reduced placental transfer of antibody (8, 9, 29, 37). Maternally derived antibody present in infants pre-vaccination may inhibit the infants' own IgG responses, leading to the observation that infants with the highest pre-vaccine levels of antibody had the lowest fold increase following vaccination (29). Although the mechanisms for this are incompletely understood in humans, animal models have shown that this inhibition is mediated by maternally derived antibody binding to vaccine antigens, which then form cross-linkage between the B cell receptor (which binds vaccine antigen) and the FcγIIB receptor (which recognizes the Fc portion of IgG). This results in inhibitory signals, reduced proliferation of B cells and decreased secretion of vaccine-specific IgG (44, 45).

Infants of mothers with HIV infection may be exposed antenatally to HIV proteins and/or maternal immune factors that have a wider effect on the development of the immune system *in utero* and early infancy. HIV-exposed, uninfected infants may have a smaller thymus, which has been associated with immune abnormalities in early infancy (38). There is some evidence that T-cells in HIV-exposed, uninfected infants show changes in proliferation and phenotype compared with HIV-unexposed infants (13). CD4 count may be significantly lower and represent a smaller proportion of total lymphocytes, and some studies have found an association between infant and maternal CD4 count (13, 46). The reduction in T-lymphocytes occurs mainly in less differentiated subsets, and cells expressing markers of replicative senescence (CD57 and PD-1) are more frequent (13). At birth and 6 weeks, the background concentration of IFNγ was reported to be significantly higher in HIV-exposed, uninfected infants than unexposed infants in one study in South Africa (41). These early changes could represent priming of some aspects of the immune response *in utero*, leading the alterations in proliferation and function of T-cell subsets in response to vaccinations in HIV-exposed infants (13, 39–41).

Another antenatal factor that may affect HIV-exposed, uninfected infants is exposure to ART. Nevirapine has been associated with slightly increased markers of immune activation in cord blood (47), and maternal ART was associated with reduced neutrophil and lymphocyte counts in HIV-exposed, uninfected infants, with the largest difference in infants of mothers on combination therapy (46). However, an association between maternal ART and infant vaccine-specific T-cell responses has not so far been demonstrated (42, 43).

Increasing maternal age is associated with higher infant levels of pertussis antibody at birth (29). This might be influenced by differing maternal exposure to circulating pertussis or to different vaccine coverage with pertussis vaccines at different times. Other maternal infections during pregnancy are likely to be important in determining infant antibody concentrations pre-vaccination and therefore potentially post-vaccination too, for example, high variability in infant hepatitis B antibody response is likely to be a result of higher prevalence of hepatitis B infection in HIV-infected mothers in some settings (37).

Postnatally both maternal and environmental factors probably have important effects on infant vaccine responses. Breastfeeding is an important conduit for transfer of IgA from mother to infant and is associated with larger thymic size, phenotypic changes to lymphocyte subpopulations and improved immune function (48). In studies of HIV-exposed, uninfected, and unexposed infants, there are often large differences in breastfeeding practices between groups (26, 29, 37), and many studies do not report data on breastfeeding (7–10, 13, 38–40, 43). One study reported that the reduction in neutralizing antibody response to OPV in HIV-exposed, uninfected infants could be accounted for by reduced breastfeeding duration (26). This could be because of reduced antibody transfer, or increased exposure to maternal infections such as CMV which are transmitted in breast milk (26).

Postnatal exposure to other infections may also affect specific antibody responses to vaccines. The large differences between studies in the proportion of infants protected against measles

following vaccination raises the possibility that differences in transmission rates of measles infection may have affected the proportions of infants protected (49). Differences in nasopharyngeal colonization with pneumococcus among HIV-exposed, uninfected infants, and unexposed infants has also been suggested to contribute to differences in their vaccine responses (7). In low-resource settings HIV-exposed, uninfected infants have poorer nutritional status than unexposed infants (50), although a trial of nutritional supplementation between age 6 and 18 months had no effect on antibody responses to OPV in HIV-exposed, uninfected infants (26).

We conclude that there is convincing evidence that reduced antibody transfer across the placenta is associated with changes in antibody responses to the initial doses of PCV, tetanus, pertussis, and Hib vaccines in HIV-exposed, uninfected infants. The effect of HIV exposure on responses to hepatitis B and measles vaccines appears more variable between populations, and prevalence of these infections may be an important factor. The functional quality of vaccine-specific antibody produced by HIV-exposed, uninfected infants requires further investigation (29, 37), but overall it is encouraging that HIV-exposed, uninfected infants do not appear to have significantly reduced levels of protection from routine infant vaccines. Breastfeeding is likely to affect responses to other vaccines besides OPV, and further studies are now more feasible following changes in WHO recommendations to support breastfeeding in HIV-infected mothers in a wider range of settings (51).

VACCINE RESPONSES IN INFANTS WITH CONGENITAL AND POSTNATAL CMV INFECTION

Effect of CMV Infection on T-Cell Populations

Congenital and postnatal CMV infection leads to a series of changes in infant CMV-specific CD4 and CD8 T-cells, as well as having an effect on the whole T-cell population. There is an initial increase in activation of the whole CD8 T-cell population, which returns to normal over 12–24 months (52–55). CMV-specific CD8 T-cells remain highly activated for at least 24 months following postnatal infection, but in congenital infection, activation may diminish more rapidly (53, 54). There is a shift toward more differentiated CD4 T-cells, but CMV-specific CD4 T-cells are infrequently found in infected infants (55, 56). In adults with CMV, these cells are common and are associated with effective control of viral replication, less severe disease, and lower risk of mother-to-child transmission (57, 58). Infant T-cells are less polyfunctional than those seen in adults, and polyfunctionality is also thought to be associated with improved control of CMV infection (55, 58–60). Therefore, congenital and postnatal CMV infection affects the whole T-cell population, and the effect is different in infants compared with adults. Infants have a longer duration of viremia than adults (55, 61), and their vaccine responses may be affected differently by CMV infection.

Effect of CMV Infection on Humoral and Cellular Vaccine Responses

Studies in elderly adults have shown that latent CMV infection leads to the expansion of $CD8^+CD28^-$ T-cells, which are thought to suppress immune responses to influenza vaccine and contribute to generalized immunosenescence in older adults (14). Few studies have evaluated the effects of CMV infection on infant vaccine responses (**Table 3**).

In a study of measles vaccine in Gambian infants, 1 week after vaccination infant CD8 T-cell responses did not vary with CMV infection acquired congenitally or postnatally, but CD4 T-cell IFN-γ responses were lower in CMV-infected infants than in infants without CMV infection (54). At 13 months of age, there were no differences in memory T-cell responses between CMV-infected and uninfected infants. However, CMV-infected infants showed significantly higher CD4 and CD8 T-cell IFN-γ responses to SEB, indicating that immune activation is present in CMV-infected infants. Furthermore, there was a positive correlation between the magnitudes of the responses to SEB and CMV (54).

A study of CMV and EBV co-infection supports the idea that the effect of CMV infection on antibody responses to measles is dependent on changes to the T-cell population. Epstein–Barr virus (EBV) infects B-cells, and EBV-infected infant IgG responses to measles vaccine and meningococcus A and C polysaccharide vaccine are reduced by approximately one third (62). In infants co-infected with CMV and EBV, the measles-specific IgG vaccine response is similar to uninfected infants. However, CMV co-infection does not have a significant effect on the IgG response to meningococcus (a T-cell independent response) in EBV-infected infants, and the vaccine response is still lower than in EBV uninfected infants (62).

Gambian infants who acquired CMV antenatal or postnatally had no significant differences in anti-Hib or anti-tetanus toxoid IgG concentration measured compared with uninfected infants at 18 months (54). A study of antibody response to OPV in Zambian infants found that neither CMV seropositivity nor viremia had a significant effect on OPV neutralizing antibody titers or frequency of vaccine failure at 18 months of age (26). However, trends in the data suggested that co-infection with HIV and CMV may have negative synergistic effects on the antibody response to OPV. CMV seropositivity at 18 months was associated with a trend toward a small decrease in vaccine failures in HIV-unexposed infants (0.4 vs 4.3% vaccine failure, $p = 0.06$), but not in HIV-exposed, uninfected infants. In HIV-infected infants antibody responses were reduced in CMV seropositive compared with CMV seronegative infants, although these results did not reach statistical significance (26).

Human immunodeficiency virus-infected mothers in this study had a mean breastfeeding duration of 6 months compared with 15 months in HIV-uninfected mothers, and longer breastfeeding duration was associated with increased mean poliovirus antibody titers in infants (26). The authors do not state whether mean breastfeeding duration differed between CMV seropositive and seronegative groups, but this is important because breastfeeding is the main route of transmission of postnatal CMV infection, and in HIV-exposed, uninfected infants in this study,

TABLE 3 | Effect of CMV infection on infant responses to vaccines.

	Vaccine	Age at vaccination	Age at blood sampling	CMV-infected infants, *n*	CMV-uninfected infants, *n*	Findings
Gambia (51)	Measles; tetanus, Hib	9 months; 2, 3, 4, and 16 months	9 months 13 months 18 months	86 90 121	46 42 11	CMV-infected vs uninfected infants (congenitally and postnatally infected infants in same cohort): • Infected infants had lower CD4 IFN-γ response to measles ($p = 0.013$) • No significant difference in CD8 T cell proliferation or IFN-γ response to measles • No difference in measles antibody titers • Infected infants' IFN-γ response to CMV correlated with measles antibody response at 13 months • No significant difference in IgG response to Hib or tetanus vaccines at 18 months
Gambia (59)	Measles, meningococcus A and C	9 months	Birth 9 months 11 months	0 115 121	224 58 51	Comparison of CMV and EBV singly infected, co-infected, and uninfected infants • CMV status had no significant effect on measles antibody titer • Infection with EBV reduced measles antibody response, except when there was co-infection with CMV (median $\log_2$ hemagglutinin antibody inhibition assay titer $EBV^+CMV^- = 3.0$, $EBV^+CMV^+ = 5.0$, $p = 0.003$) • CMV status had no significant effect on anti-meningococcus IgM or IgG
Zambia (21)	Oral polio	Birth, 6, 10, and 14 weeks, 12 months	18 months	369	75	• No significant associations between IgG response to OPV and CMV infection. • In CMV seropositive infants, % vaccine failure was slightly lower than in seronegative infants (1.4 vs 4.0%, $p = 0.14$) • In HIV^+ infants, Ab titers were lower in infants with CMV viremia than without ($\log_2$ antibody titer 3.2 vs 5.75, $p = 0.14$) • In HIV-unexposed infants, Ab titers were higher in CMV seropositive infants ($\log_2$ antibody titer 8.31 vs 7.77, $p = 0.11$) • In HIV-exposed uninfected infants, CMV had no significant effect

EBV, Epstein–Barr virus; Ig, immunoglobulin; HIV, human immunodeficiency virus; CMV, cytomegalovirus; OPV, oral polio vaccine; IFN, interferon.

the reduction in neutralizing antibody response to OPV could be accounted for by reduced breastfeeding duration (26).

Cytomegalovirus-infected infants show alterations in responses to measles and possibly polio vaccines, which are live vaccines, and no significant differences have been found in the responses to Hib or tetanus vaccines. Overall the ability to mount effective and lasting responses is preserved in otherwise well infants, at least in the short term, and responses to SEB indicate that some T-cell responses are increased in CMV infection. Interactions may occur between CMV and HIV or EBV to produce further alterations in vaccine responses, but there is still no significant impairment compared with CMV-uninfected infants. There is limited data on the effects of CMV infection on infant vaccine responses, and in light of growing evidence of poor clinical outcomes associated with CMV infection in HIV-exposed, uninfected infants, further studies are particularly important in this group.

CONCLUSION

Human immunodeficiency virus-infected infants have some impairment in their humoral and cellular responses to routine immunizations. However, as many of the infants in the studies reviewed were born to mothers who started ART a short time before delivery as part of PMTCT programs, and were not exclusively breastfed, future studies will be needed to determine whether the same changes in immune responses are present when mothers and infants undertake optimal HIV treatment, PMTCT, and feeding practices. The clinical importance of these findings is unknown, as the risk of vaccine-preventable infection in HIV-infected infants compared with HIV-unexposed infants has not been determined.

Human immunodeficiency virus-exposed, uninfected infants and those with CMV have alterations in their vaccine responses, but the evidence does not support changes to the vaccine schedule in these groups. Protecting infants from infection before their first vaccines, for example, by maternal immunization, is important in all infants, even more so in HIV-exposed, uninfected, and HIV-infected infants, who are less likely to be protected than HIV-unexposed infants. Maternal immunization is a key part of global efforts to reduce neonatal and infant infectious morbidity and mortality. A better understanding of the mechanisms by which maternal infection and immune responses influence the developing infant immune system are critical to ensuring the success of new vaccines.

AUTHOR CONTRIBUTIONS

The theme and concept were designed by M-LN and CJ. OF undertook the literature review and wrote the original draft of the paper, which was reviewed and revised by CJ and M-LN.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fimmu.2018.00328/full#supplementary-material.

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REVIEW
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Mother's Milk: A Purposeful Contribution to the Development of the Infant Microbiota and Immunity

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Breast milk is the perfect nutrition for infants, a result of millions of years of evolution. In addition to providing a source of nutrition, breast milk contains a diverse array of microbiota and myriad biologically active components that are thought to guide the infant's developing mucosal immune system. It is believed that bacteria from the mother's intestine may translocate to breast milk and dynamically transfer to the infant. Such interplay between mother and her infant is a key to establishing a healthy infant intestinal microbiome. These intestinal bacteria protect against many respiratory and diarrheal illnesses, but are subject to environmental stresses such as antibiotic use. Orchestrating the development of the microbiota are the human milk oligosaccharides (HMOs), the synthesis of which are partially determined by the maternal genotype. HMOs are thought to play a role in preventing pathogenic bacterial adhesion though multiple mechanisms, while also providing nutrition for the microbiome. Extracellular vesicles (EVs), including exosomes, carry a diverse cargo, including mRNA, miRNA, and cytosolic and membrane-bound proteins, and are readily detectable in human breast milk. Strongly implicated in cell–cell signaling, EVs could therefore may play a further role in the development of the infant microbiome. This review considers the emerging role of breast milk microbiota, bioactive HMOs, and EVs in the establishment of the neonatal microbiome and the consequent potential for modulation of neonatal immune system development.

Keywords: breast milk, microbiota, microbiome, human milk oligosaccharides, exosomes, extracellular vesicles, infant microbiome, breast milk microbiome

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INTRODUCTION

Breastfeeding confers protection against respiratory and gastrointestinal infections and is associated with a reduced risk of inflammatory diseases such as asthma, atopy, diabetes, obesity, and inflammatory bowel disease (1–7). Prolonged and exclusively breastfed infants have improved cognitive development (8, 9). Human milk continues the transfer of immunity from mother to child that started *in utero*, providing a nurturing environment that protects against infection and develops the infant intestinal mucosa, microbiota, and their own immunologic defenses. Breast milk is a specialized secretion in which immune response is highly targeted against microorganisms in the mother's gut and airway, providing an important defense against the same pathogens likely encountered by her infant (10). More recent studies suggest that breast milk not only provides passive protection

but also directly modulates the immunological development of the breastfed infant through a variety of personalized microbial and immune factors transmitted from mother to child (11–14). These early imprinting events are crucial for immunologic and metabolic homeostasis.

Breast milk immune factors are at their highest concentrations in the colostrum (15), suggesting an immunologic function of milk when the infant is at highest risk of exposure to new pathogens. However, they continue to be dynamically present throughout the lactation period. Bioactive factors transferred to the infant *via* breastfeeding including immunoglobulins, cytokines, chemokines, growth factors, hormones, and lactoferrin have been reviewed in detail elsewhere (15–17). This review will focus on the roles of breast milk microbiota in the establishment of the infant intestinal microbiota, human milk oligosaccharides (HMOs) in shaping the microbiota, and extracellular vesicles (EVs) in modulation of the host–microbe interactions. Breast milk microbiota, HMOs, and EVs are emerging as areas of potential therapeutic interests due to their implications for infant immune development, health, and scope for therapeutic manipulation.

BREAST MILK MICROBIOTA

Breast milk comprises several hundred bacterial species and harbors bacteria at concentrations of approximately 1,000 colony-forming units (CFUs)/mL (18, 19). It is estimated that breastfed infants ingest up to 800,000 bacteria daily (20). Following a dose of microbes at birth (21), breast milk is the immediate next fundamental source of microbes seeding the infant's gut (22, 23). Many epidemiologic studies have documented differences in the composition of gut microbiota in breastfed and formula-fed infants (24–26). Human milk directly contributes to the establishment of the infant intestinal microbiome (19, 20, 23, 27–29). Multiple studies have documented the sharing of specific microbial strains of *Bifidobacterium*, *Lactobacillus*, *Enterococcus*, and *Staphylococcus* species between breast milk and infant stool (30–32). During the first month of life, infants who primarily breastfeed share 28% of their stool microbes with their mother's milk microbes. The frequency of shared microbes increases with the proportion of daily breast milk intake in a dose-dependent manner (23). These findings strongly suggest the transfer of microbes from breast milk to the infant gut. Although an interindividual variation in the types and abundance of different bacteria in human milk exists, the bacteria found in the infant gut most resemble the bacteria from their own mother (23).

While early studies employed culture-dependent methods, recent development of culture-independent techniques, such as next-generation sequencing, has expanded our understanding of the composition and diversity of the breast milk microbiome (33–35). *Streptococcus* and *Staphylococcus* species are the most commonly identified bacterial families in human milk, followed by *Bifidobacterium*, *Lactobacillus*, *Propionibacteria*, *Enterococcus*, and members of the *Enterobacteriaceae* family (23, 28, 35, 36). Several hundred bacterial species have been identified with higher diversity in colostrum compared to transition and mature milk (18).

The origin of bacteria in breast milk is not well established. Breast tissue itself contains a diverse population of bacteria (37). A dynamic cycling of bacteria between mother and infant with retrograde flow from maternal commensal skin flora to infant mouth flora during breastfeeding (38) likely contributes to the bacterial communities (39). However, commensal contamination does not fully account for the diversity of human milk microbes or the presence of strictly anaerobic species such as *Bifidobacterium*, *Clostridium*, or *Bacteroides* species. Milk microbial community composition has been shown to differ from communities on the surrounding areolar skin and infant mouth (35, 40). Another proposed theory is an enteromammary pathway whereby maternal intestinal bacteria migrate to the mammary glands *via* an endogenous cellular route during pregnancy and lactation (19, 28, 41). It has been hypothesized that bacteria first translocate the maternal gut by internalization in dendritic cells and then circulate to the mammary gland *via* the lymphatic and blood circulation (42). This specialized form of mother–infant communication of transferring microbes from the mother's gut to the infant *via* breastfeeding needs further investigation.

Maternal factors affect milk microbiota composition and diversity (**Figure 1**). Higher diversity has been reported in milk from mothers who deliver vaginally compared with C-section by some groups (18, 43, 44) but not others (23, 45). Milk bacterial profiles do not significantly differ in relation to maternal age, infant gender, or race/ethnicity within a geographical region but do differ across geographic locations of Europe, Africa, and Asia (23, 45, 46). *Bifidobacterium* species concentration was higher in term deliveries than preterm deliveries (44). Total bacteria concentration using quantitative PCR is lower in colostrum than in transitional and mature milk, with increasing levels of *Bifidobacterium* and *Enterococcus* species over time (18, 44). Maternal health alters milk microbiota composition and diversity as evidenced by comparative studies of healthy mothers to those with obesity, celiac disease, and human immunodeficiency virus (HIV) (18, 47, 48). Immunomodulatory cytokines secreted in breast milk from healthy women such as transforming growth factor beta (TGFβ) 1 and TGFβ2 are associated with increased early-life microbial richness, evenness, diversity, and increased abundance of taxa protective against atopic diseases (49). Unsurprisingly, maternal antibiotic use and chemotherapy decrease bacterial diversity in breast milk (50, 51); how this impacts on the infant microbiome and immune system development in the long term is currently unknown. More studies are warranted to understand how maternal genetics, culture, environment, nutritional status, and inflammatory states from acute or chronic diseases affect breast milk microbiota.

Role of Breast Milk Microbiota in the Infant Gut

Breast milk bacteria have both immediate- and long-term roles in reducing the incidence and severity of bacterial infections in breastfed infants by multiple mechanisms. Commensal bacteria can competitively exclude or express antimicrobial properties against pathogenic bacteria. For example, *Lactobacilli* isolated from breast milk have been shown to inhibit adhesion and growth

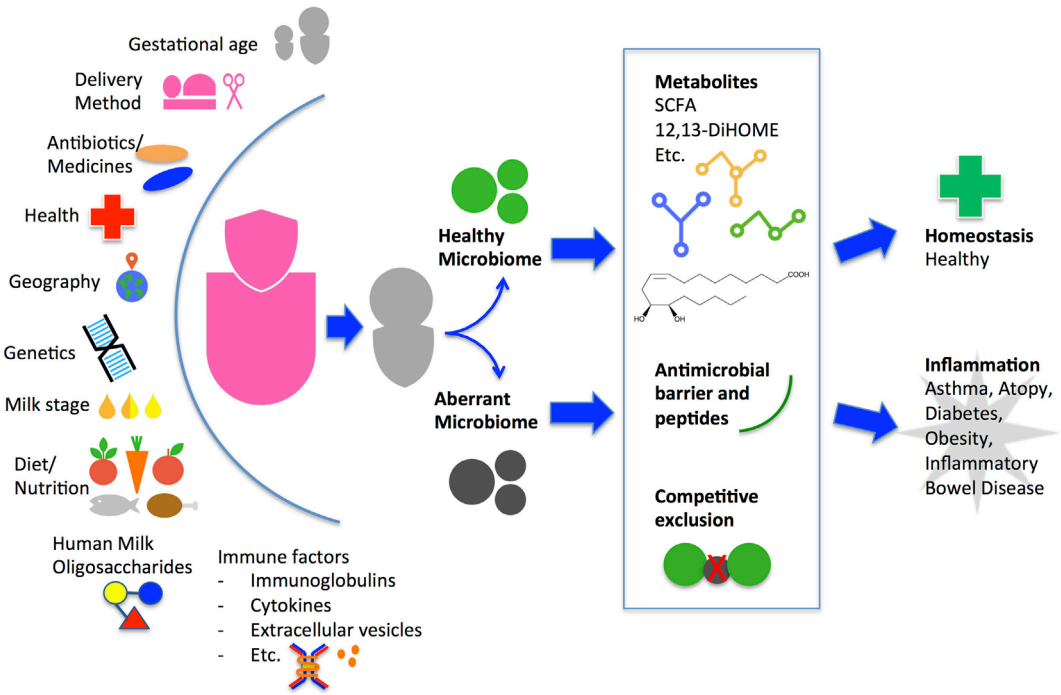

FIGURE 1 | Factors that influence maternal breast milk microbiome and proposed mechanism of how breast milk may alter the infant gut microbiome and health outcome. A myriad of environmental, genetic, and immune factors personalize a mother's milk for delivery to her infant. Starting from the initial feeding, the breast milk microbes and human milk oligosaccharides contribute to the composition and diversity of the infant gut microbiome. The initial gut microbes may continue to promote colonization of a healthy community or an aberrant community. During the critical window of immune development, the community types may induce metabolic alterations leading to differing immune phenotypes and long-term health outcomes. SCFA, short-chain fatty acids.

of gastrointestinal pathogens, including *Escherichia coli*, *Shigella* spp, *Pseudomonas* spp, and *Salmonella* spp strains (52–54). Five breast milk *Lactobacilli* strains increased mucin gene expression by intestinal enterocytes to form an antibacterial barrier (53). Administration of a breast milk *Lactobacilli* strain in a double-blind controlled trial to infants 6–12 months of age reduced the incidence of gastrointestinal, respiratory, and total infections by 46, 27, and 30%, respectively (55). The significant increase in bacterial counts of *Lactobacilli* and bifidobacteria in the experimental group compared with the controls was thought to explain the reduced clinical infection episodes although the pathogenic bacteria counts were not measured. Another study found that 30% of human milk contains nisin-producing bacteria that can survive passage through the intestine (56). Nisin is a bacteriocin used by the dairy industry to prevent spore germination and inhibit *Clostridium botulinum* and *Bacillus cereus*. *Staphylococcus epidermidis* and *Streptococcus salivarius* from expressed breast milk also possesses antimicrobial activity against pathogenic *Staphylococcus aureus* (20). Although there are many studies of antimicrobial peptides and molecules in the intestine, more studies are necessary to understand the specific antimicrobial activities of breast milk bacteria.

Increasing evidence in animals points to the instrumental role of microbiota in the development and instruction of the immune system (57, 58). In the absence of intestinal bacteria, animals have defects in lymphoid tissue development within the spleen, thymus, and lymph nodes. Germ-free intestines have reduced numbers of lamina propria CD4+ cells, IgA-producing cells, and hypoplastic Peyer's patches (59). Germ-free mice typically are Th2 skewed but achieve a balance of Th1/Th2 cytokine production after the introduction of symbiotic bacteria (60). Breast milk *Lactobacillus* strains have been shown to enhance macrophage production of Th1 cytokines including Il-2, IL-12, and TNF-alpha (61). An early human study has suggested better Th1 responses in breastfed children compared to formula-fed children with immunomodulating effects lasting beyond weaning (62). Another *in vitro* study showed that *Lactobacillus fermentum* and *Lactobacillus salivarius* were potent activators of natural killer cells affecting innate immunity as well as moderate activators of CD4+ and CD8+ T cells and regulator T cells affecting acquired immunity (63). Breastfed rhesus macaque infants develop distinct gut microbiota and robust populations of memory T cells and T helper 17 cells compared to bottle-fed infants (64). Whether these mechanisms also exists in humans is not yet known.

Critical Window of Opportunity for Immune Effects

The World Health Organization recommends exclusive breastfeeding during the first 6 months of life (65). This time period of exclusive milk ingestion is also a critical window for microbial imprinting (23, 66, 67). The infant microbiome comprises a dynamic community of bacteria that transforms throughout infancy and into early childhood, but the community assembly is

non-random and depends on early-life events (57, 66). Dysbiosis during this critical developmental window during a time of exclusive milk ingestion may have long-term health implications (57, 68). Germ-free mice have an overaccumulation of invariant natural killer (iNKT) cells leading to susceptibility to colitis, but colonization with standard microbiota before 2 weeks of life but not after, normalizes iNKT cell numbers and protected against colitis (69). Similarly, germ-free adult mice have elevated serum IgE levels associated with exaggerated allergic responses, but mice colonized with standard microbiota before 4 weeks of age, but not after, have normal IgE levels (70). Oral administration of *Bifidobacterium breve* in mice induces proliferation of FoxP3+ regulatory T cells, but only if administered during the pre-weaning stage (54). Even transient perturbations in the microbiota in early life with penicillin is sufficient to induce sustained metabolic alterations and changes in the expression of immune genes in mice (68). Longitudinal human cohorts have supported the long-term implications of early dysbiosis. Arrieta et al. showed transient gut dysbiosis during the first 100 days of life put infants at higher risk for asthma (71). The relative abundance of *Lachnospira*, *Veillonella*, *Faecalibacterium*, and *Rothia* was significantly lower in children at risk of asthma. These genera are present in breast milk (23, 36). Fujimura et al. found a microbiota conformation that was significantly associated with a higher risk of atopy; the conformation was only detectable in children younger than 6 months. By using fecal water from these infants cultured *ex vivo* with human adult peripheral T cells, the investigators showed enhanced induction of IL4+ CD4+ T cells and decreased abundance of CD4+ CD25+ FOXP3+ cells, suggesting that dysbiosis promotes CD4+ T cell dysfunction associated with atopy (72). The progressive establishment of the infant microbiota is vital for educating their immune system to tolerance and reactivity to maintain health throughout life. A recent study by Bäckhed et al. suggests that cessation of breastfeeding rather than introduction of solid foods is the major driver in the development of an adult microbiota (73). Indeed, Ding and Schloss found that history of breastfeeding as an infant dictated bacterial community composition as adults (74).

Breast Milk Virome

Viruses are also known to be transmitted through breast milk (75) and likely contribute to the gut ecology of the developing infant. The assembly of phage and eukaryotic components of the infant gut virome is affected by health and nutritional status (76). Breitbart et al. did not find similar viral sequences in maternal breast milk and the infant stool in their one infant followed over time (77). However, a recent study of 25 mother–infant pairs identified bifidobacterial communities and bifidophages in maternal milk and infant stool, strongly suggesting vertical transmission through breastfeeding (78). Because the majority of viruses inhabiting the infant and adult gut are bacteriophages (77, 79), they have the ability to kill bacteria or provide them with potentially beneficial gene functions to shape the bacterial community and long-term health. Longitudinal studies to determine the role of breastfeeding in the establishment of the infant gut virome and the viral–bacterial interactions are warranted.

HUMAN MILK OLIGOSACCHARIDES

Human milk oligosaccharides (HMO) may further influence the establishment of a healthy microbiome, by binding potentially harmful bacteria in the intestinal lumen, asserting direct antimicrobial effects, modulating the intestinal epithelial cell immune response, and thereby promoting the growth of "good bacteria" (**Figure 2**). HMOs are soluble complex carbohydrates that are synthesized in the mammary glands dependent on maternal genotype, including the genes that determine the Lewis blood group antigen.

HMO are indigestible by the infant. Instead, they function as prebiotics, encouraging the growth of certain strains of beneficial bacteria, such as *Bifidobacterium infantis*, within the infant gastrointestinal tract (80), thus preventing infection by allowing the microbiota to outcompete potential pathogenic organisms (81, 82). Once ingested by the infant, HMOs are thought to inhibit the adherence of pathogens to the intestinal epithelium by acting as a decoy receptor for pathogens, which prevents attachment to host cells, thereby preventing pathogen adhesion and invasion (83). HMOs are also thought to have direct antimicrobial effects on certain pathogens (81). Finally, HMOs have been observed to modulate intestinal epithelial cell responses, as well as act as immune modulators. HMOs alter the environment of the intestine, by reducing cell growth, and inducing differentiation and apoptosis (84). They alter immune responses by shifting T cell responses to a balanced Th1/Th2 cytokine production (85).

Genetic differences are responsible for differences in HMO profiles in breast milk (86–89), although HMO abundance changes throughout lactation. Therefore, mothers possessing different genotypes, and thus different HMO profiles, may protect their infants against certain infections to a greater or lesser extent, depending on the presence of specific HMOs. Likewise, the different HMOs produced alter the types of microbiota colonizing infants, as well as the timing of the establishment of the microbiota (90). Because of their complexity, no human milk identical HMOs have been synthesized. However, non-human milk-derived alternatives that may have similar bioactive properties are gaining interest. In a recent placebo-controlled trial of 4,556 infants from India, a plant oligosaccharide, fructooligosaccharide, was given to infants together with *Lactobacillus plantarum* and demonstrated a reduced risk of sepsis and death in those in the treatment arm (RR, 0.6; CI, 0.48–0.74) compared to those in the control arm (91). The results highlight a potential role for HMOs and non-milk oligosaccharides in preventing neonatal infection.

HMO are thought to play an important role in preventing neonatal diarrheal and respiratory tract infections (92, 93). Several HMOs have been implicated in protection against bacterial and viral infections in neonates, including fucosyltransferase enzyme (FUT3), associated with the Lewis–Secretor gene (89) and 2′-fucosyllactose (2′-FL), associated with the Secretor gene (FUT2) (87). High concentrations of 2′-FL are associated with reduced risk of infant *Campylobacter jejuni* (94) and rotavirus infections (95). However, it has been noted that there is a rotavirus strain-specific effect of different HMOs, both alone and in combination (95, 96). Lewis–secretor positive infants in Burkina

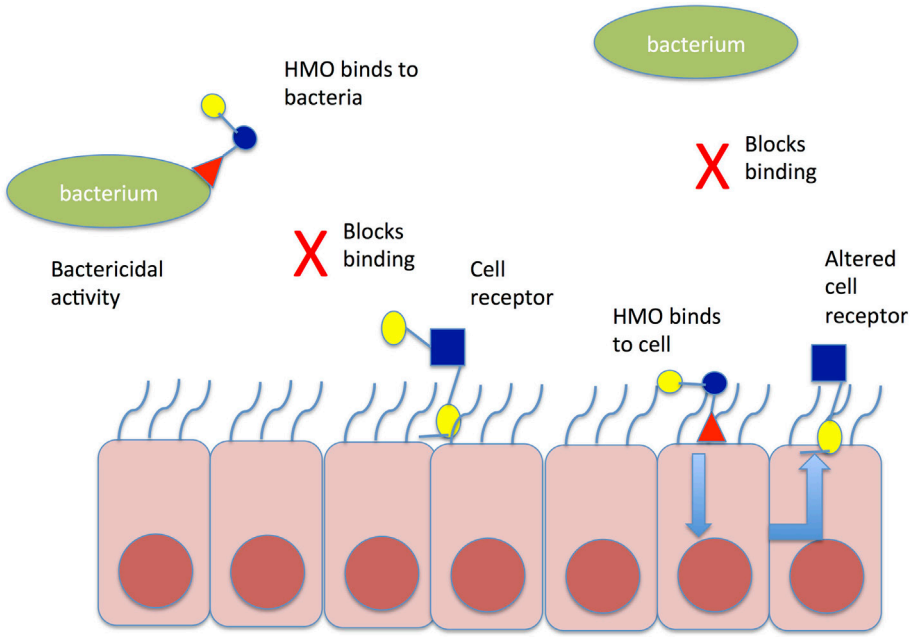

FIGURE 2 | Mechanism of action of HMO to prevent aberrant pathogen colonization. HMO may bind directly to bacteria in the gut lumen causing conformational change in bacterial binding sites and preventing binding to cell receptors; alternatively, HMO may bind directly to gut epithelial cells causing altered expression of cell receptors, which prevent pathogen binding to gut epithelial cells. HMO, human milk oligosaccharide.

Faso and Nicaragua appear to have increased susceptibility to rotavirus infection compared to Lewis-negative infants. As the Lewis antigen is partially responsible for HMO abundance, this finding may explain the reduced efficacy of the live oral rotavirus vaccine in Africa where the majority of women are Lewis–Secretor negative (96). Conversely, an observational study undertaken in the United States found severe rotavirus gastroenteritis to be essentially absent in children who had a genetic polymorphism that inactivates FUT2 expression on the intestinal epithelium, which may indicate further strain-specific adaptations of HMOs (97). Infants who received milk containing a low concentration of lacto-*N*-difucohexaose have an increased incidence of calicivirus diarrhea (98). Other HMO combinations in breast milk have also been associated with reduced risk of HIV transmission in Zambia (99).

It has been suggested that HMOs could be used therapeutically to harness these antibiotic benefits together with standard antibiotics (100, 101). Research to date has primarily focused on developing such adjuncts by investigating antiadhesive properties of HMOs *in vitro*. These include the ability of HMOs to reduce *Streptococcus pneumoniae* adherence to cells of the oropharynx (102) and gastrointestinal adherence with *Escherichia coli* (103–105). Specific HMOs such as FUT3 have been implicated in increased killing of Group B *Streptococcus* (GBS) *in vitro* (106–108). The Bode laboratories have determined that GBS requires specific HMO to proliferate *in vitro* (101), Further *in vitro* investigation revealed that GBS uses a glycosyltransferase, which incorporates HMOs into the cell membrane, preventing bacterial proliferation. The Townsend and Le Doare laboratories have also identified Lewis–Secretor status to be important in reducing biofilm associated with GBS (106, 107). Further studies have identified that HMO-2′-FL also acts as a decoy receptor for norovirus (109). Animal models also report increased Th1 responses against RSV in mice given a prebiotic containing HMOs (110). HMOs are emerging as a novel potential adjunct to antibiotic therapy, but there is much uncertainty as to individual HMO function and synthesizing individual HMOs in the laboratory for use in clinical trials has proven problematic.

EXTRACELLULAR VESICLES AND THEIR CARGO

One of the most recently identified breast milk components that may alter the intestinal immune response and subsequent establishment of the microbiota are the extracellular vesicles (EV) that contain a rich protein cargo, capable of influencing the local immune response to bacterial challenge (111, 112). Hence, the discovery 10 years ago that human breast milk contains abundant EVs has garnered a lot of attention in the field (113). EVs contain a diverse cargo, including mRNA, miRNA, and cytosolic and membrane proteins and have been demonstrated to be intricately involved in cell–cell signaling. EVs include exosomes, which form through the endosomal pathway, and are released from cells following fusion of multivesicular endosomes with the plasma membrane. The larger (0.1–2 μM), more heterogenous microvesicles are formed through direct blebbing from the cell plasma membrane. It is important to note that many breast milk studies use the term "exosomes," but do not separate exosomes from other vesicles, neither conceptually nor physically. Unless the isolation procedure takes advantage of exosomes' known size or flotation density (e.g.,

through sucrose gradients, or size exclusion chromatography) or their known markers (i.e., by immunomagnetic isolation, e.g., anti-tetraspanin beads), isolated vesicles cannot be definitively described as exosomes. Both ultracentrifugation and PEG-based reagents such as Exoquick™, commonly used in breast milk studies to date, will pellet other vesicles as well as non-vesicular proteins, including RNA-binding proteins. Studies that use these methods have still revealed exciting potential for breast milk EVs, in terms of biomarkers, or biological activity *in vivo*. The Nolte-'t Hoen group have published a useful study that compares EV isolation methods from breast milk (114).

Breast milk EVs contain RNA (115), miRNA, and long non-coding RNA (116). Several studies that profiled miRNA in breast milk exosomes found enrichment in multiple biological functions, including regulation of actin cytoskeleton, glycolysis/gluconeogenesis, aminoacyl-tRNA biosynthesis, pentose phosphate pathway, galactose metabolism, and fatty acid biosynthesis, as well as a wide range of immunological pathways (117–120). Likewise, proteomic analysis of human breast milk EVs revealed that the majority of proteins mapped to immune cell origin (121). Interestingly, a large number of these proteins had not been previously identified in human breast milk, demonstrating that exploration of EV cargoes may reveal novel biomarkers and functional pathways for further investigation. Exosomes in bovine milk are also enriched in proteins involved in immune response and growth (122).

Exosomes can mediate delivery of novel functional miRNA and mRNA to recipient cells (123). Whether miRNAs in breast milk exosomes are functional in the human digestive system is still relatively unknown; some studies show that exosomes protect miRNAs from digestion (118, 124), while others show that miRNAs are degraded by intestinal contents (125). Certainly, breast milk mRNAs and miRNAs can be taken up by cells and elicit functional effects *in vitro,* suggesting the exciting possibility that they may be able to alter protein expression at the neonatal mucosal surface, impacting on the development of the infant's immune system. These functional effects demonstrated thus far include inhibition of *in vitro* T cell cytokine production and boosting regulatory T cells (113) and inhibition of HIV-1 infection of dendritic cells (126). Liao et al. also recently demonstrated that milk-derived EVs enter human intestinal crypt-like cells, with some localization to the cell nucleus; thus, this is a potential mechanism for delivery of immunoregulatory genetic material from mother-to-infant cells (127). Administration of breast milk exosomes increases intestinal epithelial proliferation in both pigs (128) and rats (129), suggesting that they also have the potential to promote normal intestinal development and function in neonates. In addition to acting in the intestinal tract, EVs could potentially exert effects in the oropharynx and nasopharynx. Thus, breast milk EVs could alter the neonatal immune response to oral vaccines, respiratory pathogens and colonization.

Extracellular vesicles also have the potential to modulate the host–microbe interaction. Epithelial and immune cell responses to gut microbes *Lactobacillus* or *Bifidobacterium* are modulated in the presence of EVs from serum (111). These EV enhance aggregation and phagocytosis of bacteria, as well as modulating TLR responses. Whether these activities are also performed by breast milk EVs is not known. As well as human milk, EVs also have been detected in porcine (128), bovine (122), and murine (125) milk, enabling the use of animal models to explore this phenomenon, as well as raising the possibility of there being cross-kingdom cell–cell communication *via* unpasteurized milk. Studies in mice have identified that the absence of EVs decreases the diversity of the pup intestinal microbiome (130). Human studies of the role of exosomes and their cargo in modulating infant intestinal microbiome are limited. However, Kosaka et al. identified miRNA associated with immune regulation within exosomes in breast milk that are particularly abundant in the first 6 months of life, when the neonatal mucosal immune system is developing (118). Recent work investigating the role of miRNA in EVs in the ProPACT trial demonstrated an array of miRNA in human milk that differed between mothers given probiotics and those given placebo but no significant differences in atopy outcomes (131).

The few studies of exosomes in breast milk to date have often been cross-sectional (116), and there is only one study of exosomes in human colostrum (113); milk that is delivered at a key stage for early immune priming. A study of bovine exosomes shows that the immunomodulatory protein cargo changes temporally during lactation (122); thus, detailed exploration of human breast milk EV cargo across the course of lactation could yield data that are highly relevant to the development of the neonatal immune system. Isolation of exosomes from breast milk to investigate the miRNA and protein cargo that could be delivered to the infant mucosa would offer novel insight into potential delivery mechanisms for drugs with intestinal immunomodulatory factors (132). Furthermore, improved knowledge of the stability and functionality of EV cargoes *in vivo* is vital for our understanding of how breast milk improves neonatal health and immunity.

FUTURE DIRECTIONS

Breast Milk Microbiota

Many unanswered questions regarding the microbiome need further exploration. We need more studies to define the mechanism by which the microbiota impact immune development and how dysbiosis leads to gut inflammation. Greater comprehension beyond the community profile to elucidate function and metabolites produced by the microbes is integral to utilizing these pathways to improve health or alter disease outcomes. If an enteromammary pathway is confirmed, we could exert a positive influence on infant health by modulating the maternal gut microbiota. Breast milk studies to date have mainly focused on the bacterial component. We also need to further understand how the milk virome and mycobiome influence infant gut health.

Breast Milk HMOs

Further questions surround the HMOs, namely, functions of individual HMO and synthesis of HMO in the laboratory for nutrition supplementation; manipulation of HMO expression; and their delivery to establish a healthy microbiome. It is also possible that early intervention (within the first few days of life) is required for such therapies to be successful.

Breast Milk EVs

For future studies of breast milk EVs, including exosomes, it is key to ensure that the correct nomenclature is utilized, based on the isolation methods used. Utilizing the guidelines of the International Society for Extracellular Vesicles (114) and reporting isolation methods through the new EV-TRACK database (133) will greatly aid the field of breast milk EVs. Apoptotic bodies have been seen as something to deplete in breast milk studies to date. Nothing is known about their cargo nor their function in breast milk, but they could play an important biological function in the neonate, as seen in other fields. We also lack detailed understanding of how breast milk EVs change over the course of lactation in humans. We need to understand better how breast milk EVs survive *in vivo* in the oropharynx, nasopharynx, and the gut, where their delivery would be critical. Finally, a human model of EV interaction with the neonatal microbiome would also give critical insight into possible mechanisms that could be harnessed to protect infants from disease and aid intestinal immune development in term and preterm infants alike.

Summary

Future research studies should aim for enrollment of mother–infant pairs, large sample sizes, and longitudinal sample collections and include a diverse population to further elucidate variability in the breast milk microbiome, HMOs, and EVs on infant health outcomes. Studies should employ metagenomics, metatranscriptomics, and metabolomics approaches to understand the complete taxonomical, functional, and metabolic profile and create a more accurate picture of the breast milk contribution to infant health. Studies of the breast milk virome and fungome are warranted. Furthermore, ensuring that a repository of maternal and infant samples is kept for future research is useful in determining the long-term health implications of the gut microbiome present during the critical window. A repository can also present the opportunity to study the multigenerational transmission of microbes, HMOs, and EVs, facilitating a comprehensive understanding of the dynamics of the mother's contribution to the infant immune system.

AUTHOR CONTRIBUTIONS

PP conceived and designed the manuscript. KLD, BH, AB, and PP contributed to the drafting and critical revision of this manuscript. All authors approved the final copy of the manuscript.

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Adjuvant Effect of Bacille Calmette–Guérin on Hepatitis B Vaccine Immunogenicity in the Preterm and Term Newborn

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Immunization is key to protecting term and preterm infants from a heightened risk of infection. However, preterm immunity is distinct from that of the term, limiting its ability to effectively respond to vaccines routinely given at birth, such as hepatitis B vaccine (HBV). As part of the Expanded Program on Immunization, HBV is often given together with the live-attenuated vaccine Bacille Calmette–Guérin (BCG), known to activate multiple pattern-recognition receptors. Of note, some clinical studies suggest BCG can enhance efficacy of other vaccines in term newborns. However, little is known about whether BCG can shape Th-polarizing cytokine responses to HBV nor the age-dependency of such effects, including whether they may extend to the preterm. To characterize the effects of BCG on HBV immunogenicity, we studied individual and combined administration of these vaccines to cord newborn and adult human whole blood and mononuclear cells *in vitro* and to neonatal and adult mice *in vivo*. Compared to either BCG or HBV alone, (BCG + HBV) synergistically enhanced *in vitro* whole blood production of IL-1β, while (BCG + HBV) also promoted production of several cytokines/chemokines in all age groups, age-specific enhancement included IL-12p70 in the preterm and GM-CSF in the preterm and term. In human mononuclear cells, (BCG + HBV) enhanced mRNA expression of several genes including *CSF2*, which contributed to clustering of genes by vaccine treatment *via* principle component analysis. To assess the impact of BCG on HBV immunization, mice of three different age groups were immunized subcutaneously with, BCG, HBV, (BCG + HBV) into the same site; or BCG and HBV injected into separate sites. Whether injected into a separate site or at the same site, co-administration of BCG with HBV significantly enhanced anti-HBV IgG titers in mice immunized on day of life-0 or -7, respectively, but not in adult mice. In summary, our data demonstrate that innate and adaptive vaccine responses of preterm and term newborns are immunologically

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distinct. Furthermore, BCG or "BCG-like" adjuvants should be further studied as a promising adjuvantation approach to enhance immunogenicity of vaccines to protect these vulnerable populations.

Keywords: Bacille Calmette–Guérin, hepatitis B vaccine, preterm, newborn, innate cytokine profiles, HBV-specific antibodies

INTRODUCTION

Infectious diseases are a leading cause of childhood death with neonatal infection accounting for ~40% of mortality in those <5 years of age, ~7 million cases, and 700,000 deaths per year (1). Within the neonatal population, prematurity, defined as birth at <37 weeks of gestational age (GA), is the single most important cause of death in the first month of life and the second largest cause of death after pneumonia in children <5 years of age (1). Most preterm births (84%, 12.5 million) occur at >32 weeks of gestation (2). At this GA, many preterm newborns can survive with cost-effective supportive care. The mortality from neonatal infection in preterm infants has increased over the last 20 years (1). Moreover, preterm newborns remain at elevated risk of infection through 18 years of age (3). Accordingly, global progress in child survival and health to 2015 and beyond will depend on optimizing preventative care for preterm and term infants with vaccines one of the most effective biomedical approaches for disease prevention.

Vaccine-mediated prevention of infections is limited by reduced or distinct immune responses in early life (4). The current necessity for repeated vaccine booster doses to obtain full protection leaves a window of susceptibility in both the preterm and term infant during the first 6 months of life (5). Alongside efforts at maternal immunization, enhancement of responses to early-life vaccines *via* use of novel adjuvantation systems that demonstrate age-specific immune-enhancing activity is an attractive approach to address this problem. To date, development of pediatric vaccines has largely relied on *ad hoc* studies of adult vaccines, and has not taken the age-dependent development of the immune system into account (6). This holds true for vaccination of the preterm as well: the Advisory Committee on Immunization Practices (ACIP) of US Centers for Disease Control and Prevention as of 2011 has recommended that, with respect to most vaccines, preterm infants be immunized with the full-recommended dose according to the same schedule as full-term infants. However, even thought vaccine immunogenicity in preterm infants is often distinct compared with term responses (7–10), there remains a research knowledge gap. Specifically, hepatitis B vaccine (HBV) immunization is delayed in the preterm due to empiric evidence of reduced immunogenicity. In preterm newborns <1,500–1,800 g birth weight or <34–35 weeks gestation, a three-dose vaccine series of HBV induces protective antibody (Ab) titers in only ~45–85% of patients as opposed to 90–100% in more mature infants (11).

Consideration of vaccine adjuvantation in early life must take into account that newborn innate and adaptive immune cells exhibit distinct activation profiles in response to pattern-recognition receptor (PRR) agonists. However, activation of some PRRs in newborns, such as toll-like receptors (TLRs) 7/8, can induce an adult-like response (12, 13). For example, the germinal center reaction that drives the magnitude and persistence of the Ab response is impaired early in life but can be enhanced with certain TLR agonists (14, 15). Of note, preterm innate and adaptive immunity is distinct from that of both term newborns and adults. For example, preterm monocytes exhibit attenuated PRR-mediated Th1 and Th17-cytokine responses (16). Herein, we assessed whether a live-attenuated vaccine Bacille Calmette–Guérin (BCG), known to activate multiple PRRs (17, 18), might exert adjuvant activity in the context of neonatal HBV immunization.

BCG is the most commonly administered vaccine worldwide and when administered at birth is safe and effective in reducing the rates of infantile tuberculous (TB), meningitis, and disseminated miliary disease (19). It is the only routinely administered neonatal vaccine that induces a Th1-polarized immune response (20). BCG administration may also, in an age-dependent manner, induce beneficial heterologous ("non-specific" or "trained") immunity against unrelated pathogens and stimuli (21–25), impact responses to other vaccines (20, 26–28), and immune-modulate in the context of allergic diseases as well (29).

Some limited clinical studies have suggested that BCG may enhance responses to other, vaccines such as Oral Polio Vaccine and HBV (20). We posited that co-administration of BCG with HBV could induce greater innate and adaptive immune responses, including acute cytokine induction and HBV-specific Ab production. Employing *in vitro* human blood and mononuclear cell assays we demonstrated that (BCG + HBV) enhanced cytokine and chemokine production on both the protein and mRNA level. Strikingly, *in vivo* immunization of neonatal [day of life (DOL)-0 and -7] and adult mice demonstrated that combined (BCG + HBV) vaccination induced anti-HBV-specific Ab titers in all three age groups at 21 days post-immunization relative to immunization with HBV alone. Overall, our studies provide fresh insight into a vaccine–vaccine interaction that may be the basis of enhanced immunization strategies for vulnerable preterm and term newborn populations.

MATERIALS AND METHODS

Cord Blood Collection

Moderate to late preterm (28 2/7–34 6/7 weeks GA) and term cord blood was collected at The Brigham and Women's Hospital and the Beth Israel Deaconess Medical Center, both tertiary care centers for delivery and postnatal care of the preterm and term newborns. The details of our preterm study cohort are outlined in **Table 1**. The de-identified newborn cord blood (~15–60 ml) was collected immediately after caesarian section

TABLE 1 | Characteristics of human preterm, term, and adult study participants.

	Preterm	Term	Adult
Total number of individuals	10	15	14
Site of delivery	BWH (7), BI (3)	BWH (9), BI (6)	N/A
Delivery mode	7 CS, 3 VD	15 CS	N/A
Sex	6 F, 4 M	8 F, 8 M	6 F, 8 M
(Gestational) age	28/2–34/ 6 weeks GA	37/0–41 weeks GA	23–35 years old
Twins	1 set (Mono-Di)	1 set (pooled)	N/A
Chorioamnionitis	6 no/4 unknown	No	N/A
HIV positive status	No	No	No
Antibiotics	4 no/3 yes/3 unknown	No	N/A
Celestone	1 no/5 yes/4 unknown	N/A	N/A
Last celestone dose ≥48 h before delivery	2 no/3 yes/5 unknown	N/A	N/A

BWH, Brigham and Women's Hospital; BI, Beth Israel Deaconess Hospital; CS, Caesarian section; HIV, human immunodeficiency virus; VD, vaginal delivery; F, female; M, male; GA, gestational age; Mono-Di, monochorionic-diamniotic.

or vaginal delivery of the placenta from a larger placental or umbilical vein under sterile conditions, as previously described (30). No cord blood samples from newborns born to human-immunodeficiency virus-positive mothers were included. Samples were collected from both male and female newborns. Blood and blood-derived products were handled per applicable biohazard policies. As the type of anti-coagulant and length of storage prior to assay can affect cytokine production (31), we have established a routine standard of procedure in which blood was anti-coagulated with 15–20 U/ml pyrogen-free heparin sodium (Sagent Pharmaceuticals, Inc.; Schaumberg, IL, USA), and then kept at room temperature (RT) and processed within 4 hours (h) of collection (typically 1–2 h). Each preterm placenta was histologically examined for signs of chorioamnionitis, and information on the timing of prenatal steroid administration, as well antibiotic administration was collected. Peripheral blood was collected from healthy adult male and female volunteers employed at BCH.

Animals

C57BL/6 mice were obtained from Charles River Laboratories and housed in specific pathogen-free conditions in the animal research facilities at BCH. To obtain newborn mice, pregnant dams were purchased on pre-determined days of pregnancy and cages checked twice daily (~every 12 h) to assess for the presence of pups. Both male and female pups were used for experiments.

Vaccines and Whole Blood Assay

~8 ml of fresh blood was processed for the whole blood assay as previously described (32). Briefly, neonatal cord (preterm and/or term) or adult whole blood was mixed 1:1 with sterile pre-warmed (37°C) RPMI 1640 medium (Invitrogen) and 180 µl of the 1:1 suspension added to each well of a 96-well U-bottom plate (Becton Dickinson) containing 20 µl freshly prepared HBV, BCG, (BCG + HBV) at 10× final concentration, testing stimuli at a 5-point concentration–response curve based on published data (12, 33). As sources of BCG and HBV, we used the Danish Strain 1331 (Statens Serum Institut, Copenhagen, Denmark) and Recombivax® HB (Merck and Co, Inc.), respectively. Suspensions containing 200 µl/well were gently mixed by pipetting and incubated at 37°C in a humidified incubator at 5% CO_2 for 6 h.

ELISA and Multiplex Cytokine Analysis

After treatment of the preterm, term, and adult blood with the described vaccines for 6 h, the plates were centrifuged (10 min, RT, 500 *g*), and supernatants collected and stored in three aliquots at −80°C for subsequent TNF (BD Biosciences Human TNF ELISA) and IL-1β ELISA (eBioscience Human IL-1β ELISA) and for subsequent multiplexing assays for Th1 (TNF, IL-1β, IL-12p70, IFNα, and IFNγ) and Th2 (IL-6, IL-10, and IL-12p40), and Th17 (IL-6, IL-1β) polarizing cytokines (Milliplex Human Magnetic Bead Panel; Millipore; Chicago, IL, USA). Data were analyzed on the Luminex® 100/200™ System using xPOTENT® software (Luminex; Austin, TX, USA).

Isolation of Cord Blood Mononuclear Cells (CBMCs) and Peripheral Blood Mononuclear Cells (PBMCs) and *In Vitro* Stimulation

From each whole blood sample collected, matched PBMCs and CBMCs were isolated using Ficoll density gradient methodologies and cryopreserved for further downstream stimulation experiments (12, 33). MCs were stored at 50 million cells per vial in 1 ml RPMI containing 20% autologous plasma and 10% DMSO at −80°C until use. After a standardized thawing procedure, PBMCs and CBMCs isolated from human donors were resuspended at a concentration of 2×10^6 cells/1000 µl of RPMI supplemented with 10% of autologous platelet-poor plasma. Cells were stimulated for 4 h with either HBV, BCG, or (BCG + HBV) (each at 1:1,000, 1:100, 1:10 vol/vol) and cells washed with ice cold PBS prior to addition of RLT buffer (RNeasy Lysis Buffer, Qiagen, MD, USA) and storage at −80°C for subsequent RNA isolation.

Gene Expression Analysis by Quantitative Real-time PCR Array

Total RNA was extracted from lysates of vaccine-stimulated PBMCs and CBMCs using the Qiagen RNeasy Minikit and DNAse treatment performed using the Qiagen RNAase Free DNAase set all per the manufacturer's instructions. RNA concentrations were determined using the Nanodrop 1000 and cDNA generated using the Qiagen RT2 First Strand Kit. 96-well PCR array analysis was performed using the Qiagen standardized *Innate and Adaptive Immune Reponses PCR Array* (PAHS-0522A) and RT^2 qPCR roxSYBR green kit. Web-based PCR array analyses (RT^2 Profiler PCR Array Data Analysis version 3.5) was used and normalized to five reference genes (B2M, HPRT1, RPL13A, GAPDH, and ACTB). Relative quantification of gene expression was calculated by the ΔCt (relative expression $\times 10^4$). Multivariate biplots of principal component analyses were performed in R 3.4.2 using *ggplot2*, *ggord*, and *vegan* packages using log-fold transcript abundance of gene arrays in each group. Genes were sorted using unsupervised hierarchical heatmap clustering of log-fold changes using the *heatmap2* package.

Immunization and Anti-Recombinant HBV Surface Antigen (rHBsAg)-Specific Ab Quantification, Subtype Classification, and Avidity Determination

For immunization experiments, mice of three age groups were used: the first group of mice were given their first immunization (prime immunization) on DOL0; the second group of mice on DOL7, and in the third group at 6–8 weeks of life. Each of the three age groups were divided into five immunization groups: saline; BCG (Organon Teknika/Merck, Durham, NC, USA) alone; HBV vaccine alone; (BCG + HBV) as a combined admixed injection; and BCG with HBV vaccine administered separately. All immunizations were injected subcutaneously (s.c.). If one injection was performed per animal, it was performed into the right posterior thigh, if two separate injections were performed they were performed either into the right and left posterior thighs (DOL7 mice; adult mice) or in DOL0 mice into the right posterior thigh (HBV) and the scruff (BCG). The injection volumes were 50 µl of vaccine (or vaccine combination)/injection in the adult animals and 25 µl of vaccine (or vaccine combination)/injection in the newborn animals (DOL0 and DOL7). The injection dose of Recombivax® was 0.25 µg of rHBsAg for the adult animals and 0.125 µg in the newborn pups (DOL0 and DOL7) diluted in 0.9% NaCl Inj (USP). We chose to administer half HBV doses in the newborn as this is an established approach in human clinical vaccinology (ACIP recommendations for hepatitis B immunization). The injection dose for BCG was 0.4×10^6 CFU for the adult animals and 0.2×10^6 CFU for the pups (DOL0 and DOL7) diluted in 0.9% NaCL Inj (USP). We selected the BCG dose based on published literature in neonatal mice (12, 26). The selected dose of HBV was slightly lower than that routinely used in other murine studies (34) and reflected the volume limitations inherent to administration of two vaccines. We conducted preliminary experiments to confirm that we could obtain measurable Ab titers with the chosen concentration of HBV in all age groups. Mice were immunized with a prime-boost schedule; a primary immunization; and a secondary (booster) immunization, 2 weeks apart. Serum samples were obtained from blood collected *via* tail vein or artery nick as indicated for Ab detection. rHBsAg-specific IgG were quantified by ELISA. High binding flat bottom 96-well plates (Corning Life Sciences) were coated with Recombivax® diluted to 1 µg/ml in carbonate buffer pH 9.6, incubated overnight at 4°C, and blocked with PBS + BSA 1% (Sigma-Aldrich) for 1 h at RT. Then, sera from immunized mice were added with an initial dilution of 1:100 and 1:3 serial dilutions in PBS + BSA 1% and incubated for 2 h at RT. Plates were then washed and incubated for 1 h at RT with HRP-conjugated anti-mouse IgG, IgG1, IgG2c (Southern Biotech). At the end of the incubation, plates were washed again and developed with tetramethylbenzidine (BD Biosciences) for 5 min, then stopped with 2N H_2SO_4. The optical density was read at 450 nm on a Versamax microplate reader with SoftMax Pro Version 5 (both from Molecular Devices), and endpoint titers were calculated using as cutoff two times the optical density of the background (35). For assessing Ab avidity, plates were incubated 15 min with ammonium thiocyanate 0.5 M before the addition of HRP-conjugated Abs. Avidity was expressed as the $LogEC_{50}$ ratio of corresponding plates treated with or without ammonium thiocyanate (36).

Statistical Analyses and Graphics

Data were analyzed and graphed using Prism for MacIntosh v. 7.0 (GraphPad Software). Tests used for statistical comparisons are indicated in figure legends. p value < 0.05 was considered significant. An adaptation of the Loewe method of additivity (37) was used to assess whether cytokine production after stimulation with (BCG + HBV) was synergistic, additive, or antagonistic. Concentration–response curves were subjected to regression analysis to determine the slope and y-intercept of each curve in the exponential phase. The formula $D = [Ac]/[Ae] + [Bc]/[Be]$ was used, where [Ac] = the concentration of (HBV) used in the combination of (HBV + BCG) that results in half the maximal TNF production measured with the combination of both vaccines; [Ae] = the concentration of HBV used alone that results in half the maximal TNF production measured with the combination of (HBV + BCG); [Bc] = the concentration of BCG used in the combination of (HBV + BCG) that results in half the maximal TNF production measured with the combination of vaccines; and [Be] = the concentration of BCG used alone that results in half the maximal TNF production measured with the combination of (BCG + HBV). If $D = 1$: (HBV + BCG) act additively, if $D > 1$: (HBV + BCG) act antagonistically, and if $D < 1$: (HBV + BCG) act synergistically. Our laboratory has employed this interaction analysis method in other recently published studies (13, 38, 39).

RESULTS

(BCG + HBV) Synergistically Enhances IL-1β Production in Preterm, Term, and Adult Whole Blood

The *in vitro* whole blood assay is a useful tool to characterize the effects of vaccines on cytokine production as it enables testing of moderate number of different vaccine formulations, at multiple concentrations in a sample from a single individual (13, 38, 40). To assess whether (BCG + HBV) may enhance NF-κB and inflammasome-mediated cytokine induction compared to HBV alone, we compared (BCG + HBV) with identical concentrations of BCG and HBV in its ability to stimulate TNF and IL-1β production in preterm (**Figure 1A,D**) or term cord (**Figure 1B,E**) or adult peripheral blood (**Figure 1C,F**). In all three age groups, (BCG + HBV) significantly increased TNF and IL-1β secretion relative to RPMI controls and also secretion of IL-1β relative to HBV alone. Using the Berenbaum equation to assess drug–drug interactions, the interaction between HBV and BCG with regard to TNF was additive or antagonistic (**Table 2**). However, with respect to IL-1β, the combined vaccine effect was synergistic, defined as a D-value < 1, especially so in the term followed by the preterm, and least pronounced in the adult (**Table 2**). Of note, IL-1β is important to immunogencity of ALUM-adjuvanted vaccines (41), has a role in neutrophil recruitment and in Ab production, and has been used as an adjuvant (42–45). **Figure 1** also demonstrates that BCG alone is a potent inducer of both TNF and IL-1β in whole blood of

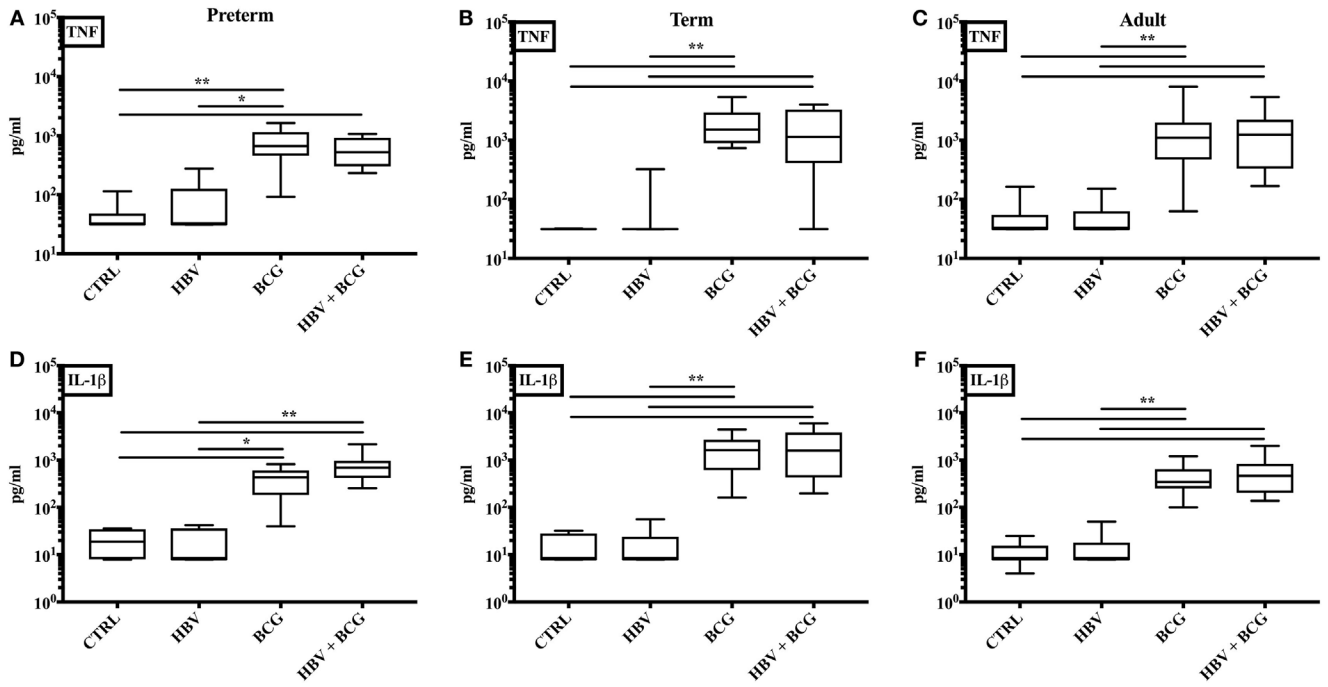

FIGURE 1 | Concurrent stimulation with [Bacille Calmette–Guérin (BCG) + hepatitis B vaccine (HBV)] synergistically enhances IL-1β production in human preterm, term, and adult whole blood. Preterm **(A, D)**, term **(B, E)**, and adult **(C, F)** whole blood was stimulated with HBV alone, BCG alone, or (BCG + HBV) at 1:10 v/v. After 6 h, supernatants were analyzed for TNF **(A–C)** and IL-1β **(D–F)** cytokine production by ELISA. Statistical significance was determined by Kruskal–Wallis with Dunn *post hoc* test. $^{*}p < 0.05$, $^{**}p < 0.01$. $N = 7$–8 preterm, $N = 10$–11 term newborns, and $N = 10$–11 adults.

all age groups and in this context is likely the driving component behind this vaccine interaction.

The Combination of BCG and HBV Vaccine Stimulates Secretion of Numerous Cytokines and Chemokines in Preterm, Term, and Adult Whole Blood

Having established that (BCG + HBV) enhance TNF and IL-1β production by preterm and term whole blood, we next characterized the cytokine profiles induced by these vaccine treatments in more detail employing multiplex cytokine analysis on the isolated supernatants. As shown in **Figure 2**, cytokines significantly induced in preterm or term cord blood by the vaccine combination relative to RPMI control and/or HBV vaccine alone included CSF2 (GM-CSF), IL-6, IL-10, CXCL8, CCL2, and CCL3. Interestingly, IL-12p70 was significantly induced by the vaccine combination in the preterm. Of note, the similarity of the BCG cytokine/chemokine profile to that induced by (BCG + HBV) suggested that the vaccine combination effect was mainly driven by BCG.

HBV, BCG, and (BCG + HBV) Induce Distinct RNA Transcription Clusters in CBMCs/PBMCs Isolated from Preterm, Term, and Adult Individuals

To investigate how addition of BCG to HBV vaccination alters gene expression patterns at the mRNA level, RNA isolated from neonatal CBMCs (term and preterm) or adult PBMCs stimulated for 4 h with vehicle (control), BCG, HBV, or (BCG + HBV) was subjected to quantitative real-time PCR array comprised of 84 genes in human innate and adaptive immune pathways. mRNA levels were quantified in 4–5 individuals/group. **Figure 3** shows that there were increases in expression of several cytokine and chemokine transcripts upon mononuclear cell stimulation with (BCG + HBV) compared to unstimulated or HBV-treated cells. Some of them reached statistical significance in all (i.e., *CSF2*) or some age groups (i.e., *CXCL8* in preterms and adults). Interestingly, some of these genes encode proteins with defined roles in vaccine efficacy (13, 42–48). **Figure 4A** demonstrates treatment-driven segregation of age groups by a principle component biplot of mRNA gene expression data. The points representing age and treatment group (open circles) approximate

TABLE 2 | Quantification of (Bacille Calmette–Guérin + hepatitis B vaccine) synergism.

Age groups	*D*-value	Interpretation
TNF		
Preterm	13.1064	Inhibitory
Term	1.3557	Additive
Adult	1.0777	Additive
IL-1β		
Preterm	0.6398	Synergy
Term	0.3506	Synergy
Adult	0.7233	Synergy

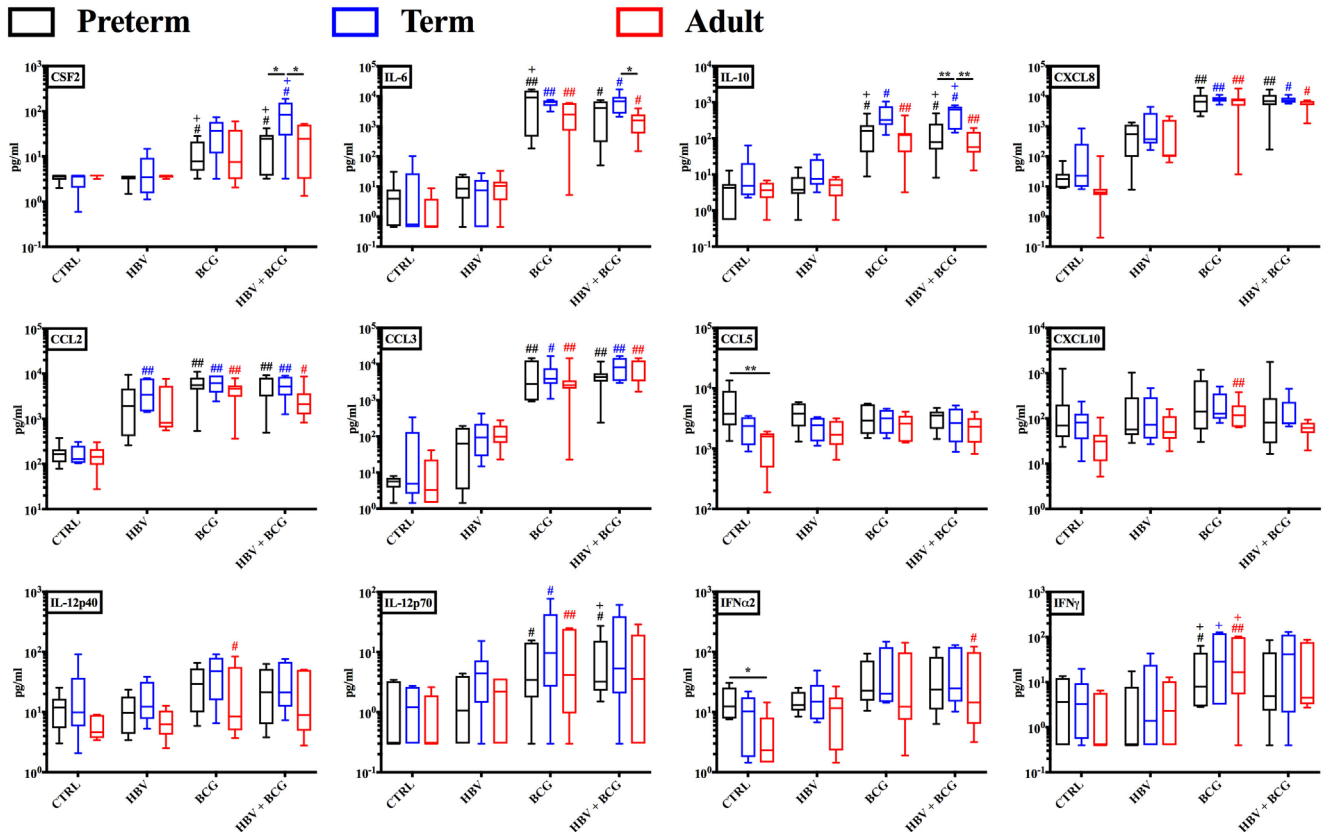

FIGURE 2 | Cytokine and chemokine profiles induced by hepatitis B vaccine (HBV) and Bacille Calmette–Guérin (BCG) in human preterm, term, and adult whole blood. Preterm, term, and adult whole blood was stimulated for 6 h with either HBV, BCG, or (BCG + HBV) and supernatants analyzed *via* Multiplex Cytokine Analysis. Statistical significance was determined by repeated measure or ordinary one-way ANOVA with Holm-Sidak *post hoc* test (or their non-parametric equivalent Friedman or Kruskal–Wallis with Dunn *post hoc* test). $^{*}p < 0.05$, $^{**}p < 0.01$ of preterm vs. term vs. adult; $^{\#,+}p < 0.05$, $^{\#\#,++}p < 0.01$ of groups indicated by the corresponding color, respectively, vs. saline or HBV. $N = 7$ preterm newborns, $N = 6$ term newborns, $N = 7$ adults.

gene expression patterns between groupings. The unsupervised hierarchical heat map in **Figure 4B** demonstrates similar clustering by treatment and outlines in a red to blue scale high to low gene expression per gene.

(BCG + HBV) Significantly Enhance the Level of Anti-rHBsAg-Specific IgG Titers in Neonatal Mice (Immunized on DOL0 and DOL7) but Not Adult Mice

To assess the impact of (BCG + HBV) *in vivo*, we turned to a murine model. Mice at DOL 0–5 have been utilized as a model for preterm innate and adaptive immunity (49, 50) while mice at DOL7 have been used to model the term newborn (4, 15, 51–53). We made use of these age-specific models to investigate whether BCG can enhance early-life immunization with HBV, for which anti-Hepatitis B sAg Ab titers are the established correlate of protection (54). **Figure 5A** demonstrates the schedule according to which the mice of all three age groups were prime-immunized and booster immunized 2 weeks later. The age at prime immunization was DOL0 (the "preterm" group, $n = 9$–14), DOL7 (the "term" group, $n = 14$–16) and 6–8 weeks for the adult group ($n = 15$–17). These data were acquired in two separate experiments, each of which included all three age groups and within each age group all five treatment groups. The linear graphs represent median anti-rHBsAg IgG titers over time post-prime immunization (**Figure 5B**). The box-and-whisker plots depict Ab titers 21 and 42 days post-prime immunization. To assess whether the potential beneficial effect of addition of BCG to HBV depends on co-administration of both vaccines into the same site, we differentiated two combined treatment groups: one in which (BCG + HBV) were combined (i.e., admixed) and injected into the right flank s.c. and one in which HBV was injected into the right thigh and BCG was injected either into the scruff (neonatal mice on DOL0) or left thigh (neonatal mice on DOL7 and adult mice). Addition of BCG to HBV significantly enhanced Ab responses at 21 days post-prime immunization in neonatal mice immunized on DOL0 and DOL7 but not in adult mice. Whereas in DOL0 mice, it was the separate injection of BCG and HBV that significantly enhanced anti-rHBsAg IgG titers, the combined injection was the administration technique that lead to enhanced Ab titers in DOL7 mice. While this effect was sustained at D42 post-prime immunization in mice immunized on DOL0, it was no longer evident in neonatal mice prime-immunized on DOL7 (**Figure 5C**). Interestingly, switching toward IgG2c was observed

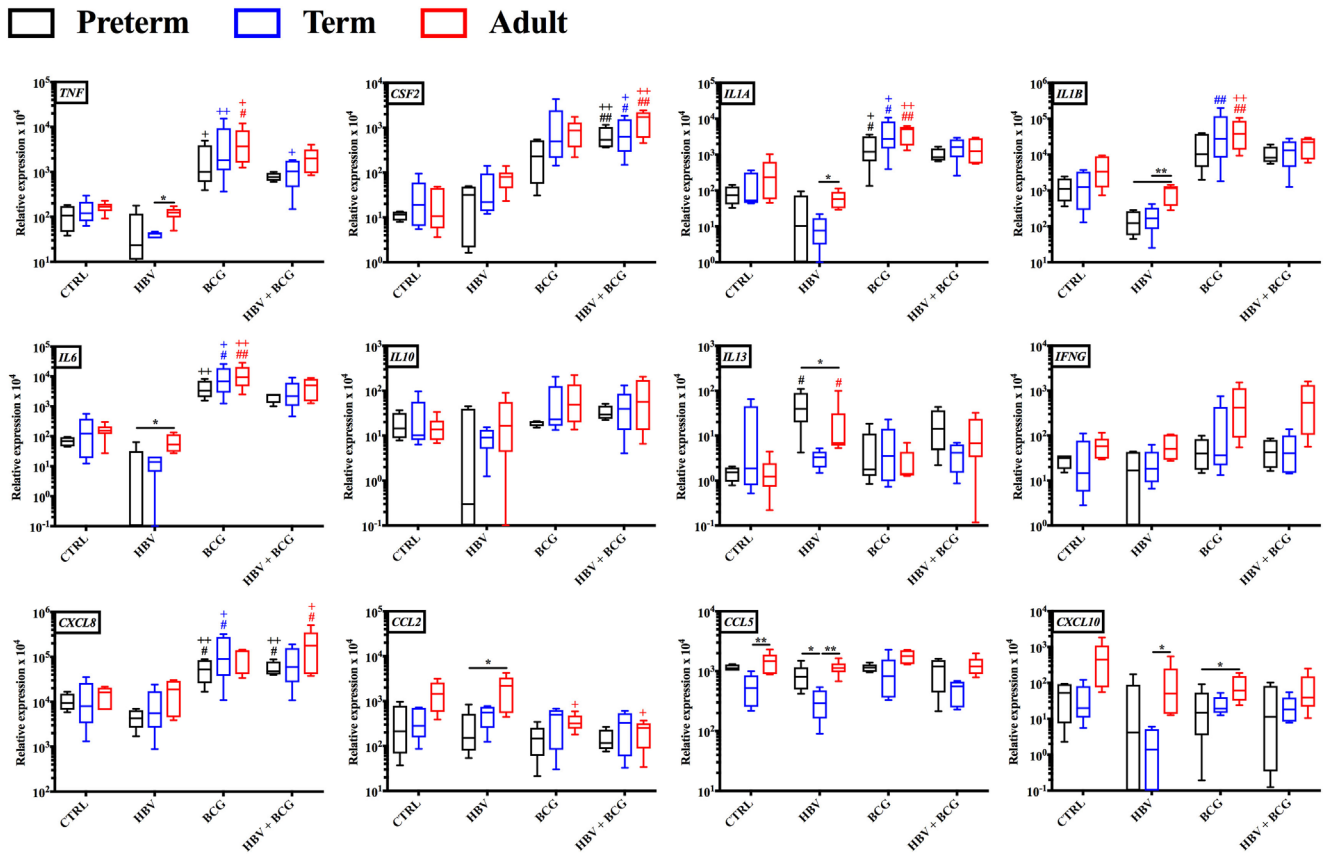

FIGURE 3 | Gene expression profiles induced by hepatitis B vaccine (HBV) and Bacille Calmette–Guérin (BCG) in human preterm, term, and adult mononuclear cells. Preterm and term cord blood mononuclear cells, and adult peripheral blood mononuclear cells was stimulated for 4 h with either HBV, BCG or (BCG + HBV) and cells were harvested for quantitative real-time PCR analysis. Statistical significance was determined by repeated measure or ordinary one-way ANOVA with Holm-Sidak *post hoc* test (or their non-parametric equivalent Friedman or Kruskal–Wallis with Dunn *post hoc* test). $^{*}p < 0.05$, $^{**}p < 0.01$ of preterm vs. term vs. adult; $^{\#,+}p < 0.05$, $^{\#\#,++}p < 0.01$ of groups indicated by the corresponding color, respectively, vs. saline or HBV. $N = 4–5$/group.

only in adult mice immunized with (BCG + HBV) (Figure S1 in Supplementary Material). In addition, BCG did not significantly modulate Ab avidity, suggesting that BCG did not affect affinity maturation of anti-rHBsAg Abs (Figure S2 in Supplementary Material).

DISCUSSION

In this study, we have demonstrated for the first time that BCG, alone or when coadministered with HBV in a neonatal context, can enhance human innate cytokine responses *in vitro*. Moreover, we show that BCG can enhance hepatitis B antigen-specific murine adaptive responses *in vivo*. These observations are important in that newborns and young infants are highly susceptible to infection with intracellular pathogens including viruses, such as hepatitis B virus. Acquisition of hepatitis B virus during the newborn period carries risks of developing both hepatocellular carcinoma and liver cirrhosis. Moreover, most licensed vaccines, including HBV, are not optimally effective at birth and require multiple booster immunizations later in life despite the fact that hepatitis B immunization in newborns induces higher primary and memory Ab responses than in adults (55). Under the current immunization schedule with an ALUM adjuvanted HBV vaccine, the term newborn does not reach a status of immunological protection against HBV, as measured by titers of anti-rHBsAg Abs, until 6 months of life. For the preterm newborn with a birth weight <2 kg the unmet need to enhance responses to HBV is exacerbated by the fact that a birth dose is not recommended as priming has been inefficient in this population. With age-specific differences in the quantity and quality of cellular and soluble factors playing a role (56), the neonatal immune system is distinct from that of infants and adults, with bias toward induction of regulatory T cell and Th2-type T cell responses. Distinct early-life immunity limits the efficacy of adjuvants that activate newborn DCs to produce Th1-polarizing cytokines. The preterm newborn, in addition to demonstrating low innate Th1 support, demonstrates a low TLR-mediated production of Th17-supporting cytokines, with robust anti-inflammatory IL-10 levels (57–60). Combined stimulation of newborn cells through certain combinations of PRRs may potentially overcome the early-life bias against Th1 responses (30, 38, 61). In summary, there is an unmet need for early-life vaccine strategies that provide earlier protection against HBV infection for the term and in particular for the preterm infant.

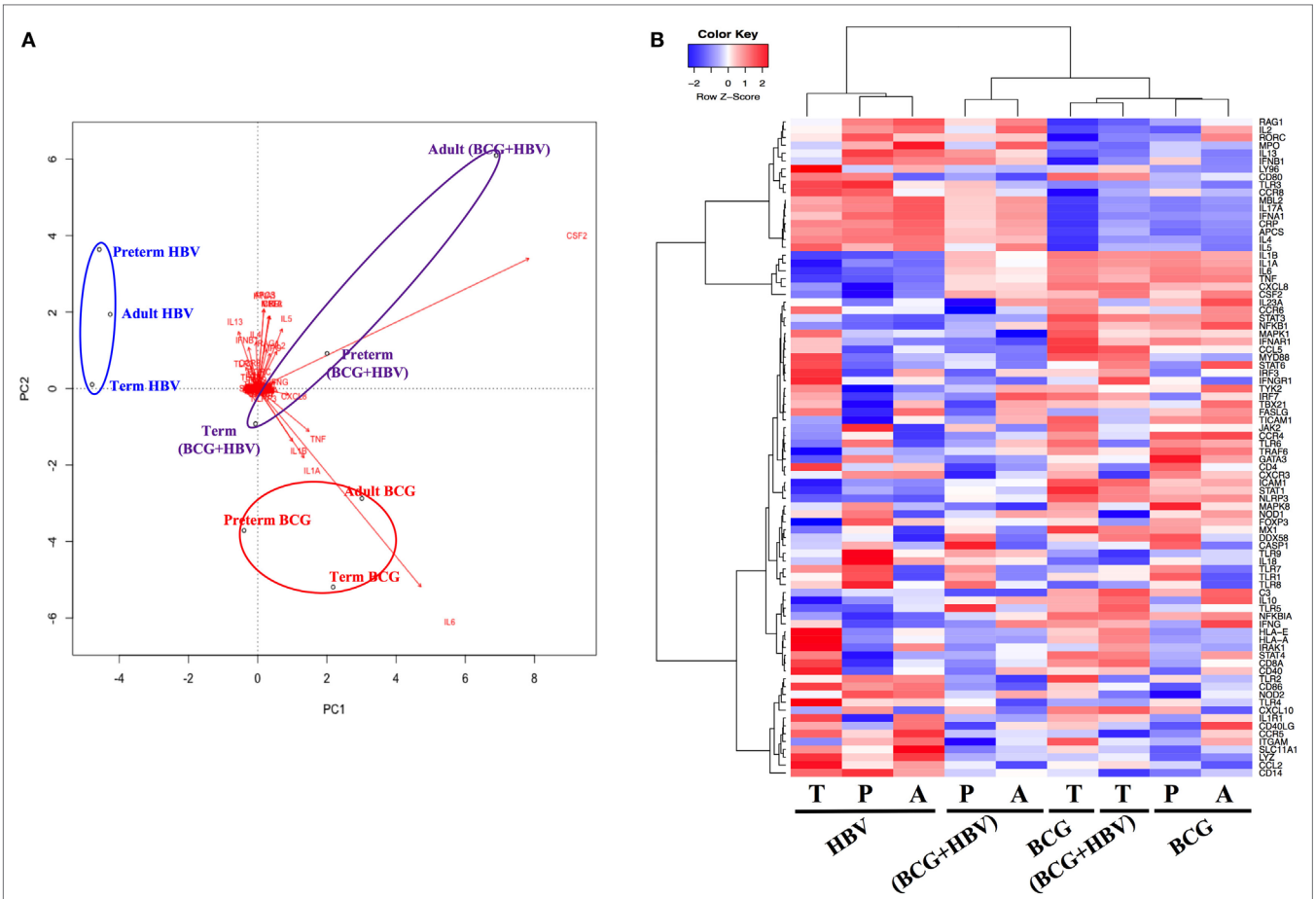

FIGURE 4 | Principal component analysis of gene expression data reveals treatment-specific segregation of different age groups. Gene expression data were generated as outlined in **Figure 3**. **(A)** Principal components analysis biplot of mRNA gene expression data where observations (samples) are points and gene expression profiles are arrows shows dominant clustering of profiles by treatment. The distance between points approximates gene expression pattern differences among groupings. Arrows indicate genes that have greater biplot scores and drives the differences between groups. Arrowheads close to a particular group indicate genes are expressed at a greater relative abundance differences in those samples. **(B)** Unsupervised hierarchical heatmap shows clustering of treatments demonstrating log2-fold changes, expression values of genes in each sample; red to blue scale represent intensity of fold changes per genes (red indicates up, blue indicates down). Each row means individual gene and each column indicates groupings of age and treatment. P, preterm; T, term; A, adult.

While HBV immunization of the term infant effectively reduces chronic Hepatitis B infection in countries with universal neonatal immunization, the window of vulnerability in term and preterm infants at birth (into infancy) is still a major concern. This concern could be addressed by maternal or an optimized (one dose) HBV immunization of the newborn at birth. The common goal among maternal vaccination programs is temporary protection of the young infant against severe illness and death by ensuring sufficient and timely transfer of protective antibodies from the mother (62). Unfortunately, while some attempt have been made, clinical studies of vaccination against hepatitis B have showed lower immunogenicity in pregnant women than in nonpregnant women (63, 64). Regarding improved immunization of the newborn, as the Center for Disease Control outlines, worldwide, most people with chronic Hepatitis B are infected at birth or during early childhood. While a positive mother is a significant source of risk for Hepatitis B infection as a newborn, the recommendation to administer Hepatitis B vaccination at birth in many countries including the United States is based on the understanding that asymptomatic chronic carriers within the family and family contacts can be a significant risk for infection in the neonatal period (ACIP, recommendations for Hepatitis B immunization).

BCG is the most commonly administered vaccine in world history with billions of doses given globally at or soon after birth to protect against disseminated TB during infancy (65). In addition to its protective effects against TB, for which there is no established correlate of protection, BCG administration may also enhance Ab responses to unrelated pathogens in human newborns and infants (20, 27, 28) and in newborn mice (26). Protective heterologous effects of certain live-attenuated vaccines including BCG have been demonstrated that reduce morbidity and mortality beyond what can be attributable to prevention of the target disease (23, 25). BCG activates TLRs, including TLR2 and TLR4 (66), as well as C-type lectin receptors (CLRs) such as Dectin-1 and macrophage-inducible C-type lectin (Mincle) (67–70), resulting in strong

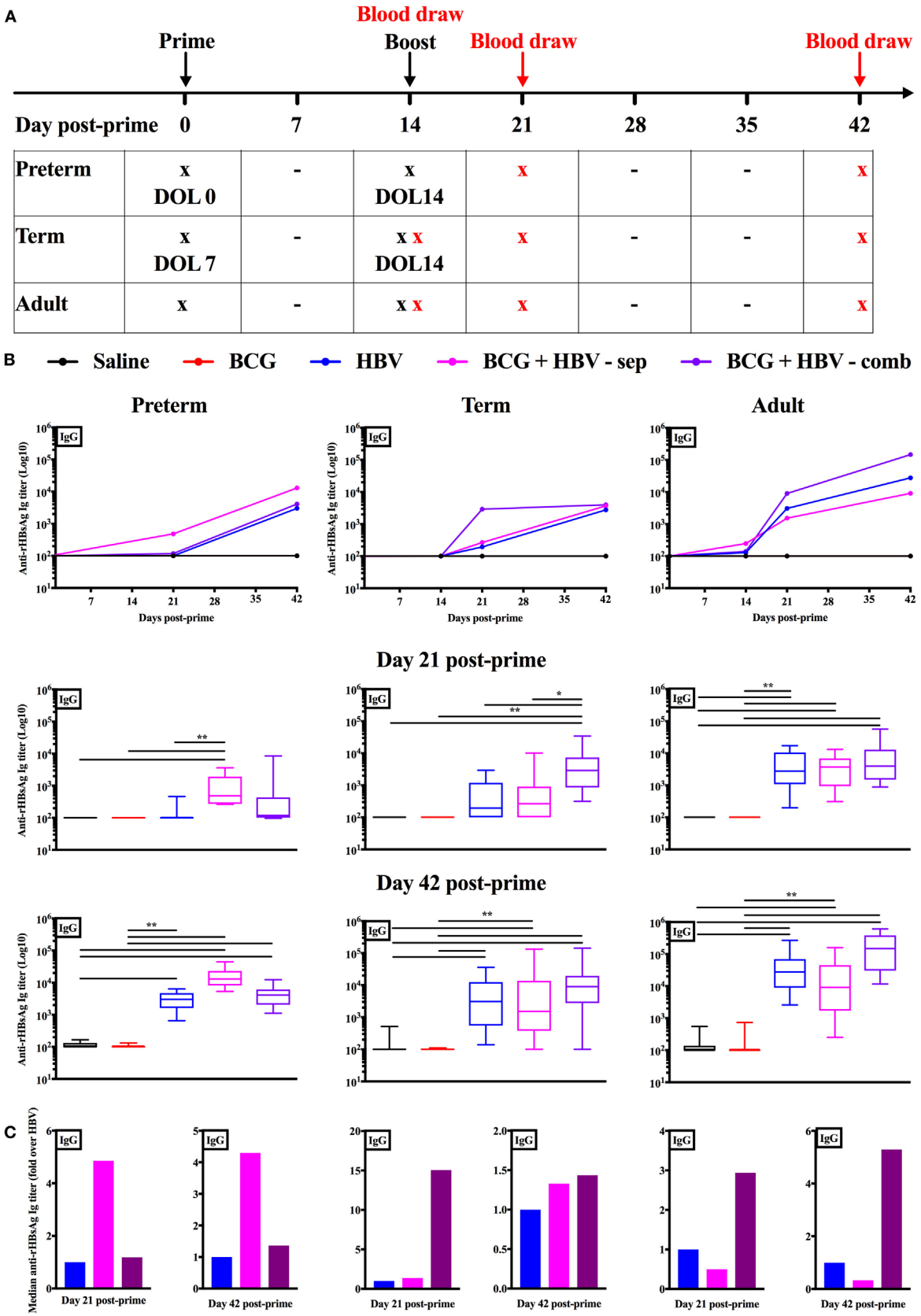

FIGURE 5 | Continued

FIGURE 5 | Combined immunization of newborn mice with Bacille Calmette–Guérin (BCG) and hepatitis B vaccine (HBV) *in vivo* enhances anti-recombinant HBV surface antigen (rHBsAg) IgG titers 21 days post-immunization. **(A)** Schematic representation of the immunization and blood draw schedule for the *in vivo* experiments: prime immunization was administered either on day of life (DOL) 0, DOL7, or at 6–8 weeks of life with either saline, BCG, HBV vaccine, BCG and HBV in a combined s.c. injection or BCG and HBV vaccine injected in separate sites. Booster immunization was performed with either saline in the mice prime-immunized with saline or BCG, or with HBV vaccine in the mice prime-immunized with either HBV vaccine alone, or BCG and HBV (either in combined or separate injection). Blood draws in the adult mice were obtained prior to prime immunization, prior to booster immunization, at 21 days and at 42 days post-prime immunization. Blood draws in the neonatal mice immunized on DOL7 were performed prior to booster immunization, at 21 days and at 42 days post-prime immunization. Blood draws in the mice immunized on DOL0 were obtained at 21 days and at 42 days post-prime immunization. Some neonatal mice from both the group immunized on DOL0 as well as DOL7 were sacrificed for a baseline blood draw prior to prime immunization. **(B)** Anti-rHBsAg IgG titers in mice immunized on DOL0, mice immunized on DOL7 and at 6–8 weeks of life. **(C)** Fold change over the HBV vaccine immunized group of the median anti-rHBsAg IgG titers. $N = 9–14$/group for the mice immunized on DOL0; $N = 14–16$/group for the mice immunized on DOL7; $N = 15–17$/group for the mice immunized at 6–8 weeks of life. Data are representative of two independent experiments each of which included all three age groups and within each age group all five treatment groups. Statistical analysis of differences between the treatment groups was performed *via* Kruskal–Wallis test with Dunn's *post hoc* test. $^{*}p < 0.05$, $^{**}p < 0.01$.

Th1-biased immune responses. Of note, combined TLR and CLR activation synergistically enhances Th1- and Th17-cytokine induction in human newborn monocyte-derived dendritic cells (38), raising the possibility that engagement of multiple PRRs by BCG may contribute to the observed enhancement of HBV immunogenicity. Accordingly, we examined the cytokine profiles induced by (BCG + HBV) relative to HBV alone in term and moderate to late preterm human infants on the protein and mRNA levels *in vitro* and then to examine the impact of cytokine polarization on the adaptive anti-rHBsAg-specific Ab production *in vivo* utilizing both mice on DOL0 as well as DOL7 to mirror different levels of immune ontogeny.

Our study characterized (BCG + HBV)-induced human leukocyte cytokine profiles at different GAs at the protein and mRNA level. Of these *IL1B*, *IL6*, *CSF2*, and *TNF* were key drivers of gene clustering in the principal component analysis by treatment instead of by age. The interleukins induced are particularly noteworthy as they have been associated with enhanced adaptive immunity: (a) IL-1β, whose production was synergistically induced by (BCG + HBV) in human preterm and term cord blood as well as peripheral adult blood *in vitro*, is an inflammasome-produced cytokine that may improve vaccine immunogenicity (42–45), having a role in neutrophil recruitment (71) and in Ab production (72) and has been used directly as an adjuvant (73). Indeed, IL-1β has been implicated as important to immunogenicity of HBV, in particular anti-HBsAg Ab responses (74–76) and (b) IL-6, a Th17-polarizing cytokine that stimulates differentiation and maturation of B cells to Ab-producing plasma cells, stimulates T cell proliferation, and is a murine adjuvant (46, 47, 77).

In neonatal mice, compared to HBV alone, we found that (BCG + HBV) induced significantly higher anti-rHBsAg-specific IgG levels at 21 days after prime immunization. In DOL0 mice, this effect was significant when BCG and HBV were injected into separate sites, in DOL7 mice this effect was significant when both vaccines were injected in combination. Although it should be recognized that the kinetics of immune ontogeny are distinct in mice compared to humans, this relatively early effect of BCG co-administration is intriguing. If such enhancing effects of BCG would extend to humans, they may fall within the window of susceptibility inherent to current term infant immunization schedules, prior to completion of HBV booster immunization. While in adult mice, the (BCG + HBV) group demonstrated higher Ab titers, the difference relative to HBV alone did not reach statistical significance. A surprising finding of this study was the observation that in the DOL0 mouse the separate injection of BCG and HBV compared to a combined injection in the same age group was the potent route of administration whereas in both the DOL7 mouse as well as in the adult mouse, the combined injection was more effective than the separate administration. This observation could reflect age-specific immunity and deserves further investigation. In summary, our study demonstrates that (BCG + HBV) synergistically induced IL-1β *in vitro* and enhanced neonatal anti-rHBsAg-specific Ab titers at an early stage post prime and first booster immunization *in vivo*. Given evidence in mice and humans that IL-1β production enhances the magnitude of HBV-induced Ab responses (74–76), we speculate that robust (BCG + HBV)-induced inflammasome activation may contribute to the observed enhancement in HBV immunogenicity.

Our study features many strengths including, to our knowledge, multiple novel aspects: (a) an age-specific approach to characterizing vaccine–vaccine interactions including study of preterm humans and newborn DOL0 mice, reflecting aspects of preterm humans who represent ~11% of all global live births and are particularly susceptible to infection (3); (b) human *in vitro* modeling of (BCG + HBV) effects, in a way that reflects immune ontological differences present in these age groups *in vivo*; (c) evaluating the impact of vaccine–vaccine interactions on innate cytokine induction using mathematical and bioinformatic approaches, and (d) characterizing age- and administration- (e.g., combined vs. separate injections) specific BCG-HBV interactions in newborn mice *in vivo*.

Our study also has a number of limitations. With regard to our *in vitro* systems, although providing potentially valuable human data that have predicted adjuvantation effects *in vivo* (13), they may not optimally reflect vaccine effects *in vivo.* The use of a whole blood assay aims to reflect *in vivo* conditions including differences in cell quantity and immunphenotype between the neonate and the adult. Consequently, whole blood data are limited with regard to the ability to ascribe cytokine differences to single cell function or cell composition (78). Moreover, there are differences in the functionality and composition of mononuclear cells from adult individuals and mononuclear cells derived from neonatal cord blood (e.g., more predominantly lymphocytic) (78). Potential confounders in the use of preterm cord blood include maternal disease such as preeclampsia, a disease not captured in our collection of data for the preterm cord blood samples. We were able to limit the effects of steroid exposure on

our sample collection for a group of preterm cord blood samples as steroids were known to have been given over 48 h prior to cord blood collection (**Table 1**). We also recognize that attention must by paid to the type of anti-coagulant used for peripheral or cord blood collections as the type of cytokine used can affect cytokine production. We chose pyrogen-free anti-coagulant heparin sodium because it is certified to be endotoxin-free. Future studies may need to compare the results obtained with other methods of anti-coagulation, such as EDTA. Our *in vivo* studies feature distinct species (mouse) and route of administration (subcutaneous) from human newborns (intradermal). Indeed, we were not able to demonstrate higher primary anti-HBV Ab responses in our neonatal mice relative to our adult mice as has been previously demonstrated in humans (55). Nevertheless, the cogent pattern of enhanced age-dependent HBV responses in the presence of BCG, mirroring those observed in some clinical cohorts (20) suggests that our data may be relevant to the effects of these vaccines in human newborns *in vivo*.

Future work will be necessary to elucidate the immunological mechanisms involved in the BCG adjuvantation phenomenon described here, and hence enable design of a new generation of vaccines that recapitulate desirable features of the live vaccine BCG as (a) a single dose effectiveness and (b) induction of both adaptive and trained immunity. At this point, it is unclear whether the observed BCG-driven phenomena relate mechanistically to "heterologous" effects that could be mediated by trained immunity (79). In addition to informing optimization of the use of BCG vaccine together with other vaccines, characterizing BCG-induced enhancement of Ab titers in response to unrelated vaccines may inform development of "BCG-like" adjuvantation systems (12). Furthermore, of importance to global health, these findings support the hypothesis, that in the appropriate context in countries in which neonatal immunization with BCG is recommended, concurrent administration of (BCG + HBV) at birth to the moderate to late preterm and term newborn may enhance the protective response to HBV immunization. Of note, in relatively small preterm studies thus far, BCG has been immunogenic and safe when administered to the moderately to late preterm infant [31–33 weeks GA] (80). Further studies of the safety, efficacy and mechanism of action of the combination of (BCG + HBV) compared to each alone in newborn animals, including humans, will shed further light into this important area crucial to the protection of the most vulnerable among us.

ETHICS STATEMENT

Local institutional review boards at The Brigham and Women's Hospital (Protocol #2000P000117/BWH) and the Beth Israel Deaconess Medical Center (Protocol #2011P-000118/BIDMC) have approved the cord blood collection protocols. Patient information concerning the collected cord blood samples was collected in a de-identified manner as approved by the onsite IRB. Peripheral blood draws were conducted at Boston Children's Hospital (BCH) from healthy adult volunteers, employed at BCH after written informed consent with approval from the Ethics Committee of BCH (protocol number X07-05-0223). All experiments involving animals were approved by the Animal Care and Use Committee of BCH and Harvard Medical School (protocol numbers 15-11-3011 and 16-02-3130).

AUTHOR CONTRIBUTIONS

AS, FB, DD, and OL designed the study. AS, DD, IB, and HC collected cord blood samples. AS, DD, MP, SJ, NL, and IB conducted the *in vitro* experiments. AS, FB, and CP conducted the *in vivo* experiments. JD-A performed the RNA data analysis. TK shared knowledge in the design of whole blood assays. AS and FB wrote the manuscript. DD and OL provided overall mentorship and assisted in writing and editing the manuscript. FB, CP, JD-A, MP, HC, SJ, NL, IB, and TK contributed to helpful discussions and review of the final manuscript. All the authors have given final approval for the version submitted for publication.

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Identification and Characterization of Stimulator of Interferon Genes As a Robust Adjuvant Target for Early Life Immunization

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Immunization is key to preventing infectious diseases, a leading cause of death early in life. However, due to age-specific immunity, vaccines often demonstrate reduced efficacy in newborns and young infants as compared to adults. Here, we combined *in vitro* and *in vivo* approaches to identify adjuvant candidates for early life immunization. We employed newborn and adult bone marrow-derived dendritic cells (BMDCs) to perform a screening of pattern recognition receptor agonists and found that the stimulator of interferon genes ligand 2′3′-cGAMP (hereafter cGAMP) induces a comparable expression of surface maturation markers in newborn and adult BMDCs. Then, we utilized the trivalent recombinant hemagglutinin (rHA) influenza vaccine, Flublok, as a model antigen to investigate the role of cGAMP in adult and early life immunization. cGAMP adjuvantation alone could increase rHA-specific antibody titers in adult but not newborn mice. Remarkably, as compared to alum or cGAMP alone, immunization with cGAMP formulated with alum (Alhydrogel) enhanced newborn rHA-specific IgG2a/c titers ~400-fold, an antibody subclass associated with the development of IFNγ-driven type 1 immunity *in vivo* and endowed with higher effector functions, by 42 days of life. Highlighting the amenability for successful vaccine formulation and delivery, we next confirmed that cGAMP adsorbs onto alum *in vitro*. Accordingly, immunization early in life with (cGAMP+alum) promoted IFNγ production by $CD4^+$ T cells and increased the proportions and absolute numbers of $CD4^+$ $CXCR5^+$ $PD\text{-}1^+$ T follicular helper and germinal center (GC) $GL\text{-}7^+$ $CD138^+$ B cells, suggesting an enhancement of the GC reaction. Adjuvantation effects were apparently specific for IgG2a/c isotype switching without effect on antibody affinity maturation, as there was no effect on rHA-specific IgG avidity. Overall, our studies suggest that cGAMP when formulated with alum may represent an effective adjuvantation system to foster humoral and cellular aspects of type 1 immunity for early life immunization.

Keywords: vaccines, adjuvants, newborn, antigen-presenting cells, germinal centers, T follicular helper cells, antibodies, stimulator of interferon genes

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INTRODUCTION

Infectious diseases represent a major cause of morbidity and mortality in neonates and young infants (1, 2). For example, each year in the US ~20,000 children <5 years old are hospitalized due to influenza complications and flu-related death may occur, especially among those with underlying chronic illness (3). Immunization strategies are fundamental to prevent infectious diseases. However, due to age-specific immunity, vaccines often demonstrate reduced efficacy in newborns and young infants compared to adults (4, 5). Newborn innate immune cells exhibit distinct activation profiles in response to pattern recognition receptor (PRR) agonists (6, 7), and only certain PRR agonists (e.g., TLR7/8 agonists) (8–14) or their combinations (15, 16) are able to induce an adult-like response. The newborn adaptive immune compartment presents distinct features that may also limit vaccine efficacy. Neonatal B cells can produce immunoregulatory cytokines (e.g., IL-10) (17–20), and the magnitude and persistence of the antibody response are reduced (21). Several mechanisms may contribute to distinct immunity in early life, including distinct activity of B and plasma cells (22, 23), the presence of maternal antibodies, impaired $CD4^+$ $CXCR5^+$ $PD\text{-}1^+$ T follicular helper (Tfh) cell differentiation and lymph node germinal center (GC) reaction (24, 25) that may adequately support the antigen-specific B cell response. Moreover, neonatal $CD4^+$ T cells produce lower amounts of IFNγ and are skewed toward Th2, Th17, and Treg polarization (6, 7). Of note, adjuvants exhibit age-specific patterns of Th-polarization (16) such that adjuvantation systems that boost adult immune responses do not necessarily lead to enhanced vaccine efficacy in newborns or young infants (26). Therefore, identification of vaccine adjuvants capable of activating neonatal and infant immune responses may inform development of adjuvanted vaccine formulations that enhance early life immunization (8, 9).

Dendritic cells (DCs) play a pivotal role in activating T cells and instructing the adaptive immune response. They express a high diversity of PRRs, whose activation leads to DC migration to lymph nodes and enhancement of immune-stimulatory functions (27). Recently, a systems vaccinology analysis of young infants vaccinated with trivalent inactivated influenza vaccine with or without the oil-in-water adjuvant MF59 demonstrated that innate immune gene signatures (e.g., antiviral and DC genes) 1 day post-immunization correlated with vaccine efficacy, highlighting the importance of robust innate immune activation in early life immunization (28). Agonists of the intracellular receptors TLR7/8, that recognize viral single-stranded RNAs, potently activate Th1-polarizing responses, including expression of interferons (IFNs), production of IL-12p70 and upregulation of co-stimulatory molecules in newborn DCs *in vitro* and enhance vaccine efficacy in newborn non-human primates *in vivo* (8–14). Moreover, adjuvantation with the TLR9 agonist CpG increases CG Tfh and B cell responses in newborn mice (25). Among intracellular PRRs, the stimulator of interferon genes (STING) is an amenable target for adjuvant discovery and development (29, 30). It binds cyclic dinucleotides (CDNs) derived from bacteria (i.e., c-di-AMP, c-di-GMP, and 3′3′-cGAMP) or synthesized in mammalian cells by cGAMP synthase in response to double-stranded DNA in the cytoplasm (i.e., 2′3′-cGAMP). Upon activation, STING induces the TBK-1-mediated phosphorylation of IRF3, which in turn modulates the expression of type I IFNs, IFN-stimulated genes, and also promotes DC maturation and type 1 (i.e., IFNγ-driven) immunity (31). Accordingly, STING agonists have demonstrated promising adjuvanticity in adult experimental models of parenteral and mucosal immunization as well as cancer immunotherapy (32–49). However, to our knowledge, STING has not yet been investigated as an adjuvant target for early life immunization.

Here, we took an unbiased approach to identify PRR-based agonists for early life immunization. We employed adult and neonatal bone marrow-derived DCs (BMDCs) to screen the activity of a comprehensive panel of PRR agonists and adjuvants, and found that the STING ligand 2′3′-cGAMP is a potent activator of newborn BMDCs. Strikingly, we found that 2′3′-cGAMP formulated with alum induces antibody isotype switching toward IgG2a/c, a subclass endowed with higher effector functions, appears to enhance the GC reaction and also promotes Th1 polarization in immunized newborn mice. Altogether, our study supports the use of STING ligands and their formulations for enhancement of early life immunization.

MATERIALS AND METHODS

Ethics Statements

All experiments involving animals were approved by the Animal Care and Use Committee of Boston Children's Hospital and Harvard Medical School (protocol numbers 15-11-3011 and 16-02-3130).

Animals

C57BL/6 and BALB/c mice were obtained from Taconic Biosciences or Charles River Laboratories and housed in specific pathogen-free conditions in the animal research facilities at Boston Children's Hospital. For breeding purposes, mice were housed in couples, and cages checked daily to assess pregnancy status of dams and/or the presence of pups. When a new litter was discovered, that day was recorded as day of life (DOL) 0. Both male and female pups were used for experiments.

Generation of Neonatal and Adult Murine Bone Marrow-Derived Dendritic Cells (BMDCs)

BMDCs were generated from newborn (5–7 days old) and adult (6–12 weeks old) C57BL/6 mice with an adaptation of previously described methods (50, 51). Briefly, mice were sacrificed and legs removed; bones were surgically cleaned from surrounding tissue, extremities of tibiae and femurs were trimmed with sterile scissors and bone marrow flushed through a 70-μm nylon mesh strainer (Corning Life Sciences). Cell number and viability was determined by trypan blue exclusion. Whole bone marrow cells were plated into non-tissue culture-treated 100 mm Petri dishes (Corning Life Sciences) at a density of 0.3×10^6 cells/ml in 10 ml total volume/plate of complete culture medium (RPMI 1640 plus 10% heat-inactivated fetal bovine serum [FBS, GE Healthcare

HyClone], 50 μM 2-mercaptoethanol, 2 mM L-glutamine, 100 U/ml penicillin/streptomycin [Gibco ThermoFisher Scientific]) supplemented with 20 ng/ml of recombinant murine GM-CSF (rmGM-CSF, R&D systems). Plates were incubated in humidified atmosphere at 37°C, 5% CO_2 for 6 days, with one supplement of 10 ml of complete culture medium and rmGM-CSF on day 3. On day 6, non-adherent and loosely adherent cells were harvested by washing the plate gently with culture medium. Adherent cells were discarded. For flow cytometry analysis, BMDCs were stained (20 min at 4°C) in PBS + FBS 2% + EDTA 2 mM, fixed with formaldehyde 4% [10 min at room temperature (RT)] and acquired on a BD LSRFortessa flow cytometer (BD Biosciences) or a Sony spectral analyzer SP6800 (Sony Biotechnology) and data were analyzed using FlowJo v.10 software (Tree Star). For a complete list of antibodies and fluorochromes used in the study, see Table S1 in Supplementary Material.

PRRs Agonists, Adjuvants, and BMDC *In Vitro* Stimulation

Rough (*Salmonella Minnesota*, R595) and smooth (*Escherichia coli*, O55:B5) lipopolysaccharide (LPS) were purchased from List Biological Laboratories. Aluminum hydroxide (Alhydrogel) and Aluminum phosphate (Adju-phos) were purchased from Brenntag Biosector. All remaining PRR agonists and adjuvants, as indicated in Table S2 in Supplementary Material, were purchased from Invivogen. All PRR agonists employed in the studies were chosen based on and verified endotoxin free as indicated by the manufacturers. For stimulation experiments, immature BMDCs generated from newborn and adult mice were plated in round bottom 96-wells non-tissue culture-treated plates at the density of 10^5 cells/well in 200 μl of fresh complete culture medium with rmGM-CSF as described above, with the appropriate stimuli at the concentrations indicated in Table S2 in Supplementary Material. Cells were incubated at 37°C for 20–24 h, then supernatant harvested and TNF, IL-6, IL-1β, and IL-12p70 concentrations were measured by ELISA (R&D Systems). IFNβ was measured with a bioluminescent ELISA kit (LumiKine, Invivogen). Alternatively, BMDCs were stained and analyzed by flow cytometry as indicated above. For experiments involving blocking antibodies, BMDCs were pre-incubated for 20 min at 37°C with anti-mouse IFNAR1 (clone MAR1-5A3, 10 μg/ml, Biolegend) or anti-mouse TNF (clone MP6-XT22, 10 μg/ml, Biolegend) antibodies or an isotype control before stimulation.

Antigens, Immunization, and Antibody Quantification

Both neonate and adult mice were immunized intramuscularly (i.m.) in the right posterior thigh with 50 μl of the 2016–2017 formulation of the FluBlok vaccine (Protein Sciences Corp.) containing 0.33 μg of each of the following recombinant influenza virus hemagglutinins (rHA): A/Michigan/45/2015 (H1N1), A/Hong Kong/4801/2014 (H3N2), and B/Brisbane/60/2008. Mice were immunized with a single dose at DOL 7 or a prime-boost schedule (two injections 1 week apart, for newborn mice at DOL 7 and 14). As indicated for specific experimental groups, the vaccine was formulated with Aluminum hydroxide (100 μg, hereafter "alum") with or without 2′3′-cGAMP (10 μg). Serum was collected at the indicated intervals for antibody detection. rHA-specific IgG, IgG1, IgG2c (for C57BL/6 mice), and IgG2a (for BALB/c mice) antibodies were quantified by ELISA. High binding flat bottom 96-well plates (Corning Life Sciences) were coated with 1 μg/ml rHA in carbonate buffer pH 9.6, incubated overnight at 4°C and blocked with PBS + BSA 1% (Sigma-Aldrich) for 1 h at RT. Then, sera from vaccinated mice were added with an initial dilution of 1:100 and 1:4 serial dilutions in PBS + BSA 1% and incubated for 2 h at RT. Plates were then washed and incubated for 1 h at RT with HRP-conjugated anti-mouse IgG, IgG1, IgG2c, or IgG2a (Southern Biotech). At the end of the incubation, plates were washed again and developed with tetramethylbenzidine (BD Biosciences) for 5 min, then stopped with 2 N H_2SO_4. The optical density was read at 450 nm Versamax microplate reader with SoftMax Pro Version 5 (both from Molecular Devices) and endpoint titers were calculated using as cutoff three times the optical density of the background.

For assessing antibody avidity, plates were incubated 15 min with ammonium thiocyanate 0.5 M before the addition of HRP-conjugated anti-mouse IgG antibodies. Avidity was expressed as the $LogEC_{50}$ ratio of corresponding plates treated with or without ammonium thiocyanate.

Quantification of 2′3′-cGAMP Adsorption onto Alum

To quantify the extent of 2′3′-cGAMP adsorption to aluminum hydroxide (Alhydrogel) we mixed 100 μg/100 μl of 2′3′-cGAMP with 1000 μg/100 μl of alum (a 1:10 cGAMP:alum mass ratio) plus 300 μl of 0.9% saline. After vortexing for 10 s the sample was placed in a 37°C incubator. Every 15 min, the sample was vortexed for an additional 5 s and placed back into the incubator. Aliquots were taken at t = 0.25, 0.5, 1, 2, 4 and 24 h and centrifuged at 3,000 RPM (rcf = 664 g) to separate the alum from the supernatant. Supernatant was immediately removed and placed into an autosampler vial undiluted for analysis by reverse-phase high-performance liquid chromatography (RP-HPLC) to determine adsorption as a function of time. RP-HPLC samples were run on a Waters 2695 HPLC equipped with a 2996 photodiode array detector at a wavelength of 254 nm. A gradient was performed using a two mobile phase system of 0.1% trifluoroacetic acid in water and 0.1% trifluoroacetic acid in acetonitrile, on an Agilent Zorbax Eclipse Plus C18, 4.6 × 150 mm, 5 μm column at 25°C. The response (peak area) of the samples were compared against a 50 μl 2′3′-cGAMP plus 200 μl 0.9% saline control and a separate 100 μl alum plus 400 μl saline control.

In Vitro Restimulation of rHA-Specific T Cell Responses

Splenocytes from immunized mice were harvested 10 days post-boost (DOL 24) as previously reported (25, 52, 53) and re-stimulated *in vitro* to assess cytokine production by flow cytometry. Spleens were mashed through a 70 μM strainer, washed with PBS, and erythrocytes were lysed with 2 min of incubation in ammonium chloride-based lysis buffer (BD Biosciences). Cells were then counted and plated 2 × 10^6 per well (round bottom

96-well plate) in 200 µl of complete culture medium with or without rHA 10 µg/ml or rHA 10 µg/ml + anti-mouse CD28 2 µg/ml (BioLegend). Plates were incubated for 18 h at 37°C with the addition of Brefeldin A (BD Biosciences) for the last 6 h. Cells were stained against for surface antigens in (PBS + BSA 0.2% + NaN_3 0.05%) for 20 min at 4°C, then fixed with formalin 2% (10 min at RT) and permeabilized with intracellular staining permeabilization wash buffer (BioLegend) for 20 min at 4°C. Finally, cells were stained with conjugated antibodies against IFNγ, IL- 2, IL-4, and IL-17. Data were acquired on a BD LSRFortessa flow cytometer (BD Biosciences) and analyzed using FlowJo v.10 software (Tree Star). For a complete list of antibodies and fluorochromes used in the study, see Table S1 in Supplementary Material.

Analysis of the GC Reaction

Draining (inguinal) lymph nodes (dLNs) from immunized mice were harvested 10 days post-boost (DOL 24) as previously reported (25, 52, 53). To prepare a single-cell suspension, dLNs were pressed using the plunger end of a syringe. Then, cells were washed and stained with the following antibodies: for GC Tfh cells, anti-CD45, anti-B220, anti-CD3, anti-CD4, anti-programmed death-1 (CD279 or PD-1), anti-CXCR5; for GC B cells, anti-CD45, anti-B220, anti-CD3, anti-GL7, and anti-Syndecan-1 (CD138) (all from BioLegend). GC Tfh cells were defined as viable singlet $CD45^+$ $B220^-$ $CD3^+$ $CD4^+$ $CXCR5^+$ $PD\text{-}1^+$ cells. GC B cells were defined as viable singlet $CD45^+$ $B220^+$ $CD3^-$ $CD138^-$ $GL\text{-}7^+$. Cells were acquired on a BD LSRFortessa (BD Biosciences) and data were analyzed using FlowJo v.10 software (Tree Star). Absolute number of cell subsets were determined using CountBright Absolute Counting Beads (ThermoFisher Scientific). For a complete list of antibodies and fluorochromes used in the study, see Table S1 in Supplementary Material.

IFNγ ELISPOT

Draining lymph nodes from immunized mice were harvested 3 days post-boost (DOL 17). Nitrocellulose 96-microwell plates (Millipore) were coated with 75 µl/well of anti-mouse IFNγ (10 µg/ml in PBS, clone R4-6A2, BD Pharmingen) overnight at 4°C, washed twice with wash buffer (PBS + Tween-20 0.05%) and once with distilled water. Wells were blocked with 200 µl of complete culture medium for 2 h at RT. Single-cell suspensions of dLNs in complete culture medium supplemented with recombinant mouse IL-2 (5 ng/ml, PeproTech) were added to the wells in the presence or absence of 10 µg/ml of Flublok and 2 µg/ml anti-mouse CD28 (Biolegend) and cultured for 18 h. Wells were then washed and incubated with 100 ml of biotinylated anti-mouse IFNγ (5 µg/ml in PBS + FBS 10%, clone XMG1.2, BD Pharmingen) for 2 h at RT, washed again and incubated with 100µl of streptavidin-alkaline phosphatase (1:1000 dilution in PBS + FBS 10%, MabTech) for 1 h prior to color development using BCIP/NBT substrate (Biorad) as per manufacturer's protocol. Spots on air-dried plates were counted on an ImmunoSpot Analyzer.

Statistical Analyses and Graphics

Data were analyzed and graphed using Prism for MacIntosh v. 7.0 (GraphPad Software). Tests used for statistical comparisons are indicated in figure legends. p-value <0.05 was considered significant.

RESULTS

Phenotypic and Functional Characterization of Neonatal BMDCs

Murine BMDCs represent a widely used model to study DC function *in vitro*. Adult BMDCs represent a heterogeneous population composed of $CD11c^+$ macrophage-like and DC-like cells with distinct phenotypic and functional profiles (54). However, murine neonatal BMDCs have never been characterized in depth. Therefore, we first sought to define the phenotypic and functional properties of neonatal BMDCs. Although the cell yield from neonatal bone marrow was lower compared to adult ones (Figures S1A,B in Supplementary Material), neonatal immature BMDCs generated from 7-day-old mice grew in culture similarly to adult cells (Figure S1C in Supplementary Material), and once fully differentiated they expressed similar levels of CD11c compared to adult cells but significantly lower levels of MHCII (Figures S1D,E in Supplementary Material). To further characterize phenotypic differences between newborn and adult BMDCs, we assessed by flow cytometry the expression of different macrophage and DC markers. As previously reported for adult BMDCs (54), neonatal BMDCs also comprised $CD11c^+$ MHCII-low and $CD11c^+$ MHCII-high cells. Of note, the percentage of MHCII-low cells was higher in neonatal BMDCs compared to adult BMDCs. Neonatal MHCII-low BMDCs also expressed higher levels macrophage-associated markers (CD64, CD115, CD11b, F4/80) compared to MHCII-high BMDCs, while this population expressed higher levels of CD117. No significant differences in surface marker expression were found between corresponding neonatal and adult MHCII-high and -low populations, except for neonatal MHCII-low BMDCs that expressed higher levels of F4/80 and neonatal MHCII-high BMDCs that expressed higher levels of CD117 compared to their adult counterparts (Figures S2A,B in Supplementary Material).

To characterize a functional response of newborn BMDCs, we next assessed cytokine production and upregulation of co-stimulatory molecules in response to the TLR4 agonist smooth LPS. While newborn BMDC production of IL-6 and TNF was, respectively, comparable or slightly lower than adult BMDCs, IL-12p70 production, albeit detectable, was markedly reduced compared to adult BMDCs (Figure S3A in Supplementary Material). The latter result might be consistent with a more macrophage-like phenotype of newborn BMDCs. As previously reported, both adult and newborn BMDCs produced IL-1β in response to rough but not smooth LPS (55), with newborn BMDCs producing slightly higher amounts of IL-1β (Figure S3B in Supplementary Material). Finally, newborn BMDCs expressed lower levels of MHCII, CD40, and CD86 in response to smooth LPS (Figures S3C,D in Supplementary Material).

Identification of STING As a Target for Inducing Neonatal BMDC Maturation

Having characterized phenotypic and functional features of neonatal and adult BMDCs, we next assessed their response to a

panel of PRR agonists and adjuvants (Table S2 in Supplementary Material). As readouts we measured cytokine production (TNF, IL-1β, IL-6, and IL-12p70) and surface expression of maturation markers (CD40, CD80, and CD86). At the most effective, non-toxic (as established in preliminary experiments, data not shown) concentration of each agonist (in bold in Table S2), neonatal BMDCs produced similar amounts of TNF, IL-6, and IL-1β compared to adult BMDCs in response to different TLR7/8 agonists, namely R848 (Resiquimod, imidazoquinoline), CL075 (thiazoloquinolone) or CL264 (9-benzyl-8 hydroxyadenine), but again failed to produce IL-12p70 (**Figure 1A**). Remarkably, the upregulation of surface maturation marker expression on neonatal BMDCs was much lower than adult BMDCs upon any PRR stimulation, with the exception of the STING agonist 2′3′-cGAMP (hereafter cGAMP) (**Figure 1B**). To assess in depth the response to STING and TLR7/8 agonists, we stimulated neonatal and adult BMDCs with different concentrations of cGAMP and R848. We confirmed that R848 induced higher production of TNF and IL-12p70 (the latter only in adult BMDCs), while cGAMP was more effective than R848 at upregulating the expression of surface maturation markers (**Figure 1C**). cGAMP also induced dose-dependent IFNβ production in both newborn and adult BMDCs (**Figure 1C**). Of note, the response of neonatal and adult BMDCs to cGAMP was comparable (Figure S4 in Supplementary Material). Using neutralizing antibodies against TNF or type I IFN receptor (IFNAR), we demonstrated that the expression of maturation markers by neonatal BMDCs mostly relies on type I IFN signaling (Figure S5 in Supplementary Material).

cGAMP Formulated with Alum Enhances Anti-rHA IgG2a/c Antibody Titers in an Early Life Immunization Model

The *in vitro* results obtained so far supported further investigation of cGAMP as adjuvant candidate for early life immunization. Therefore, we proceeded to test this hypothesis *in vivo*. We immunized newborn (7-day old) and adult (8- to 10-week old) C57BL/6 mice using a prime-boost schedule (**Figure 2A**) and employing trivalent recombinant hemagglutinin (rHA) influenza vaccine Flublok as clinically relevant model antigen that is devoid of adjuvant, alone, or formulated with alum [Alhydrogel, Al(OH)3], cGAMP or (cGAMP + alum) (**Figure 2B**). Mice were bled 14, 21, 28, and 35 days post-prime (respectively, day of life (DOL) 21, 28, 35, and 42 for newborn mice) to assess the magnitude and kinetic of the antibody response. As expected, both alum and cGAMP increased anti-rHA IgG titers in adult mice. We also investigated the titers of the IgG subclasses IgG1 and IgG2c, respectively associated with type 2 and type 1 (IFNγ-driven) immunity (56, 57). In keeping with previously published data, alum preferentially increased anti-rHA IgG1 titers (median anti-rHA IgG1 titers at Day 35 post-prime: 5.02×10^6 for alum, 0.77×10^6 for cGAMP), while cGAMP was more effective than alum at enhancing anti-rHA IgG2c titers (median anti-rHA IgG2c titers at day 35 post-prime: 0.16×10^6 for alum, 0.82×10^6 for cGAMP). (cGAMP + alum) was as effective as alum at increasing anti-rHA IgG and IgG1 titers [median anti-rHA IgG and IgG1 titers at day 35 post-prime: respectively, 4.77×10^6 and 4.46×10^6 for (cGAMP + alum)], and even more effective than cGAMP alone at enhancing anti-rHA IgG2c titers [median anti-rHA IgG2c titers at day 35 post-prime: 3.27×10^6 for (cGAMP + alum)] (**Figure 2B**, upper panels and Figure S6 in Supplementary Material). In newborn mice, we unexpectedly found that cGAMP was much less effective at increasing anti-rHA IgG, IgG1, and IgG2c titers [median anti-rHA IgG, IgG1, and IgG2c titers at day 35 post-prime (DOL 42): respectively, 20.57×10^3, 24.51×10^3, and 0.23×10^3 for cGAMP]. Alum enhanced anti-rHA IgG and IgG1 titers, but in marked contrast from adult mice it did not induce anti-rHA IgG2c titers [median anti-rHA IgG, IgG1, and IgG2c titers at day 35 post-prime (DOL 42): respectively, 48.35×10^3, 143.23×10^3, and 0.00×10^3 for alum]. Surprisingly, (cGAMP + alum) adjuvantation matched or exceeded alum at increasing anti-rHA IgG and IgG1 titers [median anti-rHA IgG and IgG1 titers at Day 35 post-prime (DOL 42): respectively, 329.19×10^3 and 167.83×10^3 for (cGAMP + alum)], and, remarkably, also induced relatively high titers of anti-rHA IgG2c as early as 14 days post-prime (DOL 21) [median anti-rHA IgG2c titers at day 14 (DOL 21) and day 35 post-prime (DOL 42): respectively, 0.14×10^3 and 4.23×10^3 for (cGAMP + alum)] (**Figure 2B**, lower panels and Figure S7 in Supplementary Material). Therefore, the addition of cGAMP to alum markedly enhanced anti-rHA antibody production (in particular IgG2c), with a more prominent effect in newborn than adult mice (~400 as compared to ~150-fold increase, respectively) (**Figure 2C**). Interestingly, newborn mice immunized at DOL 7 and 14 (as indicated in **Figure 2B**) with (cGAMP + alum) still display the highest anti-rHA IgG and IgG2c titers at DOL 90 compared to saline and alum groups (Figure S8 in Supplementary Material). Enhancement of anti-rHA IgG and IgG2a titers induced by (cGAMP + alum) was also demonstrable in the Th2-skewed mouse strain BALB/c (Figure S9 in Supplementary Material).

In light of the robust adjuvanticity of the (cGAMP + alum) formulation, we quantified cGAMP adsorption to alum by RP-HPLC (**Table 1**). We observed a rapid initial adsorption of cGAMP onto alum (63% of total cGAMP) within 15 min from the incubation. The adsorption rate dropped quickly, with the overall adsorption reaching a plateau (75.33% of total cGAMP) after 24 h of incubation. No significant degradation products were observed over this time window.

Altogether, our *in vivo* results demonstrate that (cGAMP + alum) is an effective formulation to enhance antigen-specific antibody titers (especially of the IgG2a/c subclass) for early life immunization.

(cGAMP + Alum) Fosters Th1 Polarization and GC Reaction

IgG2a/c isotype switching is driven by IFNγ *in vivo* (58), and reduced in early life, since newborns display reduced IFNγ production and Th1 polarization to many stimuli (6, 7). Therefore, we investigated whether (cGAMP + alum) was able to modulate the polarization and cytokine production of antigen-specific T cells. Accordingly, newborn mice were immunized as indicated in **Figure 2A** with alum or (cGAMP + alum). Ten days

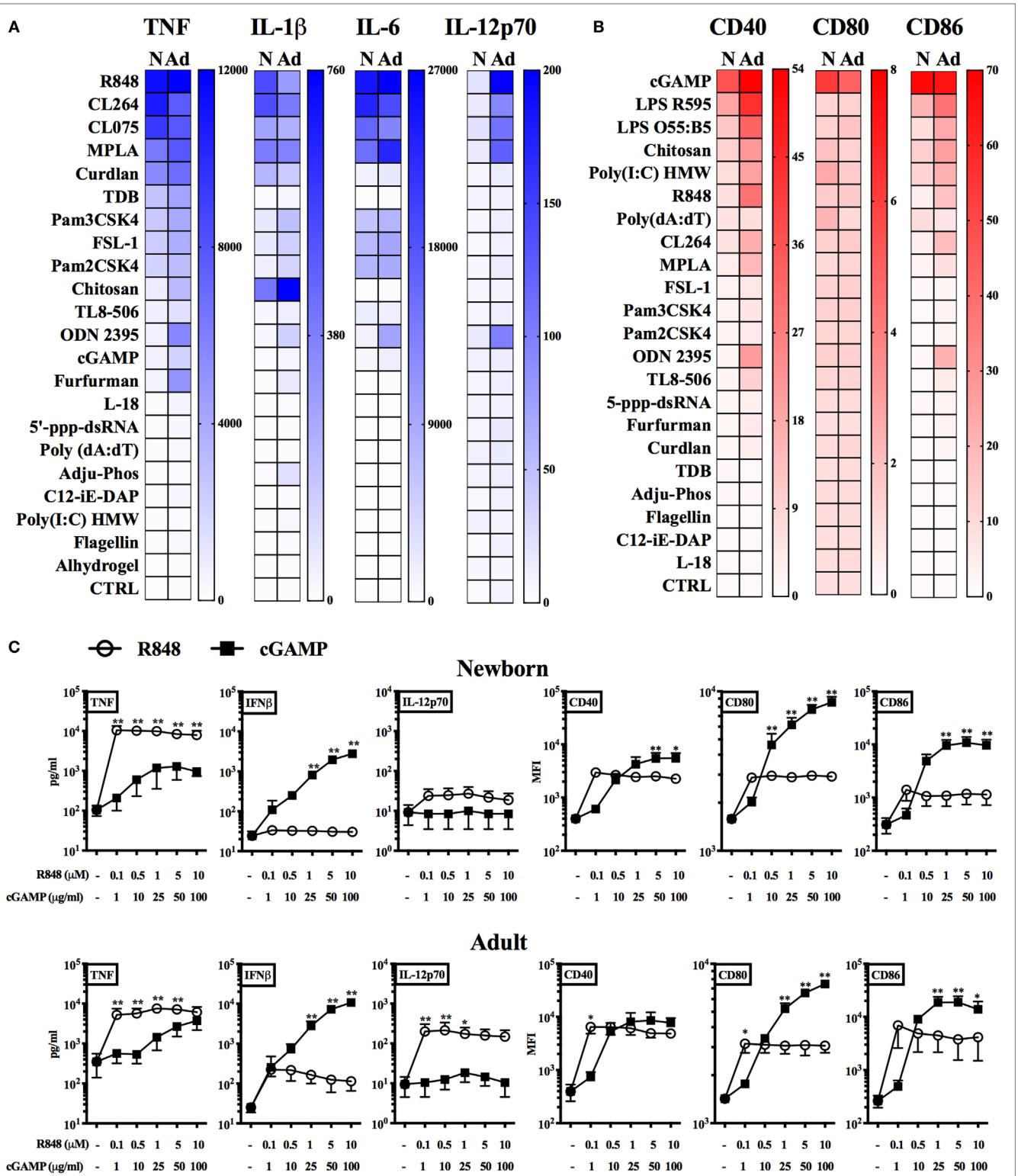

FIGURE 1 | Screening of pattern recognition receptor (PRR) agonists on neonatal and adult BMDCs. **(A–C)** Newborn (N) and adult (Ad) BMDCs were stimulated with the indicated PRR agonists or adjuvants for 20–24 h. Cytokine production **(A,C)** and MFI of surface marker expression **(B,C)** were, respectively, assessed by ELISA and flow cytometry. **(A,B)** Color intensities of the heatmaps are proportional to **(A)** mean cytokine levels (expressed as pg/ml) or **(B)** mean co-stimulatory molecule levels (expressed as fold change of median fluorescence intensity over CTRL) of 5–6 **(A)** or 3 **(B)** independent experiment. **(C)** Results are expressed as mean + SEM of 4–5 (cytokine production) or 3 (surface marker expression) independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ determined by repeated measures two-way ANOVA with Sidak *post hoc* test.

post-boost, splenocytes were harvested, re-stimulated with rHA in the presence or absence of the co-stimulus αCD28, and cytokine production by CD4+ T cells was measured by flow cytometry (**Figure 3A**). While IL-2- and IL-4-producing cells were observed in both groups, IFNγ+ CD4+ T (Th1) cells were only detected among splenocytes isolated from mice immunized

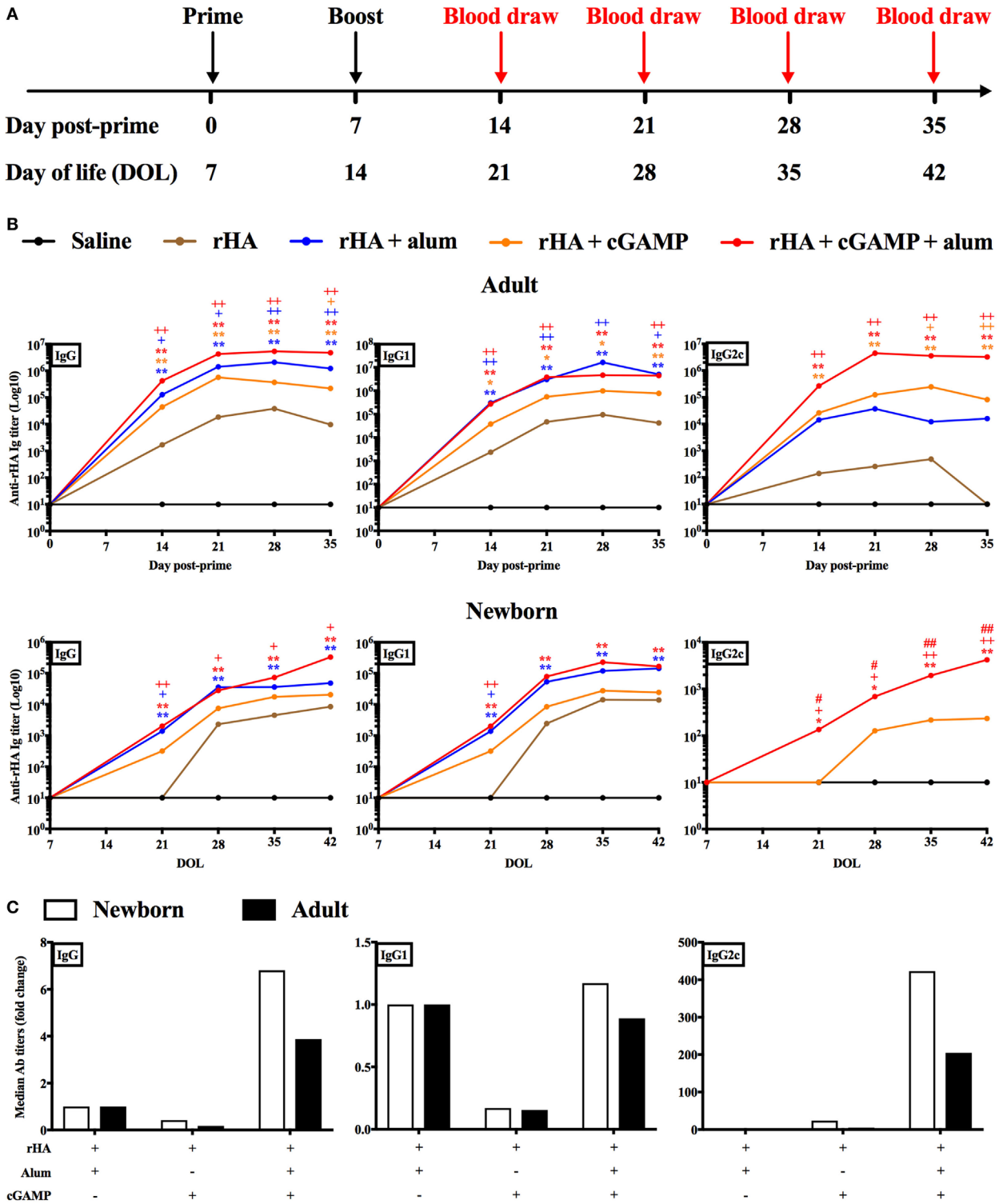

FIGURE 2 | Continued

FIGURE 2 | Immunization with recombinant hemagglutinin (rHA) formulated with cGAMP and alum induces distinct antibody profiles in adult and newborn mice. **(A)** Schematic representation of the immunization schedule for adult (day post-priming is indicated) and newborn [day of life (DOL) is indicated] mice. **(B)** Adult (top) and newborn (bottom) mice were immunized i.m. with saline (black line), rHA (brown line), (rHA + alum) (blue line), (rHA + cGAMP) (orange line) or (rHA + cGAMP + alum) (red line), and antibody titers for rHA-specific IgG, IgG1, and IgG2c were determined by ELISA in serum samples collected at the reported timepoints. **(C)** Fold change of median Ab titers over (rHA + alum) group. White bars, newborn mice. Black bars, adult mice. Results are shown as median of 9–10 (adult) or 7–8 (newborn) mice per group. *, $^{+}$, $^{\#}p < 0.05$, **, $^{++}$, $^{\#\#}p < 0.01$ of groups indicated by the corresponding color, respectively, vs. saline, rHA, and (rHA + alum) groups determined by Kruskal–Wallis with Dunn's *post hoc* test.

TABLE 1 | cGAMP adsorption onto alum as function of time as assessed by RP-HPLC.

cGAMP adsorbed onto alum—incubated at 37°C		
Time point	**Peak area (mAU)**	**% Adsorbed to alum**
15 min	22.53	63.00
30 min	22.93	62.34
1 h	22.22	63.51
2 h	20.83	65.79
4 h	21.02	65.48
24 h	15.02	75.33
Alum control (no cGAMP)	0.53	ND
Saline	0.55	ND

RP-HPLC, reverse-phase high performance liquid chromatography.

with (cGAMP + alum) [median percentages of IFNγ^{+} CD4^{+} T cells upon rHA re-stimulation: 0.000 for saline, 0.031 for alum, and 0.295 for (cGAMP + alum) groups; upon rHA + αCD28 re-stimulation: 0.009 for saline, 0.021 for alum, and 0.280 for (cGAMP + alum) groups]. No IL-17 production was observed in any of the tested conditions (**Figure 3B**). To corroborate this evidence, upon *in vitro* re-stimulation with rHA + αCD28 we found by ELISPOT a higher number of IFNγ-producing cells in the dLNs of mice immunized with (cGAMP + alum) 3 days post-boost (**Figure 4**).

T cell-dependent antibody generation is initiated in GCs and guided by Tfh cells (59, 60). Since GCs are major sites for isotype switching, we reasoned that immunization of newborn mice with (cGAMP + alum) might promote the GC reaction, thereby inducing IgG2a/c switching. To this aim, we assessed by flow cytometry the percentages and absolute numbers of GC Tfh and B cells (respectively, identified as viable singlet CD45^{+} B220^{-} CD3^{+} CD4^{+} CXCR5^{+} PD-1^{+} and CD45^{+} CD3^{-} B220^{+} GL-7^{+} CD138^{-} cells) in dLNs 10 days post-boost of newborn mice immunized with alum or (cGAMP + alum). Interestingly, we found a significant increase in the percentage [median: 0.275 for saline, 0.42 for alum, and 0.925 for (cGAMP + alum)] and absolute number [median: 1,360 for saline, 2,558 for alum, and 5,754 for (cGAMP + alum)] of GC Tfh cells and the percentage [median: 14.4 for saline, 19.7 for alum, and 27.35 for (cGAMP + alum)] and absolute number [median: 10,975 for saline, 19,878 for alum, and 42,524 for (cGAMP + alum)] of GC B cells only in the (cGAMP + alum) group (**Figure 5A**). Immunization with alum induced a small increase in the percentage (but not absolute number) of GC B cells, while only minor modifications of the percentages and absolute numbers of total CD4^{+} T cells and B cells were observed across different immunization groups (**Figures 5A,B**).

The GC is also the site where the processes of somatic hypermutation of antibody variable region genes and generation of high-affinity antibodies take place (60). To verify whether cGAMP modulates antibody affinity maturation, we measured rHA-specific IgG avidity of newborn mice immunized with alum or (cGAMP + alum) as indicated in **Figure 2A**. Although we observed a steep increase in antibody avidity 21 days post prime (DOL 28) which reached a plateau later on [28 (DOL 35) and 35 (DOL 42) days post-prime], no differences between the two groups were detected at any time point (**Figure 6**).

Overall, these results demonstrate that the addition of cGAMP to alum promoted the induction of IFNγ-producing T cells and appeared to foster the GC reaction, which might in turn drive IgG2a/c isotype switching in our early life immunization model.

Single-Dose Immunization with (cGAMP + Alum) Induces rHA-Specific IgG2c Antibodies

The results obtained so far supported the efficacy of (cGAMP + alum) as an adjuvantation system in a prime/boost model of neonatal murine immunization. Of note, a single-dose immunization strategy capable of enhancing antigen-specific antibody titers would be highly desirable early in life. To this end, we immunized newborn mice with rHA formulated with alum, cGAMP, or (cGAMP + alum). Distinct from its effects in prime/boost immunization, cGAMP without alum did not induce detectable anti-rHA IgG, IgG1, and IgG2c titers. Alum and (cGAMP + alum) significantly increased anti-rHA IgG and IgG1 titers [median anti-rHA IgG and IgG1 titers: respectively, 26.74×10^3 and 52.08×10^3 for alum; respectively, 1.07×10^6 and 1.48×10^6 for (cGAMP + alum)]. Interestingly, only (cGAMP + alum) induced detectable levels of anti-rHA IgG2c (median: 571.9), albeit at lower levels compared to prime/boost immunization (**Figure 7**). Altogether, these results demonstrate that (cGAMP + alum) is an effective adjuvantation system also for single dose early life immunization.

DISCUSSION

Over the past decades, many PRRs and their agonists have been identified, and the molecular definition of their mechanisms of action and immunostimulatory properties has paved the way for new classes of adjuvants (26, 61). For example, the TLR4 agonist monophosphoryl lipid A is employed in different FDA-approved vaccine formulations. Despite this wealth of knowledge, the portfolio of adjuvants approved or in clinical development for

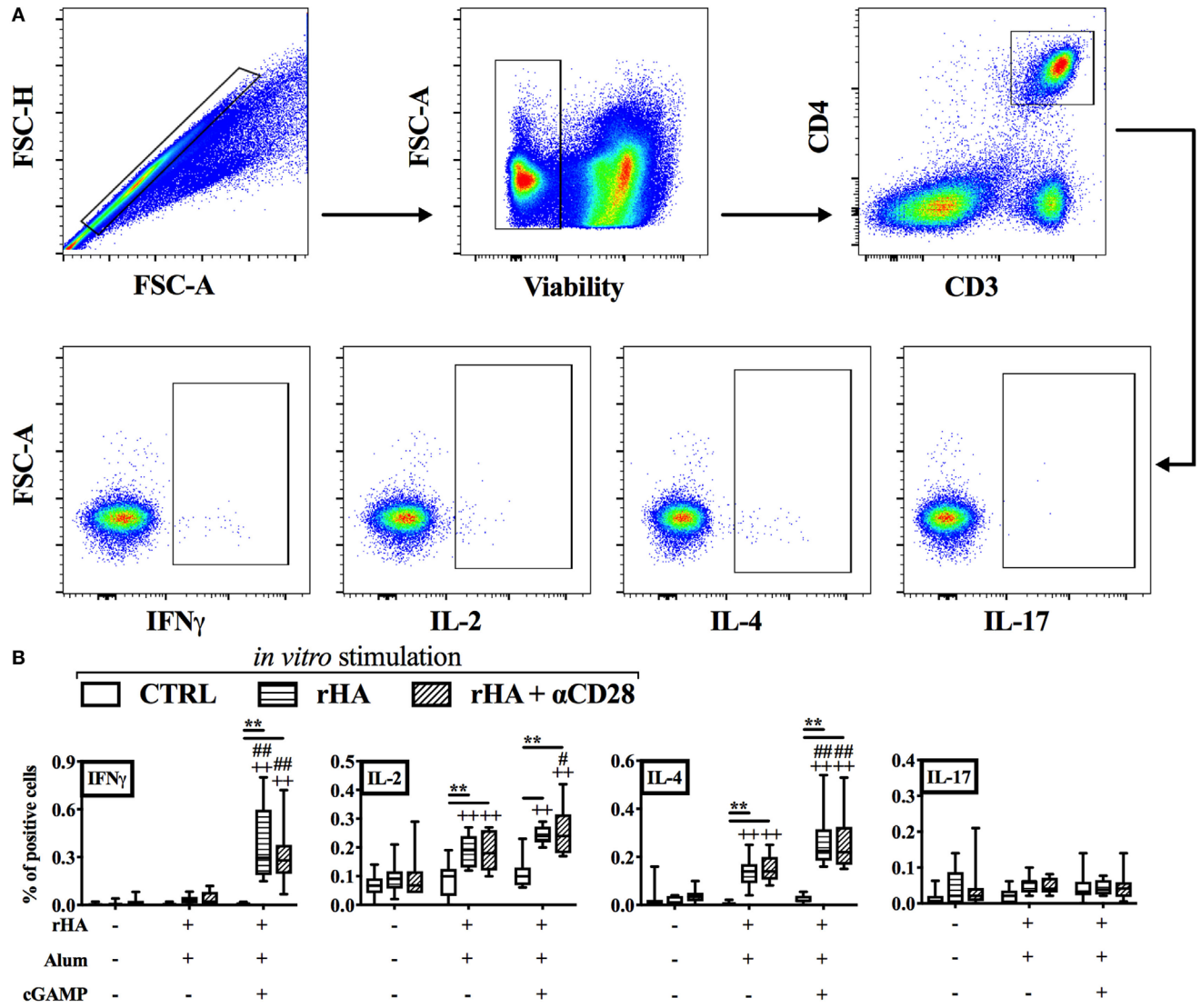

FIGURE 3 | Immunization with (cGAMP + alum) induces Th1 polarization in early life. Newborn mice were immunized with alum or (cGAMP + alum) as indicated in **Figure 2A**. Ten days after boost [day of life (DOL) 24] splenocytes were harvested, re-stimulated for 18 h with recombinant hemagglutinin (rHA) in the presence or absence of the co-stimulus αCD28, and cytokine production by $CD4^+$ T cells was assessed by intracellular flow cytometry. **(A)** Representative gating strategy. $CD4^+$ T cells were defined as viable singlet $CD3^+$ $CD4^+$ cells. **(B)** Results are shown as the median, the 25th and 75th percentiles (boxes) and the 5th and 95th percentiles (whiskers) of 9–10 mice per group. $^{**}p < 0.01$ of *in vitro* CTRL vs. rHA vs. rHA + αCD28, $^{++}p < 0.01$ of respective *in vitro* conditions compared to *in vivo* saline group, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ of respective *in vitro* conditions compared to *in vivo* alum group, determined by two-way ANOVA with Tukey's *post hoc* test.

the newborn and the young infant is much narrower, in part due to our limited knowledge of the immune system early in life (6, 7, 62). Notwithstanding these limitations, *in vitro* and pre-clinical *in vivo* studies have shown that targeting some PRRs, in particular TLR7/8 (8–14), potently activates newborn immune cells and markedly enhances vaccine efficacy early in life. Here, by combining an *in vitro* analysis of newborn BMDC activation in response to PRR agonists and *in vivo* immunization models, we identify the STING agonist cGAMP as adjuvant candidate for early life immunization. In particular, we demonstrate that immunization of newborn mice with cGAMP formulated with alum appears to foster the GC reaction as well as features of IFNγ-driven type 1 immunity, namely switching toward IgG2a/c subclass and Th1 polarization.

Although there is no comprehensive consensus on whether and how *in vitro* models can predict the *in vivo* effect of candidate adjuvants, the use of DCs has some advantages for assessing their activity *in vitro* (6, 27, 63, 64). First, DCs are the most prominent subset of antigen-presenting cells. Second, they express many PRRs. Third, DCs can be employed to recapitulate age-specific differences. Although isolating primary DCs from spleen and lymph nodes of neonatal mice would be ideal, this approach is cumbersome if not impossible due to low cell yield (65, 66). Therefore, we developed and characterized a neonatal BMDC

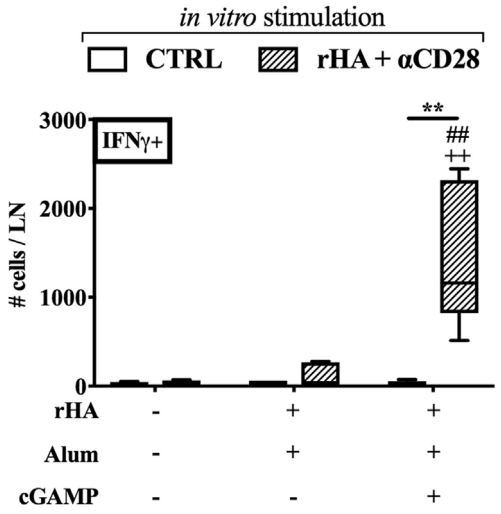

FIGURE 4 | Immunization with (cGAMP + alum) induces IFNγ-producing cells in draining lymph nodes (dLNs) of newborn mice. Newborn mice were immunized with alum or (cGAMP + alum) as indicated in **Figure 2A**. 3 days after boost [day of life (DOL) 17] cells were isolated from dLNs, re-stimulated for 18 h with rHA + αCD28, and the number of IFNγ-producing cells per LN was assessed by ELISPOT. Results are shown as the median, the 25th and 75th percentiles (boxes) and the fifth and 95th percentiles (whiskers) of 4–5 mice per group. $^{**}p < 0.01$ of *in vitro* CTRL vs. rHA + αCD28, $^{++}p < 0.01$ of respective *in vitro* conditions compared to *in vivo* saline group, $^{\#\#}p < 0.01$ of respective *in vitro* conditions compared to *in vivo* alum group, determined by two-way ANOVA with Sidak's *post hoc* test.

model, and found phenotypic and functional differences between neonatal and adult BMDCs. Most importantly, by comparing the activation profiles of neonatal and adult BMDCs we identify that the STING agonist cGAMP induces their maturation (e.g., upregulation of CD40, CD80, and CD86). Although we proceeded in assessing the *in vivo* adjuvant activity of cGAMP, we cannot exclude that other PRR ligands that did not activate newborn BMDCs *in vitro* might act as adjuvants *in vivo*. Therefore, further studies, especially of combination formulations, are required to define the predictive value of the *in vitro* newborn BMDC system.

Cyclic dinucleotides including cGAMP have been tested as candidate adjuvants in experimental models of parenteral or mucosal adult immunization (32–49). In the present work, mice were immunized by the intramuscular route as it is commonly employed for pediatric vaccines: a new formulation specific for intramuscular injection may fit easily with other vaccines in the pediatric vaccination schedule, while intranasal immunization against influenza virus, for example, is currently not recommended by the CDC (67). We found that free cGAMP, simply injected together with the model antigen, is much less effective in newborn than in adult mice at increasing antigen-specific antibody titers. Remarkably, cGAMP formulated with alum induces relatively high titers of antigen-specific IgG2a/c compared to alum or cGAMP alone, especially in newborn mice immunized with prime/boost or single dose schedules. The explanation for this might be that about 60% of cGAMP adsorbs onto alum *in vitro*, which also suggests that there is still the possibility of further optimizing this formulation and increasing the percentage of adsorbed cGAMP by modification of the adsorption pH, buffer, and alum to cGAMP ratio. Interestingly, it has already been reported that CDNs tend to diffuse in the bloodstream after injection, while their nanoparticle formulations deliver CDNs to the dLNs (40). It is tempting to speculate that the same phenomenon might explain the differences in the efficacy between cGAMP and (cGAMP + alum). In addition, it will be interesting to compare the effect of optimized (cGAMP + alum) and nanoparticle-based cGAMP formulations in our early life immunization model.

Newborns and young infants have a distinct immunity with an impairment of IFNγ-driven type 1 immunity, which in turn leads to reduced vaccine efficacy and higher risk of infections (6, 7). By using (cGAMP + alum) as adjuvantation strategy for early life immunization, we were able to induce cardinal features of type 1 immunity: (1) IFNγ production by antigen-specific $CD4^+$ T cells and (2) relatively high titers of antigen-specific IgG2a/c. As IFNγ promotes isotype switching toward IgG2a/c *in vivo* (58), these two events are likely linked. The importance of inducing this antibody subclass relies in its higher affinity toward Fcγ receptors expressed on myeloid cells, which endows this subclass with greater effector functions (e.g., induction of phagocytosis, complement fixation) that may be important for protecting from infections (56, 57). Our results also suggest that (cGAMP + alum) increases the magnitude of the GC reaction, known to be impaired in early life (24, 25), by inducing higher percentages and absolute numbers of GC Tfh and B cells in dLNs. Although we cannot exclude that the GC reaction induced by alum follows a different kinetics, these results might represent the cellular correlate of the isotype switching and early IgG2a/c production observed in the (cGAMP + alum) group. Altogether, our data point to a relevant effect of the (cGAMP + alum) formulation on the humoral and cellular immune responses elicited upon immunization early in life.

Overall, our study features several strengths, including (a) the first immunophenotypic characterization of murine neonatal BMDCs, (b) an unbiased screening of PRR agonists for activity toward neonatal BMDCs, and (c) identification of a novel adjuvantation system active *in vitro* and *in vivo* with evidence supporting potential utility in enabling single-dose immunization at birth. Our study also has limitations, including (a) the neonatal BMDC model studied represents a mix of cells generated by treatment with cytokines *in vitro* such that they may not fully reflect *in vivo* biology, (b) the potential effects of (cGAMP + alum) on GCs are intriguing but until such time as they are verified by microscopy are inferential, (c) although our studies demonstrated robust increases in antibody titers and features of type 1 immunity elicited by immunization with (cGAMP + alum), future functional studies (e.g., pathogen challenge) are required to assess the efficacy of this adjuvantation system, and (d) due to species specificity, results in mice may not accurately reflect those in humans.

In conclusion, we demonstrate that cGAMP is a promising and robust adjuvant candidate for early life immunization. We also show that cGAMP formulated with alum potently enhances humoral and cellular aspects of type 1 immunity in early life. Since we employed the rHA influenza vaccine throughout our work,

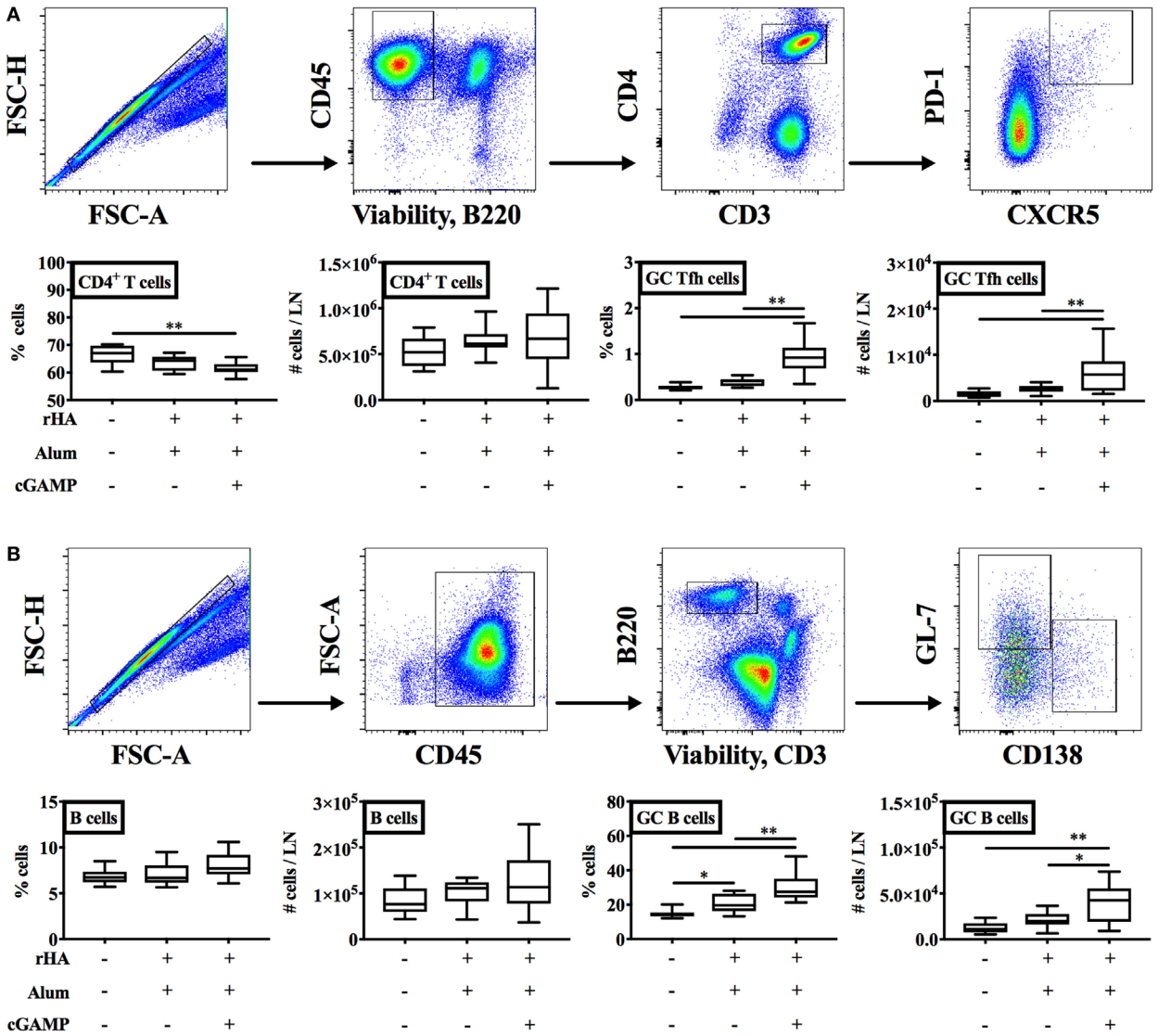

FIGURE 5 | Immunization with (cGAMP + alum) fosters the germinal center (GC) reaction. Newborn mice were immunized with alum or (cGAMP + alum) as indicated in **Figure 2A**. Ten days after boost [day of life (DOL) 24] cells were isolated from draining lymph nodes and the percentages and absolute numbers of $CD4^+$ T cells, B cells, GC Tfh, and B cells were assessed by flow cytometry. [**(A,B)** top panels] Representative gating strategies. $CD4^+$ T cells were defined as viable singlet $CD45^+$ $B220^-$ $CD3^+$ $CD4^+$ cells. GC Tfh cells were defined as viable singlet $CD45^+$ $B220^-$ $CD3^+$ $CD4^+$ $CXCR5^+$ $PD\text{-}1^+$ cells. B cells were defined as viable singlet $CD45^+$ $B220^+$ $CD3^-$ cells. GC B cells were defined as viable singlet $CD45^+$ $B220^+$ $CD3^-$ $GL\text{-}7^+$ $CD138^-$ cells. **(B)** Results are shown as the median, the 25th and 75th percentiles (boxes) and the 5th and 95th percentiles (whiskers) of 9–10 mice per group. $^*p < 0.05$, $^{**}p < 0.01$ determined by one-way ANOVA with Holm–Sidak's *post hoc* test.

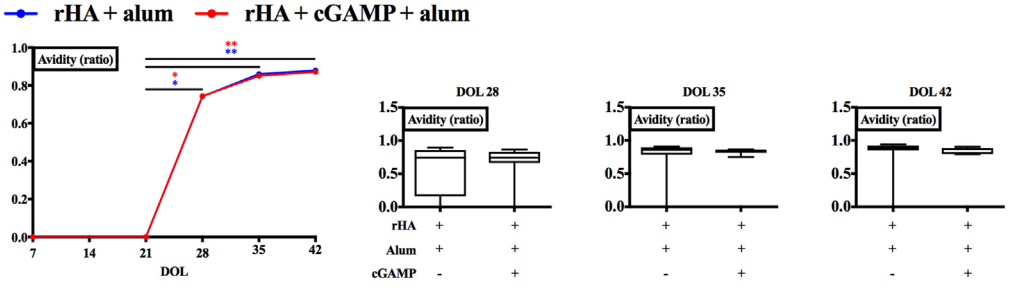

FIGURE 6 | Continued

FIGURE 6 | Immunization with (cGAMP + alum) does not modulate recombinant hemagglutinin (rHA)-specific IgG avidity. Newborn mice were immunized with rHA formulated with alum or (cGAMP + alum) and serum samples were collected as indicated in **Figure 2**. Avidity of rHA-specific IgG was measured by ELISA and expressed as the ratio between the LogEC50 values obtained with and without ammonium thiocyanate treatment (0.5 M). Results are shown as median (left panel) or as the median, the 25th and 75th percentiles (boxes) and the 5th and 95th percentiles (whiskers) (right panels) of 7–8 newborn mice per group. *$p < 0.05$, **$p < 0.01$ determined by two-way ANOVA with Sidak's *post hoc* test (left panel) or Mann-Whitney test (right panels).

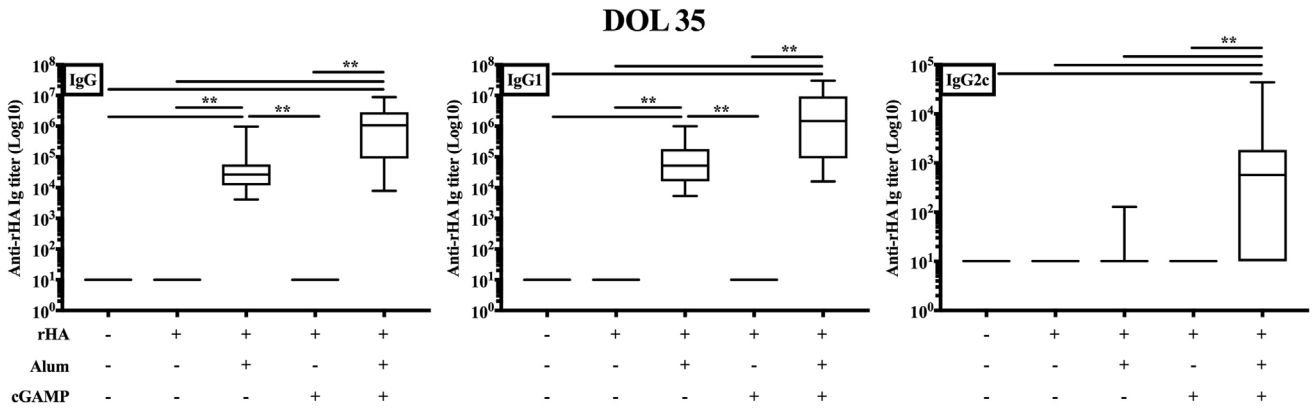

FIGURE 7 | Single-dose immunization of newborn mice with (cGAMP + alum) significantly increases anti-recombinant hemagglutinin (rHA) IgG2c titers. Newborn mice were immunized i.m. with saline, rHA alone or formulated with alum, cGAMP or (cGAMP + alum) and antibody titers for rHA-specific IgG, IgG1, and IgG2c were determined by ELISA in serum samples collected 28 days after boost [day of life (DOL) 35]. Results are shown as the median, the 25th and 75th percentiles (boxes), and the 5th and 95th percentiles (whiskers) of 9–13 mice per group. **$p < 0.01$ determined by Kruskal–Wallis with Dunn's *post hoc* test.

our results may be applicable to influenza immunization. Use of (cGAMP + alum) may also represent a general strategy to elicit type 1 immunity toward protein antigens for early life immunization.

ETHICS STATEMENT

All experiments involving animals were approved by the Animal Care and Use Committee of Boston Children's Hospital and Harvard Medical School (protocol numbers 15-11-3011 and 16-02-3130).

AUTHOR CONTRIBUTIONS

FB, CP, DD, and OL designed the study. CP, DD, and FB conducted the *in vitro* experiments. FB, CP, and JL conducted the *in vivo* experiments. LW and DB conducted the adsorbance experiments. FB and CP wrote the manuscript. DD and OL provided overall mentorship and assisted in writing the manuscript. FB, CP, JL, LW, PS, DO, JR, SB, LP, FM, DB, DD, and OL contributed to helpful discussions, review, and approval of the final manuscript. All the authors have given final approval for the version submitted for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fimmu.2017.01772/full#supplementary-material.

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REVIEW
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They Are What You Eat: Can Nutritional Factors during Gestation and Early Infancy Modulate the Neonatal Immune Response?

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The ontogeny of the human immune system is sensitive to nutrition even in the very early embryo, with both deficiency and excess of macro- and micronutrients being potentially detrimental. Neonates are particularly vulnerable to infectious disease due to the immaturity of the immune system and modulation of nutritional immunity may play a role in this sensitivity. This review examines whether nutrition around the time of conception, throughout pregnancy, and in early neonatal life may impact on the developing infant immune system.

Keywords: nutrients, immunity, ontogeny, neonatal, pregnancy, infection, supplements

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INTRODUCTION

Nearly 3 million deaths occur annually in children less than 30 days old, principally in low and middle-income countries (1). Improvements in neonatal mortality rate have proved difficult to achieve. Low-cost, easily implementable interventions are urgently needed.

Infections directly account for approximately one-third of neonatal deaths and are likely to contribute to deaths from other causes such as prematurity and in cases where babies are stillborn (1). Neonates show heightened susceptibility to infectious diseases due to a functionally immature immune system (2). Innate immune components are compromised by impaired mucosal surface integrity (3), lower levels of complement proteins (4), and reduced phagocytic capacities (5). Adaptive immune responses to pathogens are attenuated compared to adult responses, with children under 2 months old tending toward more regulatory responses with strong Th-2 and Th-17 cell polarization and weak Th-1 polarization (2, 6, 7). This is partly necessary to produce a tolerogenic environment, stopping rejection at the maternofetal interface and reducing reactions to self-antigens, and partly due to lack of primary exposure to antigens necessary to build up the adaptive immune responses. This functional immaturity of responses leaves the neonate particularly vulnerable to infectious pathogens. Decades worth of research has been directed at identifying interventions to improve neonatal immune responses to infections.

Various organs are sensitive to nutrition during embryonic and fetal development. Nutritional status can have short-term impacts on both fetal and childhood growth and development and longer term influences on adult health. Infants born following periods of nutritional deprivation, such as the Dutch Hunger Winter and identified in The Hertfordshire cohort, show increased risks of coronary heart disease, stroke, type-2 diabetes and metabolic syndrome when subsequently exposed to periods of nutrient sufficiency (8, 9). The concept that undernutrition during gestation may contribute to adult disease by having permanent effects on the structure, function and metabolism of

the developing fetus, is known as the Developmental Origins of Health and Disease (DOHaD) theory. It has subsequently been shown to extend to a range of other diseases including psychiatric illnesses and cancers (10). Excess macronutrient consumption in mothers has also been associated with long-term sequelae in their offspring (11). Micronutrient deficiencies have long been known to have impacts on organogenesis, with iodine deficiency leading to congenital hypothyroidism (12) and folate deficiency increasing the risk of neural tube defects (13). Therefore, it has been hypothesized that the developing immune system is likely to be similarly sensitive to nutrition and that optimizing a mother's nutritional state during pregnancy will have long-term benefits for the immune responses during the neonatal period and beyond.

Early human evidence that nutritional factors during gestation might specifically influence adult immune responses came from longitudinal studies carried out in The Gambia in the 1990s (14). The Gambia has a strong bimodal seasonality that has major effects on the nutritional status of the population. The dry season, running from November to June, is a time of relative nutrient security. With the previous seasons crops being harvested, macronutrients are in greater supply and manual labor levels tend to be lower. In contrast, the rainy season, running from July to October, is characterized by declining levels of food availability and higher manual labor demands as the next season's crops are planted but the previous seasons supply is running short. This leads to deficits of both energy and micronutrient intakes that are particularly pronounced for women, who bare the brunt of much of the agricultural work (15). Analysis of demographic surveillance data available for the population from the 1940s revealed that people born during the "hungry" rainy season had a threefold higher risk of mortality from infectious diseases as adults than those born during the dry season (14). These findings were independent of subsequent nutritional status, as demonstrated by anthropometric and hematological status at 18 months of age, suggesting that the effector of these changes occurred earlier on in development. These data suggested that environmental factors, most likely nutrition, during conception, gestation and early postnatal life can have marked effects on the immune system that are stable, durable and not susceptible to modification by later improvements in nutritional status.

Nutrient intake of the mother and neonate is theoretically amenable to modification *via* supplements, which represent low-cost, easily implementable public health interventions. As such, there has been huge interest in the provision of nutritional supplements during gestation and early infancy to improve neonatal outcomes. This review summarizes the evidence regarding the impact of early life nutrition on biochemical immune markers and clinical infectious diseases outcomes in neonates.

POTENTIAL MECHANISMS FOR NUTRITIONAL INFLUENCES ON THE DEVELOPING NEONATAL IMMUNE SYSTEM

Studies in older children and adults have demonstrated the important influence that different nutrients have on the immune system. These effects, and the impacts of deficiencies on susceptibility to infectious diseases, are summarized in **Table 1**. Although the influence of nutrients on the developing immune system *in utero* and in early neonatal life may be similar to that of older children and adults, the impact of the nutritional state of the mother on the neonatal immune system is less well described.

Mother's nutritional status may hypothetically affect the neonatal immune system by influencing:

- *The mother's own immune system:* Optimizing maternal nutrition could directly enhance the neonatal immune system by increasing the quality and quantity of antibody and other immune factors available for passive transfer across the placenta and in breast milk. It could also indirectly improve neonatal immunity, by reducing the likelihood of maternal infections that may lead to preterm birth, a known cause of IgG deficiency in neonates due to reduced third-trimester antibody transfer (57). Increased maternal infections may also influence neonatal immune development *via* effects on the hypothalamic–pituitary–adrenal (HPA) axis (see below).
- *Placentation:* Maternal nutrient availability has been shown in animal and human studies to affect placentation, with affects on size, morphology, nutrient transfer receptors and vascular flow (58–63). This may theoretically affect passive transfer of antibodies and other immune factors to the fetus as well as altering the efficiency of nutrient transfer for fetal immune system development.
- *The maternal HPA axis:* The HPA axis is activated in times of low nutrient availability [particularly protein-energy malnutrition (64) and zinc deficiency (65, 66)] leading to increased circulating glucocorticoids. Increased cortisol levels can lead to both immunosuppression and altered placental function in the mother, with downstream effects for the fetus as described above, as well as directly impacting on the fetal immune system *via* actions on its own HPA axis.
- *The maternal gut microbiome:* The human intestinal tract contains more than 10^{14} bacteria and other organisms (67). These commensal microflora have evolved a complex symbiotic relationship with humans, and are increasingly recognized as essential for many aspects of human health (68). Nutrient intake influences the composition of the gut microbiota, which in turn can influence the availability of nutrients for absorption from food (69–71). The gut microbiome is crucial for the development and functioning of the mucosal immune system (72). Healthy gut flora help to promote mucosal tolerance to non-pathogenic antigens, reduce the overgrowth of pathogenic microorganisms and enhance absorption of nutrients that are potentially important for systemic immune system development (68). Dysbiosis (altered microbiome) has been associated with increased risk of immune-mediated diseases such as allergy, asthma, and inflammatory bowel diseases, as well as increased risk of infections (73). Animal models suggest that the immune development of the offspring may be influenced by the maternal microbiota in the following ways [reviewed in detail in Ref (74)]: (1) alteration of nutrient uptake having direct effects on maternal immunity and hence the availability of antibodies and immune factors for transfer to the offspring,

TABLE 1 | Nutrients and their effects on immunity.

Nutrient	Effect on immunity	Effect of deficiency on clinical immune outcomes	Reference
Protein energy	*Innate* Epithelial integrity Complement levels NK-cell activity *Adaptive* T-lymphocyte number and function, particularly Th1-type cytokines Delayed type hypersensitivity Effect on B-lymphocytes less clear	Increased bacterial, viral, and fungal infections	(16, 17)
n-3 PUFAs	Activity is largely immunosuppressant with reductions in: *Innate* Leukocyte chemotaxis and adhesion NK-cell function Innate cytokine production *Adaptive* T-lymphocyte signaling	Theoretical increases in inflammatory-mediated diseases and allergy. Trials suggest that supplementation reduces the risks of inflammatory-mediated diseases such as rheumatoid arthritis and improves responses to infectious disease	(18–25)
Vitamin A	*Innate* Epithelial integrity Neutrophil, monocyte, macrophage, and NK-cell number and function *Adaptive* T-lymphocyte differentiation and migration T-lymphocyte numbers, especially CD4 B-lymphocyte numbers Antibody production and may affect the balance of production of different IgG subclasses	Increased susceptibility to infections, particularly diarrhea, respiratory infections and measles. Supplementation of children from 6 months to 5 years in areas at risk of deficiency reduces all cause mortality, diarrhea incidence and mortality and measles incidence and morbidity on meta-analysis	(26–28)
B vitamins	*Vitamin B2 (riboflavin)* Phagocyte activation *Vitamin B6* Dendritic cell function Lymphocyte maturation and growth T-lymphocyte activity and delayed type hypersensitivity B-lymphocyte activity and antibody production *Vitamin B9 (folate)* Epithelial integrity NK-cell activity T-lymphocyte proliferation and response to mitogenic activation Cytotoxic T-lymphocyte activity *Vitamin B12* NK-cell activity CD8+ T-cell activity B-lymphocyte activity and antibody production		(29–39)
Vitamin C	*Innate* Epithelial integrity Phagocyte production Antioxidative functions *Adaptive* T-lymphocyte maturation Interferon production	Association with increased incidence and severity of pneumonia. Supplementation in the elderly shows possible reductions in pneumonia incidence and duration	(40)
Vitamin D	*Innate* Macrophage activity (cathelecidin antimicrobial peptide expression, induction of reactive oxygen intermediaries, activation of autophagy) *Adaptive* T-lymphocyte number and function Th1/Th2 balance skewed to Th2 Unclear effect on B-lymphocytes (in humans)	Increased susceptibility to infections, particularly of the respiratory tract. Meta-analysis shows reduced acute respiratory tract infections when routine supplementation is given in the context of deficiency	(41–43)
Vitamin E	*Innate* Epithelial barrier integrity NK-cell activity *Adaptive* T-lymphocyte proliferation and function Delayed type hypersensitivity reactions Vaccine-mediated antibody responses	Supplementation is suggested to lead to reduced respiratory tract infections in the elderly	(37, 44, 45)

(Continued)

TABLE 1 | Continued

Nutrient	Effect on immunity	Effect of deficiency on clinical immune outcomes	Reference
Zinc	*Innate* Epithelial barrier integrity Proinflammatory cytokine production Neutrophil oxidative burst NK-cell function *Adaptive* T-cell maturation and proliferation Th1/Th2 balance skewed to Th1	Increased bacterial, viral and fungal infections: particularly diarrhea and pneumonia. Routine supplementation of children in at-risk areas leads to reductions in duration of diarrhea and incidence of pneumonia, in children >6 months on meta-analysis, but not in children 2–6 months old	(46–50)
Selenium	*Adaptive* CD4+ T-lymphocyte proliferation and function	Increased viral virulence	(51–54)
Iron	*Innate* Neutrophil, NK-cell, and macrophage activity Innate cytokine production *Adaptive* T-lymphocyte numbers No apparent effect on B-lymphocyte number and function	May enhance or protect from infections with bacteria, viruses, fungi and protozoa depending on the level of iron. Although supplementation may theoretically enhance immunity to infectious diseases, untargeted supplementation may increase availability of iron for pathogen growth and virulence and increase susceptibility to, particularly, malaria and bacterial sepsis	(55, 56)

(2) alteration of the repertoire of antibodies passively transferred to the neonate, which may alter the degree of mucosal tolerance in the neonate, and hence its own microbiome composition (75, 76), (3) bacterial metabolites derived from the microbiota may be transferred to offspring across the placenta and in breastmilk and may impact on the offspring's developing immune system (77), and (4) organisms from the maternal microbiota can be found in placental tissue (78) and this exposure may impact directly on the developing infant immune system and indirectly by altering gestational length.

The mother's nutritional status may also affect the neonatal immune system by directly altering the nutrients available to the developing embryo/fetus. This may theoretically have long-term effects on offspring immunity *via*:

- *Epigenetic modification:* Epigenetic modification is the process by which stable alterations to gene expression, and thus the phenotype of cells, are induced without changes to the primary DNA sequence (79, 80). These modifications may be altered in response to environmental factors, persist following cell division, and, in some cases, are heritable—providing a means by which the environment may have permanent and multigenerational impacts on phenotype (81). The three main types of epigenetic modification are (1) DNA methylation; where the degree of methylation at, primarily, CpG dinucleotide rich sites in gene-specific promoters affects the degree of expression of that gene, (2) histone modification; where the accessibility of promoter regions of genes to transcription machinery is altered by additions to protein tails, affecting the degree to which DNA transcription occurs, and (3) non-coding RNAs, where small lengths of RNA bind to target mRNA, altering its subsequent translation (81). Of these, DNA methylation has emerged as a strong candidate effector mechanism to explain the DOHaD theory as it largely occurs during embryogenesis or early postnatal life, and produces durable effects (82). Alterations in DNA methylation of key metabolic genes induced by famine exposure in early life persist for at least six decades (83, 84). Epigenetic modification could theoretically have similar long-term impacts on the expression of genes important for the immune system.
- *Organogenesis and lymphopoiesis:* The process by which organs develop during embryonic and fetal life is highly sensitive to environmental influences. It has long been known that exposure to adverse factors at critical windows of organogenesis can lead to permanent changes in organ growth and function. Development of the infant immune system is likely to be similarly susceptible to environmental influences, including nutrient levels. In older children, both the thymus and hematopoietic branches of immunity are acutely sensitive to undernutrition, with reductions in thymus size and blood cell functioning shown to occur in both acute and chronic starvation conditions (85). As both immune compartments undergo massive expansion during the gestational period, with the thymus being at its largest as a proportion of body size at birth, it is highly plausible that nutritional conditions *in utero* would impact on the neonatal immune system. Studies in animals support a link between maternal macro/micronutrient deficiency and reduced thymic size and function (86–88), which may not be fully reversible by later improvements in nutrition (89).
- *Immunoregulatory mechanisms, e.g., the neonatal HPA axis:* Maternal cortisol levels (which may be altered by nutrient availability, see above), can influence the development of the fetal HPA axis, with long-term consequences for neuroendocrine-immune interactions (90, 91). Although the developing fetus is generally protected from maternal cortisol fluctuations by the function of 11 B-hydroxysteroid dehydrogenase in the placenta, levels of this enzyme are decreased by undernutrition (92). Evidence from animal studies suggests that stimulation of the fetal HPA axis can lead to lower lymphocyte proliferation, reduced NK-cell activity, and reduced antibody responsiveness in offspring (93), as well as increasing the responsiveness of the HPA axis to stressors later in life. These effects are hypothesized to be mediated through epigenetic programming of glucocorticoid receptors (91).

- *The neonatal gut-microbiome:* The neonatal gut microbiome is strongly influenced by the maternal microbiome. Colonization of the gastrointestinal tract occurs around the time of birth (and possibly even earlier) with organisms acquired from the mother's gastrointestinal tract, vagina, skin, and breast milk, and is influenced by delivery type, gestational age, and feeding method among other factors (94). Modification of the maternal microbiome may thus be hypothesized to influence the developing neonatal immune system both directly, by altering the neonatal microbiome composition, and indirectly, by altering the nutrient status of the mother and hence the availability of nutrients for immune system development during fetal life.

A conceptual framework for the potential influences of early life nutrition on the developing infant immune system is shown in **Figure 1**. Evidence for such effects occurring in humans is discussed below.

EVIDENCE FOR THE INFLUENCE OF PRE- AND PERICONCEPTIONAL NUTRITION ON THE INFANT IMMUNE SYSTEM

Epigenetic Modification of the Early Embryo

Specific evidence for the impact of periconceptional nutrition on later immune functioning through epigenetic modifications has been suggested from the previously described Gambian cohort. The plasma levels of 1-carbon metabolites crucial for DNA methylation undergo seasonal variations in pregnant women. Higher levels of folate, methionine, and riboflavin, and reduced homocysteine levels occur in the nutritionally challenged rainy season (95–97). Although counterintuitive, this may be due to increased consumption of green leafy vegetables during this period, due to the need to food diversify (98). The increased level of these methyl-donor intermediaries correlates with increases in DNA methylation seen at metastable epialleles (MEs) (see **Box 1**) in children conceived in the rainy season (and thus born in the dry season, correlating with reduced later infectious disease mortality) (96, 99). A metastable epiallele VTRNA2-1, involved in tumor suppression and viral immunity, has been identified that is differentially methylated according to season of conception (and hence nutritional status), and is stable for at least 10 years (100). This provides the first in-human evidence that periconceptional nutrition could directly influence subsequent immune functioning. Although the clinical relevance of the variability in methylation of this ME in susceptibility to infections has yet to be proven, it provides a tantalizing suggestion that the seasonal variation in adult infectious disease mortality is mediated, at least in part, through nutritionally sensitive epigenetic modifications.

A number of epidemiological studies have now linked DNA methylation status at the promoter region of inflammatory mediators to nutritional status in pre- and early postnatal life (107–109), although the timing of nutritional influences causing these epigenetic modifications is difficult to prove. Methylation status of these genes has been correlated with later markers of biochemical inflammation, though effects on clinical outcomes have yet to be shown (107). Intriguingly, animal models have shown that alterations to paternal diet can alter DNA methylation in offspring, with resultant phenotypic changes increasing the risk of obesity and metabolic syndromes (110–113). The

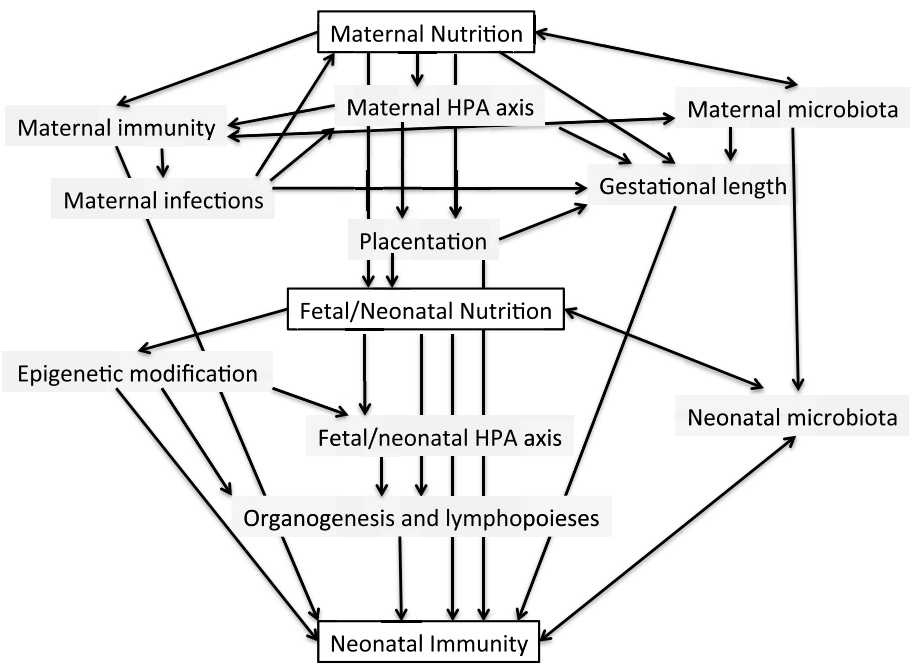

FIGURE 1 | Conceptual framework for the potential interactions between maternal and early neonatal nutrition and the developing infant immune system.

BOX 1 | Metastable epialleles. A tool for investigating the influence of the periconceptional environment on offspring epigenomes.

The inherent tissue specificity of many epigenetic changes creates challenges for the study of the influence of epigenetic modifications on adult phenotypes (99). While epidemiological association studies between gene variants and risk of disease may use easily obtainable peripheral blood draws, studies investigating epigenetic influences on disease etiology may require tissue-specific samples that are often not as accessible. Metastable epialleles (MEs) are regions of DNA where methylation is established stochastically in the early embryo and is subsequently maintained throughout all three germ-layer lineages (101). Thus, methylation of MEs occurring in the early embryonic period (pregastrulation) may be determined from peripheral blood samples.

Differential methylation of MEs in mice has been shown to have dramatic phenotypic consequences including alterations in fur color (102), tail-kinking (4, 103), and propensity to obesity (104). Methylation of murine MEs is strongly influenced by maternal nutrition and other environmental factors in the periconceptional period (105, 106). MEs in humans may have effects on adult disease and provide an easily accessible method of investigating the epigenetic pathways that may be involved in the DOHaD theory.

potential transgenerational influence of paternal diet on the health outcomes of offspring has also been suggested in humans from epidemiological studies carried out in Sweden. These showed a correlation between reduced food availability during the father's, and even grandfather's, preadolescence and increased life expectancy, with reduced risk of cardiovascular and diabetes-related mortality (114). Other studies have linked early onset of paternal obesity with increased liver enzymes and long-term changes in percentage body fat in offspring. These effects are likely to be mediated by epigenetic modification of spermatozoa, and may be sex specific (115). Thus, it may be that paternal diet is also ultimately shown to produce lasting effects on the immune system of offspring.

Although most human studies have focused on DNA methylation as a mediator of long-term effects of periconceptional environment on the health of off-spring, animal studies suggest that histone modification (116) and microRNAs (117, 118) may also play a role in the developmental origins of disease, though their importance in immune system development has yet to be investigated. Thus, it appears likely that immune system functioning is influenced by interacting and overlapping epigenetic modifications induced by nutritional status, and other environmental factors, occurring around the time of conception, during gestation and in early postnatal life.

Placentation

Although evidence for the importance of several micronutrients including vitamin D, zinc, folate, calcium, and iron on placental growth and function exists (58, 59), studies directly investigating the effects of periconceptional maternal nutrition on placentation and subsequent fetal immunity are limited. One study that randomized non-pregnant women of child-bearing age to a multiple-micronutrient (MMN) supplementation or placebo and followed up subsequent pregnancies, showed minimal improvements in placental vascular function with MMN supplementation, but no improvements in other markers of placental function (plasminogen activation inhibitor 1 and 2 ratio) and transfer of maternal measles antibody at birth (119).

EVIDENCE FOR THE INFLUENCE OF GESTATIONAL NUTRITION ON THE INFANT IMMUNE SYSTEM

Macronutrients

Protein Energy

The relationship between maternal nutrition and fetal growth is complex, involving maternal metabolic and endocrine, as well as placental, functioning (2, 120). However, the neonatal presentation of protein-energy malnutrition is assumed to be infants who are born small-for-gestational age (SGA). Infants born SGA or low-birth weight (LBW) have an increased risk of infectious mortality in the neonatal period and beyond (121–124). SGA/LBW infants show altered immunology, with lower complement and IgG (125), lower plasmacytoid dendritic cells, higher NK-cells and higher IgM (126), and higher inflammatory activation and T-cell turnover (127), compared to those delivered at an appropriate weight. Gambian infants born in the nutritionally deprived rainy season (a presumptive marker of reduced macronutrient supply in late gestation) show smaller neonatal thymus size (128), and have some changes to thymic function (129). These immune changes do not appear to be long lasting, however, and a seasonal effect of infectious disease incidence may contribute to these findings (130, 131). Intrauterine growth restriction has been associated with reduced vaccine responses in childhood, though inconsistently (132–135).

Given the suggested link between macronutrient deficiencies and neonatal morbidity, a number of maternal protein supplementation strategies have been evaluated (136). Balanced protein energy supplementation (containing up to 20% of energy as protein) leads to modest increases in birth weight (up to 324 g) (137), and reduces the number of SGA infants born by around a third (136). Reductions in neonatal deaths as a result of supplementation have not been clearly shown, however, with meta-analysis of the three published studies reporting neonatal mortality showing only non-significant improvements in neonatal outcomes (136, 138–140). Even if these non-significant reductions in mortality are true findings, the causal mechanisms underlying such effects are unknown, with reductions in prematurity likely to play a significant role. No clear link between maternal protein energy supplementation and improvement in neonatal immunity has been demonstrated. Maternal protein supplementation has no proven impact on later vaccine responses, mucosal immunity and delayed-type hypersensitivity reactions (130) or thymus size (141), although impacts on thymic function at the cellular level were not assessed. The lack of substantial demonstrable neonatal benefits from maternal protein energy supplementation may reflect the heterogeneous etiologies of SGA and LBW, with factors such as poor placentation and environmental toxin exposure not addressed by supplementation. It may also be due to challenges with targeting the intervention to the most at-risk subjects within populations. Subgroup analysis of supplementation studies suggest that the intervention is only beneficial when provided to malnourished individuals, and that high protein supplements may even impair fetal growth when given in the context of adequate diets (136).

Lipids

Maternal PUFA supplementation during gestation is associated with reductions in preterm births and small increases in birth weight (142) on systematic review. However, impacts on the immune system are less clear. Most research has been directed on the effect of fish-oil supplementation on reduction in atopy risk in offspring. Systematic reviews have suggested reductions in offspring IgE-mediated allergy and eczema following gestational/lactational n-3 PUFA supplementation, though the duration of these effects is not clear and the relative importance of the timing of supplementation during gestation or lactation is difficult to determine (143, 144). Murine studies suggest that n-3 PUFA supplementation of mothers can improve offspring responses to infections, with enhanced vaccination responses shown in mice fed high n-3 PUFA diets during gestation and lactation (145). In humans, docosahexaenoic acid (DHA) supplementation during gestation and lactation was associated with reductions in CD8+ T-cells, increases in naive CD4CD45RA+ helper cells and reductions in lymphocyte IFNγ production (146). However, this trial did not show changes to immunoglobulin levels, vaccination responses or clinical outcomes and may have been confounded by the high baseline dietary DHA levels of all participants. One trial of prenatal DHA supplementation has shown reduction in incidence and duration of cold symptoms during infancy (147). No significant evidence of reductions in neonatal outcomes such as sepsis, morbidity or mortality have been shown in systematic review of human studies, though adequately powered trials to assess these outcomes are lacking (148).

Micronutrients

Micronutrient deficiencies are estimated to affect approximately 2 billion people worldwide. They are often particularly severe in women of reproductive age due to the high demands of pregnancy and lactation (149). Optimization of micronutrient levels in pregnant women has therefore been proposed as a strategy to enhance neonatal immunity.

Specific Micronutrient Supplementation during Gestation

Zinc

Overt zinc deficiency is now rare but moderate deficiency is common worldwide (150). Zinc supplementation of mothers leads to biochemical improvements in their zinc status and that of their offspring (151, 152). Thymus size in infants correlates with cord-blood zinc levels (153), although a recent study showed no impact of maternal zinc supplementation on infant thymic size (154). Improved hepatitis B vaccine antibody responses and delayed type hypersensitivity reactions to BCG vaccination have been shown following maternal zinc supplementation (154), but no effect on haemophilus influenza B conjugate vaccine responses has been found (155). Theses studies suggest some influence of maternal zinc supplementation on infant immune development, but the clinical impact of this is uncertain. A recent systematic review of 21 trials (>17,000 mother–infant dyads) suggests no benefit of maternal zinc supplementation for IUGR, LBW, stillbirth, and neonatal death, though small reductions in preterm birth were shown (156). No significant reduction in neonatal infective outcomes, including neonatal sepsis, umbilical infections, fever, and necrotizing enterocolitis (NEC), was seen but the number of studies reporting these outcomes was small. One study from Bangladesh showed reduced acute diarrheal and impetigo episodes in the first 6 months of life following maternal zinc supplementation, though no difference in persistent diarrhea, cough, and LRTI (157, 158). A study from Indonesia similarly reported reduced diarrheal incidence in infants <6 months old following maternal supplementation with zinc, but this was at the expense of increased episodes of cough (159). Conversely, a study in Peru did not report any benefit for diarrheal prevalence (160).

Vitamin D

Vitamin D deficiency is common worldwide due to lack of UV exposure in northern latitudes, darker skin pigmentation in southern latitudes, covering the skin with clothes, and vegetarian diets. There are strong correlations between maternal and umbilical cord vitamin D with deficiency or insufficiency in the mother likely to cause deficiency in offspring (161). Systematic reviews of supplementation in pregnancy suggest reduced risk of vitamin D deficiency in offspring and slight increases in birth weight (162, 163). However, no evidence for improvement in any other neonatal outcomes including neonatal mortality has been shown (162). Impacts of vitamin D deficiency on the developing immune system have been shown with reduced thymus size in offspring (164) and an association with increased CRP [although this trend is reversed with vitamin D sufficiency (>50 nmol/L) (165, 166)]. Maternal vitamin D supplementation during gestation results in increased Th1 and Th2 cytokine gene expression and reduced pattern recognition receptor expression in cord blood, following stimulation with PHA (167). Clinically, vitamin D deficiency in cord blood has been associated with increased risk of lower respiratory tract infections, wheeze, and eczema in a number of observational studies, suggesting long-term impacts on immune ontogeny, although causation is difficult to prove (168, 169). Of four studies assessing the impact of maternal vitamin D supplementation on infant risk of respiratory infections and wheeze (170–173), only one showed significant reductions in incidence of acute respiratory tract infections in offspring (170). In this study the intervention was combined with postnatal vitamin D supplements so the contribution of maternal supplementation *per se* is difficult to assess. A recent systematic review of vitamin D supplementation in pregnancy and early life did not show any reduction in the risk of persistent wheeze, eczema, or asthma, though the quality of available evidence was low (174).

Vitamin A

Vitamin A deficiency is associated with increased susceptibility particularly to diarrhea, respiratory infections, and measles (27). Infants born to mothers with low serum retinol had increased all-cause neonatal mortality in a study in Malawi (175). Nepali infants born to mothers with xeropthalmia (the clinical manifestation of severe vitamin A deficiency) had a 63% increased mortality within the first 6 months of life, which was reduced following maternal supplementation (176). However, large randomized controlled trials of vitamin A supplementation

including more than 310,000 mother–infant pairs have failed to show benefits for perinatal and all-cause neonatal mortality on systematic review, despite reductions in maternal night-blindness and possible reductions in maternal infections (177). There is some evidence, though, that vitamin A supplementation of women may lead to long-term enhancement of natural antibody levels in offspring, perhaps acting through impacts on early lymphopoiesis (178). This suggests that long-term alterations to the neonatal immune system may occur following vitamin A supplementation, but that more sensitive outcome measures are required to identify these changes than all-cause neonatal mortality.

Iron

Fetal iron acquisition occurs actively across the placenta, mainly in the last trimester of pregnancy, and is highly regulated (179, 180). Direct correlations between maternal and fetal iron status are not consistently seen, as neonatal iron levels are likely to be preserved at the expense of maternal stores, but severe maternal anemia is associated with reductions in neonatal iron (181). Iron deficiency is thought to be the most prevalent micronutrient deficiency worldwide (182). It occurs particularly in low-income countries where diets tend to be low in absorbable iron and parasitic burden can be high. Systematic reviews support the use of daily or intermittent iron supplementation during pregnancy for improvement of maternal iron status and reduction in anemia (182, 183). However, no evidence for improvements in other maternal or neonatal outcomes has been found. There is a current paucity of evidence regarding specific impacts, whether beneficial or detrimental, of maternal oral iron supplementation on neonatal infection risks (184). Similarly, studies investigating a direct impact of fetal iron status on immune system ontogeny are lacking.

B-Vitamins, Including Folic Acid

Folate (vitamin B9) has been widely studied as a pregnancy supplement, due to its role in the reduction of neural-tube defects. A systematic review of 31 studies, mainly carried out in Europe in the 1960s and 1970s, showed a modest increase in birth weight (136 g) following maternal folate supplementation, but no reduction in preterm birth, still-birth, or neonatal death (all cause) (185). The impact of folate supplementation in pregnancy on neonatal immune parameters and infective outcomes has not been investigated. More recently, concerns have been raised that folate supplementation given beyond the first trimester, or in excessive doses during pregnancy, may be linked to an increased risk of allergy/asthma, but the evidence is largely from observational studies and is not yet conclusive (186).

Vitamin B12 deficiency is associated with an increased risk of preterm birth (187), but its supplementation in pregnancy has been little studied. One study in Bangladesh confirmed that maternal oral vitamin B12 supplementation during pregnancy and lactation led to significant increases in infant B12 levels, but this was not associated with improvements in passive transfer of influenza antibodies or levels of acute inflammation markers (188). A significant reduction in number of infants with raised CRP was shown, but the number of infants with the outcome was small and the influence of timing of supplementation during pregnancy or lactation could not be distinguished.

A systematic review of three randomized controlled trials of maternal supplementation with vitamin B6 has been shown to result in a significant reduction in mean birth weight (217 g) (189). The impact of supplementation on neonatal mortality or infections has not been studied (190).

One study of vitamin B2 supplementation during pregnancy and lactation exists, which showed modest increases in infant riboflavin levels, but did not report neonatal outcomes (191). Sole supplementation with other B-vitamins has not been studied in the context of pregnancy and their impacts on the developing neonatal immune system are unknown.

Other Vitamins and Trace Elements

A number of other micronutrients with known immunomodulatory effects in adults have been little studied in neonates. Longitudinal studies of the influence of maternal diet on infant respiratory outcomes have suggested inverse associations between maternal vitamin E intake and infant asthma/wheeze (192–194), however, this has not been borne out in randomized controlled trials of maternal supplementation (195). Maternal selenium deficiency leads to low selenium status of neonates and is associated with reduced circulating adaptive immune cells and *in vitro* thymocyte activation (196). Observational studies have associated maternal selenium deficiency with enhanced risk of infant infections in the first 6 weeks of life, but these studies are at high risk of confounding (197). One supplementation study of selenium in HIV positive mothers showed a possible reduced risk of all-cause child mortality after 6 weeks of life, but a non-significant increase in fetal deaths (198). No studies have yet investigated maternal vitamin C, vitamin E, or selenium supplementation for neonatal immune outcomes specifically. There is also no current evidence for reductions in the more gross markers that may be associated with neonatal immune function (IUGR, LBW, preterm birth, perinatal, or neonatal death) from supplementation in pregnancy of vitamin C (199), vitamin E (200), copper (201), or selenium (198).

Multiple Micronutrient Supplementation during Gestation

When micronutrient deficiencies exist they are often multiple, due to poor quantity and diversity of available foodstuffs (149). Identification and targeted treatment of specific deficiencies in pregnant women is expensive and programmatically challenging. Therefore many studies aiming to enhance micronutrient levels in pregnancy use multiple micronutrient (MMN) supplements that provide the recommended daily allowance of all vitamins and minerals in one tablet (202). However, the evidence supporting the use of MMNs for neonatal outcomes in general, and neonatal immunity specifically is not clear. Meta-analysis of studies involving more than 135,000 women showed modest increase in birth weight (22–54 g), with corresponding reduction in babies born SGA or LBW, following MMN supplementation compared to standard iron and folic acid supplementation (203). These improved birth outcomes did not translate into improvements in neonatal and infant morbidity/mortality including from

infectious disease (204). No MMN supplementation studies to date have investigated neonatal immune parameters specifically, although one randomized controlled trial from The Gambia is due to report shortly (205).

Probiotics, Prebiotics, and Synbiotics

Studies of maternal supplementation with probiotics (live microorganisms that contribute to a "healthy" gut microbiota), prebiotics [nutrients that promote growth of healthy bacteria, such as non-digestible oligosaccharides (206)], and synbiotics (a combination or pro- and prebiotics), for modulation of the neonatal immune system have been conducted in humans, but are relatively limited. A number of randomized controlled trials have shown that maternal consumption of probiotics or synbiotics can lead to measurable changes in the composition of their offspring's microbiome (207–210) and to changes in immune markers in the mother (211). However, alterations in infant immune markers following maternal supplementation, such as vaccine responses and cytokine levels, have been harder to show (212). Reduced incidence of eczema, though not asthma and wheeze, in infants has been suggested from systematic reviews of trials of prenatal supplementation but the effects may not be durable (72, 213–216). One small trial has shown reduced gastrointestinal infections in infants born to mothers supplemented with probiotics (211), and another a reduction in respiratory infections (217), but these findings need to be confirmed in larger studies.

EVIDENCE FOR THE INFLUENCE OF EARLY POSTNATAL NUTRITION ON THE INFANT IMMUNE SYSTEM

The major nutritional influence on neonatal immunity is breast milk, which contains immunological components such as antibodies, anti-inflammatory cytokines and other antimicrobial factors, as well as the macro and micronutrients to support neonatal immune system development (218). Its benefits over formula milk for protection against various infections, atopy, and allergy are well reviewed elsewhere (219, 220). Here, we focus on the potential impact of supplementary nutritional interventions for the breastfeeding mother and neonate on the developing neonatal immune system.

Lactational Supplementation

The composition of breast milk is highly regulated according to the neonate's needs with the concentrations of many components maintained independently of maternal nutritional status and diet (221). Some immunomodulatory micronutrients, such as iron, folate and zinc (222, 223) and macronutrients such as arachadonic acid (224, 225) are not altered in the breast milk according to maternal diet. Therefore, maternal supplementation of these nutrients would likely have little or no impact on neonatal immune outcomes and they are not discussed further in this section. However, some immunoactive nutrients in breast milk are impacted by diet and their concentrations in milk vary worldwide. These include vitamin A, vitamin D, B vitamins, selenium, and PUFAs, particularly DHA (34, 221).

Micronutrient Supplementation of Lactating Mothers

Vitamin A

Vitamin A is not only necessary for the developing neonatal immune system, its presence in breast milk is also important for the regulation of a number of breast milk proteins important for host defense (226). Infants are born with low vitamin A stores in the liver, and breast milk is the main source of vitamin A for infants during the first 6 months of life (227). Numerous reports have shown decreased breast milk vitamin A concentration with maternal deficiency, and increased concentrations with high exogenous vitamin A levels (228, 229). However, the results of postnatal maternal vitamin A supplementation studies for neonatal outcomes have been inconclusive. Systematic reviews of both lower dose (200,000 IU) and higher dose (400,000 IU) postpartum maternal vitamin A supplementation have shown only small increases in breast milk retinol concentrations (230) and a lack of supporting evidence for reduced infant morbidity (including from infections) to 6 months of age (230, 231). As a result, WHO no longer recommends routine postpartum vitamin A supplementation for women in low- and middle-income countries (WHO 2017). Studies on the effects of postpartum vitamin A supplementation on immunological outcomes specifically are limited and inconclusive. Studies variously report increases and no change to sIgA following postpartum vitamin A supplementation (226, 232). Further studies looking at a wider array of immunological parameters, and altering the timing of vitamin A supplementation are ongoing (226).

Vitamin D

Vitamin D deficiency is relatively common in breastfed infants, with low concentrations in milk even from vitamin D sufficient mothers (233). Studies investigating maternal postpartum supplementation have shown variable results, though on balance suggest supplementation may enhance infant vitamin D status (234–238). At present, however, direct neonatal supplementation of with vitamin D is the preferred method of enhancing neonatal vitamin D status (see below). Studies investigating the impact of vitamin D supplementation in breast-feeding women for neonatal immunological outcomes are lacking.

B-Vitamins

B-vitamins levels in the breast milk are largely amenable to improvements with supplementation of the mother (with the exception of folate) (34, 239), but there are no studies looking at the impact of lactational B-vitamin supplementation on neonatal immune outcomes.

Selenium

Selenium levels in breast milk are sensitive to dietary intake (240) and can be increased by supplementation (240, 241) [although these effects have not been consistently shown (197, 242)] and alter infant selenium status (243). Although selenium deficiency in infants has been associated with increased risk of respiratory infections in the first 6 weeks of life (197), large studies investigating maternal postpartum selenium supplementation for infant infectious morbidity have not been conducted.

Multiple Micronutrients

Given the high prevalence of coexisting micronutrient deficiencies world-wide, there is a surprising lack of studies investigating the impact of multiple micronutrient supplements in breastfeeding mothers for infant outcomes (34). Only two small trials (52 women total) have compared MMN supplementation with nothing/placebo in breast feeding mothers, and reported on neither infant morbidity nor immunological outcomes (34, 232).

Lipid Supplementation of Lactating Mothers

The concentration of PUFAs, particularly DHA, in breast milk is highly affected by maternal diet (244), and PUFA supplementation increases levels in breast milk (245). Breast milk n3:n6 ratios have been associated with risk of allergy and atopy in infants in observational studies (246–248) although not consistently (249). Fish oil supplements provided during lactation alter cytokine production in the infant for at least 2.5 years, favoring faster immune maturation and Th1 polarization (250). Given the increasing existence of imbalanced n3:n6 ratios in westernized diets, there has been interest in providing PUFA supplements to lactating women for allergy prevention in infants, although concerns exist about potential negative impacts on infectious disease susceptibility (251, 252). However, at present only two studies (667 participants) have investigated postnatal maternal PUFA supplementation specifically, and although persisting alterations in cytokines have been shown, the studies were underpowered to detect differences in infant atopic disease or infectious morbidity (143, 250).

Probiotic, Prebiotic, and Synbiotic Supplementation of Lactating Mothers

Supplementation of lactating mothers with probiotics has been associated with alterations to breast milk cytokines and infant fecal IgA (253), and changes to the breast milk and infant microbiomes (254). Studies supplementing mothers with probiotics during lactation suggest a reduced risk of dermatitis, but interventions tended to combine pre- and postnatal supplementation, so the specific impact of lactational supplementation is difficult to determine (255). As with prenatal maternal supplementation, effects on infant immune outcomes following lactational supplementation require further evaluation (72, 256).

Neonatal Supplementation

Direct supplementation with crucial nutrients in the neonatal period has also been assessed as a strategy to protect infants from deficiency. However, in the majority of cases, despite improvements in the nutrient status of infants, no clear evidence for improvements in clinical or biochemical immune outcomes has been shown.

Micronutrient Supplementation of the Neonate

Zinc

Zinc use in older infants has been associated with reductions in diarrhea duration (48) and lower respiratory tract infections incidence (47), but results following supplementation in the neonatal period have been more equivocal (257–261). One small study of zinc supplementation as an adjunct to antibiotics in neonates with sepsis showed a reduction in treatment failures and a non-significant 43% reduction in mortality (262). A larger study to investigate this is currently ongoing (263). Studies directly investigating the impact of neonatal zinc supplementation on immunological markers are limited. Routine zinc supplementation has not been associated with improvements in OPV seroconversion rates (264), although its use as an adjunct to antibiotics in neonatal sepsis has been associated with significantly reduced serum calprotectin, IL-6, and TNFα and a non-significant reduction in mortality (265).

Vitamin D

Vitamin D supplementation is recommended routinely in many countries for its impact on calcium and bone metabolism, but large-scale evidence for postnatal supplementation on any immunological disease outcomes (infection or allergy) is lacking (266). A recent systematic review of supplementation in children below 5 years of age did not show reductions in diarrhea and pneumonia incidence despite raised vitamin D levels in supplemented children, though supplementation in the neonatal period was not looked at specifically (42). One trial of maternal and infant vitamin D supplementation has suggested lower numbers of respiratory infection primary care visits following high dose maternal and infant supplementation, compared to low dose (170). A large trial to investigate immunological outcomes following neonatal vitamin D supplementation in breastfed infants is currently underway (266).

Vitamin A

Vitamin A supplementation in children from low- and middle-income countries aged 6 months to 5 years is associated with reductions in all-cause mortality of around one-third on systematic review (28). In contrast, a large systematic review of trials including more than 168,000 infants from low- and middle-income countries did not show any benefit of vitamin A supplementation when given in the neonatal period (267). Effects of supplementation may differ by underlying vitamin A status of the population, as reductions in all-cause mortality were suggested in the South Asian studies but not in the African studies. The African studies also showed concerning side-effects with increased transient bulging of the fontanelle and interactions of vitamin A with routine immunizations, particularly in female infants (268, 269). Studies investigating the effects of neonatal vitamin A on immunological parameters are limited. One study conducted in Guinea Bissau showed no effect of neonatal vitamin A supplementation on BCG vaccination responses at 6 months of age (270), although some evidence of reduced TNFα and IL-10 production in girls who have not received DTP vaccination (271). Two RCTs are currently ongoing to specifically investigate the effects of neonatal vitamin A supplementation on the immune system, but these have yet to report (226, 272). Routine vitamin A supplementation in children below 6 months of age is not currently recommended.

Iron

The provision of iron supplements to neonates deserves special mention due to its potential for increasing susceptibility to

infections by enhancing iron availability for pathogens (55). Studies conducted in the 1970s showed that injecting neonates with iron dextran at birth significantly increased the risk of *Escherichia coli* meningitis and sepsis (273) and enhanced *in vitro* bacterial growth (274, 275). This may have been partly due to the mode of delivery, as parenteral iron administration is not subject to regulated uptake in the gut and therefore may overwhelm iron homeostatic mechanisms in iron replete children, but similar concerns exist with the untargeted provision of oral iron supplements. Older children given iron supplements from 4 months of age have increased risk of gastrointestinal infections (276), adult studies show increased *in vitro* bacterial growth in serum after oral iron supplementation (277) and there are suggestions that malaria risk is increased when oral iron is provided to iron replete children in endemic countries (55, 278). Human breast milk contains low levels of iron and has specific iron chelating agents such as lactoferrin. Our group and others have also shown that serum iron drops rapidly and profoundly in the first 12 h of life that and persists at low levels for at least 4 days. This low serum iron is associated with reduced *ex vivo* bacterial growth (279, 280). Taken together, this evidence suggests that humans may have evolved to mitigate against the enhanced pathogen susceptibility and oxidative stress that results from high iron loads. Therefore provision of exogenous iron to the neonate, except in specific situations where severe iron deficiency anemia has been diagnosed, may do more harm than good. In fact, there is increasing interest in novel therapeutics, such as lactoferrin and hepcidin agonists, that reduce serum iron in the context of neonatal infections (281–283). However, as preterm and growth-restricted infants have lower iron stores from birth, routine iron supplementation is often given, starting from 4 weeks of age, in high-income countries (284). In these settings, where infectious disease burden is low, no adverse infective outcomes have been shown on systematic review (285).

Other Vitamins and Trace Elements

Parenteral selenium supplementation of very LBW infants in NICU has been shown to increase selenium levels and reduce the incidence of neonatal sepsis, but systematic review of available evidence does not show improvements in survival (286, 287). No similar studies of oral supplementation in normal weight, term, breastfed infants in areas of selenium deficiency have been conducted. Studies looking at the effects of neonatal selenium, B-complex vitamins, vitamins C and E, or combined micronutrient supplements on immunological parameters specifically are lacking.

Probiotic, Prebiotic, and Synbiotic Supplementation in the Neonate

Interest in the provision of probiotics, prebiotics, or synbiotics directly to neonates that are at risk of dysbiosis of the gut microbiome has exploded in recent years (255). Preterm infants are at particular risk of dysbiosis, not only due to gut immaturity, but because they often have reduced or delayed enteral feeds and increased exposure to antibiotics. Failure to establish normal gut flora is linked to higher risk of NEC and nosocomial sepsis (288). Systematic review of studies providing probiotics to low-birth weight infants in neonatal units, suggest a reduction in grade II or III NEC and all-cause mortality, though no significant reductions in sepsis (289, 290). Not all studies have shown clear benefits for NEC, however, and multistrain probiotics appear more beneficial than single strain organisms (291). Prebiotic supplements have not been shown to result in significant reduction in NEC, all-cause mortality or sepsis when given to preterm infants (292). The long-term health implications of use of pre- and probiotic supplements in preterm infants are not currently known. Provision of probiotics and prebiotics to formula fed infants, in attempts to produce a gut microbiome profile similar to breastfed infants, has also been extensively studied. Although beyond the scope of this review, these studies suggest reductions in atopic disease (though few studies have follow-up of sufficient duration to assess long-term effects) (293) and some limited evidence on systematic review for reductions in gastrointestinal and respiratory infections (294, 295). More excitingly, a recent randomized controlled trial in breastfed infants in rural India showed that synbiotic administration during the first 7 days of life led to a 40% reduction in sepsis and all-cause mortality in the first 60 days of life (296). This suggests that in certain situations even the breastfed microbiome may be altered for immunological benefits in the early neonatal period. However, further studies to examine the effect of different strains, dosages and durations, as well as the long-term consequences of synbiotic administration, will be needed before synbiotics could be considered as a public health intervention for neonatal sepsis.

SUMMARY

Despite multiple animal and human studies associating nutrient deficiencies with adverse immunological outcomes, there is strikingly little evidence to suggest nutritional supplementation during gestation and early infancy has benefits for neonatal responses to infection or allergic disease prevention.

There are a number of plausible explanations for the lack of significant and consistent impacts of individual or combined nutrient supplements on neonatal outcomes. First, it may reflect the heterogeneity of the studied populations in-terms of their underlying nutritional status. Improvements in clinical outcomes are likely to be most where deficiencies are highest. The transfer of many nutrients across the placenta, such as vitamin A (177) and iron (179), occurs actively and is regulated by the fetus, meaning that even in the context of maternal insufficiency the fetus remains relatively protected. As a result, maternal supplementation might only benefit infants born to mothers with critical deficiencies. Large population studies including non-deficient participants will have reduced power to detect clinical benefit. Maternal vitamin A supplementation, for instance, had larger effects on maternal and neonatal outcomes in Nepal (297), where severe deficiency is common, compared to Ghana (298) and Bangladesh (299) where levels of deficiency are more moderate (177). Second, in many studies iron and folate were provided to mothers in the non-intervention arm. As these can also impact on neonatal infective outcomes, this may have confounded the results (156). Third, the optimal level of supplementation of micro- and macronutrients for neonatal outcomes is not known and dosages often differ between studies (300). Micronutrients

have nutrient–nutrient interactions that may alter the availability of other immunity modulating nutrients and have a rate-limiting effect on immune development (301). High levels of iron, zinc, and protein, for instance, can have counterintuitively negative effects on the immune system, and may have detrimental outcomes when given to sufficient women (302). If this is the case, then population-based treatment as a public health intervention becomes challenging and less measurably effective. Fourth, it may be that the onset of maternal supplementation in the studies was too late in gestation to have lasting effects on immune system development. Supplementation was commenced after 12 weeks of age in many studies, which would miss an early programming effect of nutrients if one exists. As a number of supplementation studies reported improvements in mothers nutrient status following supplementation, but no improvements in clinical outcome for the offspring, it would be interesting to know whether this enhanced nutritional status had positive impacts on future pregnancies, by improving nutrient status during the periconceptional period. Lastly, despite the large number of studies investigating maternal nutrient supplementation, those designed specifically to look at the effects on neonatal immune development and infectious/allergic disease outcomes are limited and further research with more sensitive outcome markers is warranted.

Although the evidence for the benefits of nutritional supplements in pregnancy and early infancy has so far been disappointing, some exciting possibilities remain. The persisting epigenetic changes induced by nutritional factors around the time of conception, which may impact on immune functioning in later life, warrants further study to assess their impact on neonatal infections, allergy and the amenability to supplementation. The potential benefit of probiotics and synbiotics for infectious disease and allergic outcomes in infancy is also extremely exciting. The World Allergy Organisation has recently recommended probiotic use during gestation, lactation and early life for infants at high risk of atopic disease (303), but further work to determine the most effective strains, dosage and duration, and whether these vary by geographical region, will be needed before their widespread use as a public health intervention against neonatal infections can be recommended.

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SP was responsible for all parts of this article.

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Innate Immunity to Respiratory Infection in Early Life

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Early life is a period of particular susceptibility to respiratory infections and symptoms are frequently more severe in infants than in adults. The neonatal immune system is generally held to be deficient in most compartments; responses to innate stimuli are weak, antigen-presenting cells have poor immunostimulatory activity and adaptive lymphocyte responses are limited, leading to poor immune memory and ineffective vaccine responses. For mucosal surfaces such as the lung, which is continuously exposed to airborne antigen and to potential pathogenic invasion, the ability to discriminate between harmless and potentially dangerous antigens is essential, to prevent inflammation that could lead to loss of gaseous exchange and damage to the developing lung tissue. We have only recently begun to define the differences in respiratory immunity in early life and its environmental and developmental influences. The innate immune system may be of relatively greater importance than the adaptive immune system in the neonatal and infant period than later in life, as it does not require specific antigenic experience. A better understanding of what constitutes protective innate immunity in the respiratory tract in this age group and the factors that influence its development should allow us to predict why certain infants are vulnerable to severe respiratory infections, design treatments to accelerate the development of protective immunity, and design age specific adjuvants to better boost immunity to infection in the lung.

Keywords: respiratory, neonatal, infection, respiratory syncytial virus, innate immunity

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INTRODUCTION

Respiratory infection is one of the leading causes of mortality in children under 5 years of age (1, 2). Early life respiratory viral infections are most commonly caused by rhinovirus, respiratory syncytial virus (RSV), influenza, parainfluenza virus, and coronavirus (3). Infection is frequently restricted to the upper respiratory tract but may develop into severe lower respiratory tract infection, such as RSV bronchiolitis, the leading cause of hospitalization of infants worldwide (4–7). Bacterial pneumonia in infants, caused by agents such as *Haemophilus influenzae* and *Streptococcus pneumoniae*, is estimated to cause a million deaths in infants under 5 years of age annually (8, 9). Maternal antibodies afford some protection against infection but wane over the first months of life, and neonates and infants respond poorly to vaccination, leaving early life as a window of particular vulnerability to respiratory infection (10, 11). Experiences during the crucial neonatal and infant window may shape respiratory health in the long term (12–14). Severe RSV infection in infants is associated with the development of wheeze and asthma in childhood (15–19) and even respiratory disease that occur late in life, such as chronic obstructive pulmonary disease, are associated with early life events (20–24).

At birth, the neonate emerges from the sheltered intrauterine environment into a plethora of antigenic challenges from pathogens, commensals, and harmless environmental antigens. Neonatal immunity is, in general, attenuated compared to that of adults (4, 25–29). Differences in immunity in early life are due to tissue leukopenia, cell intrinsic hyporesponsiveness, and inhibitory mechanisms, such as CD71+ immunosuppressive erythroid cells and high levels of adenosine in extracellular fluids (26, 28–31). Protective Th1 polarized responses and antibodies are produced less well in early life than in adults, along with a propensity to develop unwanted, Th2 or Th17 biased, or dysregulated inflammation (28, 31–33), for example, following vaccination or allergen exposure (34, 35). TLR stimulation of cord blood leukocytes results in a lower production of proinflammatory, Th1-associated cytokines (IL-12p70, TNF-α, IFN-α), and greater production of IL-10 and the Th17-promoting IL-6 and IL-23 when compared to stimulation of adult blood cells, although equivalent responses to TLR 7/8 ligand R848 occur (29, 36, 37). Over the first few years of life, antiviral and Th1-biasing cytokine production increases (38, 39).

In the face of an inexperienced adaptive response, innate immunity is likely to play a more dominant role in protection against infection in early life than in adulthood. This is supported by the findings that many gene polymorphisms associated with severe RSV infection in infants encode components of the innate immune response (4, 40–43). The importance of TLR signaling in early life is illustrated by individuals with genetic deficiencies in components of the TLR signaling pathway such as MyD88 or IRAK-4. These patients are at high risk of bacterial infection in childhood, including in the respiratory tract; however, their condition improves dramatically with age (44). This review will focus on describing our current knowledge of innate immunity in the neonatal lung as a first line of defense against infection. Some potentially important mechanisms underlying susceptibility to lung infection in infants are summarized in **Figure 1**.

RESPIRATORY IMMUNITY IN EARLY LIFE

It is relatively difficult to obtain samples from the lower airways of healthy infant subjects, so many studies have been carried out in murine and other animal models. Information on the cellular composition of the neonatal lung in humans has come from analysis of bronchoalveolar lavage fluid composition (46–49), immunohistochemistry (50), and more recently, extensive phenotypic analysis of leukocyte subsets in pediatric tissues (51–53).

Adaptive Immunity

Fetal airways are essentially devoid of lymphocytes, they are seeded from birth, and lymphocytes increase as a proportion of airway cells over the first few years of life (48, 54). There is a relative paucity in CD4+ cells (46, 50), and memory T cells are less abundant in infant lungs than in adults, though they are more abundant in the lungs than many other tissues (51). Tregs are relatively abundant in pediatric tissues and may have a higher suppressive capacity than those from adults (28, 51) and a transient increase in regulatory T cells, associated with microbial colonization, protects from hyperresponsiveness to allergen (35). A failure of regulation may underlie excessive inflammation in infection, as in RSV bronchiolitis (43), and RSV infection in early life can increase susceptibility to allergic inflammation in the mouse model through an impairment of regulatory T cells (4, 55). CD8+ T cells in the lung correlate with disease severity in infants with respiratory failure due to respiratory viral infection (52) and in neonatal mice infected with RSV, a CD8+ T cell epitope hierarchy emerges, which is distinct to that of adults (56). Distinct phenotypes of adaptive lymphocytes are found in early life. A subset of Th cells in human cord blood produce the neutrophil chemoattractant interleukin-8 upon activation (57) and, during RSV infection, a regulatory phenotype in the neonatal B cell compartment may dampen protective immunity (58).

Lung Dendritic Cells (DCs)

There is some evidence that neonatal T cells have the capacity to mount adult-like protective responses to lung infection. Adoptive transfer of neonatal CD4+ T cells into *Pneumocystis carinii*-infected adult SCID mice allowed for adult-level pathogen clearance and cytokine production (59, 60), suggesting that the neonatal environment in the lung influences T cell responses. This may be due in part to the function of neonatal antigen-presenting cells. Neonatal mouse lungs contain relatively fewer conventional DCs (cDCs), which are immature and poorly functional (56, 61, 62), although mature functions *ex vivo* have been reported (63). During neonatal RSV infection, migratory cDCs are dominated by CD103+ DCs, while the CD11b+ contribution increases with age (64). These CD103+ DCs are phenotypically immature and poorly functional (65), and this may influence the magnitude and epitope hierarchy of the CD8+ T cell response (64–66), although these are also influenced by T cell intrinsic differences and regulatory T cells (56, 67). As well as stimulating protective responses, lung DCs in neonates must promote tolerance to harmless environmental antigens. CD11b+ cDCs in the lung induce Th2 responses to allergens, but transiently express high levels of PD-L1, which promotes tolerance, following acquisition of the microbiota (35, 68). In contrast to murine studies, the relative frequency of different DC subsets in the human lung appears to be relatively stable over the life course (53).

In the murine neonatal lung, potent IFN-α-producing pDC cells are scarce (61), and there is limited recruitment of pDCs and IFN-α production following RSV infection (69).

Alveolar Macrophages (AM)

Lung resident macrophages, which include AM and the less well-characterized interstitial macrophages (70–72), are an important component of the first line of defense in the lung. In the steady state, AMs remove debris and maintain a tolerogenic environment; during infection, they secrete proinflammatory cytokines and contribute to pathogen clearance; and after infection, they aid resolution of inflammation (45). AMs are the predominant cell type in the neonatal airway, they appear in the alveolar compartment from just before birth and throughout the first week of life, and are relatively abundant and self-renewing, persisting for at least 11 weeks in mice (47–50, 73, 74).

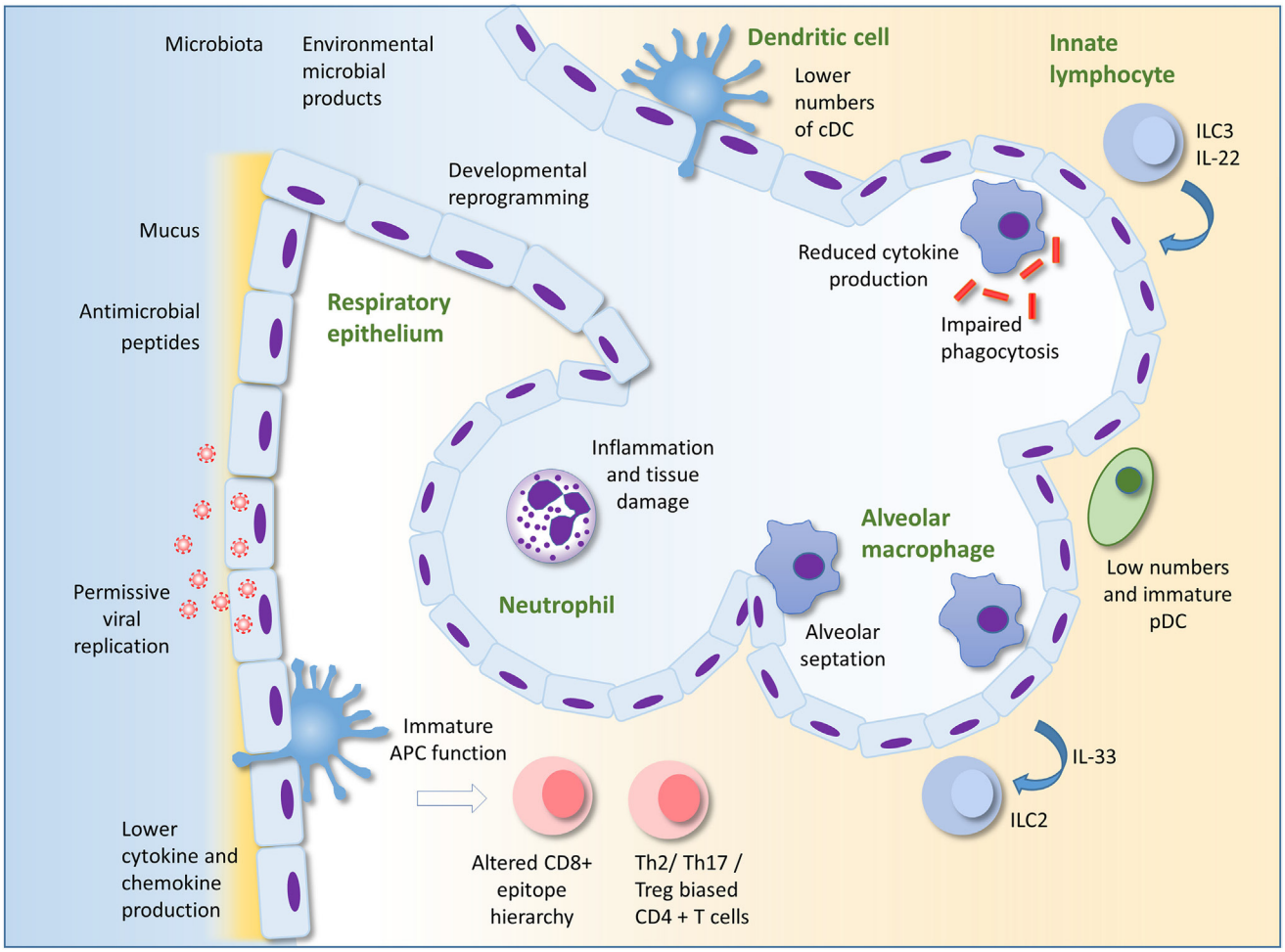

FIGURE 1 | Innate immunity to infection in the lung in early life. Alveolar macrophages (AM) are the most numerous leukocyte in the lungs in early life. Reduced cytokine production and phagocytic ability in AM in early life compared to those of adults could underlie susceptibility to infection. AM also promote pre- and post-natal lung development and remodeling. The respiratory epithelium protects against infection through the production of mucus and antimicrobial peptides. Production of type I IFNs may be lower in infant than adult epithelial cells, perhaps permitting greater viral replication. Epithelial cells may interact with innate lymphocytes to both initiate and regulate inflammation. Developmental reprograming in the epithelium in early life may also alter the nature of the epithelial response to infection. There are low numbers of pDC in the lungs compared to adults. Recruitment of neutrophils to the lung occurs less readily in early life compared to adults in some circumstances, but in other situations, excessive recruitment of inflammatory cells can lead to lung inflammation, tissue damage, and impairment of gaseous exchange. Immaturity and lower numbers of dendritic cells, the environment as well as intrinsic differences in T cells in early life may result in the development of skewed helper T cell responses and an altered epitope hierarchy in CD8+ T cells. Innate immunity in the lung in early life is influenced by acquisition of the microbiota, exposure to microbial products and other environmental factors, as well as the infant genome. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology (45), copyright 2014.

Stimulation of cultured cells has been used to interrogate the relative antimicrobial functions of neonatal and adult AMs. LPS stimulation of rodent or ovine AMs results in similar or even enhanced upregulation of TNF-α and CXC-chemokines in neonatal compared to adult cells (75–77), though others demonstrated a reduced translocation of NF-κB to the nucleus of AM from neonatal mice (78). Enhanced phagocytosis by neonatal compared to adult rat AM has been observed (75), but others have reported impaired phagocytosis and subsequent killing of yeast particles in neonatal rhesus monkey AMs; and impaired phagocytosis of opsonized red blood cells in neonatal rat AMs in comparison to adults (79, 80). In a murine model of *Pneumocystis* infection, neonatal AMs were delayed in their expression of activation markers *in vivo* in comparison to adults (81). Similarly, during murine neonatal RSV infection, there was reduced and delayed AM activation compared to adult infection (82), but intranasal IFN-γ was able to promote AM maturation (82). Little is known about responses in human infant AMs. Cultured cells obtained by bronchoalveolar lavage from infants <2 years of age produce lower IL-1 and TNF-α following LPS stimulation compared with cells from children aged 2–17 (54). The apparent contradictions in the data on AM function in early life may reflect differences in the species, age, experimental conditions, and assays used. Various macrophage functions are likely to mature at different

rates. Neonatal and adult AMs are likely to behave differently in their respective lung environments, which is a limitation of these *in vitro* studies.

Respiratory Epithelial Cells

The respiratory epithelium is the principal site of replication of respiratory viruses. It is in close communication with AM and acts an immune sentinel producing inflammatory mediators, such as type I and III interferons, mucus, and antimicrobial proteins (45, 83). Relatively little is known about the immunological functions of the airway epithelium in early life. In cultured tracheobronchial epithelial cells from Rhesus macaques of different ages (infant, juvenile, and adult), IL-8 production on exposure to LPS positively correlated with age (84). Furthermore, epithelial cells from juveniles housed in filtered air produced higher cytokine responses than those in conventional housing suggesting the microbial richness of the environment may influence epithelial responsiveness. The same group demonstrated that infant Rhesus monkey primary epithelial cell cultures are more permissive for the H1N1 influenza virus than those from adult airways, while producing less IL-1α (85).

In humans, type I IFNs are detected at only low levels in the airways of RSV-bronchiolitic infants. This may be due to inhibition of the host anti-viral response by the viral non-structural proteins but alternatively may reflect the timing of sampling, and an IFN-induced gene signature is detectable in blood (86–88). Pediatric nasal and airway epithelial cells cultured from bronchial brushings are readily infected with RSV (89–91) and poor induction of type I IFNs by RSV is reflected in these cultures (92, 93). Instead, the type III interferon IL-29 (IFN-λ) is detected both in the airways of bronchiolitic infants and in cultures of RSV infected airway epithelial cells, and IL-29 pretreatment of cultured epithelial cells attenuates RSV growth (92, 93). Epithelial cells are probably a key source of inflammatory cytokines in respiratory tract secretions of infants with acute RSV (92, 94, 95), including the type-2 immunity promoting cytokine IL-33 (96). The cells used in many *in vitro* experiments on pediatric respiratory epithelial cells were originally taken from the conducting airway and data surrounding lower airway and ATII cells in early life is even sparser.

Antimicrobial proteins are a first line of defense at barrier sites and are produced primarily by epithelial cells and innate leukocytes, particularly neutrophils (97, 98). In the lung, they include surfactants as well as S100s, β-defensins, and cathelicidin and they may provide protection against important infant respiratory infections, including RSV (99–102). Cathelicidin has direct antiviral activity against RSV, can prevent infection *in vitro* and *in vivo* and in children hospitalized with bronchiolitis, those with low serum cathelicidin were significantly more likely to have RSV infection and a longer hospital stay (97, 103–107).

Innate Lymphocytes

Neonatal murine lungs show no quantitative deficiency in γδ T cells as a proportion of CD3+ T cells (61, 108). Exposure to allergen in neonatal mice can stimulate innate ILC2 lymphocytes, a major source of type 2 cytokines (109). Colonization by the microbiota in neonates protects against the accumulation of potentially pro-inflammatory mucosal iNKT cells in the lung and gut (110). Colonization of the gut of neonatal mice can also lead to intestinal DC mediated upregulation of CCR4 on IL-22 producing ILC3, which allows their migration into the lungs of neonatal mice, and promotes protection against bacterial pneumonia (111).

Neutrophils

Recruitment of innate leukocytes and, in particular, neutrophils, is likely to play an important role in the innate response to infection in the neonatal lung following microbial recognition. Both TLR4 gene and protein expression are present in the murine lung in the fetus and increase with age through to adulthood (112, 113). TLR2 expression is also present in the human fetal lung and increases with gestational age (114). It appears that there is an immaturity of chemokine production at baseline in the respiratory mucosa. Expression of CXCL2 is low in neonatal mice compared with adults (115) and in uninfected infants (newborn to 18 months), the concentration of IL-8 in nasal washes positively correlates with age (116). There is a dramatically reduced and delayed neutrophil influx in neonatal lung in response to administration of LPS or bacteria in comparison to adult animals (75, 117–119). In the neonatal murine lung, infection with the paramyxovirus Sendai virus results in a minimal early influx of neutrophils and low production of pro-inflammatory cytokines compared with the adult lung; similarly in murine RSV infection, early pro-inflammatory cytokine production is impaired (108, 115). Diminished recruitment of neutrophils may also be due to an impaired chemotaxic ability of infant neutrophils (25, 120, 121).

In severe RSV bronchiolitis in infants, neutrophils can account for the majority of cells recovered from the airways, associated with increased neutrophil elastase (122–125) and IL-8 (94, 126), although others have reported a lower inflammatory cytokine response in infants with severe vs mild RSV bronchiolitis (127). There is a considerable influx of neutrophils into *S. pneumoniae*-infected lungs of neonatal and adult mice, with the neonatal influx even occurring at a lower bacterial dose (128). It is unclear under what circumstances the neonatal lung will produce an equivalent or exacerbated inflammatory response compared to that of adults, whether this simply requires a high level of stimulation or whether additional factors are involved.

FACTORS INFLUENCING THE DEVELOPMENT AND MATURATION OF LUNG IMMUNITY

Despite the apparent absence of a mature adult-like immune system, neonates are able to produce effective immune responses that defend against infection and indeed excessive inflammation can occur. The neonate must strike a balance between protection against infection and potential damage to the developing lung and may use alternative mechanisms of protection against infection to those that predominate in adults.

Exposure to microbial products from the environment, the microbiota, or infection may be beneficial in terms of their ability

to promote immune maturation and more adult like innate and adaptive immunity (28, 30). Treatment with TLR agonists CpG or LPS during RSV infection alters the CD8+ T cell response toward a more adult-like immunodominance (66) and treatment of neonatal mice with CpG prior to RSV infection shifts the secondary response to re-infection away from a type 2 response (129). Furthermore, administration of BCG shifts lung CD4+ responses away from a Th2 bias and cDC from BCG treated lungs promote Th1 responses (61).

The microbiota is acquired from the mother at birth and in early life and an adult-like microbiome is established by around 3 years of age (130). The composition of the microbiota and microbial richness of the environment in which children develop have been linked to susceptibility to severe respiratory infections and the development of wheeze and asthma (131–133). Environmental microbial exposure may influence lung health by establishing the set-point of immunological responsiveness of the lung, as seen by the attenuation of allergic lung inflammation by airway exposure to LPS or endotoxin rich dust samples (133, 134). Additionally, commensal bacteria may influence neonatal respiratory immunity indirectly. For example, sensing of commensal bacteria by gut DCs promotes resistance to bacterial pneumonia in neonatal mice (111). Factors that shape the microbiota, such as delivery by cesarean section and antibiotic use in early life and pregnancy, are likely to profoundly influence the developing immune system (14, 135). Other environmental factors that regulate the balance of immunity in the infant respiratory tract may include diet, vitamin D status, breast feeding, maternal immunity, and exposure to environmental pollutants.

Significant stages of lung development occur both before and after birth and hyporesponsiveness to immune stimuli may have evolved to protect the developing lung from the disruptive and damaging effects of inflammation (136, 137). This is evidenced in mouse models of chorioamnionitis, where exposure of the fetal lung to LPS results in abnormal development of the distal airways (138, 139). In addition, IL-1β expression in the fetal or newborn lung impairs normal postnatal development (140). Reciprocally, the developmental programmes active in resident lung cells, which drive cell growth and differentiation may also influence immune responses (141, 142). Macrophages take on important roles in lung development and remodeling including septation and vascularization of the alveoli after birth (137, 143). Macrophages associate with sites of branching morphogenesis where they assume a tissue remodeling phenotype and promote development through production of growth factors and matrix metalloproteases (143). Polarization of macrophages away from this phenotype might, therefore, be a mechanism by which pro-inflammatory signals disrupt lung development (138, 140). As with lung macrophages, the respiratory epithelium will be subject to lung developmental programmes extending into the postnatal period, which regulate epithelial cell proliferation and differentiation, and these may potentially also alter epithelial immunological function. Foxa2 is an epithelially expressed member of the forkhead family of transcription factors. In the developing lung, it regulates epithelial differentiation and controls goblet cell hyperplasia. It also has immunoregulatory functions and limits type-2 immunity through inhibition of the cysteinyl LT signaling pathway (83, 141, 144).

CONCLUSION

The mechanisms that regulate inflammatory responses to microbial stimulation in the lung need to be more fully elucidated. Increasing our knowledge of how the developing immune system responds to infectious challenge is of importance for development of neonatal vaccines and treatments for exaggerated respiratory inflammation during infection. In certain circumstances, the immune system in early life is capable of adult-level responses, and perhaps boosting responses in at-risk infants—in treatment for acute infectious disease or as adjuvant for vaccination—would be a beneficial protective strategy. Additionally, selectively harnessing the protective innate mechanisms that are already expressed at adult or greater than adult levels in the neonate could be a safe therapeutic method. Thus, while early life is clearly a period of immunological vulnerability for the developing lung, it is also an opportunity for effective intervention strategies, which could benefit respiratory health not only in infancy, but into adulthood.

AUTHOR CONTRIBUTIONS

LL researched the literature and wrote the review. FC wrote the review, edited, and updated it.

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REVIEW
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Codevelopment of Microbiota and Innate Immunity and the Risk for Group B Streptococcal Disease

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The pathogenesis of neonatal late-onset sepsis (LOD), which manifests between the third day and the third month of life, remains poorly understood. Group B *Streptococcus* (GBS) is the most important cause of LOD in infants without underlying diseases or prematurity and the third most frequent cause of meningitis in the Western world. On the other hand, GBS is a common intestinal colonizer in infants. Accordingly, despite its adaption to the human lower gastrointestinal tract, GBS has retained its potential virulence and its transition from a commensal to a dangerous pathogen is unpredictable in the individual. Several cellular innate immune mechanisms, in particular Toll-like receptors, the inflammasome and the cGAS pathway, are engaged by GBS effectors like nucleic acids. These are likely to impact on the GBS-specific host resistance. Given the long evolution of streptococci as a normal constituent of the human microbiota, the emergence of GBS as the dominant neonatal sepsis cause just about 50 years ago is remarkable. It appears that intensive usage of tetracycline starting in the 1940s has been a selection advantage for the currently dominant GBS clones with superior adhesive and invasive properties. The historical replacement of Group A by Group B streptococci as a leading neonatal pathogen and the higher frequency of other β-hemolytic streptococci in areas with low GBS prevalence suggests the existence of a confined streptococcal niche, where locally competing streptococcal species are subject to environmental and immunological selection pressure. Thus, it seems pivotal to resolve neonatal innate immunity at mucous surfaces and its impact on microbiome composition and quality, i.e., genetic heterogeneity and metabolism, at the microanatomical level. Then, designer pro- and prebiotics, such as attenuated strains of GBS, and oligonucleotide priming of mucosal immunity may unfold their potential and facilitate adaptation of potentially hazardous streptococci as part of a beneficial local microbiome, which is stabilized by mucocutaneous innate immunity.

Keywords: *S. agalactiae*, Group B *Streptococcus*, cellular innate immunity, microbiome, colonization, sepsis

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INTRODUCTION

Neonatal sepsis occurs as two distinct clinical entities either in the first 72 h of life as early-onset disease (EOD), resulting from *in utero* or intrapartum infection, or during the following 3 months as late-onset sepsis (LOD). In both cases, the Gram-positive, β-hemolytic Group B *Streptococcus* (GBS) is one of the most prevalent bacterial species in blood and cerebrospinal fluid. As a consequence,

pregnant women undergo routine or targeted screening for GBS in the last third of pregnancy in many Western European countries and the USA. In case of positive testing, women receive preventive intrapartum antibiotics during delivery (1). Since approximately 20–30% of all pregnant women are colonized, this prevention strategy affects an estimated 1 million women every year in the US alone. In other countries such as the Netherlands, a risk-based approach has been adopted, i.e., antibiotics are only administered in case of additional risk factors such as premature labor, intrapartum fever, bacteriuria, prolonged membrane rupture or previous children with GBS disease.

Before the use of antibiotic prophylaxis, the GBS sepsis incidence exceeded 1 in 1,000 children with high case fatality rates (2, 3). The role of GBS in neonatal sepsis may be due to (i) it being one of the most prevalent colonizers of the birth canal and thus among the first bacteria to get into contact with the newborn (4, 5), (ii) GBS carrying highly invasive properties, and (iii) a particular neonatal immunopathology induced by GBS. In EOD, the size and deposition site, e.g., the lung, of the GBS inoculum may be decisive factors. However, it is unresolved why GBS establishes as a harmless mucocutaneous colonizer in approximately 10% of infants in the first weeks of life, and overcomes epithelial barriers and cellular innate immunity only in less than one in thousand infants to cause LOD. In other words, it remains a puzzle which specific factors at the level of mucosal immunity and the local microbiome allow GBS to leave its colonizing niche, thus facilitating invasion in the individual child.

At the beginning of life, the developmental lines of the microbiota and of the local cellular innate immunity have to run with substantial interdependence. Both areas are subject to factors *in cis* and *in trans*, i.e., specific bacteria are influenced by the microbiota and by host immunity, and host cells are modulated by other host and microbial cells (6). In order to guarantee long-term ecologic stability, adaptation on either side of the host–microbe interface is required, both at the population level and in the individual cell. The putative contribution of variations in specific innate immune genes to neonatal sepsis has recently been discussed (7). The authors suggested that affected children may suffer from yet to be identified minor primary immunodeficiency. This is a tempting hypothesis, given the enormous gain in knowledge on single gene alterations leading to susceptibility to a narrow spectrum of microorganisms. On the other hand, there is no indication for inheritance of a specific neonatal sepsis risk. Moreover, LOD typically remains the only "suspicious" episode in the individual infection biography. Finally, preterm birth is a well-recognized risk factor of GBS sepsis. In preterm infants, several factors impact on the individual codevelopment of microbiota and immunity, in particular cesarean section and formula feeding, which modify the microbiome (8, 9), and antibiotic usage, which affects both the microbiome and myeloid cell development (10, 11).

The hypothesis underlying this review holds that aberrations in the codevelopment of microbiota and host immunity, rather than genetic variations in immune genes alone, shape the individual risk for neonatal GBS sepsis, in particular LOD.

GBS: COLONIZATION AND VIRULENCE FACTORS

Neonatal GBS sepsis is a global problem with an overall incidence of around 0.5/1,000 live births. In contrast to the situation in Europe, American and African countries, GBS are reported to be a rare cause of neonatal colonization and sepsis in Southeast Asia (12, 13). However, the epidemiology in developing countries often suffers from constraints related to early deaths outside hospitals and low microbiological sensitivity of detection methods (13). In many, but not all Western European and North American countries, intrapartum antibiotic prophylaxis (IAP) has been associated with a decreased incidence of EOD while LOD rates remained unchanged (14–16). Notably, a substantial proportion of mothers whose infants developed EOD were tested negative before birth (1). It is unclear whether this phenomenon is due to false-negative test results or very recent GBS acquisition. Although, as outlined above, incidence and fatality rates are significantly higher in preterm than term infants (16–18), most cases occur in term infants (1) without clinical or laboratory evidence for immunodeficiency. LOD alone has an incidence of about 0.3–0.4 per 1,000 children and can develop randomly within the first 3 months after birth (19). It manifests more frequently as meningitis than EOD (17, 20). Conceptionally, these observations indicate that EOD and LOD originate from distinct biological processes or disturbances thereof.

Group B *streptococcus* is classified into 10 serotypes based on chemical structure and conformation of capsular polysaccharides. Serotyping relies on latex agglutination or multiplex PCR (21). In the past 30 years about 50% of the reported neonatal GBS sepsis cases worldwide were caused by serotype III strains (13). This indicates a considerable genetic homogeneity and stability in the pathogenic potential of GBS despite antibiotic selection pressure. Notably, Islam et al. did not detect any colonization by GBS of serotype III in their cohort of more than 600 infants in Bangladesh, while 6% of all infants were colonized by other serotypes, predominantly VII and Ia (22). It is very plausible yet uncertain that low circulation of highly invasive GBS strains underlies the low incidence of invasive neonatal GBS in several Asian countries (13).

In addition to the serotypes, GBS can be further classified by multilocus sequence typing, with more than 700 identified types (ST). The majority of human isolates belong to six clonal complexes (23, 24). EOD is significantly associated with serotype Ia strain ST-23 and closely related ST-24 as well as the ST-17 strain of serotype III (25, 26). LOD on the other hand is largely caused by ST-17 (20, 25). Moreover, ST-17 causes most cases of meningitis in EOD and LOD (27). In EOD, the distribution of invasive strains mainly corresponds to those colonizing the mothers (26). However, ST-17 shows an elevated disease-to-colonization ratio in EOD and LOD, i.e., it causes more cases of invasive disease than expected from its colonization rate of pregnant women (28–30). These observations, together with the characteristic expression of several virulence factors, have led to the term of a "hypervirulent" strain. Two of these factors, the hypervirulent GBS adhesin HvgA (27) and the serine-rich

repeat glycoprotein Srr2 (31), are surface-anchored proteins which allow for adherence to epithelial cells and host plasma proteins. ST-17 strains also often carry the 2b pilus variant which contributes to invasion in mouse models (32, 33).

Most GBS strains produce surface-associated β-hemolysin which can damage membranes and promote barrier penetration (34). β-Hemolysin was found to be identical to the orange to red pigment of GBS, an ornithine rhamnolipid called granadaene (35). Both factors rely on the *cyl* operon which is controlled by the CovR/S two-component system. Strains mutated in CovR/S show hyperhemolysis and increased virulence (34, 35). For further detailed descriptions about GBS virulence factors, we refer to recent reviews (36, 37).

ROUTES OF INFECTION

In EOD, GBS is usually transmitted from the colonized maternal vaginal tract during birth to the infant. Aspiration of contaminated fluids allows for bacterial entry *via* the respiratory tract in many cases, resulting in sepsis or pneumonia during the first days of life (38). The route of infection in LOD is less well understood. The gastrointestinal tract is considered to be a natural reservoir for sepsis pathogens in neonates (39, 40). GBS shares this niche with *Escherichia coli*, the second typical organism in neonatal sepsis. Yet, the point of time when GBS establishes colonization is highly variable. 50–70% of colonized mothers transfer GBS to their offspring during delivery (41, 42) and 50% of infants which later developed LOD were colonized with GBS at birth (43). It remains unknown how many of these infants were stably colonized between the first contact with GBS and the disease onset. Unfortunately, large-scale and longitudinal colonization data of mother-infant pairs before and after disease onset, which would allow resolving this LOD puzzle, are not available. In a case series, Carl et al. found that 7 out of 11 children with LOD by GBS, *E. coli* or *Serratia marcescens* produced at least one stool with the matching organism before bloodstream infection (39). However, only two infants with GBS sepsis contributed to this study and they showed a GBS positive stool only briefly before sepsis, indicating recent colonization or overgrowth in the gastrointestinal tract. Another longitudinal case study on LOD also found that GBS occurred in the stool 2 days before sepsis onset (44). In contrast, it has been shown for other infections, e.g., enterococcal or staphylococcal bloodstream infections, that children often have a pathogen-dominated gut flora before disease onset (44, 45). Thus, it is conceivable that GBS exposure constitutes a particular LOD risk to infants who failed to firmly establish GBS colonization after birth (46). However, it seems important to note that stool samples do not always adequately mirror the actual intestinal community (47).

Meningitis caused by serotype III strains is often linked to high-level bacteremia. Factors that enable serotype III strains to survive in the blood stream, i.e., escape of adaptive and innate immune mechanisms, such as antibody or complement-mediated phagocytosis may be responsible for this effect (48). While the route of infection has not been resolved with certainty in infants, several studies showed bacterial dissemination to the blood and CNS after intraperitoneal (49), subcutaneous (50, 51) and intragastral (27, 52) inoculation of GBS serotype III in neonatal mice and rats. ST-17 is also specifically found in cases of GBS meningitis after 3 months of age (53), indicating that this clonal complex has an increased capability of overcoming colonization site barriers and blood borne immunity and of invading the CNS.

THE NEONATAL MICROBIOME

The microbiome, defined as the microbial flora inhabiting the human body, constitutes an important factor in individual health and development. The composition of the microbiome is complex, distinct between individuals and subject to environmental changes and adaptation to host factors. Each body site contains a unique microbial community. Even within one niche such as the skin the composition varies depending on the exact location, i.e., the back skin shows a different microbial signature than the foot pad or the axillary vault (54). It seems self-evident that exposure to bacteria in the birth canal impacts on the colonizing flora in the infant. However, the fetus may be less sterile than thought, i.e., that the microbiome might develop already *in utero*. 16S rDNA sequencing of amniotic fluid, placenta samples and meconium revealed prenatal presence of bacteria with a predominance of *Escherichia* spp. (9, 55, 56). Of note, the *Streptococcus* genus was also detected in these samples, yet at very low abundance (56). Intrauterine colonization data have to be interpreted with some caution, since microbial viability is usually not confirmed and the risk of contamination is high in many of the investigated samples (57). Accordingly, the contribution of colonization *in utero* to microbiome development is still unclear, whereas that of colonization after rupture of fetal membranes is beyond doubt. As an example, vaginal delivery and cesarean section result in different bacterial communities on skin, nares, and gingiva (9). Yet, the impact of the delivery mode on the expansion and functional diversification after the first 6 weeks of life is surprisingly modest (9, 58). Instead, the infant's microbiome follows a rather predictable successive colonization pattern and reaches a stable state resembling the adult microbiome already at 1–3 years of age (59–61). Oxygen abundance in the neonatal gut facilitates the colonization by facultative anaerobes, e.g., *Lactobacillus* and *Streptococcus* followed by *Enterobacteriaceae*. After oxygen is consumed and anaerobic conditions are established, obligate anaerobic species, e.g., *Bifidobacterium*, *Bacteroides*, and *Clostridium* spp. populate the intestine (62, 63). Administration of antibiotics, on the other hand, heavily affects the postnatal microbiome (8, 64, 65). Postnatal exposure to antibiotics alters the gut microbiome in the first 2–3 years of life by delaying microbiome development and altering phylogenetic diversity, e.g., affecting early colonization with *Lactospiraceae* spp. (8, 65). In addition, antibiotics reduce the stability of the microbiota composition as indicated by an increased variation between consecutive samples as compared to controls (65). Notably, very preterm infants with a gestational age of <33 weeks, who in many cases receive antibiotics within 24 h of birth, showed a 10-fold reduced bacterial diversity in comparison to term infants (66).

GBS AS PART OF THE HUMAN MICROBIOME

Streptococcus is, together with *Lactobacillus*, *Staphylococcus*, and *Propionibacterium*, one of the most commonly found bacterial genera in the neonatal intestine and oral cavity (9). Streptococcal species account for up to 10% of total bacteria in fecal samples during the first months of life (67–69). In pregnant women, GBS colonization is found in up to 30% of rectovaginal samples (28, 70, 71) and stable colonization with the same clone for several years has been demonstrated (4, 70). Spread from the gastrointestinal tract to the genital tract is considered to be a probable colonization sequence for GBS (4). Since strains might be lost or reacquired in relatively short time periods (72, 73), GBS screening is recommended relatively late in pregnancy, i.e., between gestational weeks 35 and 37 (74).

Colonization by GBS is not exclusively confined to humans. Instead, GBS was first described in the 1880s as a cause of mastitis in goats and cows and it is a frequent commensal in seals and fish (75, 76). Although rare, invasive GBS disease can be a zoonotic disease as outbreaks in adults have been linked to raw fish consumption (77). Moreover, the hypervirulent ST-17 strain, which emerged 40 years ago, shares greater genetic similarity with bovine than with many human strains, indicating that it originated from a bovine lineage. Therefore, GBS may—under very specific conditions—cross species barriers (28, 78). However, since virulent strains in humans are distinct from those causing disease in animals (26, 75), person-to-person transmission plays the primary role in human GBS dissemination. Data on GBS spread are largely confined to mother-infant pairs. In contrast, the contribution of fecal-oral transmission by other family members than the mother to GBS colonization of the infant remains unclear. While strains are largely shared between sexual partners (79, 80), cohabitation appears to play a minor role in transmission (81).

Intrapartum antibiotic prophylaxis during delivery may transiently increase the GBS colonization risk of the infant yet probably does not affect the relative abundance of *Streptococcus* spp. in the stool beyond the first few weeks of life (72). While a number of studies longitudinally analyzed the development of the microbiome after birth on the level of phylum, class or order, studies on species or even genus level, e.g., with a specific focus on Group A *Streptococcus* (GAS) or GBS are rare and do not allow for robust statements on this level of resolution. Infants which were tested negative for GBS after IAP administration frequently acquire maternal GBS strains at later time points (82). Breast milk is hence a probable source of GBS in LOD. Several LOD case studies detected GBS in breast milk (46, 83). However, it is often unclear whether GBS in breast milk results from maternal colonization or infant oropharyngeal contamination. Mutated strains from infants which have been detected in the maternal breast milk (84) support the latter hypothesis. On the other hand, positive cultures of breast milk correspond to heavy colonization of the newborn (82), which is in turn a risk factor for LOD, especially in the case of mastitis (18). Bacterial expansion in breast milk and subsequent uptake by the infant may favor heavy colonization and LOD recurrences. Finally, nosocomial GBS transmission can occur in the case of children with invasive devices (82), indicating again that LOD can be a smear infection in some cases.

COMPETING MICROBES: GBS NEEDS TO FIND ITS (NEONATAL) NICHE

Although GBS is the most prevalent streptococcal strain in neonatal sepsis, other streptococci, notably Groups A, D, and G, are isolated from blood cultures of newborns as well (22, 85, 86). Indeed, the connection of GBS and neonatal sepsis was only found in the 1960s and its predominance was established in the 1970s (24, 78). Prior to that, GAS and *Streptococcus pneumoniae* accounted for most neonatal sepsis cases (3, 87). As in other ecological niches, competition for nutrition and space occurs between bacterial species on colonized human body sites (88). Indeed, examples of mutual exclusion are found in the genus *Streptococcus*, e.g., in the case of *Streptococcus mutans*, the predominating cause of caries. The presence of other streptococcal species in the oral cavity, namely *Streptococcus sanguinis* and *Streptococcus oligofermentans*, is inversely correlated with the abundance of *S. mutans* which has been linked to the production of hydrogen peroxide *in vitro* (89, 90). Another example is the observation that *Corynebacterium* and *Dolosigranulum* in the upper respiratory tract are protective against colonization with *Streptococcus pneumonia*, which causes otitis media in infants after colonization of the airways (91). More importantly in the context of this review, growth of GBS is inhibited by *Streptococcus salivarius* both *in vitro* and in a vaginal colonization mouse model (92). Competitive growth was also shown for *Bifidobacterium* and GBS *in vitro* (93) and lactobacilli inhibited growth (94) and attachment of GBS to vaginal epithelial cells (95). In addition, *Lactobacillus reuteri* reduced vaginal colonization in a mouse model (96) and—importantly—as a probiotic in a placebo-controlled trial in pregnant women (97). These findings are in line with a very recent randomized, double-blind, placebo-controlled trial from Indian, where *Lactobacillus plantarum* plus fructooligosaccharide protected newborns from sepsis (98). In general, however, the presence of GBS appears not to be linked to an abnormal microbiome or a reduction of the predominant *Lactobacillus* genus in the vaginal tract of the mother (99–101). Interestingly, a small study found significant taxonomic differences in stools of 6-month infants, when mothers were GBS carriers, as compared to non-carriers (102). Yet, robust epidemiological evidence for a correlation of neonatal colonization with GBS and that of other specific intestinal commensals such as other streptococcal species is not existent.

Next to streptococci, staphylococci cause bacteremia and sepsis in newborns. Indeed, coagulase-negative staphylococci are the most common cause of nosocomial sepsis in newborns, yet do not play a role in healthy term infants. The generally more virulent *S. aureus* is isolated in variable frequency from neonatal blood cultures, but it is rarely found in cerebrospinal fluid (86). Furthermore, in view of the omnipresence of *S. aureus* as a colonizer in up to 50% of neonates, infants of this age group are not specifically susceptible to staphylococcal infections, unless they are subject to medical interventions such as indwelling catheters

or surgery (85, 103). Hence, the contact with GBS and potentially other (beta-hemolytic) streptococci and the establishment of coexistence with these bacteria appears to impose a greater risk to the infant compared to other genii.

THE IMPACT OF ANTIBIOTIC PRESSURE AND RESISTANCE ON LOD

The majority of GBS strains isolated from humans are resistant to the antibiotic tetracycline. Indeed, the insertion of tetracycline resistance (TcR) elements, i.e., the ribosomal protection proteins Tet(M) and Tet(O), in few GBS clones led to their selection and expansion after the onset of extensive tetracycline usage since 1948 (24). These clones have since replaced a prior diverse GBS population, concurrent with the rise of GBS as major cause of neonatal sepsis. Notably, TcR elements are the most widely spread resistance genes in the human gut microbiota (104). Moreover, a subset of GBS strains, especially ST-1, carry genes which confer general resistance to macrolids and lincosamides, i.e., the methylases erm(B) and erm(TR) (24). Resistance rates to clindamycin (lincosamid) and erythromycin (macrolide) range up to 30 and 50%, respectively (30, 71, 105, 106). A rise of resistance to fluoroquinolones has been described in serotype V strains (105, 107). In addition, GBS with reduced penicillin susceptibility due to mutations in the penicillin-binding proteins are isolated with increasing frequencies in Japan (108, 109) and were also reported to occur spontaneously in an American patient after prolonged penicillin treatment (110). In this context, it seems likely that the frequent use of antibiotics other than tetracyclines may also lead to selection of hypervirulent strains. In the Netherlands, the incidence of EOD caused by ST-17 has significantly increased after implementation of a risk-based approach of antibiotic prophylaxis (15). ST-17 strains are also significantly more prevalent in women with IAP as compared to other strains (72). Thus, a relatively short course of intrapartum antibiotics, usually penicillin and ampicillin, may allow for seeding and expansion of hypervirulent GBS strains, which may not affect the majority of infants but propagate LOD development in few colonized individuals.

In addition, the capsular serotypes of GBS are not fixed but subject to frequent exchange by conjugative transfer between strains, explaining for the diversity of serotypes within clonal complexes. Lately, serotype IV has emerged as a causative agent of adult GBS disease in the US (106, 111). This seems important, as serotype IV is not included in the latest efforts in vaccine development to capsular antigens of GBS. Sequencing has revealed that a predominating serotype IV strain acquired large genomic fragments by horizontal gene transfer from the hypervirulent ST-17 and ST-23 strains (112). Additionally, ST-17 strains with capsular switching to serotype IV have been identified in several countries (29, 113, 114). Since maternal antibodies can impact on colonization with the antibody-specific GBS strains in mothers and early infants (115–117), it remains an open question whether targeting certain serotypes may eventually select for strains which have acquired novel capsule genes and allow for their expansion.

Interestingly, single-nucleotide polymorphisms (SNPs) in virulence-associated genes were detected in neonatal invasive GBS strains in comparison to the respective colonizing strains from the mothers, possibly contributing to the transition from a maternal commensal to a neonatal pathogen (84). This suggests that mutations are positively selected for in the neonatal environment. Moreover, mutations in the virulence regulator CovR/S leading to hyperhemolytic activity were found in invasive isolates of women in preterm labor (35). The acquisition of antibiotic resistance, serotype switching and SNPs can therefore lead to microevolution in the individual newborn, which may explain the pathogenicity of GBS in only a very small number of infants.

THE ROLE OF ANTIBIOTICS AND DYSBIOSIS IN THE DEVELOPMENT OF GBS SEPSIS

The microbiota may have beneficial but also detrimental, acute, and chronic effects on infant health. Dysbiosis may predispose the neonatal intestine to inflammation (63) and facilitate the expansion of otherwise infrequent pathobionts (118, 119). Dysbiosis with lower bacterial diversity and decreased density of *Propionibacterium* spp. was found to precede the onset of necrotizing enterocolitis (NEC) (120, 121). Moreover, lactate-producing bacilli such as staphylococci and streptococci were reduced after birth in infants with NEC (68). Even though the increased prevalence of opportunistic pathogens such as uropathogenic *E. coli* (122) and Clostridium perfringens (68) has been linked to NEC, a common bacterial signature has not been found (121, 123). In addition, it is often unclear whether dysbiosis and the development of organ pathology are causally linked or whether they both depend on upstream disturbances, which may be diverse. Mai et al. found signs of dysbiosis in preterm infants already 2 weeks before onset of sepsis (124). Dysbiosis meant a delayed colonization with *Proteobacteria* and decreased density of *Bifidobacteria* spp. This observation receives support by the finding that *Bifidobacterium* spp. in the gut are protective for LOD (44), although the data on this issue are not fully consistent between studies (40). During sepsis, anaerobic *Bacteroides* and *Bifidobacterium* spp. were found to be decreased and aerobic *Enterobacteria* to be increased in affected infants as compared to non-septic twin controls (125). In view of these observations, a reduced intestinal *Bifidobacterium* density in infants whose mothers received IAP constitutes an important warning sign for the most careful usage of antibiotics in this sensitive period (93). In support of this notion, the risk for LOD caused by various pathogens including GBS in preterm infants is threefold higher after prolonged empirical antibiotic treatment (126). Antibiotics can affect the composition of the microbiome in many ways, including the depletion of competitive microbes, a delay in immune cell maturation (see below) and dysbiosis, all of which widen the niche for pathogenic bacteria.

CELLULAR INNATE IMMUNITY AND RESISTANCE TO GBS

Group B *streptococcus* is also recognized as an important health threat in immunocompromised adults, i.e., the elderly and patients with diabetes mellitus or HIV infections. Notably, the most common manifestations are skin/soft tissue infections and bacteremia (127–129), indicating that in these patients barrier immunity is important for the normal containment of GBS, similar to the situation in infants. The immaturity of the neonatal immune system in comparison to that of the adult was reviewed in detail elsewhere (130–132) and we will therefore focus on selected GBS-related aspects.

Neonatal rodents show exquisite sensitivity for GBS. Neonatal rats succumb to doses as low as 10 CFU intraperitoneally, while adult rats require approximately 6-log higher inoculums for a similar mortality rate (49) even if their body weight is taken into account (50). Neonatal mice, which normally die after i.p. infection within 48 h, were protected by transfer of specific antiserum to the pregnant dam before delivery (133). This experimental data is in line with the protective role of maternal GBS antibodies in the protection from GBS EOD, which is the basis for the development of a maternal vaccine (36, 134). In contrast, the role of maternal antibodies in the prevention LOD development is less clear. Recently, it has been inferred that high antibody levels also prevent GBS colonization (42, 116, 117). Women with high serotype-specific titers had a significantly lower risk of rectovaginal colonization with the respective GBS strains (42). However, GBS antibody levels do not inversely correlate with the sepsis risk *per se*. Thus, it remains puzzling why only very few of the GBS exposed and/or colonized infants with low antibody levels develop LOD.

In the innate arm of the immune system, the family of Toll-like receptors (TLRs) is essential for the defense against invasive streptococcal infections. Children with genetic deficiency in MyD88, an essential adaptor for all TLRs but TLR3, or IRAK4, a kinase downstream of MyD88, have an approximately 50% risk of dying from invasive bacterial infections in the first 8 years of life. In most cases, streptococci are the causative organisms (135, 136). Furthermore, roughly one third of the affected children suffer from a sepsis episode in the first 3 months of life. Thus, the risk for early and late neonatal sepsis is approximately 1,000-fold higher in these infants than in newborn infants overall. It seems noteworthy that most isolates are either pneumococci or GAS, whereas only few cases of late neonatal sepsis and meningitis caused by GBS have been reported so far (135, 137). Whether this predominance of other streptococcal species is due to an altered microbiome in MyD88- and IRAK4-deficient individuals has not been explored so far. In mice with MyD88 deficiency, a gross deviation in microbiome composition cannot be observed (138, 139), although a generally increased risk for the invasion and dissemination of intestinal commensals was observed (140). Moreover, MyD88-deficient neonatal mice have not been studied in this context. The already exceptional susceptibility of neonatal mice for local GBS infections, with a 100,000-fold decreased LD90 (cfu/g bw) in 2-day-old mice as compared to adult mice, is further significantly increased in MyD88 deficiency (141, 142).

Within the MyD88-dependent TLR family, TLR2 activation by GBS lipoproteins (143, 144) and endosomal TLR-activation by single-stranded RNA are equally important. TLR13 is a common receptor of 16S rRNA from Gram-positive bacteria including GBS in mice (10, 145), whereas TLR8 is the incomplete analog in humans (146–148). TLR recognition by myeloid cells is highly site-specific, i.e., RNA sensing and TLR13 are crucial for recognition of GBS by resident mouse macrophages but not circulating blood monocytes (142). Interestingly, recognition of GBS and Gram-positive bacteria appears to rely more on endosomal TLRs than recognition of Gram-negative bacteria (149). This seems intriguing in the context of human neonatal mononuclear cells, which are particularly responsive to TLR8 ligands (150). Accordingly, recognition of bacterial RNA by TLRs is not only particularly important at the beginning of life, but may result in distinct immune activation patterns induced by *Streptococcaceae* and *Enterobacteriaceae*. It remains an appealing yet unproven hypothesis that TLR8-dependent immunopathology contributes to myeloid cell-mediated disturbance of mucocutaneous barrier integrity. In addition, TLR8 and 13 do not hold exclusive roles in the recognition of GBS RNA or nucleic acids in general. First, the NLRP3 inflammasome mediates GBS-induced formation of IL-1β and IL-18 in macrophages *via* recognition of ssRNA (151, 152). NLRP3 activation requires the induction of potassium efflux by a rhamnolipid of GBS, which also mediates cytolysis (35). Proper inflammasome activation is essential for the neonatal resistance against GBS (151). Next, GBS DNA engages the cytosolic signaling of cGAS and STING which leads to interferon (IFN)-β production and contributes to GBS immunity (153, 154). In addition, conventional dendritic cells secret type I IFNs in response to endosomal GBS RNA interacting with TLR7 (155). GBS may subvert nucleotide sensing *via* expression of ectonucleotidases (154, 156) (**Figure 1**). Similarly, the GBS hyaluronidase HylB blocks cellular activation by degrading host hyaluronic acid into fragments which bind and inhibit TLR2 (157). HylB was shown to promote vaginal colonization and ascending infections in mice (157, 158). How these enzymes impact on the sensing of colonizing GBS and of competing bacteria in neonates is currently unclear. It furthermore remains to be determined how the relatively increased TRIF-dependent pathway in neonates impacts on barrier defense against GBS (159). Any effect can be assumed to be indirect, since TRIF is redundant in GBS-mediated activation of phagocytes, although a role as a signaling intermediate in other (immune) cells cannot be excluded (149, 160).

Understanding the distinct TLR, inflammasome and cGAS engagement in the monocyte-macrophage lineage by GBS is of utmost importance, since macrophages are the dominant resident immune cells at mucocutaneous barriers, i.e., the dermis and the gut. They are crucially involved in barrier maintenance (161, 162), both by executing direct antimicrobial actions and by cytokine and chemokine dependent recruitment and activation of other immune cells. Development of the neonatal macrophage compartment is particularly well understood in the neonatal

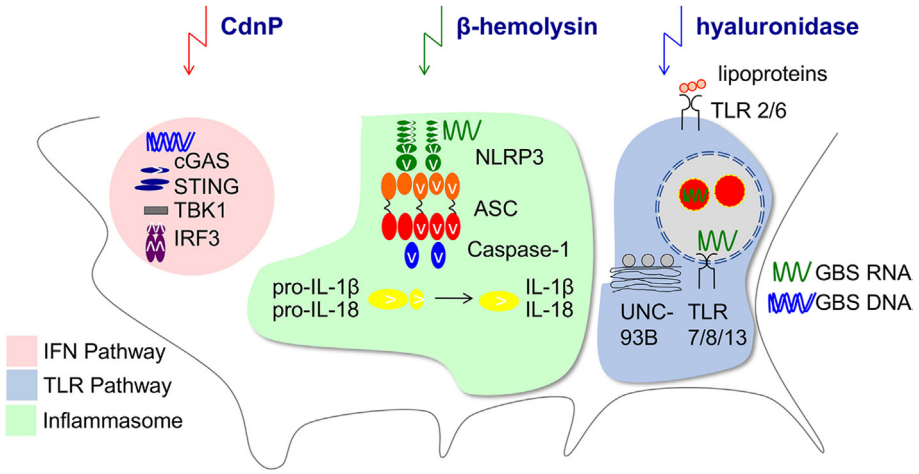

FIGURE 1 | Innate immune pathways manipulated by Group B *Streptococcus*. Depicted is the impact of GBS on type I interferons (IFN) (153, 155), Toll-like receptor (TLR) (10, 149), and inflammasome (151) pathways by secreted bacterial factors. The ectonucleotidase CdnP hydrolyzes bacterial cyclic dinucleotides which otherwise activate STING and IFN-β production (154). Hemolysin contributes as second signal to the NLRP3 inflammasome activation (152). The GBS hyaluronidase can degrade pro-inflammatory hyaluronan polymers during tissue injury which normally bind to TLR2 and the resulting fragments block TLR2 signaling in the host (157).

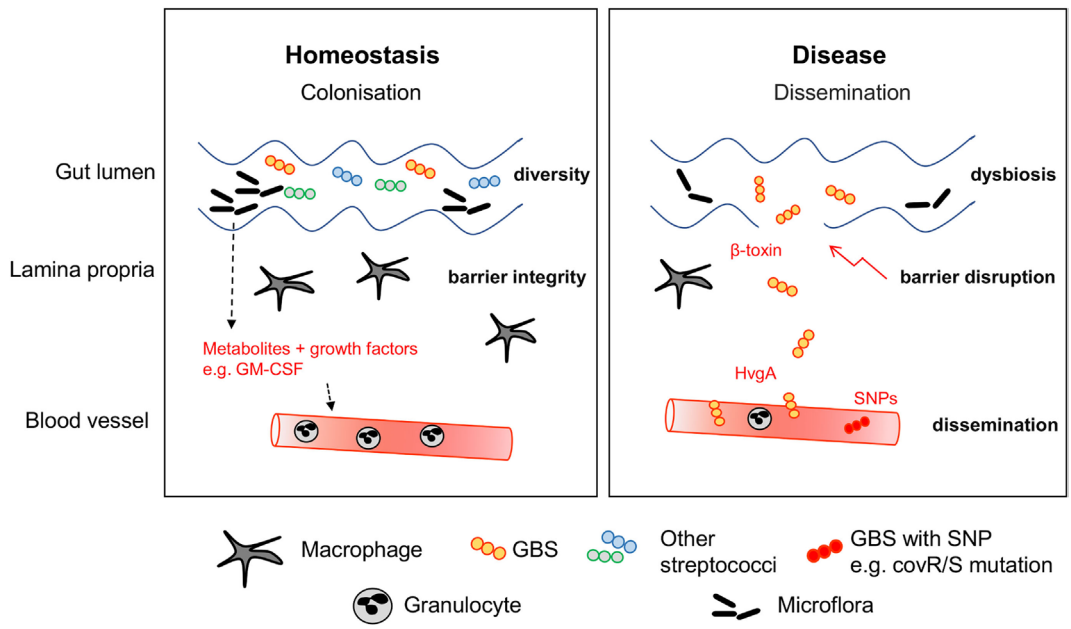

FIGURE 2 | Stabilization of the mucocutaneous niche. During homeostasis, GBS colonizes the intestine of healthy infants. Macrophages and other immune cells guarantee barrier integrity by surveillance. Other commensal bacteria including streptococcal species form the niche. Disease can be preceded by multiple factors leading to dysbiosis, expansion of GBS and barrier disruption. Expression of virulence factors such as HvgA and β-toxin facilitate adhesion to epithelial cells and barrier disruption. Dissemination is often concurrent with mutations of the CovR/S virulence repressor.

intestine, where the population of embryonic macrophages is replaced by monocyte-derived macrophages starting at weaning (163). It is tempting to speculate that macrophage maturation in the lamina propria directly impacts on the macrophage-driven recognition and elimination of invading GBS. Another TLR-based mechanism promoting susceptibility to GBS is the increased production of anti-inflammatory cytokines. Enhanced IL-10 concentrations in serum and cord blood are correlated with mortality in septic infants (164). Moreover, IL-10 has a major impact on intestinal barrier immunity, both in humans and mice. Yet, whereas too little IL-10 leads to spontaneous inflammation and colitis, increased IL-10 production impairs neutrophil recruitment into infected organs and thus decreases GBS clearance (164, 165). How increased IL-10 formation

impacts on keeping GBS in a colonization—as opposed to an invasion—state is currently not known.

IMPACT OF THE MICROBIOME ON THE DEVELOPING IMMUNITY

Numerous studies were initiated to understand the impact of the colonizing flora on the function of intestinal cells in general and the immune system in general. Research is usually based on germ-free mice and antibiotic treatments in order to understand the consequences of a reduction or absence of microorganisms. Evidence for immunological consequences of alterations in the microbiome was even found in cells very distant to the gastrointestinal tract such as brain microglia (166). In a highly interesting mouse study, exposure of the pregnant dam to antibiotics not only led to neutropenia in newborn mice, but subsequently increased the susceptibility to Gram-negative sepsis (10). A reduction in *Gammaproteobacteria* may mediate these effects, since their effector LPS induces granulocyte colony-stimulating factor production and consequently granulopoiesis. Recently, Josefsdottir et al. suggested that the microbiota is the cause of neutropenia and general depletion of hematopoietic stem cells across multiple lineages in antibiotic-treated mice (11). The phenotype could be partially rescued by fecal transfer. This experimental data is in line with the observation that administration of ceftalorine and β-lactam antibiotics can lead to neutropenia in patients (167, 168). Consequently, antibiotics appear to indirectly impact on the maturation of the immune response (169) and the resistance against neonatal sepsis pathogens. An overall smaller granulocyte pool in neonates (132) may further propagate the negative effects of antibiotics. Therefore, it seems that the immaturity of neonatal blood cells, including phagocytes and adaptive immune cells, might restrict the ability to fight off pathogens. Hence, in the stochastic event of pathogen invasion through the mucocutaneous barrier, which may be potently responded to by the adult immune system, neonatal immunity may be overwhelmed, resulting in bacterial spread and sepsis (**Figure 2**). It remains incompletely understood whether the protection in the adult usually involves the resident immune cells at mucocutaneous sites, e.g., the lamina propria in the gut or the dermis in the skin, or whether circulating leukocytes are necessary for efficient barrier defense.

CONCLUSION

The challenge to understand and ultimately prevent neonatal GBS sepsis comprises (i) the control of GBS transmission during and immediately after birth leading to EOD and (ii) the subsequent control of GBS as a mucocutaneous colonizer, when failure results in LOD. Whereas high maternal antibody titers, as induced by GBS vaccines, and IAP are established strategies to prevent EOD, similar strategies with proven efficacy for LOD reduction are missing. Based on experimental and observational evidence, it seems worth considering—and thus requires careful studies—whether antibiotic pressure during primary colonization of the intestine facilitates dysbiosis on the strain level and transient immunodeficiency in the individual child. Furthermore, capsular polysaccharide based vaccines may select for serotype-switched virulent strains as observed with ST-17 and allow for the expansion of other β-hemolytic streptococci than GBS.

The vast recent gain in knowledge on the coevolution of microbiome and cellular barrier defense make the design of novel approaches for neonatal sepsis prevention conceivable, although much preclinical work remains to be done first. Examples are designer probiotics, containing—among others—strains which occupy the streptococcal niche without risk of invasion. Immunomodulators that accelerate the maturation of the phagocyte population resident at mucocutaneous sites may be another strategy that holds potential. Yet, the variable conditions and demands at the beginning of life, e.g., that of very preterm infants or those requiring antibiotic therapy early on, make one-fits-all solutions to the neonatal sepsis conundrum unlikely and rather ask for individualized approaches.

AUTHOR CONTRIBUTIONS

JK and PH wrote and edited the manuscript.

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SIgA, TGF-β1, IL-10, and TNFα in Colostrum Are Associated with Infant Group B *Streptococcus* Colonization

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Background: Group B *Streptococcus* (GBS) is a major cause of mortality and morbidity in infants and is associated with transmission from a colonized mother at birth and *via* infected breastmilk. Although maternal/infant colonization with GBS is common, the majority of infants exposed to GBS remain unaffected. The association between breastmilk immune factors and infant colonization and disease prevention has not been elucidated.

Objectives: We have investigated the association between SIgA and cytokines in breastmilk and infant GBS colonization and clearance.

Methods: Mother/infant GBS colonization was determined in a prospective cohort of 750 Gambian mother/infant pairs followed to day 89 of life. Anti-GBS secretory IgA bound to the surface of whole bacteria was assessed by flow cytometry and a panel of 12 cytokines quantified by mesoscale discovery in colostrum, breastmilk and serum.

Results: Compared with infants receiving low anti-GBS SIgA in colostrum, infants receiving high anti-GBS SIgA were at decreased risk of GBS colonization for serotypes III and V. Infants colonized at day 6 were twice as likely to receive colostrum with high TGF-β1, TNFα, IL10, and IL-6 compared to uncolonized infants. Infants receiving high colostral TGF-β1, TNFα, and IL-6 had two-fold enhanced GBS clearance between birth and day 89.

Conclusion: Our results suggest that the infant GBS colonization risk diminishes with increasing anti-GBS SIgA antibody in breastmilk and that key maternally derived cytokines might contribute to protection against infant colonization. These findings might be leveraged to develop interventions including maternal vaccination that may reduce infant GBS colonization.

Keywords: breast milk, antibody, cytokines, neonatal immunity, microbiome, Group B *Streptococcus*

Abbreviations: GBS, group B *Streptococcus*; SIgA, secretory Ig A; FI-C, fluorescence intensity; STGG, skim-milk tryptone glucose glycerol; ST, serotype; PBS, phosphate buffered saline; BSA, bovine serum albumin.

INTRODUCTION

Given the limited ability of newborns to respond efficiently to infectious agents, including at the mucosal surface, there is increasing interest in identifying maternal factors that may influence protection from infections in the early months of life. Neonatal immunity can be influenced by maternal factors through transfer of specific antibody across the placenta as well as *via* breastmilk (1). Several studies have shown that breastfed infants are better protected from infections of the gastrointestinal and respiratory tracts compared to formula-fed infants (2–4). The neonatal gut immune system is constantly modulated by colostrum and breastmilk, promoting the growth of certain bacteria over others and thereby influencing the neonatal immune response (1). Evidence now supports a connection between the mother's gastrointestinal tract and the mammary glands *via* an "entero-mammary circulation," which may play a role in immune priming (5).

One of the most important factors in breastmilk is secretory IgA (SIgA). SIgA mediates protection *via* binding to mucosal pathogens as well as neutralization of toxins and virulence factors. SIgA antibodies can prevent bacterial adhesion by binding to pili and other adhesins found on the surface of group B *Streptococcus* (GBS) (6, 7).

It is also established that cytokines in breastmilk vary according to maternal exposure to bacteria (8). TGF-β, IL-6, and IL-10 in breastmilk may enable the development and differentiation of neonatal cells that produce IgA (9) and are involved in the maturation of the neonatal gut immune system (10). It has also been shown that TGF-β1 and TGF-β2 in colostrum are positively correlated with infant IgA in neonatal serum (11, 12).

Little is known about the impact of cytokines and SIgA in breastmilk on bacterial colonization and clearance in general and in relation to GBS in particular. We tested the hypothesis that SIgA and breastmilk cytokines could influence infant colonization and clearance over the first three months of life in a cohort of Gambian infants.

MATERIALS AND METHODS

Samples were collected in an urban clinic in The Gambia, West Africa as part of a wider study of GBS infection previously reported (13). Ethical approval was given by the Gambia Government/MRC Joint Ethics committee (reference SCC 1350v4).

Sample Collection

Sample collection and microbiology have been previously described (13). Briefly, 750 pregnant women between the ages of 18 and 40 years who had a low-risk, singleton pregnancy were recruited after giving informed consent. The study excluded very preterm (<32 weeks gestation) or very low birth weight (<2.0 kg) infants. Colostrum was collected within 12 h of birth and breastmilk was collected at day 6 and 60–89 days of life. After washing their hands, mothers wiped each breast with an alcohol wipe beforehand expressing 2–3 mL of colostrum and 4–5 mL of breastmilk. Milk samples were stored on cold packs at 4°C and transported to the laboratory within 6 h of collection. The lipid and whey layers were separated by centrifugation at 3,200 rpm for 30 min. The solid lipid layer was removed using a sterile scalpel and the whey stored separately in 1 mL aliquots at −70°C.

Midwives or field nurses collected rectovaginal swabs (Copan, UK) after cleaning of the perineum when women presented to the hospital in labor. Infant nasopharyngeal (calcium alginate swabs, Sterilin, UK) and rectal swabs (Copan, UK) were taken at 4 h of life, on day 6 and at day 60–89. Swabs were stored in 5 mL skim-milk tryptone glucose glycerol (STGG) on cold packs at 4°C and transported to the laboratory within 6 h of collection. The swab in STGG was vortexed briefly before being stored at −70°C (13).

Microbiological and Molecular Quantification of GBS Colonization

The protocol was adapted from the Public Health England (PHE) protocol for the pre-incubation of swabs in enrichment broth before plating on solid media to enhance the yield of GBS (14). Swab samples were processed within 7 days of collection in batches of 100. Swabs in STGG were thawed on wet ice and transported to the microbiology laboratory. Samples were vortexed for 10–20 s and 100 µL of vortexed specimen was transferred to 2.5 mL Todd Hewitt Broth for 24 h pre-culture at 37°C in an atmosphere of 5% CO_2. A Colombia Agar plate was divided into four segments, and 10 µL of neat suspension and each of three 1:10 dilutions were dispensed into each of the four quadrants and incubated overnight at 37°C in 5% CO_2. From the primary plate, presumptive beta hemolytic Streptococcus colonies were streaked onto a blood agar plate and reincubated at 37°C in 5% CO_2 for 24 h. Beta hemolytic streptococci appeared colorless or gray, about 2 mm in diameter with or without a surrounding zone of beta-hemolysis. Streptex® (Oxoid, UK) was used in the qualitative identification of GBS. Samples were serotyped using PCR as previously described (13).

All infants were exclusively breastfed and all samples were collected before feeds to ensure samples were not contaminated with oral bacteria.

Cytokine Quantification

The concentrations of cytokines IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, IL-13, IFN-γ, TNFα, and TGF-β1 were determined in the cord serum, colostrum, and breastmilk of a subset of 100 randomly selected Gambian mothers and serum from their infants at day 60–90, using electroluminescence *via* the Meso-Scale Discovery system and the Proinflammatory Panel 1 (human) MSD Multi-Spot Kit (MSD, Rockville, MD, USA). First, multi-analyte calibrator solution containing recombinant human cytokines at known concentrations, which have been expressed in *E. coli* or Sf21 insect cells, was reconstituted using the diluent provided. The calibrator was diluted fourfold six times to generate a series of seven reference calibrators to which unknown serum/breastmilk samples were compared. All serum/colostrum/breastmilk samples were diluted two-fold in the same diluent before addition to the plate. 50 µl of the diluted serum or

breastmilk and calibrators were added to each well. Plates were incubated at room temperature for 2 h and then washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween-20. 25 µl of a solution containing detection antibody to each cytokine was added to each well. Plates were then incubated for a further 2 h at room temperature. After the incubation, plates were washed and 150 µl read buffer added. Plates were then read using the MESO QuickPlex SQ 120 MSD instrument. Samples below the limit of quantification were allocated a value for each cytokine of half the lower limit of detection as per manufacturer's instructions. Details of each lower limit of quantification and the associated allocations for analysis are found in Table S2 in Supplementary Material. The coefficient of variance (CoV) for the assay was 25%.

SIgA Binding to GBS Bacteria

The assay was adapted from a serological assay described by Le Doare et al. (15) which quantified IgG binding to GBS bacteria using serum samples. GBS isolates used in this study were H092040676 (ST Ia), H092120162 (ST III), and H091780506 (ST V), which were kindly provided by Professor Androulla Efstratiou, PHE, Colindale. 5 µl of the breastmilk sample was added to a 96 well micro-titer plate and incubated with 195 µl of killed GBS of STs Ia, III, and V diluted to OD 0.05 (at 600 nm) in PBS with 2% bovine serum albumin (BSA) for 30 min at 25°C while shaking at 900 rpm. After centrifuging at 3,060 *g* for 5 min, excess fluid was removed. The pellet was washed with 200 µl of PBS with 2% BSA before centrifuging. The pellets were then re-suspended in rabbit anti-human SIgA polyclonal FITC antibody (BIOSS, Woburn USA) diluted to 1:100 in PBS with 2% BSA. This was incubated at 4°C for 20 min. After centrifugation, the pellet was washed twice. The final pellet was re-suspended in PBS with 1% formaldehyde to kill any sample bacteria in the breastmilk before SIgA binding was analyzed using a Beckman Coulter Cyan flow cytometer with attached Cytek 96 well plate loader (High Wycombe, UK) calibrated with Ultra rainbow beads (San Diego, CA, USA). A secondary gate was set to 10% of the histogram of the bacteria and conjugate wells providing a percentage gated value for each of the samples. The percentage gated and the mean fluorescence for that population were multiplied together to give a fluorescence index (FI). The FI of the conjugate control was subtracted from each sample's mean Fl to remove background, non-specific binding. This is referred to as florescence intensity (FI-C) (see Figure S1 in Supplementary Material). For the measurement of SIgA deposition, raw data were plotted and used in the analysis. Where no antibody deposition was detected, these data were given a value of ND and excluded from analysis; all samples were run in duplicate and samples were accepted if the standard error was < 5%. Over the course of the study, the mean SD of FI for zymozan controls was 8,831 (CoV, 31.6%).

Statistical Analysis and Sample Size Calculation

To ensure that the study avoided bias, we followed the statistical design of experiments for cluster analysis method (16). Based on estimates from the original study in The Gambia [21% infants colonized (158/750); 6.5% prevalence of the lowest serotype GBS STIa] (13) a sample size of 750 women would be required to give at least ten women/5 infants colonized with GBS STIa. Five infants for each GBS ST would give 80% power to detect a correlation of 70% or greater between GBS ST-specific antibody and colonization.

STATA version 12 (StataCorp 2013, California, CA, USA) and GraphPad Prism version 6.0 (GraphPad Software Inc., La Jolla, CA, USA) were used for statistical analysis. Additional statistical support was provided by Fiona Warburton (PHE) who verified all statistical analysis.

Potential differences between cytokines in colostrum and breastmilk were calculated using the Mann–Whitney *U* Tests or Wilcoxon Rank sum tests. Radar plots were generated to demonstrate the distribution of cytokines between colonization groups. Odds ratios, adjusted odds ratios, and analysis of variance (ANOVA) were calculated to compare groups. Potential differences in antibody concentrations between colonized and non-colonized mothers and infants were evaluated by Student's *t*-test after log transformation of data. Three groups were compared (mother colonized/infant non-colonized; mother colonized/infant colonized; and neither mother nor infant colonized) using ANOVA following log-transformation of data. Comparison of log-transformed serum FI-C values at day 6 and 60–89 days was performed using a paired *t*-test. Linear regression was calculated from log transformed data to determine IgG in serum and IgA in breastmilk.

Definitions

Maternal colonization was defined as the identification of a GBS-positive rectovaginal swab at time of delivery. Infant colonization was defined as the identification of a GBS-positive nasopharyngeal and/or rectal swabs at 4 h (birth), day 6 (early colonization), or day 60–89. We identified infants colonized both at birth and day 6 as the baseline colonization point to ensure we captured only true colonization episodes. Infant acquisition of colonization was defined as a positive nasopharyngeal and/or rectal swab at day 6 and/or day 60–89 when the associated swab from the previous visit was negative, and loss was defined as a negative nasopharyngeal and/or rectal swab when the previous swab was GBS-positive. Persistently colonized infants were defined as infants where nasopharyngeal and rectal swabs at birth, day 6, and day 60–89 were all GBS positive.

RESULTS

The maternal GBS colonization rate was 32% ($n = 237$), with 21% of infants colonized at birth ($n = 158$), 20% colonized at day 6 ($n = 152$), and 7% of infants colonized on day 60–89 ($n = 50$) (13). 680 colostrum and 750 breastmilk samples were available for analysis.

We examined the presence of GBS in breastmilk from all 750 women and found 10 to be GBS colonized in breastmilk.

Relationship between SIgA in Breastmilk and Maternal GBS-Colonization Status

Overall, 324/680 (47.6%) colostrum samples had detectable SIgA against any GBS ST. There was no significant difference in SIgA concentration between non-colonized mothers and those colonized with STs Ia [GBS + mother FI-C 1300 (1,000–1765) GBS-mother 1500 (1000–1886) $p = 0.3$], III [GBS + 1713 (1,545–4,837), GBS-1995 (1,798–2,223) ($p = 0.3$)], or V [GBS + 2089 (1,883–5,896) GBS-2691 (2,426–2,985) ($p = 0.8$)].

There was no difference in IgA antibody concentrations found between those women colonized in breastmilk and those uncolonized.

Relationship between SIgA in Colostrum and Maternal/Infant Colonization on Day 6

152 infants [152/680 (22.4%)] were colonized both at birth and on day 6 of life. Colonized infants born to colonized mothers received colostrum with lower SIgA binding to GBS than any other colonization group. Non-colonized infants born to colonized mothers received colostrum with significantly higher SIgA than colonized mother/infant pairs for STIII and STV, when adjusted for maternal age, maternal weight, maternal anemia, and gestation ($p < 0.0001$) (**Figure 1**).

SIgA in Colostrum Is Associated with Absence of Infant Colonization at Days 60–89

Compared to infants who remained colonized on day 60–89, non-colonized infants received higher SIgA concentrations against STV in colostrum ($p < 0.001$). There were no infants who remained colonized with STIa and only three infants remained colonized with STIII. Infants who cleared colonization between birth and day 6 also received colostrum with higher concentrations of SIgA than those infants who remained colonized for STV (**Figure 1**). In addition, non-colonized infants at 60–89 days were more likely to have higher antibody concentrations in cord blood and in infant serum ($p < 0.001$). There was a positive correlation between SIgA in colostrum and complement-mediated antibody deposition in cord blood and in infant serum found in the main study ($R = 0.67$, $p < 0.0001$) (17).

Cytokines in Colostrum, Breastmilk, and Infant Serum

Significantly higher concentrations of TNFα, IFNγ, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, IL-13 ($p < 0.0001$ for all) were found in colostrum in comparison with breastmilk, cord blood, or infant serum at days 60–89. Breastmilk had lower concentrations of TNFα, IL-1β, IL-6, and IL-13 ($p < 0.0001$) than infant serum. Low levels of IL-2, IL-4, and IL-12 were found in all samples. Associations between cytokines in colostrum, breastmilk, cord blood, and infant serum are highlighted in **Figure 2**.

Relationship between Cytokines in Colostrum and Early Infant Colonization

Infants who were still colonized on day 6 of life were more likely to receive colostrum with high concentrations of TGF-β1 [OR 1.45 (1.1–1.9), $p = 0.02$], IL-10 [2.8 (1.1–7.5), $p = 0.05$], TNFα [2.4 (1.1–5.0), $p = 0.02$], and IL-6 [2.4 (1.2–5.0), $p = 0.02$] than non-colonized infants (**Figure 3**). Multivariate logistical regression adjusted for other cytokines showed that colonized infants were more likely to receive colostrum with high concentrations of TGF-β, IL-10, TNFα, and IL-6 than non-colonized infants [AOR 3.2 (1.8–9.2), $p = 0.03$]. There was no association with any other cytokine at day 6.

Relationship between Cytokines in Colostrum and Acquisition and Clearance of Infant Colonization between Birth and Days 60–89

Median concentrations of ten cytokines in colostrum were compared for all four mother/infant groups. As shown in **Figure 4**, infants who cleared colonization between birth and days 60–89 ($n = 27$) received colostrum with higher concentrations of TNFα, IL-6, and TGF-β1 ($p = 0.01$) than infants who acquired colonization between birth and day 60–89 [AOR 2.4 (1.2–5.0), $p = 0.02$]. Infants who were persistently colonized ($n = 7$) received higher concentrations of IFN-γ in colostrum than infants who acquired colonization ($n = 28$) [AOR 5.1 (2.4–11.0), $p < 0.001$]. There was no association between other cytokines in breastmilk and infant colonization at day 60–89 (**Figure 4**).

DISCUSSION

The results from our large cohort of mother/infant pairs show for the first time that SIgA and key immunomodulatory cytokines TNFα, IL-6, IL-10, and TGF-β in colostrum may be associated with infant GBS colonization, acquisition, and clearance up to 3 months of life.

Few studies have focused on the relationship between SIgA in breast milk and infant colonization with potential pathogens. Given that infant GBS colonization is the pre-requisite for disease, investigating factors that can reduce infant colonization is important, as manipulation of such factors could reduce the risk of invasive disease. Our data, obtained using an assay that measures SIgA that binds to whole GBS bacteria of STs Ia, III, and V, demonstrate that compared to all other mother/infant groups, the lowest anti-GBS SIgA levels were found in colonized mother/infant pairs. We also show that infants receiving the lowest concentration of anti-GBS SIgA in breastmilk were less likely to clear GBS colonization. Several studies have investigated anti-GBS antibody levels in breastmilk. Lagergard et al. (1992) identified IgA antibodies to CPS type III GBS in 63% of a cohort of 70 Swedish women (18), while Weisman and Dobson (1991) determined anti-STIa, II or III CPS IgG in a cohort of 46 USA women and found levels at approximately 10% of those in maternal serum (19). The most recent study of SIgA was conducted by Edwards et al. (2004). The study investigated IgG and IgA in breast milk to GBS ST III CPS in 9 colonized and 9 non-colonized women with antibody titers less than or equal to 1 μg/mL and those greater than 1 μg/mL and also found that detectable levels of anti-STIII SIgA in breastmilk correlated with high levels in maternal serum (20).

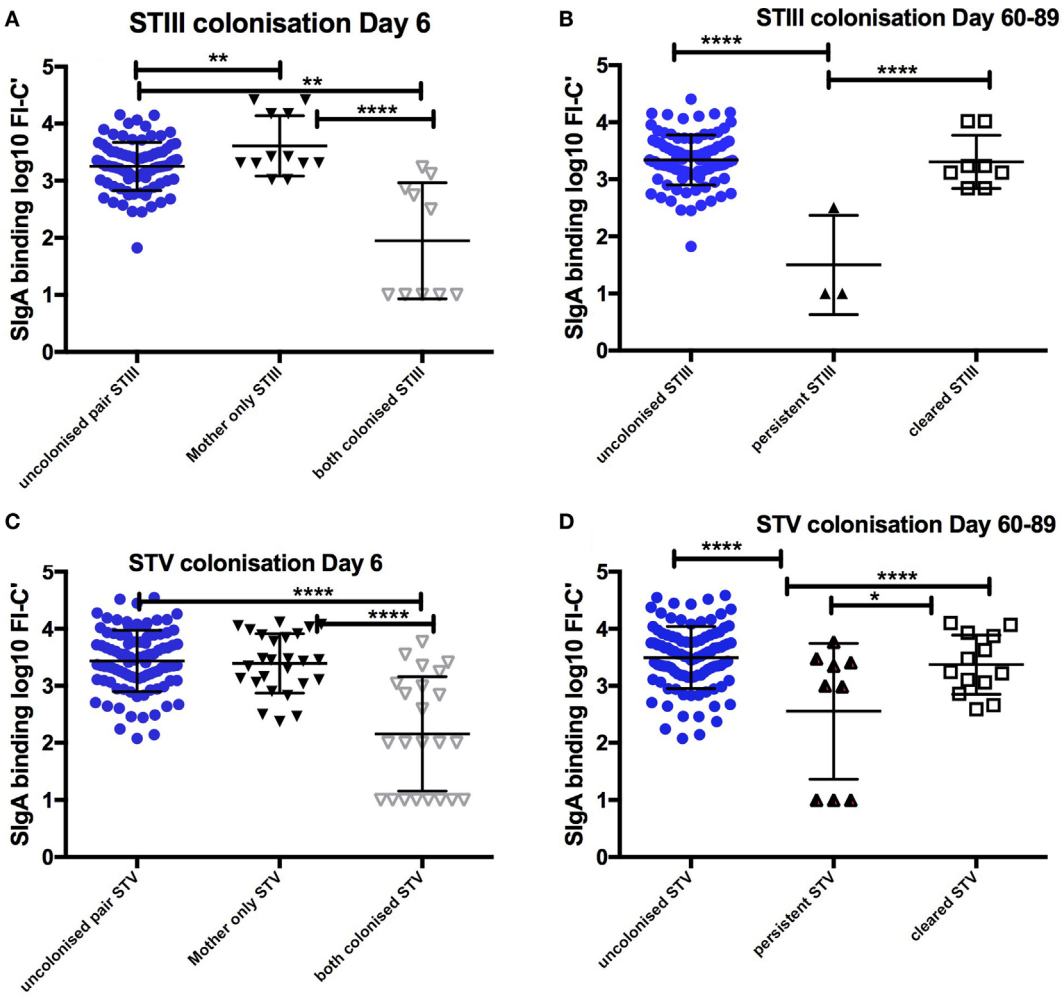

FIGURE 1 | Relationship between SIgA in colostrum and maternal and infant colonization group for STIII and STV. Mean and SD of log transformed florescence intensity (FI-C) of SIgA deposition onto the surface of whole group B *Streptococcus* (GBS) bacteria STIII and STV at day 6 **(A,C)** and clearance of colonization between day 6 and day 60–89 **(B,D)**. STIII ($n = 3$ persistent, $n = 12$ cleared colonization, $n = 106$ non-colonized any serotype) STV ($n = 9$ persistent colonization, $n = 33$ cleared colonization, $n = 106$ non-colonized any serotype). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$. Table S1 in Supplementary Material outlines the numbers in each group by colonizing serotype.

In line with our own observations, animal models of GBS ST Ib and III disease (21–23) demonstrate an association between increased SIgA in breastmilk and pup survival. There have been few studies of the effect of breastmilk antibody on infant colonization, but a small study of breastfed infants challenged postnatally with non-pathogenic *E. coli* demonstrated a reduction in colonization with high breastmilk SIgA concentrations (24), whilst studies of *H. influenzae* (25), *S. pneumoniae* (26), and *N. meningitides* (27) demonstrate that increasing antibody concentrations in serum are associated with reduced risk of pharyngeal colonization with these bacteria. If the same is true of SIgA in breastmilk and GBS colonization, then increasing maternally derived antibody in breastmilk in conjunction with increasing serum antibody through vaccination could interrupt GBS colonization and subsequently development of GBS disease.

The highest concentration of SIgA antibody in milk was found in mother/infant pairs where the mother but not the infant was colonized. This could be explained by the development of an immune response in the mother, triggered by a new GBS challenge. This antibody is then passed to the infant and acts in a protective fashion. Timing of acquisition of maternal GBS colonization is likely to be important but our study was not able to assess this aspect. The fact that mother/infant pairs who remain completely non-colonized also have high concentrations of anti-GBS SIgA antibody indicates a long half-life of IgA during lactation due to previous colonization, as has been seen in mothers vaccinated with *N. meningitidis* vaccines (28). Alternatively, this could also be due to another cross-reactive antigen that we have not identified.

Our finding of the association between TNFα and *de novo* GBS colonization fits with the results of the only other published study of breastmilk cytokines and infant disease by Riskin et al. (2012). This study analyzed the breastmilk of exclusively breastfeeding mothers of 31 sick infants under 3 months of age and

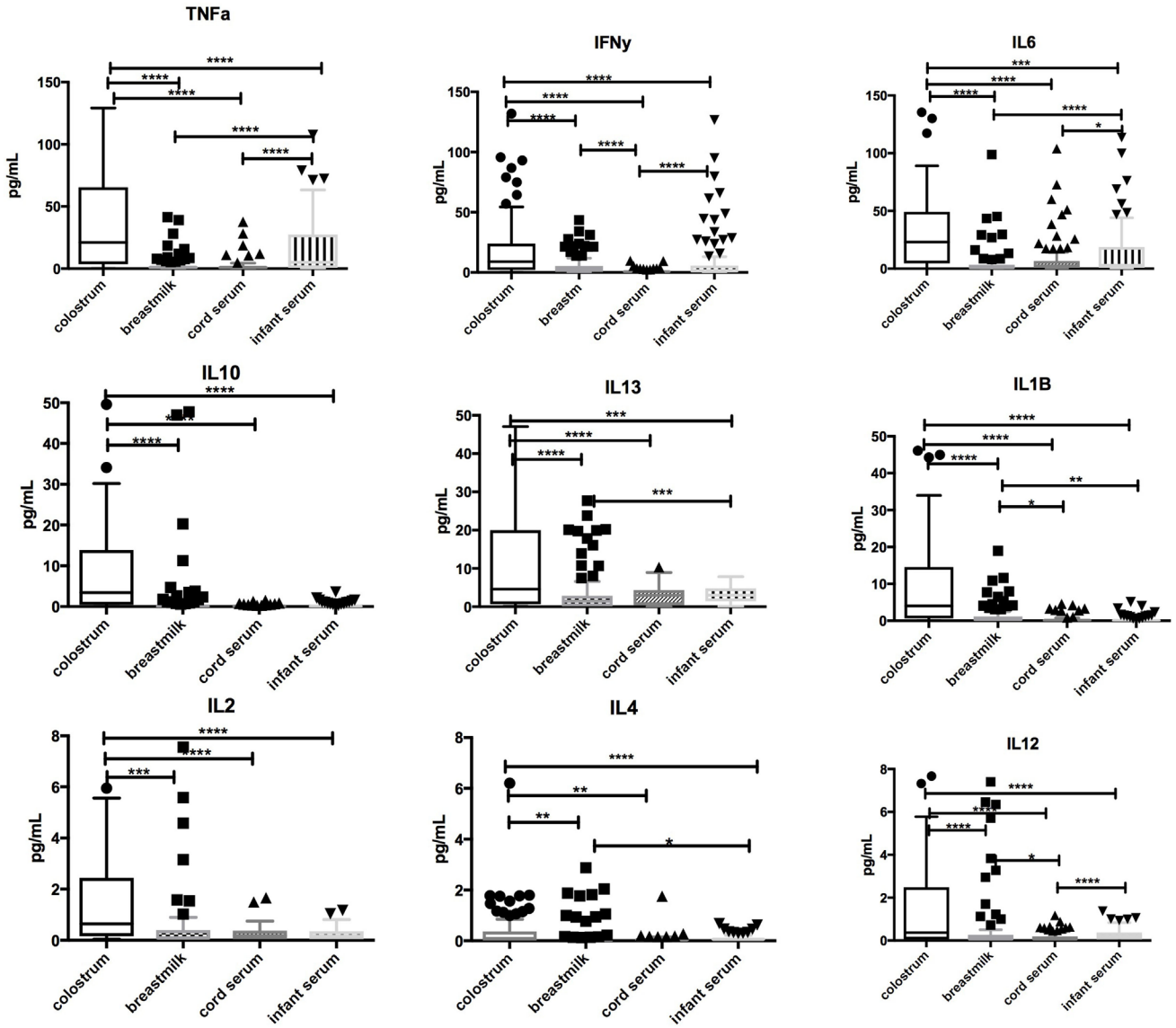

FIGURE 2 | Comparison of cytokine concentrations in colostrum, breastmilk, cord blood, and infant serum. Box-and-whisker plot of concentrations of TNFα, IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, and IL-13 in colostrum, breastmilk, cord, and infant serum in picograms per milliliter (pg/mL). 100 samples were assessed. Median and interquartile range is demonstrated. Solid shapes indicate outliers. $^{*}p = <0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

demonstrated that increases in TNFα in colostrum were associated with acute infant disease, whether or not the mother was also sick (29). A possible explanation is that colostral TNFα induced an increase of MHC class II expression on antigen presenting cells (30) and expression of receptors that facilitate transcytosis of SIgA into exocrine fluids in the respiratory and gastrointestinal tracts to counteract infection (31). *In vitro* studies have similarly demonstrated that increased TNFα was associated with altered binding of *E. coli* in intestinal cells (32). If the same is true with GBS, then increased colostral TNFα could reduce GBS binding to neonatal gut epithelium and reduce the risk of systemic invasion by inducing IgA at the mucosal surface.

Our observation that early infant colonization was associated with increased concentrations of IL-10 and TGF-β might be explained by IL-6-driven synthesis of IgM and IgG together with TGF-β as part of the maternal immune response to a pathogen during active infection, which equally increases IgA synthesis from naive B cells (33). However, it is also possible that the cytokines in breastmilk result from local immune activation in the mammary glands due to GBS in breastmilk. These hypotheses remain speculative at present and future longitudinal studies of breastmilk and assessment of function of infant gut epithelial cells in response to pathogen challenge would be required to provide more insight into this phenomenon.

Our study has several limitations. As cytokines vary within and between mothers, our data can only provide a cross-sectional snapshot and a larger cohort with longitudinal maternal sampling is needed to fully appreciate the implications of these results.

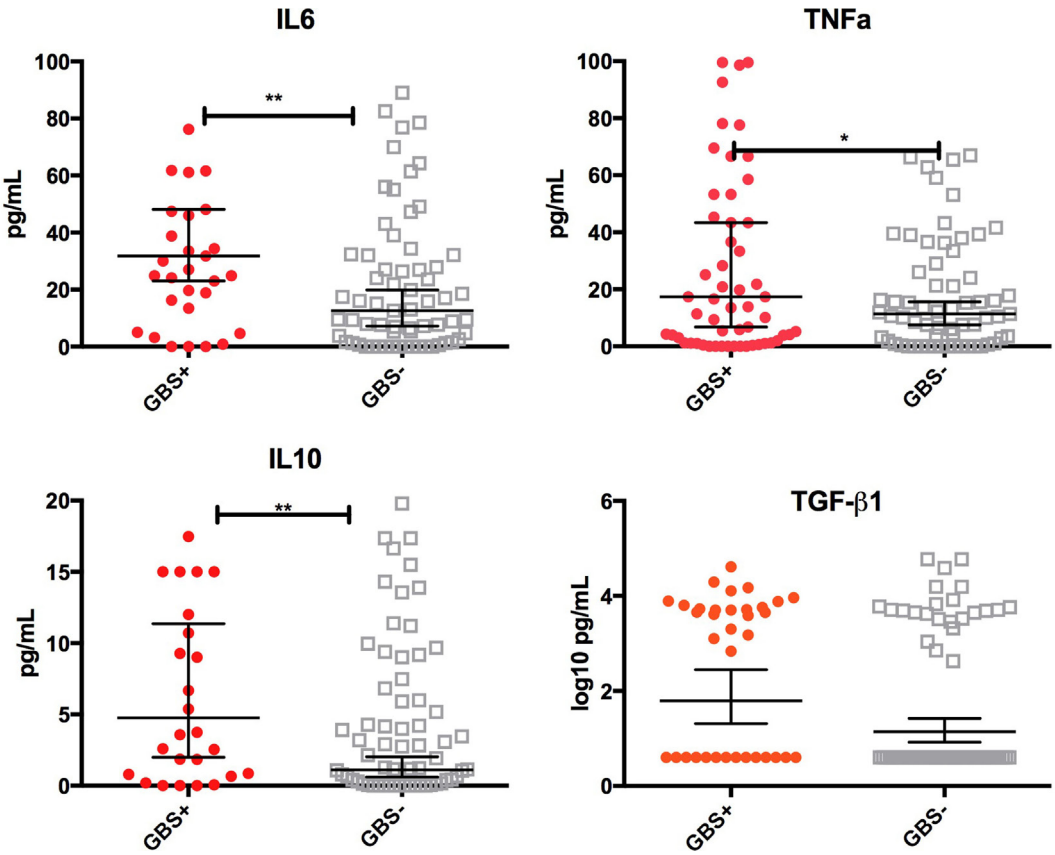

FIGURE 3 | Cytokine concentrations in colostrum by infant group B *Streptococcus* (GBS) colonization status at day 6. Box-and-whisker plot of colostrum concentrations of TNFα, IL-6, IL-10, and $\log_{10}$ values of TGF-β1 in picograms per millilitre (pg/mL). 65 of the 100 samples had cytokine concentrations above the lower limit of detection. Geometric mean and 95% confidence interval is demonstrated. Solid shapes indicate outliers. *p = <0.05, **p < 0.01.

On average 10 of the 100 samples analyzed for each cytokine had levels below the limit of detection of our assay and we have allocated an arbitrary level of half the lower limit of detection into our results as the most robust way of dealing with these data. There is the possibility that this may introduce bias into our results but this is reduced by the analysis of logged data.

Additionally, the data surrounding the associations between acquisition and loss of infant GBS colonization do not take into account maternal colonization at each time point, as this was only measured in mothers at delivery. Maternal colonization is transient (34) and we cannot be certain that mothers who were not colonized at birth did not acquire GBS colonization themselves during the study. The collection of repeat rectovaginal swabs from the mothers in addition to breastmilk and infant samples was beyond the scope of our study. In future studies, it might be possible to consent for longitudinal swabbing in order to better understand the dynamics between maternal colonization, breastmilk SIgA, cytokine production and subsequent infant colonization over time.

We did not conduct any analysis of the association between cytokines and SIgA antibody and GBS colonization as the complexity of the model and the sample size needed was outside of the scope of this study. However, we have demonstrated that infants who remained non-colonized received high concentrations of SIgA in colostrum and high functional antibody in cord blood and infant serum (17), suggesting a role for both systemic and mucosal antibody in preventing GBS colonization.

The low numbers of mother/infant pairs colonized with GBS STIa and III precluded detailed analysis of these GBS serotypes, which are important causes of infant disease in many countries and represent two of the serotypes being targeted by the current vaccine candidates. The serotype distribution in The Gambia is different from that in the USA and Europe, and highlights the need to understand GBS serotype prevalence in different regions (13).

The lack of standardized protocols available for the measurement of specific SIgA in breastmilk remains a limitation not just of this study, but in general, restricting comparison with other studies. Going forward, it will be very important for the field that assay standardization is undertaken in order to demonstrate consistency in our predictions of antibody-mediated protection from colonization and disease between studies, particularly when assessing novel vaccines against GBS and their potential impact on breastmilk factors.

We did not seek to measure cellular or functional mucosal immunity as part of our cytokine analysis, therefore the data do not offer mechanistic insights into the role of cytokines in breastmilk and interactions at the mucosal surface.

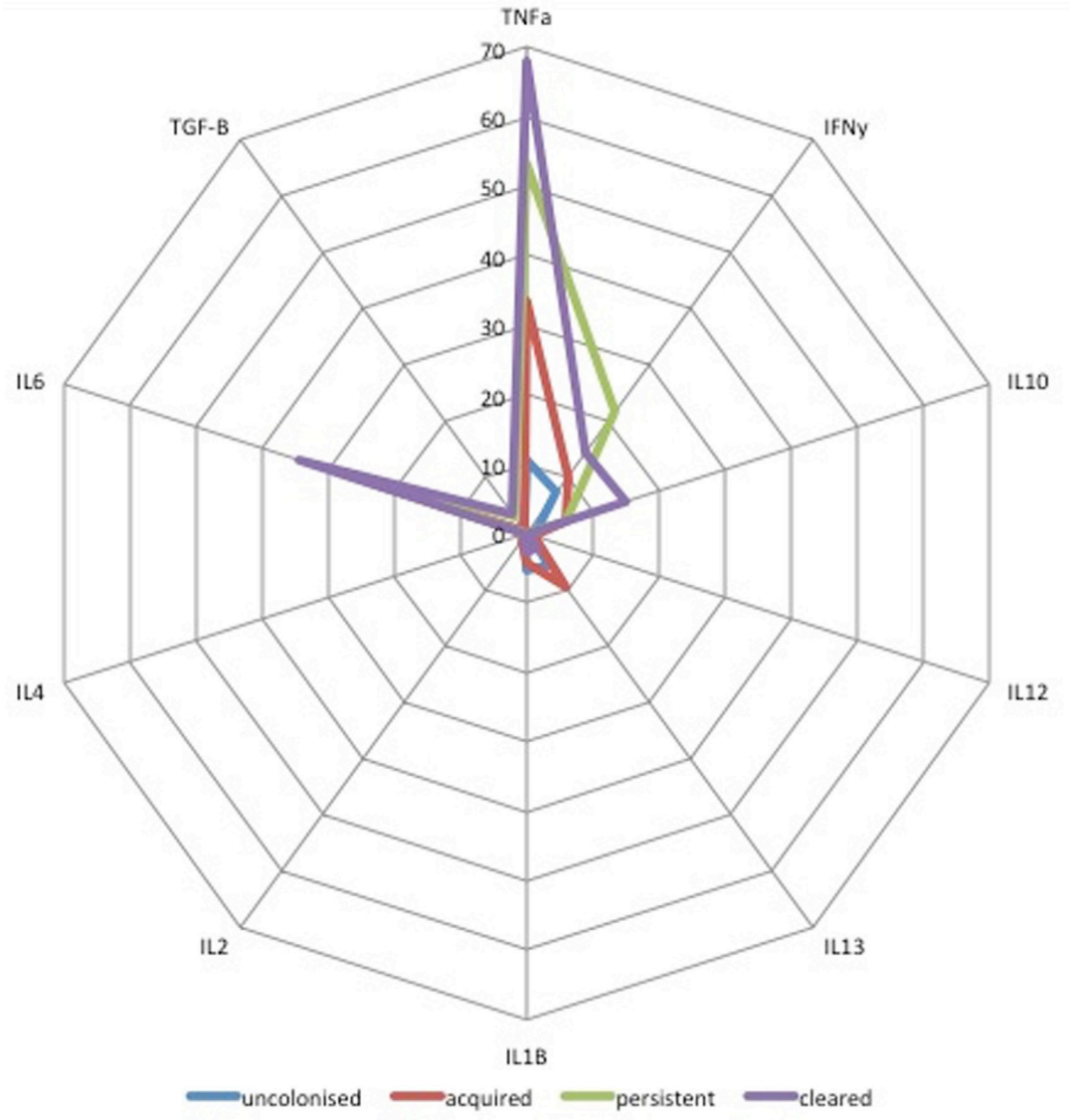

FIGURE 4 | Radar plot of cytokine concentration in colostrum by group B *Streptococcus* (GBS) colonization status by colonization status of infants. Radar plot of median cytokine concentration in colostrum in pg/mL from: blue = non-colonized, green = persistently colonized infants, red = infants who acquired colonization and purple = infants who cleared colonization between birth and day 60–89. TGF-β values are shown as $\log_{10}$ pg/mL values.

Finally, we made the assumption that the reduction in colonization is due to breastmilk cytokine and SIgA antibody concentrations. It is probable that protection from colonization at the mucosal surface is more complicated and includes both innate and adaptive factors associated with breastmilk (35) or the infants' developing microbiome (1). Measurement of the complexity of breastmilk immunity is extremely challenging, as there are few methods available and to assess these additional aspects requires sophisticated phenotyping of breast milk immune cells. However, samples have been stored for a comprehensive proteomic interrogation of breastmilk components and further work is ongoing.

In conclusion, our data support the notion that the risk of GBS colonization in infants diminishes as naturally acquired SIgA antibody in breastmilk increases as part of the maternal immune response to GBS, and that the presence of key cytokines such as TNFα, IL10, and TGF-β might further contribute to protection. Our findings support the idea that increasing SIgA through vaccinating mothers in pregnancy against GBS could have the same effect, provided the antibody levels and function induced by vaccination are similar or greater than that of naturally acquired antibody.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the MRC Gambia joint research ethics committee with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the MRC Gambia joint research ethics committee SCC1350v4.

AUTHOR CONTRIBUTIONS

KLD was responsible for original data, study design, statistical analysis, and manuscript writing; KB, AF, and JB were responsible for the laboratory analysis and had input into the manuscript writing; DM, HH, and ST were responsible for overseeing the laboratory analysis and had input into study design and the final manuscript; FW was responsible for statistical analysis plan and the overall statistical analysis. PTH, AG, and BK had intellectual input into study design and final manuscript. All authors had access to the data.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/article/10.3389/fimmu.2017.01269/full#supplementary-material.

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Conflict of Interest Statement: KLD, KB, AF, JB, FW, HH, ST, and AG declare no conflict of interests. PTH is an advisor to Pfizer and GSK vaccines; BK is an advisor to Pfizer and GSK vaccines. The MRC Unit The Gambia has previously received funding for vaccine trials, including vaccines produced by Pfizer and GSK.

frontiers in Immunology

REVIEW
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Factors Affecting the FcRn-Mediated Transplacental Transfer of Antibodies and Implications for Vaccination in Pregnancy

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At birth, neonates are particularly vulnerable to infection and transplacental transfer of immunoglobulin G (IgG) from mother to fetus provides crucial protection in the first weeks of life. Transcytosis of IgG occurs *via* binding with the neonatal Fc receptor (FcRn) in the placental synctiotrophoblast. As maternal vaccination becomes an increasingly important strategy for the protection of young infants, improving our understanding of transplacental transfer and the factors that may affect this will become increasingly important, especially in low-income countries where the burden of morbidity and mortality is highest. This review highlights factors of relevance to maternal vaccination that may modulate placental transfer—IgG subclass, glycosylation of antibody, total maternal IgG concentration, maternal disease, infant gestational age, and birthweight—and outlines the conflicting evidence and questions that remain regarding the complexities of these relationships. Furthermore, the intricacies of the Ab–FcRn interaction remain poorly understood and models that may help address future research questions are described.

Keywords: neonatal Fc receptor, placenta, antibody, immunoglobulin G, pregnancy, maternal, vaccination

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INTRODUCTION

Despite medical advances, infection continues to be a leading cause of neonatal and infant morbidity and mortality worldwide (1). At birth, neonates encounter a wide range of new pathogens and have an inexperienced immune system, making them particularly vulnerable to infection (2). The transfer of antibodies from the mother to the fetus across the human placenta is central for providing immunity in early life. Vaccination in pregnancy is a strategy that aims to protect mother and infant by increasing the concentration of maternal vaccine-specific antibody, and thereby the quantity transferred to the infant by transplacental transfer (3). This serves to protect the newborn until the time of infant vaccination, or until the window period of greatest susceptibility has passed.

In the human placenta, a histological barrier separates the blood in the maternal and fetal circulations. This barrier consists of two layers: the multinucleated synctiotrophoblast and the endothelial cells of the fetal capillaries. Wide ranges of substances are transferred, either actively or passively, across the placenta from mother to fetus, including the nutrients and solutes needed for normal fetal growth and development. Many compounds of low molecular weight (<500 Da) will simply diffuse across the placental tissue, whereas substances of very high molecular weight are usually not able to transverse the placental barrier (4). One of the exceptions is immunoglobulin G (IgG), which has a molecular mass of 160 kDa, yet is actively transported from mother to fetus (5). Of the five antibody

classes in humans, IgG is the only one to be transferred across the placenta in significant quantities, and this process begins at around 13 weeks of gestation (6).

Transplacental antibody transfer occurs *via* binding with the neonatal Fc Receptor (FcRn) in the placental synctiotrophoblast (7). A better understanding of mechanisms underlying FcRn-mediated transplacental antibody transfer, and the factors that affect these, is thus crucial for the optimization of maternal vaccination strategies, especially for developing countries where the burden of maternal and neonatal morbidity and mortality is highest (3). This review therefore sets out to summarize our current understanding of this field, review factors affecting FcRn-mediated transport of relevance to vaccination in pregnancy, and highlight gaps in our knowledge to direct future research.

THE ROLE OF VACCINATION IN PREGNANCY

Increasingly, vaccination in pregnancy is being recognized as a vital strategy to protect mother, fetus, and infant from infection and the associated adverse consequences. A number of vaccines are now routinely offered to pregnant women in several countries, including tetanus, influenza, and pertussis (8). Other vaccines may be offered to women in special circumstances (such as foreign travel and during outbreaks) and include meningococcus, inactivated poliovirus, and hepatitis A and B. Live vaccines are contraindicated in pregnancy. Vaccines currently progressing through the vaccine pipeline with a specific indication of use in pregnancy or pre-pregnancy include respiratory syncytial virus (RSV) (9), group B streptococcus (GBS) (10), and cytomegalovirus (11). Vaccination in the neonatal period is challenging as neonates may mount ineffective protective immunity, and the presence of maternal antibodies can blunt vaccine responses (2, 12).

Maternal vaccination is a highly effective approach to protect infants from infection. Early evidence comes from a study of tetanus vaccination in pregnancy in Papua New Guinea in the 1960s. Ten percent of infants born to mothers who received either no doses or one dose of tetanus developed neonatal tetanus compared to 0.57% of infants whose mothers had received three doses (13). More recent observational (14) and randomized controlled trials (RCTs) (15–17) conducted in both developed and developing countries have demonstrated that infants of influenza vaccinated mothers were 45–63% less likely to have episodes of proven influenza illness in early infancy (4–6 months of age). Furthermore, two of these RCTs showed that influenza vaccination reduced the incidence of maternal respiratory illness by 36 and 50.4% (15, 16). Maternal vaccination with a pertussis-containing vaccine is now routinely recommended in several countries and has been shown to be safe and to result in high concentrations of antibody in the infant over the first 2 months of life (18–21). Furthermore, maternal vaccination against pertussis has been demonstrated to have an effectiveness of over 90% at preventing disease in infants up to 3 months of age (22–24). Little is known regarding the beneficial effects of vaccination in pregnancy on breast-feeding, in which the transfer of secretory immunoglobulin A (IgA) antibodies serve to protect infants in the first few months of life by binding and opsonizing pathogenic microorganisms (25). However, recent studies have demonstrated that higher concentrations of secretory IgA to various diseases exist following maternal vaccination (26), with the strongest evidence coming from studies of influenza vaccination (27, 28).

Underpinning maternal vaccination is the effective FcRn-mediated transplacental transfer of vaccine-induced maternal IgG. A better understanding of the mechanisms of transplacental antibody transfer and the factors that affect this is crucial to optimize maternal vaccination strategies. Factors discussed below include IgG subclass, IgG glycosylation, maternal IgG concentration, maternal disease, gestational age at birth, and birthweight, all of which may all affect the protection conferred to the infant by maternal vaccination.

IgG AND THE FcRn

The human IgG molecule is a heterodimer of two identical 50 kDa heavy chains and two identical 23 kDa light chains (5) (**Figure 1A**). The heavy chains are of five different classes: μ, γ, δ, α, and ε, with four subclasses of γ and two of α. The light chains are of two classes: κ and λ (29). Together, the light and heavy chains form a Y-shaped structure, consisting of two fragment antigen-binding (Fab) arms, which contain the antigen-binding site and one crystallizable (Fc) tail region (30). The Fab region consists of constant and variable regions of the light chain, constant region 1 of the heavy chain (C_H1), and variable region of the heavy chain (V_H). Constant regions two and three of the heavy chain (C_H2 and C_H3) form the fragment crystallizable (Fc) tail region (30). A flexible hinge of disulfide bonds connects the C_H1 and C_H2 domains, to allow the Fab arms freedom of movement from the fragment crystallizable (Fc) tail. The outward-facing part of the interface between the C_H2 and C_H3 domains is where binding with FcRn occurs.

On the basis that whole IgG molecules and the Fc portion of IgG pass into the fetal circulation more readily than antigen-binding Fab fragments, it was hypothesized in the 1960s that receptors for the Fc part of IgG (FcγR) may be involved in the placental transfer of IgG (31). A functionally distinct FcγR was first proposed to mediate this specific transport of IgG by Brambell (32, 33), and this was later established to be the neonatal Fc receptor (FcRn)—termed as such due to its identification in the gut epithelial cells of neonatal rats (34). Its existence was confirmed by further work in mice (35, 36), and direct evidence of its involvement in the delivery of maternal IgG came from *ex vivo* perfused placenta studies comparing the maternofetal transfer of a recombinant IgG1 with that of a variant containing a mutation in the Fc region that did not bind to FcRn (37).

The structure of FcRn is unlike other Fc receptors and is markedly similar in structure to major histocompatibility complex (MHC) class I, with which it shares 22–29% sequence homology (37) (**Figure 1B**). It is a heterodimer consisting of a complex of two chains: a polypeptide α-chain (heavy chain) and β2-microglobulin (light chain) (38). The heavy (45 kDa) α-chain is encoded on chromosome 19 and consists of three extracellular domains (α1, α2, and α3), a transmembrane region, and a

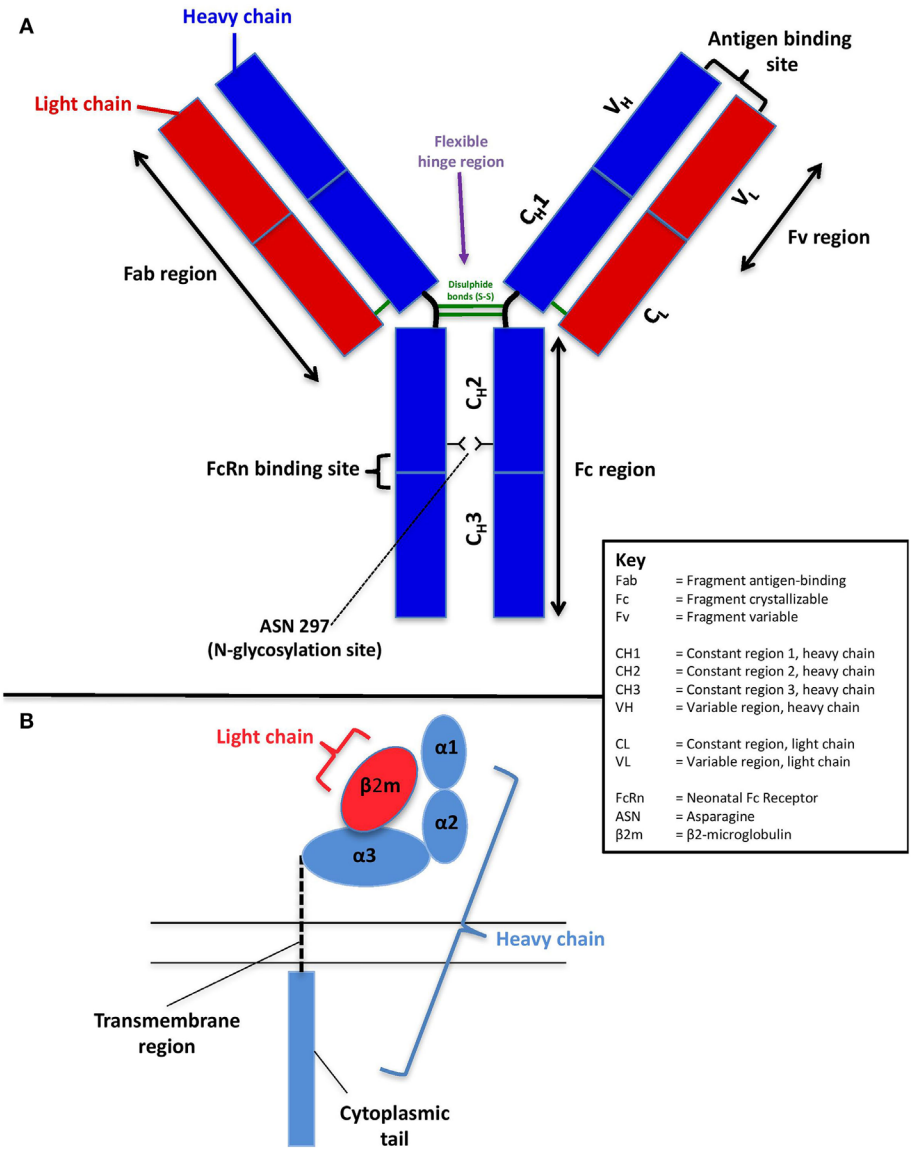

FIGURE 1 | Schematic of the structure of human immunoglobulin G **(A)** and the neonatal Fc receptor **(B)**.

short cytoplasmic tail. While the α-domains are closely related to MHC class I, the transmembrane and cytoplasmic domains distinguish FcRn from other receptors of the same class (39). The light (12 kDa) chain, β2-microglobulin (β2m), is encoded on chromosome 15 and is non-covalently associated with the α3 domain (38).

The role of FcRn extends beyond its role in placental FcRn transport. It is central to the homeostatic maintenance of both serum IgG and albumin levels by protecting them from lysosomal degradation and is thereby responsible for their long serum half-lives relative to other plasma proteins (40, 41). Furthermore, FcRn is increasingly recognized to have a wide role in modulating humoral and cell-mediated immunity (42). It is involved in the bidirectional transcytosis of IgG and IgG immune complexes across various human epithelia (43–45), and its expression in hematopoietic cells (46, 47) is essential for the enhancement of IgG-mediated phagocytosis (48), anti-tumor immunosurveillance (49), and the direction of immune complexes to lysosomes in dendritic cells in order to facilitate antigen presentation (50, 51).

MECHANISMS OF FcRn-MEDIATED IgG TRANSCYTOSIS IN THE PLACENTA

The placenta is a complex organ of which the basic functional unit is the chorionic villus (52, 53). Villi are highly branched vascular projections of fetal tissue, through which fetal blood flows from the umbilical cord. The villi are surrounded chorion, which consists of two layers: the outer syncytiotrophoblast (which is in direct contact with maternal blood flowing through the intervillous space) and the inner layer of cytotrophoblast progenitor

cells. Under the chorion lies the stroma and the fetal capillaries (**Figure 2A**).

Unlike other FcγRs, the interaction of FcRn with IgG displays a strong pH dependence, such that high-affinity binding occurs at pH 6.0, but little or no binding occurs at physiological pH 7.4 (54). This pH-selective binding is key to the effective transport of IgG across the synctiotrophoblast of the placenta from the maternal to fetal circulation. Various crystallography studies have found structural modifications in the FcRn α-chain that might contribute to this pH dependence. This characteristic is likely mediated, at least in part, *via* protonation of histidine residues (the only amino acid that changes between pH 5.5 and 7.4) at the C_H2–C_H3 domain interface of IgG (54–56). Additionally, thermal denaturation studies have shown that the FcRn heterodimer is significantly more stable at pH 6 than pH 8 (57).

To be successfully transferred across the placenta, maternal IgG must cross the synctiotrophoblast layer, the villous stroma, and the fetal vessel endothelium. The mechanisms of FcRn-mediated IgG trancytosis across the synctiotrophoblast have been elucidated by the use of the BeWo choriocarcinoma cell line (a model for placental trophoblast) (58) and fluorescence microscopy of FcRn-green fluorescent protein-transfected live human endothethial cells, which enable analysis of the intracellular trafficking of IgG in real time (**Figure 2B**) (59, 60). These studies suggest that IgG is taken up from the extracellular fluid on the apical side of the synctiotrophoblast by endocytosis. Within the acidic environment of endosomes, IgG binds with membrane-bound FcRn and is protected from proteolytic degradation by lysosomal enzymes. IgG is then transcytosed to the basal cell surface, where a return to physiological pH causes dissociation of IgG from FcRn. FcRn may then be recycled back to the maternal membrane to perform more cycles of transcytosis.

The mechanisms underlying the initial endocytosis of IgG, and onward transport of IgG across the villous stroma and the fetal vessel endothelium remain somewhat of a gap in our knowledge. It is controversial as to whether FcRn is also expressed in fetal vessel endothelium. Various studies using immunohistochemical staining of placental sections with anti-FcRn antibodies have shown a mix of some (61, 62) or no (36). FcRn expression in fetal endothelium, and some evidence, points toward alternative Fc receptors in the further movement of IgG (63, 64).

FACTORS ASSOCIATED WITH CHANGES IN TRANSPLACENTAL ANTIBODY TRANSFER

How Does the Structure of IgG Vary between Subclasses and How Might This Affect FcRn Binding and Transplacental Transfer?

Human IgG can be divided into four subclasses (IgG1, IgG2, IgG3, and IgG4), named in order of decreasing abundance (65). IgG subclasses are over 90% identical at the amino acid level; however, each subclass has a unique functional profile. In human serum, FcRn prolongs the half-life of IgG1, IgG2, and IgG4 equally. It is thought that FcRn does not prolong the half-life of IgG3 in the same way, because IgG3 has an arginine at position 435 instead of the histidine found at the same position in the other subclasses, except for individuals expressing a natural IgG3 variant (H435) (66). IgG1 is preferentially transported across the placenta, followed by IgG4, IgG3, and IgG2 (37, 67). Placental IgG transport has been estimated by comparing cord and maternal concentrations of IgG subclasses. These studies have shown that concentrations of IgG1, IgG4, and H435-containing allotypes of IgG3 exceed maternal levels; however, levels of IgG2 do not (68–70). This suggests that the placental transport of IgG2 is significantly less efficient.

One explanation for this difference in placental transport relates to the IgG hinge region, as differences in the length and flexibility of the hinge region are found in the subclasses.

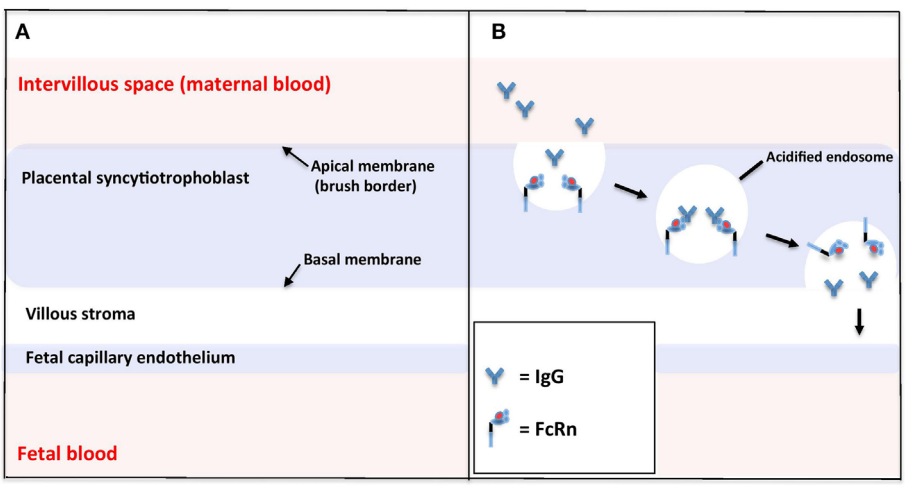

FIGURE 2 | Schematic of the microstructure of the human placental barrier at term **(A)** and the neonatal Fc receptor (FcRn)-mediated endocytosis of immunoglobulin G (IgG) across the placental syncytiotrophoblast **(B)**.

The length and flexibility of the hinge region affects the orientation and movement of the Fab arms in relation to the Fc tail (5). The relative flexibility differs as follows: lgG3 > lgG1 > lgG4 > lgG2 (71). IgG2 has been demonstrated to have a uniquely short hinge region, comprising 12 amino acids and containing a poly-proline double helix, stabilized by four inter-heavy chain disulfide bridges (72). This causes the Fab arms to be relatively close to the Fc tail and enables its κ isotope, but not λ isotope, to form three disulfide isoforms that differ from each other with relation to their disulfide bridges in the hinge region (73). It has therefore been postulated that one these IgG2κ isoforms may have decreased interaction with FcRn and account for the reduced placental transport displayed by IgG2. However, recent studies in humans have found that FcRn binding does not seem to vary among these different disulfide isoforms (74) and that no preference occurs for recycling and placental transport of IgG2λ or IgG2k (69).

The question therefore remains over the mechanism underlying the reduced placental transport of IgG2 relative to other subclasses. One possible explanation relates to recent findings suggesting that different proteins are involved in regulating FcRn-mediated IgG transcytosis (actin motor myosin Vb and Rab25) and recycling (Rab11a), respectively (75). While IgG2 transport through the placenta is indeed low, its recycling and half-life extension in the adult circulation are even better than IgG1 (69). It is therefore a possibility that regulation by these proteins varies depending on IgG subclass, but how the stoichiometry of IgG2 may affect these intracellular processes requires further study. Another possible explanation is that another Fc receptor, FcγRIIb, may play a role in modulating transplacental antibody transport (76, 77). A role for FcγRIIb might provide a plausible explanation for the reduced transport of IgG2 because, unlike other subclasses, IgG2 has almost non-existent binding affinity to FcγRIIb (78).

The discrepancy between transfers of different IgG subclasses may have key implications for maternal vaccination. IgG2 is crucial for the opsonization and killing of polysaccharide-encapsulated pathogens and is induced by polysaccharide vaccines (69). Conversely, vaccines that contain protein antigens, such as tetanus, predominantly elicit production of IgG1 and IgG3. Therefore, transcytosis of some vaccine-induced IgG subclasses is more efficient than others. Future work to optimize placental transfer of IgG2 has the potential to better protect infants against important polysaccharide-encapsulated pathogens such as GBS, Haemophilus influenzae B (HiB), and *Neisseria meningitidis* (79).

How Does Glycosylation of IgG Affect FcRn Binding and Transplacental Transfer?

Glycosylation involves the covalent addition of sugar moieties (such as fructose, galactose, and sialic acid) to proteins. The dynamics and binding affinity of IgG can be influenced by its glycosylation (80), and IgG exists in a number of glycosylated variants (glycoforms) (81). Both pregnancy and disease may have an impact on IgG glycosylation. Pregnancy is associated with increased Fc and Fab region galactosylation and sialylation (82). Interestingly, pregnancy is also associated with clinical improvement of autoimmune disease (such as rheumatoid arthritis), which, as well as infectious disease, is associated with a reduction in galactosylation of IgG in human serum (83).

Neonatal Fc receptor binds to the outward-facing part of the C_H2 and C_H3 domains of the Fc region of IgG. The *N*-glycosylation site occupies the inner part of the Fc region at asparagine 297, helping to maintain its quaternary structure and stability [**Figure 1A**; Ref. (84)]. It has therefore been suggested that IgG glycosylation may affect the IgG–FcRn interaction and that that there may be a preferential placental transport for glycosylated IgG. Supportive evidence for the hypothesis of preferential transport of glycosylated IgG comes from studies in the 1990s, which demonstrated reduced concentrations of non-glycosylated IgG and higher concentrations of galactosylated IgG in newborn infants (85, 86). More recently, Dashivets et al. studied enzymatically engineered glycosylation variants and showed that deglycosylated IgG1 had a slightly diminished binding to FcRn, with digalactosylated IgG demonstrating superior binding than monogalactosylated and agalactosylated variants (87). Furthermore, *in vivo* pharmacological studies have also shown an impact of the glycan on the half-life mediated by FcRn (88).

Evidence to the contrary, however, includes a study by Bakchoul et al. that showed agalactosylated IgG was transported equally well across the placenta (89). In addition, Einarsdottir et al. studied Fc region glycosylation for all IgG subclasses in 10 pairs of fetal and maternal IgG samples. They demonstrated comparable Fc region glycosylation for all IgG subclasses (including galactosylation, sialylation, bisecting G1cNAc, and fucosylation), suggesting that transplacental IgG transfer does not favor certain Fc glycoforms (90). However, another more recent study by the same group in 2016 found clear, albeit minor, differences in the *N*-glycosylation profile of IgG between maternal and umbilical cord plasma in 42 mother–newborn pairs (91). Levels of galactosylation were slightly higher for cord IgG, with lower levels of bisection, sialylation, and sialylation per galactose. Possible reasons for the differences observed between studies include a IgG subclass-related transport bias (discussed previously), as well as the method of measurement, which was at the released glycans level in the 2016 study, rather than by analyzing IgG-derived Fc-glycopeptides (92). It is therefore possible that it is the quality of Ab glycosylation, rather than the total quantity of glycosylation that determines transplacental transfer. It is not known how vaccination in pregnancy might affect glycosylation of IgG and the efficacy of transplacental transfer of vaccine-specific IgG and is an area where more research is needed.

How Does Total Maternal IgG Concentration Affect Transplacental Transfer of Specific IgG?

It is well established that maternal antibody levels play a role in determining transfer efficiency. Neonatal IgG levels usually correlate with maternal ones; however, it has been suggested that once maternal total IgG levels reach a threshold (>15 g/L), FcRn can become saturated (37, 93). IgG must then compete for a finite number of FcRn receptors. Unbound IgG molecules are subsequently destroyed through the lysosomal degradation process within cells. This is supported by African studies showing

that reduced IgG transfer ratios were associated with the higher maternal total IgG levels (94, 95). Furthermore, a number of more recent studies have demonstrated negative correlations between maternal IgG levels and placental transfer ratios for both total and antigen-specific IgG (96–98).

Very high concentrations of vaccine-specific antibodies could potentially result in a reduced proportion of maternal IgG being transferred across the placenta to the infant, resulting in a lower transplacental transfer ratio. However, the concentration of antibody in cord blood is still likely to be significantly higher in infants born to vaccinated women compared to infants born to unvaccinated women and therefore may not have implications for protective infant immunity, and to date, no adverse clinical outcomes have been observed.

How Does Maternal Disease Affect the Ab–FcRn Interaction and Placental Transfer of IgG?

Maternal Infectious Disease

It is now well established that maternal chronic infection can reduce the transplacental transfer of IgG specific to a variety of important childhood pathogens, including RSV, measles, tetanus, and HiB (37, 99–103). The majority of these studies have focused on placental malaria and HIV, which are particularly prevalent in developing countries and continue to exert a significant burden of morbidity and mortality globally. These include studies of HIV-exposed but uninfected infants, which showed reduced transplacental transfer ratios and lower concentrations of specific antibodies than HIV-unexposed infants did to HiB, pertussis, pneumococcus, and tetanus at birth (104).

The mechanisms behind this reduced transfer are poorly understood, and current models remain speculative. Infections may impact on IgG transfer directly through infection and inflammation of the placenta, or a reduction FcRn-antibody binding avidity, or as detailed above, *via* induction of hypergammaglobulinemia (IgG > 15 g/L) leading to saturation of placental FcRn (105). Studies assessing the impact of infection and hypergammaglobulinemia have had a great deal of overlap between these populations (>90%), complicating the interpretation of these effects independently (95). One Malawian study demonstrated that reduced antibody transfer in placental malaria may occur independently of hypergammaglobulinemia using multivariate regression analysis (106); however, more recent conflicting evidence from Papua New Guinea showed that only hypergammaglobulinemia, and not placental malaria, was associated with impaired transport of RSV antibody (99). Further studies are therefore clearly needed to understand the complexities of these relationships.

Interestingly, non-pregnant individuals with infectious diseases such as HIV have been shown to have significantly higher levels of galactose-deficient IgG than healthy controls. If glycosylation does indeed impact on the Ab–FcRn interaction as discussed above, then this may represent a further possible mechanism by which HIV could impact on placental IgG transfer and thus the effectiveness of maternal vaccination (107, 108).

Maternal Nutrition and Non-Communicable Diseases

Maternal malnutrition can have adverse implications for the neonate, and it has been demonstrated that neonatal immune responses may be modulated by the nutrition of a mother during gestation (108). One study reported a 14% reduction in antibody transfer among malnourished pregnant women compared to controls (109); however, the reasons for this are unclear and possibly relate to differences in placental size, morphology, and vascular development (110, 111). Other studies of micronutrients include a recent review of antenatal zinc supplementation that did not find significant evidence for the positive effect of zinc on antibody transport (112).

Another significant maternal morbidity is diabetes mellitus, which can either be pre-existing or gestational and affects 0.2–0.3 and 2–5% of pregnancies, respectively (113). To date, the effect of maternal hyperglycemia on FcRn and IgG transfer remains unclear. Stach et al. (98) demonstrated an increased rate of IgG transfer in hyperglycemic mothers for all antigens they studied (GBS, Klebsiella LPS, and Pseudomonas LPS), as did França et al. (114). More recently, De Souza et al. investigated both the transfer of IgG and expression of FcRn expression (measured by flow cytometry), in normo- and hyperglycemic mothers (115). They found that mothers with pre-existing type 2 diabetes had lower total levels of IgG, and reduced leukocyte FcRn expression across maternal blood, cord blood, and placental samples (collected at delivery) compared with normoglycemic mothers. Interestingly however, FcRn expression increased with mild gestational hyperglycemia. There was no statistically significant difference in total IgG levels in newborns between groups of mothers. Differences were observed on subclass analysis however, with significantly lower transfer of IgG1, IgG3, and IgG4 in women affected by diabetes, but significantly higher transfer of IgG3 in women with mild gestational hyperglycemia.

This decrease in FcRn expression may explain the reduced transfer of some IgG subclasses in mothers with diabetes. Furthermore, high levels of glycated IgG have been demonstrated in the plasma of patients with diabetes, and this may have an effect on the avidity of binding with FcRn and its transfer across the placenta (116, 117). The question also remains over why higher transfer might occur for IgG3 in the context of mild gestational hyperglycemia. Hyperglycemia is associated with a variety of alterations to placental structure, including increased numbers of glucose transporters (118) and a discontinuity in the trophoblastic layer (119), which may both facilitate the passage of glucose, and possibly some immunoglobulins, across the placenta (120). Additionally, greater placental villous capillarization has been noted in women with mild gestational hyperglycemia, and may facilitate placental transfer of a variety of substances (121).

Another common complication in pregnancy is maternal hypertension, affecting 2–3% of pregnancies (122). One study has examined the effect of pregnancy-induced hypertension on IgG transfer and, interestingly, found that hypertension was associated was increased transfer of IgG against *Klebsiella* spp. (98). This might be considered paradoxical given the immune-pathological damage observed in the placenta of hypertensive women (123).

Clinical trials of vaccination in pregnancy typically enroll healthy women, without chronic infections or co-morbidities.

As these factors may influence transplacental transfer of antibody and therefore the protection afforded to the infant, it is important to also design studies, which assess vaccines in pregnancies in "real-life" settings, without the extensive exclusion criteria applied to early phase clinical trials. These data also suggest that optimization of maternal health for the benefit of mother and infant is important.

How Does the Ab–FcRn Interaction Change across Gestation and Birthweight?

Placental transfer of IgG occurs in an exponential fashion as pregnancy progresses, with minimal transfer in the first trimester (6). In the second trimester, the use of cordocentesis has demonstrated that fetal IgG rises from roughly 10% of the maternal concentration at 17–22 weeks of gestation, to 50% at 28–32 weeks (124). In the third trimester, the rate of IgG transfer rises significantly (particularly from 36 weeks), with the increase of fetal IgG concentrations between 29 and 41 weeks of gestation doubling that of 17–28 weeks. At term, fetal levels vary, however, usually exceed maternal levels by 20–30% (64, 125, 126).

It follows therefore that a reduced transfer of IgG in preterm infants compared with term infants has been demonstrated for a variety of pathogens (97, 127, 128) particularly for infants born at less than 36 weeks of gestation (126). This knowledge has significant implications for the optimal timing of vaccination in pregnancy and has shaped the development of maternal vaccination strategies, reviewed by Calvert et al. (129). In order to protect preterm infants, a vaccine would need to be given early in pregnancy to ensure sufficient time of transport of IgG to the infant. However, later vaccination could be more desirable to more closely match the peak antibody response with the peak of transplacental transport of IgG to the infant. There remains debate in the published literature about the optimal timing of vaccination in pregnancy. It is worth noting that, given the increased susceptibility of premature infants to serious early-life infections, the optimal strategy may require a compromise between giving the best protection to term babies, versus protecting all viable infants.

Birthweight may also affect IgG transfer, with studies demonstrating a reduced transfer of antibodies in term low birthweight infants (65, 130). Interestingly, on subclass analysis, the reduced transfer seen in premature and low birth weight infants has been shown to be specific to IgG1 and IgG2, which may in part explain the higher susceptibility of premature infants to infections caused by polysaccharide-encapsulated pathogens which predominantly elicit IgG2 production, such as GBS (97, 127).

It is thought that this change in rate of transplacental transfer may partly occur because of increased expression of FcRn throughout gestation; however, this is yet to be formally demonstrated and our understanding of the evolving expression of FcRn remains poor. Whether alternations in the Ab–FcRn interaction may also play a role in this effect is unknown. It is worth noting that preterm labor and low birthweight are associated with numerous maternal pathologies, such as gestational hypertension, diabetes, and preeclampsia, which may also have a direct or indirect effect on placental function and the Ab–FcRn interaction. Thus, interpreting their independent effects may therefore be challenging.

WHAT MODELS OF PLACENTAL FUNCTION ARE CURRENTLY AVAILABLE TO STUDY TRANSPLACENTAL TRANSFER OF IMMUNITY?

Over the years, several models of placental function have been developed to study the transplacental transfer of substances, including IgG. Mouse and rat models have been central to the discovery of FcRn (34) and have provided useful insights into the possible mechanisms of FcRn-mediated IgG transfer in situations where human studies are considered invasive or impractical (131). However, they differ from humans in many key features including levels of FcRn expression (132), immunological function (133), and placental anatomy (77). Another major model has been paired maternal–cord samples, which have been used widely and offer the possibility of comparing blood samples from the mother at the time of delivery with umbilical cord blood. The ratio of cord:maternal antibody concentration has been used as a proxy for placental transport (104).

In addition, several *ex vivo* and *in vitro* placental models are available to study transplacental transfer at a more mechanistic level. The cell line most commonly used is the choriocarcinoma-derived BeWo (b30) cell line, which can be cultured to form polarized, confluent monolayers with tight junctions for use in directional transport studies. These trophoblast cells serve as an *in vitro* model of the rate-limiting barrier of maternal–fetal exchange and can be used to study placental metabolism and transport of numerous substances, including IgG (134–136). BeWo cells also demonstrate hormone secretion properties and characteristics of third trimester trophoblasts; however, the model lacks connective tissue and fetal endothelium, which are present in the *in vivo* human placenta. Also, as single cytotrophoblast cells with tight junctions, they do not fully recapitulate the multinucleate syncytiotrophoblast, which is the cell type in contact with maternal blood. Forskolin treatment has sometimes been used, as it can induce fusion of BeWos to form syncytia (137). However, this fusion is variable and never reaches 100%, so is unable to create a complete syncytiotrophoblast barrier for transfer studies. Culture of isolated primary term cytotrophoblasts, which differentiate in culture to model syncytiotrophoblasts can be employed to overcome this issue (138).

The gold standard for placental transfer studies is the placental perfusion model. For this, a term placental cotyledon is cannulated and re-perfused to model the fetal and maternal circulations, enabling the study of the placental transfer of a chosen substance (139). Compared to placental transfer *in vivo*, this model is obviously simplified and does not take into account some of the possible maternal/fetal physiological variables. It does however offer the best technique to study the transplacental exchange of substances across the intact human placenta (134, 140).

The BeWo and placental perfusion models have shown good comparability in studies comparing the transport of different

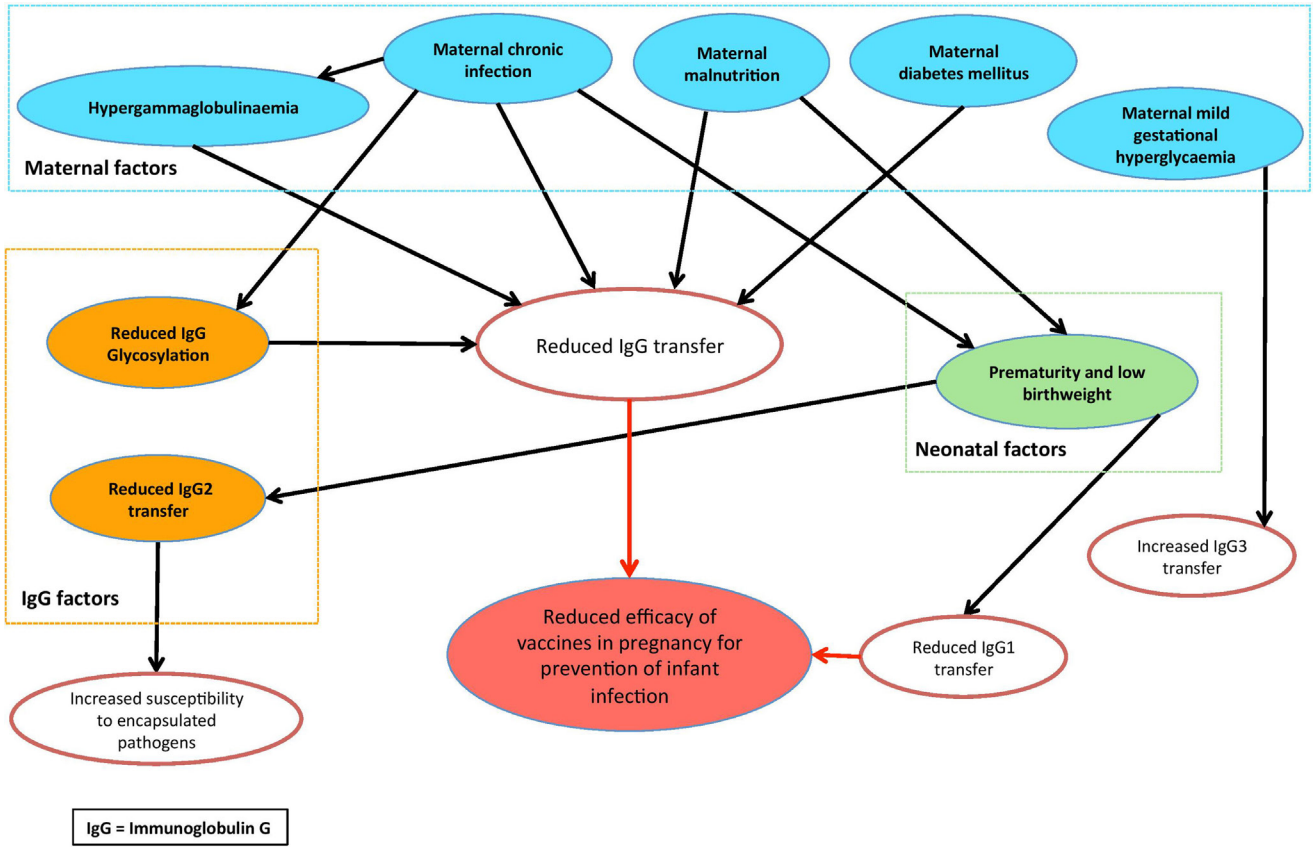

FIGURE 3 | Conceptual diagram of the factors that may modulate placental antibody transfer of relevance to maternal vaccination.

compounds across the placenta in terms of rank order; however, the transfer rate is much slower in BeWo cells (141, 142). This could be due to the higher pressure and flow in the circulation pump setup of the perfusion model, and while the BeWo cell monolayer can be placed on a shaking plate to create flow, this is not in the same magnitude as the placenta and there is a lack of hydrostatic pressure on the fetal side (140). Also, as mentioned above, the BeWo is a cytotrophoblast model, not a syncytiotrophoblast model, and thus, uptake rates and receptor expression may differ. Despite its limitations, the BeWo model is far less technically challenging to perform than the perfusion model, which requires very rapid access to fresh placenta samples and has a high failure rate (134). It therefore may present a useful first-step model for those wishing to investigate placental transfer, before progressing to the more complex placental perfusion model, particularly for the study of inter-individual differences or disease states (139, 143).

One other consideration is that both the BeWo model and the placental perfusion models only enable the modeling of term placenta. This represents a gap in our knowledge, particularly as maternal vaccines are often given in the first and second trimester. One way to overcome this issue could be through the use of the placental explant model. For this, small placental villous explants are dissected and cultured *in vitro*. This model can be performed with placental tissue of any gestation and thus is a commonly used model for early placental function, as samples are obtainable from termination of pregnancies. The explant model enabled the first demonstration of Zika virus infection of the first trimester placenta *in vitro* (144) and has been used to investigate placental uptake of other substances, including glucose (145), amino acids (146), and exosomes (147). The explant model has not been extensively used for antibody investigations, except for in the study of antiphospholipid antibodies (148); this is likely due to the fact that it does not fully model maternal to fetal transfer. Nevertheless, the ability to demonstrate uptake into intact human placental tissue from across gestation could provide useful information regarding maternal antibody uptake and interaction with the FcRn throughout pregnancy, both requisite steps for transfer of antibody to the fetus.

CONCLUSION

Since its first identification in 1989, it has become increasingly apparent that FcRn plays a lifelong role in immunity. Importantly for neonates, FcRn is crucial for establishing humoral immunity *via* transplacental IgG transfer, and this exciting research field continues to expand.

This review has highlighted a number of factors that may affect the effective FcRn-mediated transplacental antibody transfer,

which are summarized in **Figure 3**. These include IgG subclass, IgG glycosylation, maternal IgG concentration, maternal disease, gestational age at birth, and birthweight—yet there is conflicting evidence and many questions remain regarding the complexities of these relationships. Furthermore, while the role of FcRn in IgG transfer is well recognized, the intricacies of the Ab–FcRn interaction and how binding varies across subclass, gestation, glycosylation, and disease states remain poorly understood. Future research platforms will therefore benefit from utilizing a combination of placental models, as well as affinity studies of Ab–FcRn binding using approaches such as surface plasmon resonance and biolayer interferometry (149, 150), which represent an exciting new avenue for research.

As maternal vaccination becomes an increasingly important strategy for the protection of young infants, improving our understanding of the mechanism of transplacental antibody transfer and thus the factors that could impact vaccine effectiveness will be increasingly important, especially in developing countries where the burden of morbidity and mortality is highest.

AUTHOR CONTRIBUTIONS

CW designed and wrote the article. BH designed and critically revised the article. CJ (senior author) conceived, designed, and critically revised the article. All authors approved the final copy of the manuscript.

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Pertussis Maternal Immunization: Narrowing the Knowledge Gaps on the Duration of Transferred Protective Immunity and on Vaccination Frequency

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Maternal safety through pertussis vaccination and subsequent maternal–fetal-antibody transfer are well documented, but information on infant protection from pertussis by such antibodies and by subsequent vaccinations is scarce. Since mice are used extensively for maternal-vaccination studies, we adopted that model to narrow those gaps in our understanding of maternal pertussis immunization. Accordingly, we vaccinated female mice with commercial acellular pertussis (aP) vaccine and measured offspring protection against *Bordetella pertussis* challenge and specific-antibody levels with or without revaccination. Maternal immunization protected the offspring against pertussis, with that immune protection transferred to the offspring lasting for several weeks, as evidenced by a reduction (4–5 logs, $p < 0.001$) in the colony-forming-units recovered from the lungs of 16-week-old offspring. Moreover, maternal-vaccination-acquired immunity from the first pregnancy still conferred protection to offspring up to the fourth pregnancy. Under the conditions of our experimental protocol, protection to offspring from the aP-induced immunity is transferred both transplacentally and through breastfeeding. Adoptive-transfer experiments demonstrated that transferred antibodies were more responsible for the protection detected in offspring than transferred whole spleen cells. In contrast to reported findings, the protection transferred was not lost after the vaccination of infant mice with the same or other vaccine preparations, and conversely, the immunity transferred from mothers did not interfere with the protection conferred by infant vaccination with the same or different vaccines. These results indicated that aP-vaccine immunization of pregnant female mice conferred protective immunity that is transferred both transplacentally and *via* offspring breastfeeding without compromising the protection boostered by subsequent infant vaccination. These results—though admittedly not necessarily immediately extrapolatable to humans—nevertheless enabled us to test hypotheses under controlled conditions through detailed sampling and data collection. These findings will hopefully refine hypotheses that can then be validated in subsequent human studies.

Keywords: pertussis, *Bordetella pertussis*, pregnancy immunization, acellular vaccine, protection

INTRODUCTION

Pertussis or whooping cough is a respiratory disease mainly caused by the Gram-negative coccobacillus *Bordetella pertussis*. This disease affects all individuals regardless of age, but with higher morbidity and mortality rates among infants that have received either no vaccine or an incomplete vaccination schedule (1–3). Pertussis has resurged as a major public health concern in many countries (4, 5). Until two decades ago, the control of the disease was mainly carried out through a vaccination scheme consisting in a three-dose primary series, with the first dose administered as early as at 6 weeks of life, with subsequent doses being completed by 6 months of age (6). In order to accomplish the three-dose primary series, two types of vaccines are currently available: a whole-cell vaccine based on standardized cultures of *B. pertussis* strains (wP) and an acellular form [acellular pertussis (aP)] composed of purified *B. pertussis* immunogens. Acellular vaccines, originally developed to reduce the side effects associated with wP vaccination (7, 8), have since replaced wP, especially in industrialized countries. Unfortunately, the duration of the immunity conferred by these two vaccines is not lifelong (9). Moreover, recent data indicated that protection from aP vaccines wears off faster than that induced by wPs. This weakness in the current vaccines together with the lack of optimal vaccination coverage and the evolution of the causal agent to greater vaccination resistance have contributed to the recent rise in *pertussis* incidence and fatalities (10–12). While coverage is improved and better vaccines are designed, many countries have added vaccination boosters beyond the primary doses with the main aim at reducing both the disease burden and the incidence in the most vulnerable populations.

Maternal pertussis immunization during the third trimester of every pregnancy is one of the recent strategies recommended in several countries to improve pertussis control in infants (13, 14). The reported safety of the acellular vaccine when used during pregnancy and the placental transfer of *pertussis* antibodies from mothers to their infants that has been detected argue in favor of this strategy (15–17). Nevertheless, the question of whether or not this approach is able to effectively protect neonates against pertussis and how the transmitted maternal immunity affects the protection conferred by subsequent infant vaccination are still insufficiently clear. Recently, Amirthalingam et al. reported the effectiveness of maternal immunization in preventing infant pertussis, as evaluated 1 year after the introduction of the maternal-*pertussis*-immunization program in England in 2012 (18). Moreover, in the 3 years following its introduction vaccine effectiveness against confirmed pertussis has been sustained >90% with a vaccine effectiveness against infant deaths estimated at 95% (95% confidence interval, 79–100%). Furthermore, the authors reported that the protection conferred by maternal immunization was retained in infants who received the first dose of the primary series (18). Though all these data are highly promising and support the successfulness of maternal immunization, the reported number of cases in that study was, unfortunately, small; and therefore an ongoing assessment is still needed.

The use of animal models has also indicated an effective protection against pertussis in the offspring of mothers immunized during pregnancy. In a primate model (baboons), it was observed that both maternal and neonatal vaccinations were found to confer protection against the pathogen (19). The authors of those baboon experiments suggested that the transmitted maternal antibodies alone would be sufficient to confer protection against pertussis symptoms. In pigs, protection seems to be related to the presence of anti-*B. pertussis* antibodies in the colostrum (20, 21). We need to emphasize here that although in mice a protection transmitted to the pups *via* the placenta has been duly demonstrated, a transmission *via* the breast milk cannot be discarded since that *via* has not yet been completely investigated. Oda et al. (22) and Quinello et al. (23) reported some data on that topic.

We thus used this mouse model in order to substantially enhance our understanding of the efficacy of maternal pertussis immunization in the protection of subsequent offspring as well as determine the potential interference of maternal immunity with the eventual protection of those offspring by the primary vaccination against *B. pertussis*.

MATERIALS AND METHODS

Mice

BALB/c mice (4 weeks old), obtained from the Instituto Biológico Argentino SAIC (Biol Argentina), were kept in ventilated cages and housed under standardized conditions with regulated daylight, humidity, and temperature. The animals received food and water *ad libitum*. Day 1 of gestation was determined when vaginal plugs were observed. Breeding cages were checked daily for new births, and the pups were kept with their mothers until weaning at the age of 4 weeks. The animal experiments were authorized by the Ethical Committee for Animal Experiments of the Faculty of Science at La Plata National University (approval number 004-06-15 and 003-06-15).

B. pertussis Strain and Growth Conditions

Bordetella pertussis Tohama phase I strain CIP 8132 was used throughout this study as the strain for challenge in the murine model of protection. *B. pertussis* was grown in Bordet–Gengou agar supplemented with 15% (v/v) defibrinated sheep blood (BG-blood agar) for 72 h at 36.5°C. Isolated colonies were replated in the same medium for 24 h and then resuspended in phosphate-buffered saline (PBS: 123 mM NaCl, 22.2 mM Na_2HPO_4, 5.6 mM KH_2PO_4 in MilliQ® nanopure water; pH 7.4). The optical density (OD) at 650 nm was measured and serial 10-fold dilutions plated onto BG-blood agar to determine the density of the challenge inoculum.

Vaccines

The maternal immunization protocols were performed with the three-valent pertussis aP BOOSTRIX® (GSK, GlaxoSmithKline), with composition per human dose (HD): pertussis toxoid (8 μg), pertactin (2.5 μg), filamentous hemagglutinin (8 μg), tetanic toxoid (20 IU), and diphtheria toxoid (2 IU). For all experiments, immunization was carried out through the use of a 1/10 HD of that vaccine, hereafter referred to as a mouse dose (MD). The vaccinations of infant mice were performed with 1 MD of the

aP, a commercial wP vaccine (DTP vaccine, PT. BIO FARMA, Indonesia), or the *B. pertussis*-outer-membrane-vesicle-based vaccine formulated by us as previously described (24), to be referred to as the OMV vaccine.

Experimental Protocol

Maternal Immunization and Offspring Protection

Female BALB/c mice ($n = 10$) were intraperitoneally immunized with three doses of commercial acellular vaccine (aP) Boostrix™ 1/10 HD at days 0 and 14. Before applying the third vaccine, dose females were housed with males within the same cage and daily checked for pregnancy, when mucosal vaginal plug was detected a third vaccine dose was applied. Pregnancy eventually occurs after detection of vaginal mucosal plug. Mice couples stayed cohoused until the end of the experiment. Non-immunized mice were used as negative control of protection. Offspring born to either immunized or non-immunized mothers were intranasally challenged with a sublethal dose (10^6–10^8 CFU 40 μl^{-1}) of *B. pertussis* Tohama phase I at 21 days of life. Seven days after challenge, mice were sacrificed, and their lungs were harvested, homogenized in PBS and plated in serial dilutions onto BG-blood agar to count CFUs after incubation at 37°C for 3–4 days. At least three independent assays were performed.

Passive Immunization through Lactation

To investigate the protection of infant mice by means of passive immune transfer through lactation; after giving birth, aP-vaccinated mothers were separated from their own pups and exchanged with non-immunized mothers that had given birth at the same time. The changeling pups were then breast-fed by the surrogate mothers until weaning at the age of 4 weeks. Finally, the mice were infected with *B. pertussis* and protection assessed on day 7 as described above.

Adoptive Transfer

To study protection conferred by passive transfer, pooled serum (100 µl) or spleen cells ($20–50 \times 10^6$) from mice born from non-immunized or immunized dams were transferred intraperitoneally to 4-week-old naïve mice. Twenty-four hours later, the mice were infected with *B. pertussis* and protection assessed on day 7 as described above.

Effect of Infant Vaccination on Protection in Mice Born to Vaccinated Mothers

To study the effect of active immunization of infant mice born to vaccinated mothers on protection from subsequent pertussis infection, the offspring were immunized at 4 weeks of age with an MD of the commercial aP, a commercial wP vaccine, or with the OMV vaccine. Non-immunized offspring from aP-immunized mothers or aP-immunized mice at 4 weeks of age were used as controls. Mice were challenged with *B. pertussis* 2 weeks after receiving the vaccine dose and protection assessed on day 7 as described above.

Enzyme-Linked Immunosorbent Assay

As we previously described (25), plates (Nunc A/S, Roskilde, Denmark) were coated with sonicated *B. pertussis* Tohama phase I (whole-cell lysates), designated Bp, or with the purified recombinant pertussis toxin (PTxA), each at 3 µg/ml in 0.5 M carbonate buffer pH 9.5, by means of an overnight incubation at 4°C. The rinsed plates were then blocked with 3% milk in PBS (2 h 37°C) and incubated with serially diluted samples of mouse serum (1 h 37°C). In the experiments described above, the samples of blood used were collected from mothers at delivery, from mothers and pups at weaning, from mothers and pups before pup challenge, and from pups 13 days after immunization. The sera were obtained after leaving the blood samples to clot for 1 h at 37°C followed by centrifuging for 10 min at $7{,}000 \times g$. IgGs from individual serum or pooled sera bound to the plates were detected after a 2-h incubation with goat anti-mouse-IgG-linked horseradish peroxidase (1:8,000 Invitrogen, USA). As substrate 1.0 mg/ml *o*-phenylenediamine (Bio Basic Canada, Inc.) in 0.1 M citrate-phosphate buffer, pH 5.0 containing 0.1% hydrogen peroxide was used. ODs were measured with Titertek Multiskan Model 340 microplate reader (ICN, USA) at 492 nm, and the OD was plotted as a function of the log of the $(\text{serum dilution})^{-1}$. A successful assay produced for each antibody sample a sigmoidal curve in this type of plot. The titer of each antibody sample was determined from this curve by identifying by GraphPad Prism® software the concentration (expressed as inverse of the dilution of the antibody) that provokes a half way between the basal response and the maximal response.

Of the experimental protocol—it performed in triplicate—one representative experiment is presented in Section "Results."

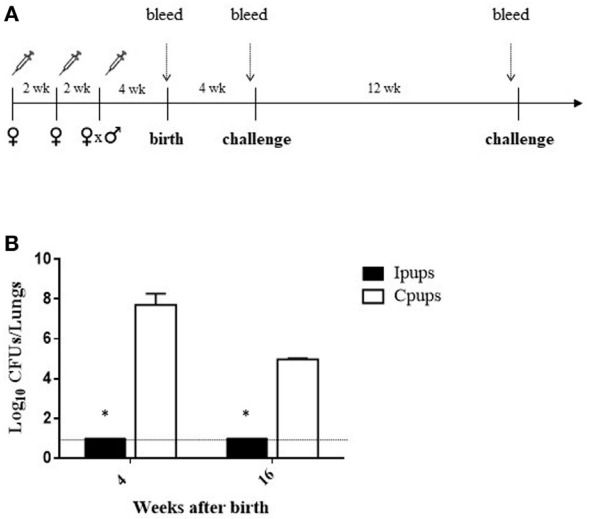
Statistical Analysis

The data were evaluated statistically by one-way analysis of variance followed by the Tukey test *post hoc* (*via* the GraphPad Prism® software). Differences were considered significant at a $p < 0.05$.

RESULTS

Maternal Immunization and Protection of the Offspring against *B. pertussis* Infection

To evaluate the protection of the offspring against *B. pertussis* infection through a maternal-vaccine-induced immunization, female mice were vaccinated twice with a commercial aP within an interval of 2 weeks, then mated with male mice and a third aP-vaccine dose administered when a vaginal plug was detected. Non-immunized females were mated with male mice at the same time to serve as the negative-control dams. Mice born to aP-immunized (hereafter referred to as Ipups) or non-immunized females (hereafter referred to as Cpups, for negative control) were challenged (intranasally) with 10^6 CFUs of *B. pertussis* at 4 and 16 weeks after birth (**Figure 1A**). We observed significant differences in the lung *B. pertussis* bacterial counts between mice born to immunized mothers and the negative-control group (**Figure 1B**; $p < 0.001$). Differences in CFUs of six to seven orders of magnitude were detected between the Ipups and the Cpups challenged at 4 weeks after birth and of about four to five orders of magnitude after challenge at 16 weeks of age. Once again, the CFU counts in the Ipups were non-detectable. What is notable,



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		At birth	4 wk after birth	16 wk after birth
Mother IgG Titers	a-PTxA	56.09	53.03	48.95
	a-Bp	144.00	34.94	32.25
Ipups IgG Titers	a-PTxA	-	73.00 ± 14.87	9.04 ± 2.98
	a-Bp	-	65.91 ± 10.84	nd
Cpups IgG Titers	a-PTxA	-	nd	nd
	a-Bp	-	nd	nd

FIGURE 1 | Effect of maternal immunization on protection of offspring against *Bordetella pertussis* infection. **(A)** Schematic representation of the maternal vaccination and challenge protocols. Female mice were vaccinated twice with a commercial acellular pertussis (aP) at a 2-week interval; mated with male mice; and when the vaginal plug was detected, a third aP dose was administered. Non-immunized females were mated with male mice at the same time as the immunized females. Mice were born 3–4 weeks after the last immunization. Mice born to aP-immunized or non-immunized females (controls) were challenged with *B. pertussis* at 4 and 16 weeks after birth. Mothers and pups were bled at different time points as indicated by the dotted vertical arrows. **(B)** Short- and long-term protection of offspring through maternal immunization. Mice born to aP-immunized (Ipups $n = 6$, black bars) or non-mmunized (Cpups $n = 6$, white bars) females were challenged with *B. pertussis* Tohama I at 4 and 16 weeks after birth. The number of bacteria recovered from mouse lungs, expressed as the $\log_{10}$ of CFUs per lungs, is plotted on the *ordinate* for the times after birth in weeks indicated on the *abscissa*, with the data representing the means ± the SD. The dotted horizontal line indicates the lower limit of detection. *$p < 0.001$ Ipups versus Cpups. **(C)** The anti-whole-cell-*B. pertussis* (a-Bp)- and anti-pertussis toxin (a-PTxA)-specific IgG titers were determined in the mother and in the offspring at the indicated time points. The titers are expressed as the geometric mean of the data from each group. $p < 0.001$ a-PTxA and a-Bp IgG titers in Ipups at 4 weeks after birth versus those detected at 16 weeks after birth. The results from one representative experiment are shown. nd, not detected.

however, is that the difference found at 16 weeks after birth was not as high as after 4 weeks after birth (four to five versus six to seven orders of magnitude) probably because of the age of mice since at 16 weeks mice seem to be less susceptible to pertussis infection (**Figure 1B**; cf. CFUs recovered from Cpups at 4 versus 16 weeks of age). As to the antibody titers, we performed a quantification of both the anti-whole-cell-*B. pertussis* (a-Bp) and the anti-PTxA (a-PTxA) antibodies in the mothers and in the offspring at the times indicated in **Figures 1A,C** lists the a-Bp- and a-PTxA-antibody titers detected in the immunized mothers and in their offspring. In contrast to the undetectable titers in the Cpups born to non-immunized females, significant levels of a-Bp and a-PTxA IgGs were present in the serum of the Ipups (**Figure 1C**). The antibody titers in the Ipups were lower at 16 weeks than at 4 weeks after birth, although the titers were still high enough to afford complete protection against *B. pertussis* infection (**Figure 1B**). With respect to the time elapsed between the administration of the last dose of vaccine to the mother and the evaluation of the protective capacity in the offspring, it is important to point out that the immunity transferred to the offspring still provided protection against *B. pertussis* infection even for the pups born to mothers whose last vaccination was given at up to 5 weeks before pregnancy (**Table 1**).

Another significant result observed in relation to maternal vaccination and immune protection of the successive litters was that the immunity acquired during the first pregnancy proved to be capable of conferring protection to the offspring born in later pregnancies (**Figures 2A,B**). For example, we detected a protection against *B. pertussis* infection in the Ipups born in the second pregnancy in which the reduction in CFUs recovered from the lungs was by more than six orders of magnitude below the levels determined in the Cpups (**Figure 2B**). Moreover, we also detected differences of 3.9–5.2 orders of magnitude between the CFUs recovered in the Ipups born in the third through the fifth pregnancies and those measured in the Cpups (**Figure 2B**). Of particular interest to us was that the antibody titers against PTxA detected in the pups born in the later pregnancies were lower than those recovered from the pups born in the earlier ones; but nevertheless, as we observed here, those titers were still high enough to protect the pups against *B. pertussis* infection (**Figure 2B**). In this sense, the technique produced a degree of redundancy of protection that constitutes a benefit in this model system simulating a clinical situation in generating a certain margin of error that would be of pragmatic value in the latter circumstance.

Donor Pups for Examination of Effector Mechanisms of Passive Immunity Transfer

To evaluate the contribution to protection of the transferred maternal antibodies, we performed transfer experiments of immune sera from Ipups to naïve mice. As a negative control of this protection, an equal volume of non-immune sera (100 µl) from Cpups was transferred to groups of five of those naïve female

BALB/c mice. Twenty-four hours after transfer, all the mice were infected with a sublethal dose of *B. pertussis* and sacrificed 7 days later to determine the number of CFUs in the lungs. Transfer of 100 µl of sera from Ipups either with high titer (sera from pups from the first and second deliveries, the earlier pregnancies) or with low titer (sera from pups born from the third through the fifth deliveries, the later pregnancies) was found to confer protection (**Figure 3**). A reduction of 2.76 logs or 1.17 logs in CFU counts was detected relative to the control group (**Figure 3**) in the mice thus passively immunized with a high and a low titer of immune sera, respectively ($p < 0.05$).

TABLE 1 | Protection of offspring through maternal immunization before pregnancy.

	Time elapsed between the last immunization and pregnancy			
Log_{10}CFU/lungs	**0 weeks**	**1 week**	**2 weeks**	**5 weeks**
Cpups	7.00 ± 0.86	7.52 ± 0.42	7.12 ± 0.54	7.72 ± 0.64
Ipups	1 ± 0.0	1 ± 0.0	1.07 ± 0.15	1 ± 0.0

$p < 0.001$ Ipups versus Cpups at each tested time.

Similar transfer experiments were also performed with spleen cells removed surgically from Ipups born from the earlier deliveries and injected into naïve mice, with the spleen cells from non-immunized mice being used as a negative control for protection. One day after the transfer, the recipient mice were challenged with *B. pertussis*, and 7 days after the challenge the CFUs in the lungs

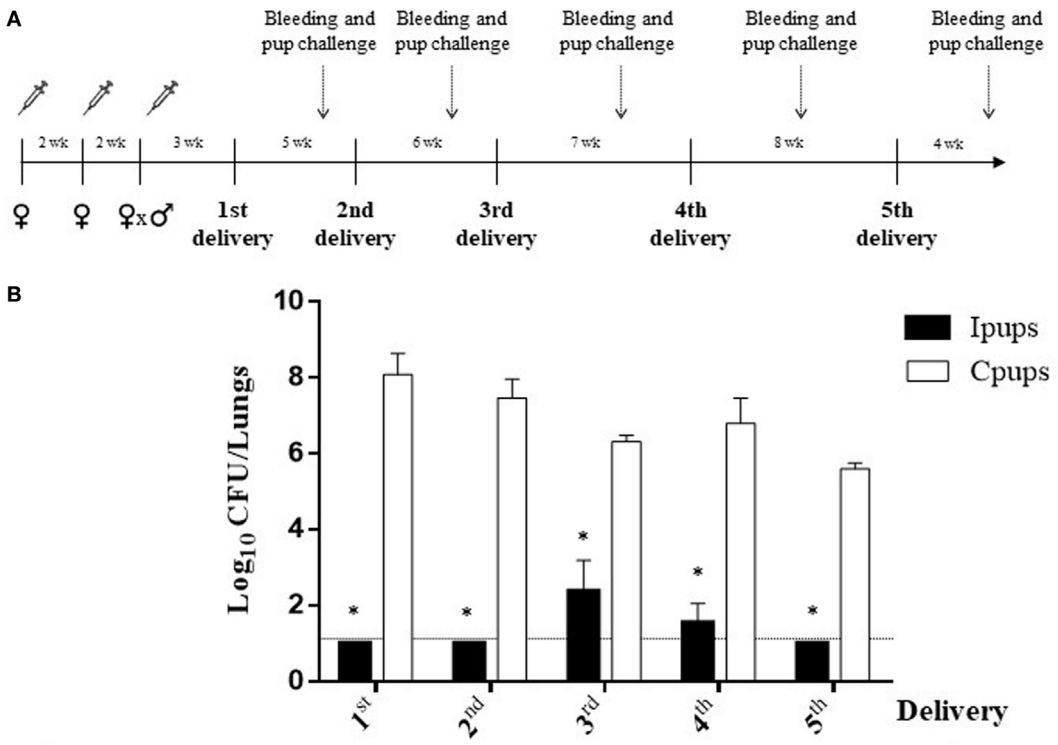

FIGURE 2 | Duration of protective immunity conferred by maternal immunization beyond the first pregnancy. **(A)** Schematic representation of the maternal vaccination and pup-challenge protocol. Female mice were vaccinated twice with commercial acellular pertussis (aP) at a 2-week interval; mated with male mice; and when the vaginal plug was detected, a third aP dose was administered. Non-immunized females were mated with male mice at the same time as the immunized females. The first birth was 3–4 weeks after the last immunization. Successive births occurred at intervals of 5–8 weeks. Mice born to aP-immunized or non-immunized females (the negative controls) were challenged with *Bordetella pertussis* at 4 weeks after birth. Mothers and pups were bled 1 day before pup challenge as indicated by the dotted vertical arrows. **(B)** Protection conferred to the offspring born in later pregnancies by maternal immunity acquired during the first pregnancy. Mice born to aP-immunized (Ipups $n = 5$) or non-immunized (Cpups $n = 5$) females were challenged with *B. pertussis* Tohama I 4 weeks after birth. The protection conferred to the offspring through maternal immunization during the first pregnancy was estimated by determining the number of bacteria recovered from mouse lungs. The bacterial counts expressed as the $\log_{10}$ of CFU per lungs, is plotted on the *ordinate* as a function of the number of births after the initial delivery indicated on the *abscissa*. *$p < 0.001$ Ipups versus Cpups. The anti-pertussis toxin (a-PTxA) IgG titers in the mothers and the offspring determined at the time of challenge are listed below the *abscissa*. The titers are expressed as the geometric mean of the data from each group ($n = 5$). $p < 0.001$ a-PTxA IgG titers in Ipups from the first and second deliveries versus those detected in Ipups from the third through the fifth deliveries. The results from one representative experiment are presented.

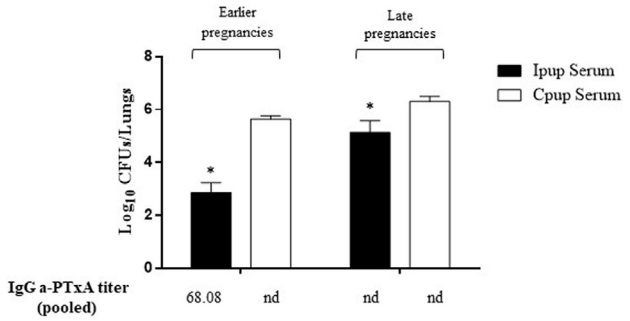

FIGURE 3 | Effect of passive immunization on protection through sera collected from Ipups. Pooled sera from either early pregnancy Ipups with high anti-pertussis toxin (a-PTxA) titer or late-pregnancy Ipups with low a-PTxA titer were tested by transfer to naive (Cpup $n = 5$) female mice. Pooled sera from Cpups transferred to naive female mice were used as negative control of protection. Twenty-four hours after transfer, the mice were infected with *Bordetella pertussis* and sacrificed 7 days later to determine the CFUs in the lungs. In the figure, the number of bacteria recovered from mouse lungs, expressed as the $\log_{10}$ of CFUs per lungs, is plotted on the *ordinate* for each of the two serum-donor groups (early- and late-pregnancies) indicated above the bars. $^*p < 0.05$ Ipup serum versus Cpup serum. The IgG a-PTxA titers are listed for each pool below the *abscissa* (nd, not detected).

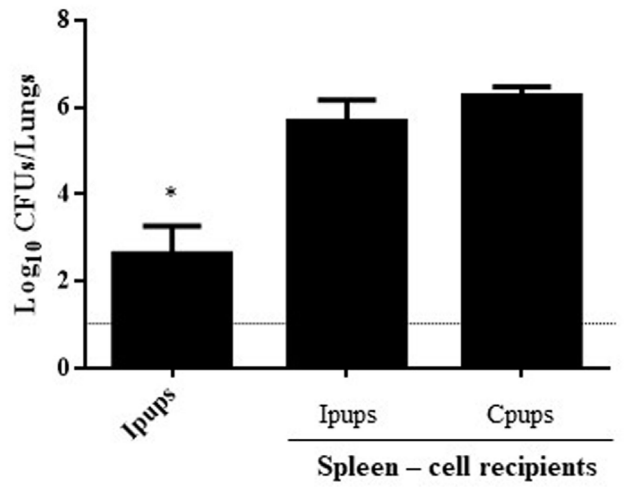

FIGURE 4 | Effect of passive immunization through spleen cells collected from Ipups on protection from infection. Whole spleen cells ($20–50 \times 10^6$) from Ipups or Cpups (controls) were tested as possible vehicles of immune protection by passive transfer to naïve mice. Twenty-four hours after transfer, the mice were infected with *Bordetella pertussis* and sacrificed 7 days later to determine the CFUs in the lungs. Ipup-recipients were used as a positive control. In the figure, the number of bacteria recovered from the mouse lungs, expressed as the $\log_{10}$(mean CFUs ± SD of per lungs), is plotted on the *ordinate* for each of the spleen-cell-recipient groups ($n = 7$) indicated on the *abscissa*. The dotted horizontal line marks the lower limit of detection. $^*p < 0.05$ Ipups versus both spleen-cell recipient groups.

of the recipients were counted. In this instance, no significant difference was found between the control group and the group injected with spleen cells from the Ipups (**Figure 4**).

Passive Immunization through Lactation

We also evaluated passive immunization through lactation. To achieve this aim, pups born to immune mothers (Ipups) were fostered to non-immune mothers (Ipups–Cmother), and pups born from control mothers were fostered to immune mothers (Cpups–Imother). Ipups breast-fed by their immune mother (Ipups–Imother) and Cpups breast-fed by their non-immune mother (Cpups–Cmother) were used as the respective positive and negative controls. After all the pups were suckled for 21 days, the pups' sera were collected to analyze the titers of a-PTxA and a-Bp IgGs before *B. pertussis* challenge. Pups were then challenged with a sublethal dose of *B. pertussis* to analyze the protection against infection. Through this experimental protocol and the resulting assays, we detected that pups from the Ipups–Imother and Cpups–Imother groups exhibited high titers of a-PTxA and a-Bp IgGs (**Figure 5A**) and also possessed a high degree of protection against *B. pertussis*—i.e., a reduction in the CFUs recovered from the lungs by more than six orders of magnitude (**Figure 5B**). In contrast, pups from the group Ipups–Cmother contained low titers of a-PTxA and a-Bp IgGs and retained a low degree of protection. The lowest titers of a-PTxA and a-Bp IgGs and degrees of protection were detected in the pups from the Cpups–Cmother group (**Figures 5A,B**).

Effect of Infant Vaccination in Mice Born to aP-Vaccinated Mothers on Protection

To evaluate the possible interference of maternal immunization with subsequent infant immune boosting, offspring born to aP-vaccinated mothers, upon weaning from their mothers, were split into four groups at 4 weeks after birth. Three groups were treated with a single dose of either the aP vaccine, a commercial wP vaccine, or the OMV vaccine (**Figure 6A**), while the remaining group was left untreated. Cpups that received one dose of the aP vaccine at 4 weeks after birth were also used as control. Two weeks after vaccination of the infant mice, the antibody titers to PTxA were measured. We observed that the titers in aP-vaccinated Cpups (given a single dose) were slightly higher than those detected in the non-vaccinated Cpups. In the mice born to aP-vaccinated mothers (i.e., the vaccinated Ipups), the titers detected after vaccination with any of the three vaccines tested were lower than those quantified in Ipups left untreated (blunting effect) (**Figure 6B**). Moreover, that the titers in the non-vaccinated Ipups were higher than those detected in the vaccinated Cpups was most notable (**Figure 6B**). All the mice in this experiment, including the Cpups used as the negative control for protection, were then challenged with $10^6–10^7$ CFUs of *B. pertussis* 2 weeks after the postnatal immunization of the Ipups. We observed that at 7 days after challenge, the vaccinated Cpups exhibited a reduced bacterial burden in the lungs by 1.5 logs (32-fold) compared with the non-vaccinated Cpups (**Figure 6C**). Furthermore, aP vaccination of the infant mice born to vaccinated mothers did not interfere with the maternally transmitted protective immunity, as evidenced by a comparable reduction in the CFUs detected in the lungs of approximately 5 logs compared with the burden of the non-vaccinated Cpups. In these experiments, we also observed that the immunization of Ipups with vaccines of different antigenic compositions from that used in

A

	aP Mother		Pups			
	At delivery	At pup weaning	Ipups-Imother	Cpups-Imother	Ipups-Cmother	Cpups-Cmother
a-PTxA IgG Titers	71.50 ± 6.54	71.10 ± 11.56	53.66 ± 4.53	155.2 ± 98.63	nd †	nd †
a-Bp IgG Titers	162.9 ± 70.87	178.5 ± 65.15	98.07 ± 32.77	119.9 ± 7.25	20.7 ± 1.99	9.45 ± 0.74

*Titers of the pups at weaning
†nd: not detected

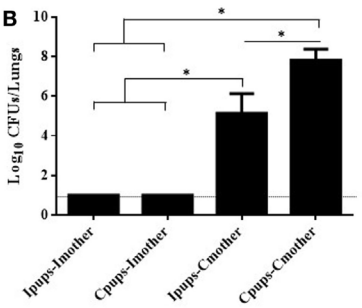

FIGURE 5 | Effect of passive immunization through lactation on pertussis protection. Pups born from immune mothers (Ipups) were fostered to non-immune mothers (Ipups–Cmother), and pups born from control mothers were fostered to immune mothers (Cpups–Imother). Ipups breast-fed by their immune mother (Ipups–Imother) and Cpups breast-fed by their non-immune mother (Cpups–Cmother) were used as the respective positive and negative controls. All the pups ($n = 6$ for each group) were suckled for 21 days. **(A)** Sera from mothers and pups were collected at different time points to analyze the titers of anti-pertussis toxin (a-PTxA) and anti-whole-cell-*B. pertussis* (a-Bp) IgGs. The titers are expressed as the geometric mean of the data for each group ($n = 6$). **(B)** Pups were challenged with a sublethal dose of *Bordetella pertussis* Tohama phase I to analyze protection. In the figure, the number of bacteria recovered from the mouse lungs, expressed as the $\log_{10}$(mean CFUs ± SD of per lungs), is plotted on the *ordinate* for each of the dam-suckling groups indicated on the *abscissa*. The dotted horizontal line marks the lower limit of detection. Two independent experiments were performed. *$p < 0.05$ for Ipups–Imother and Cpups–Imother versus the other groups and for Ipups–Cmother versus Cpups–Cmother.

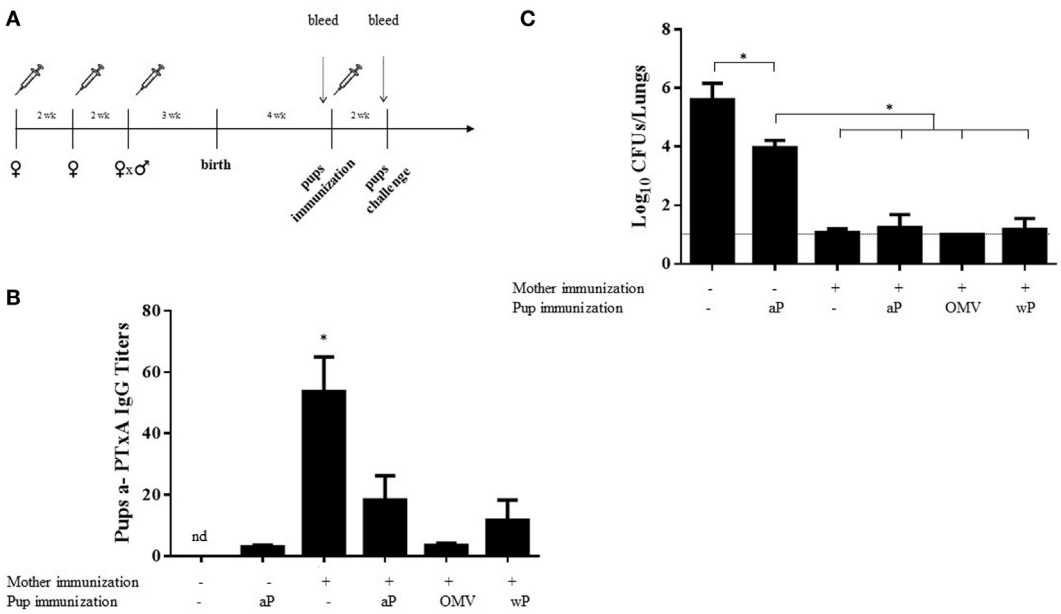

FIGURE 6 | Effect of heterologous mother–pup vaccination on protection against *Bordetella pertussis* challenge. **(A)** Schematic representation of vaccination and challenge protocols. Female mice were immunized with three doses of commercial aP vaccine on days 0 and 14; mated with male mice; and when the vaginal plug was detected, a third aP dose was administered. Four weeks after birth, the offspring received either no vaccine ($n = 8$) or one dose of the aP, OMV, or wP vaccine ($n = 8$ in each group), as indicated below the *abscissas* of panels **(B,C)**. Two weeks later, the pups were challenged with *B. pertussis* and the bacterial burden in the lungs measured 7 days after challenge. Pups born to vaccinated or non-vaccinated mothers and receiving no postnatal vaccine served as controls. The pups ($n = 5$ for each group) were bled and the anti-pertussis toxin (a-PTxA) IgG titers determined 1 day before the challenge as designated by the dotted vertical arrow. **(B)** The titers, plotted on the *ordinate*, are expressed as the geometric mean of the data from each group specified on the *abscissa*. *$p < 0.05$ pups born to immunized mother versus others groups, and the nd signifies not detected. **(C)** In the figure, the number of bacteria recovered from the mouse lungs, expressed as the $\log_{10}$(mean CFUs ± SD per lung), is plotted on the *ordinate* for each group indicated on the *abscissa*. *$p < 0.05$ for negative control versus other groups, and aP-immunized pups born to non-immunized mother versus others groups. The dotted horizontal line marks the lower limit of detection. Of the three independent experiments that were performed, the results from a single representative one is presented.

the maternal immunization reciprocally did not interfere with the maternally transmitted protective immunity since after postnatal vaccination with either a commercial cellular vaccine (wP) or our previously designed OMV vaccine the protection conferred was similar to that seen in infant mice born to aP-vaccinated mothers with or without postnatal aP vaccination (**Figure 6C**).

DISCUSSION

The vaccination of women with aP during pregnancy is expected to provide infants with a certain degree of protection from pertussis until they are old enough to be vaccinated themselves. Because of this strategy and the data reported on its safety, the Advisory Committee on Immunization Practices (ACIP) recommended in 2011 that unvaccinated pregnant women receive a dose of tetanus toxoid, reduced diphtheria toxoid, and aP vaccine (26). In an effort to reduce the pertussis burden in infants, in 2012, the ACIP recommended the use of aP during every pregnancy (27). In their publication of August 2015, the WHO stated that they considered the vaccination of pregnant women to be most likely the greatest cost-effective additional strategy for preventing disease in infants too young to be vaccinated, with that approach appearing to be more effective and favorable than the so-called *cocooning* tactic, the vaccination of adults in close contact with infants (6). Indeed, WHO recommended that the national programs might consider the vaccination of pregnant women with one dose of aP administered during the second or third trimester or at least 15 days before delivery as a control strategy in addition to routine primary infant pertussis vaccination either in entire countries, or in other more limited settings having a high or increasing infant morbidity and/or mortality from the disease. Although the present time is still early to assess the definitive effect of implementing this strategy on the disease in infants, reports in support of that approach have already appeared in the literature (28, 29). In particular, several studies evidenced the placental transfer of anti-*B. pertussis* antibodies from aP-vaccinated mothers to their infants where the infants born to those immunized mothers had a high level of antibodies during their first months of life (28, 29). A further report in England related the key observation that vaccine effectiveness against laboratory-confirmed pertussis had been sustained during the 3 years following the vaccine's introduction in 2012 (18). Also highly significant was the finding that the disease incidence in infants less than 3 months of age had remained low despite a high persistence in those aged 1 year and older (18). In 2017, a retrospective cohort study appeared that was designed to evaluate whether or not pertussis-infected infants born from 2011 through 2015 whose mothers had received aP vaccine during pregnancy had less severe pertussis than infants born to unvaccinated mothers. The authors concluded that the infants with pertussis whose mothers had been aP-vaccinated during pregnancy had a significantly lower risk of hospitalization and admission to intensive-care units as well as shorter hospital stays (30). Another promising aspect found in the Amirthalingam et al. (18) study was that additional protection from maternal immunization was retained in infants who subsequently received their first dose in the primary Amirthalingam series (18).

Animal models had been used earlier to obtain information about that strategy. Although the data reported once again had been scarce, evidence was nevertheless garnered for protection by the aP vaccine when used during pregnancy against intracerebral infections of *B. pertussis* and those contagions transmitted by aerosols to infant mice (22). In those studies, mice of from 6 to 10 days of age born to aP-immunized mothers were protected against an aerosol challenge with *B. pertussis* strain 18323. The authors ascertained that the protection was transferred from the dams to their offspring first through the placenta and then through the milk (22). More recently, Feunou et al. (31) confirmed that maternal immunization protected the offspring against *B. pertussis* challenge; but in their experimental paradigm, the protection waned and was eventually lost after the postnatal vaccination of the infant mice with the selfsame vaccine (31).

In the experiments reported here, we used just such a mouse model to enhance our understanding of that specific form of maternal immunization. In agreement with previous reports, we detected that maternal immunization with an aP vaccine—in our paradigm, administered in three doses (one being during the pregnancy)—led to offspring protection against *B. pertussis* infection (**Figure 1**). Moreover, we confirmed that the antibody levels to *B. pertussis* and PTxA were accordingly higher in those neonates (the Ipups) than in mice born to non-immunized females (the Cpups). The antibody titers in the offspring declined at 16 weeks relative to the levels at 4 weeks after birth, although the titers were high enough to protect the neonates against *B. pertussis* infection. Furthermore, the transferred antibodies, but not the spleen cells, from Ipups to naïve mice were sufficient to confer protection (**Figure 3**).

What was interesting to us was the observation that the immunity transferred to the offspring had a protective capacity even for pups born to mothers whose last dose of vaccine was given some weeks before pregnancy. Moreover, we detected that the immunity acquired during the first pregnancy was even capable of conferring protection to the offspring born in later pregnancies. We noted that although the titers of antibodies against PTxA were low, protection against pertussis in the Ipups born in those later deliveries was still significantly elevated (**Figure 2B**). These results, though having been obtained in a murine model, would underscore the need to revise the frequency with which human maternal immunization should be conducted.

In agreement with Oda et al. (22), we detected that the main protection was transferred through colostrum and/or milk. Those authors found that challenged infant mice born to mothers immunized with either the aP or the wP vaccine twice before mating evidenced the lowest increase in the number of CFUs per lung. Moreover, out of eight mice, seven deaths were registered in the non-immunized group, whereas five deaths occurred in the transplacentally immunized group, but only two in the transcolostrally immunized mice (22). In our protocol involving a schedule that included the administration of a third aP dose during pregnancy, we detected high degree of protection against *B.* pertussis through lactation (**Figure 5B**). In particular, we detected that the Ipups–Imother and Cpups–Imother experimental groups exhibited high a-PTxA and a-*B. pertussis* IgG titers (**Figure 5A**) in combination with high resulting protection against *B. pertussis* infection (more than a 6-log reduction in the CFUs recovered from the lungs: cf. **Figure 5B**). The results obtained by Quinello et al., in agreement with ours, demonstrated that the *pertussis*-absorbed serum and the colostrum pools protected only 30% of the immunized mice, whereas purified IgGs protected some 65% (23). Although IgA was not measured in this study or in our work, its presence could contribute at least in part to protection. In fact, it was reported

that IgA induced by oral or nasally delivered pertussis antigens formulated with mucosal adjuvants confers protection although at levels not so high than the equivalent parenterally delivered vaccines [reviewed in Ref. (32)].

Although the data garnered from our murine model underscored the significance of breastfeeding in protecting infants against pertussis infection, we must point out that this protection could be less substantial in humans since in *Homo sapiens* the majority of the maternal IgG is transferred to the fetus *in utero* during pregnancy (33) and not *via* the milk.

We also observed that, when infant mice born to aP-immunized mothers (the Ipups) were vaccinated at 3 weeks of age with the same aP vaccine or a different one (i.e., a commercial wP or the OMV vaccine); the titer of IgG against a-PTx decreased (**Figure 6B**), but the maternally derived protection was not reduced upon that subsequent vaccination, regardless of the type of vaccine administered. These results seem to be contradictory in principle to those reported by Feunou et al., but we must bear in mind that those authors applied two vaccine boosters, one at 7 days and another at 3 weeks of life (31). The blunting effect on protection that the authors observed could have resulted from the application of those boosters—and particularly the ones performed during neonatal life—at a time when the antibody titers in the pups were still high. In contrast, our results are in agreement with those observed in humans in whom the protective capacity conferred by maternal immunization was retained in neonates receiving their first dose of the primary series (18).

Though the use of mouse models to research maternal vaccination is not expected to completely replicate human physiology, the results obtained with a model of this design will enable a test of the proposed hypotheses under controlled conditions, where the forthcoming results can then refine those hypotheses for further validation in subsequent human studies. Here, in the present mouse model, we have demonstrated that immunization with aP during pregnancy or up to 5 weeks prior effectively protects newborns against pertussis. Although the titer of maternal antibodies in the infant offspring diminishes with time, protection is not reduced for at least up until 4 months of age. Moreover, subsequent vaccination of the infant mice with the same vaccine or a different one from the type used during pregnancy did not affect the transferred maternal protection (**Figure 6**). The potential blunting of protection conferred by maternal immunization through infant vaccination could be developed depending on the antibody levels in the infants. Another important finding here described was that maternal-vaccination-acquired immunity from the first pregnancy still conferred protection to offspring up to the fourth pregnancy. The results presented here reinforce the need to continue studying that blunting effect in humans as well as to revise the frequency of vaccination in successive pregnancies according to the time between each one.

ETHICS STATEMENT

The animal experiments were authorized by the Ethical Committee for Animal Experiments of the Faculty of Science at La Plata National University (approval number 004-06-15 and 003-06-15).

AUTHOR CONTRIBUTIONS

DH planned the study, made the laboratory analysis, interpreted data, and drafted manuscript. DB, MG, and MZ planned the study, interpreted data, and revised figures and the manuscript. DS-M, EB, FC, PA, CC, and MSB performed certain experiments and laboratory analyses. All authors approved the final manuscript.

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Whole Blood Profiling of Bacillus Calmette–Guérin-Induced Trained Innate Immunity in Infants Identifies Epidermal Growth Factor, IL-6, Platelet-Derived Growth Factor-AB/BB, and Natural Killer Cell Activation

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Vaccination of infants with bacillus Calmette–Guérin (BCG) activates both the innate and adaptive arms of the immune response. The antimycobacterial effects of these responses most likely account for the ability of BCG to protect against childhood forms of tuberculosis (TB). There is also evidence for a heterologous protective effect of BCG vaccination against TB-unrelated mortality in low birth weight infants. A possible mechanism of action of this effect, the induction of trained innate immunity, has been demonstrated when cells from BCG-vaccinated adults are restimulated *in vitro* with non-related microbial stimuli. Our aim was to examine an extensive panel of secreted immune biomarkers to characterize the profile of trained innate immunity in infants. Stimulation of whole blood for 48 h was performed 4 months after BCG vaccination, or in control unvaccinated infants. Stimulants were lipopolysaccharide; Pam3Cys (P3C); heat-killed *Candida albicans*, *Staphylococcus aureus*, *Escherichia coli*, and a lysate of *Mycobacterium tuberculosis*. Culture supernatants were tested for secreted cytokines and chemokines by 42-plex bead array and monocytes and natural killer (NK) cells assessed for expression of activation markers by flow cytometry. BCG-vaccinated infants displayed increases in 11 cytokines and chemokines in response to different non-specific innate immunity stimuli: epidermal growth factor (EGF); eotaxin; IL-6; IL-7; IL-8; IL-10; IL-12p40; monocyte chemotactic protein-3; macrophage inflammatory protein-1α; soluble CD40 ligand and platelet-derived growth factor (PDGF)-AB/BB. Although each stimulant induced a distinct response profile, three analytes, EGF, IL-6, and PDGF-AB/BB, were commonly higher after stimulation with Pam3Cys, *C. albicans*, and *S. aureus*. Conversely, certain cytokines such as interferon gamma-inducible protein-10, IL-2, IL-13, IL-17, GM-CSF, and GRO were suppressed in BCG-vaccinated infants, while no increases in TNFα or IL-1β production were detected. We did not observe a concomitant, BCG-associated change in monocyte surface activation markers in response to non-specific stimuli, but we detected a significant increase in CD69 expression on NK cells in response to Pam3Cys.

Pam3Cys-induced NK cell activation correlated with the magnitude of IL-12p40 and IL-10 responses to the same stimulant. This study reveals a novel cytokine/chemokine biomarker signature of BCG-induced trained innate immunity in infants and the involvement of NK cells in these responses.

Keywords: bacillus Calmette–Guérin, vaccination, heterologous effects, trained immunity, infants, cytokines, chemokines, natural killer

INTRODUCTION

Mycobacterium bovis bacillus Calmette–Guérin (BCG) is currently the only licensed vaccine for tuberculosis (TB). Although its protective efficacy against adult pulmonary TB is variable (1–3), BCG affords more reliable protection against childhood forms of the disease when administered to infants (4). The immune mechanisms responsible for this protection are not fully understood, although many studies have described BCG-induced, antigen-specific immune responses that may play a part (5–12).

Evidence also exists of a beneficial effect for the BCG vaccine against several different diseases and outcomes other than TB, including as a therapeutic agent against bladder cancer when instilled directly into the bladder (13) and protection against all-cause mortality in low birth weight infants (14). These effects are heterologous (non-specific) as they do not rely on mycobacteria-specific adaptive immune responses. Exactly how BCG mediates these effects is not clear, although different pathways are probably involved. Non-specific protective effects against mortality in low birth weight infants are a result of resistance to pathogenic microorganisms that are responsible for death due to neonatal sepsis and respiratory infections (15, 16). The immune responses mediating this effect could be either heterologous T-cells (i.e., T-cells induced by an epitope from one organism but with cross-reactivity to others) or the ability of BCG to potentiate the responsiveness of the innate immune system to later infections: a biological process termed trained innate immunity which represents a *de facto* innate immune memory (17–19). Such a phenomenon is thought to have existed for millions of years as acquired systemic resistance in plants and also in invertebrates, neither of which have adaptive immune systems (17). Several studies have revealed enhancements of the neonatal innate immune response to Gram-positive and Gram-negative infections following previous encounters, mediated by different toll-like receptor pathways (20) or initiated by *in utero* inflammatory conditions such as histologic chorioamnionitis (21). Studies in adults show that trained innate cells, including monocytes and natural killer (NK) cells, appear to be epigenetically and metabolically reprogrammed to produce increased amounts of pro-inflammatory cytokines and display higher levels of surface activation markers in response to restimulation with toll-like receptor ligands or different whole microorganisms (22–25).

If trained immunity is to provide a mechanism of action for the non-specific protective effects of BCG, evidence of trained immunity in infants or infant innate cells is needed. The immune system of the newborn infant differs from that of the adult in its constitution as well as in its propensity to respond to different stimuli; differences that reflect the unique physiological challenge of transitioning from the intrauterine environment to the outside world where some microorganisms are beneficial commensals and some are life-threatening pathogens (26, 27). Newborn innate immune responses are characterized by a reduced capacity for pro-inflammatory cytokines and dendritic cell differentiation and activation, but a greater propensity to produce regulatory cytokines (28, 29). More data are needed on how these differences impact upon the generation of trained innate immunity by BCG.

Our aim in this study was to probe the infant immune response to mycobacteria-unrelated stimuli following BCG vaccination for potential mediators of trained immunity. We used a multiplex bead array approach for the detection of secreted cytokines and chemokines from diluted whole blood following stimulation with a panel of innate stimuli. The rationale for this was to maximize the variety of immune cells available for stimulation as well as the potential to detect a broad array of soluble mediators. Our data provide a description of previously unreported profiles of trained immunity in infants and a role for activated NK cells.

MATERIALS AND METHODS

Study Participants and Sample Collection

Healthy, UK-born infants were recruited following ethical approval from the National Research Ethics Service Committee London-East (11/LO/0363) and from the Ethics Committee of the London School of Hygiene and Tropical Medicine (ref. 4068). Written consent was obtained from parents prior to recruitment. Infants were recruited from two regions of South East England: Redbridge, where a single dose of intradermal BCG (BCG Vaccine Danish Strain 1331, Staten Serum Institute, Copenhagen, Denmark) was administered to infants at approximately 6 weeks of age in local vaccination clinics and West Essex where infants do not routinely receive BCG. Heparinized venous blood was obtained 4 months post-vaccination or from unvaccinated infants at an age-matched time point. This exploratory study was part of a larger study (11) and 4-month post-vaccination samples available were $n = 11$, vaccinated infants; and $n = 10$, unvaccinated infants for Luminex analysis and $n = 10$ and $n = 8$, respectively, for flow cytometry analysis.

Diluted Whole Blood Assays for Cytokine Responses to Innate Stimuli

Venous blood was diluted 1/5 in RPMI 1640 (Invitrogen) supplemented with 2 mM L-glutamine (Invitrogen) and cultured

at 37°C for 48 h in 96-well U-bottomed plates in a final volume of 200 µl. Duplicate wells were incubated alone (medium only negative control) or with the following stimuli: lipopolysaccharide (LPS; 10 ng/ml); (S)-(2,3-bis(palmitoyloxy)-(2RS) -propyl)-*N*-palmitoyl-(R)-Cys-(S)-Ser(S)-Lys_4-OH, trihydrochloride [Pam3Cys; 10 µg/ml]; heat-killed (HK) *Candida albicans* (*C. albicans*; 10^6 microorganisms/ml); HK *Staphylococcus aureus* (*S. aureus*; 10^6 microorganisms/ml); HK *Escherichia coli* (*E. coli*; 10^6 microorganisms/ml); and sonicated *Mycobacterium tuberculosis* H37Rv [*Mtb* lysate; 1 µg/ml end concentration]. Concentrations used for each stimulus were optimized in previous experiments.

Tissue Culture Supernatant Harvest and Multiplex Bead Array Assay

After 48 h, plates were centrifuged at 400 *g* for 5 min. Supernatants were removed from duplicate wells, pooled, and stored in aliquots at −80°C prior to analysis. Thawed supernatants were subjected to multiplex bead array analysis using the human cytokine/chemokine Milliplex™ MAP 42-plex premixed kit (Merck Millipore) and following the manufacturer's instructions. The pre-mixed bead set included the following panel: IL-1α, IL-1β, IL-1Ra, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17, epidermal growth factor (EGF), eotaxin, Flt-3L, FGF-2, fractalkine, G-CSF, GM-CSF, GRO, IFNα2, IFNγ, IP-10, MCP-1, MCP-3, MDC, MIP-1α, MIP-1β, platelet-derived growth factor (PDGF)-AA, PDGF-AB/BB, RANTES, sCD40L, sIL-2Ra, TGFα, TNFα, TNFβ, and VEGF. Data were acquired using the Biorad Luminex® 100 system and Bioplex Manager Software version 6.1 (Biorad).

Peripheral Blood Leukocyte (PBL) Harvest and Flow Cytometry

Following the removal of supernatants from diluted whole blood assay plates, remaining PBLs were harvested and cryopreserved for later analysis. EDTA in PBS (2 mM) was added to assay wells, which were incubated at room temperature for 15 min to detach adherent cells. Wells were mixed and cells from duplicate wells were pooled and then incubated with 10× volume of 1× FACS Lysing Buffer (BD Biosciences) at room temperature for 10 min. Following red cell lysis, PBLs were pelleted, resuspended in fetal bovine serum with 10% dimethylsulfoxide, and cryopreserved in liquid nitrogen. For flow cytometric analysis, thawed cells were washed in PBS with 0.1% bovine serum albumin and 0.01% sodium azide (both Sigma Aldrich) and stained for 30 min at 4°C with the following antibodies: CD3-BV510 (clone UCHT1); CD25-PerCP-Cy™5.5 (clone M-A251); CD56-PE-Cy™7 (clone B159); CD206-PE-CF594 (clone 19.2); HLA-DR-BV605 (clone G46-6) (all from BD Biosciences); CD11b-FITC (clone ICRF44); CD69-PE (clone FN50); CD163-APC (clone eBioGHI/61) (all from Affymetrix/eBiosciences); CD14-BV650 (clone M5E2; Biolegend). Following a further wash, cells were resuspended in PBS with 1% paraformaldehyde for acquisition. Cells were acquired using a BD LSR II flow cytometer (BD Biosciences) equipped with blue (488 nm), red (633 nm), and violet (405 nm) lasers and FACSDiva 6.1.3 software. Compensation was carried out using BD™ CompBead Plus compensation particles stained separately with each antibody conjugate and fluorescence minus one control stains were used to determine the position of phenotype and activation marker analysis gates.

Data Analysis, Management, and Statistical Analysis

Multiplex bead array data for each stimulation condition were background subtracted using values measured in unstimulated controls. Background levels of each cytokine/chemokine are given in Table S1 in Supplementary Material. Final concentrations below the lower limit of quantitation were adjusted to the value of the lowest standard (3.2 pg/ml) and data above the upper limit of quantitation were adjusted to the highest standard (10,000 pg/ml). The compensation matrix was generated and flow cytometric data were analyzed using FlowJo™ 10.2 software (FlowJo LLC). Monocytes were gated based on CD14 and HLA-DR expression as well as on size and granularity. NK cells were gated based on CD56 expression and lack of CD3 expression (see Figure S1 in Supplementary Material). Multiplex bead array data were analyzed using IBM® SPSS® Statistics version 23 software (IBM Corp.) and Prism 7 for Windows (GraphPad Software Inc.). Flow cytometric data were further analyzed using a combination of Spice 5.35 (30) and Prism. Statistical comparisons between vaccinated and unvaccinated infant groups for both multiplex bead array and flow cytometry data were by Mann–Whitney *U* test and correlations were by Spearman's rank correlation coefficient.

RESULTS

Infant BCG Vaccination Is Associated with Enhanced Cytokine and Chemokine Responses to Non-Specific Innate Stimuli

Diluted whole blood from BCG-vaccinated and unvaccinated infants was stimulated with a panel of non-specific innate stimuli and with a preparation of *Mtb* lysate. Secreted cytokines and chemokines were measured in tissue culture supernatants by multiplex bead array after 48 h.

Previous reports described TNFα, IL-6, and IL-1β as the characteristic cytokines of BCG-induced trained innate immunity in adults (24, 31). However, when we investigated this signature in infants, only IL-6 was significantly increased in response to non-specific stimuli. Re-stimulation with the mycobacterial stimulant *Mtb* lystae did produce a significant increase in TNFα (**Figure 1**). As expected, *Mtb* lystae induced the most extensive upregulation of responses in BCG-vaccinated infants including 27 cytokines and chemokines that characterize both innate and adaptive T-cell responses (**Figures 1** and **2**; **Table 1**). We detected 11 cytokines and chemokines that were significantly increased and 6 that were significantly decreased in BCG-vaccinated infants in response to non-specific stimulants (**Figure 2**; **Table 1**). Each non-specific stimulant induced a distinct response profile. For example, increases in 10 cytokines and chemokines were detected following Pam3Cys stimulation

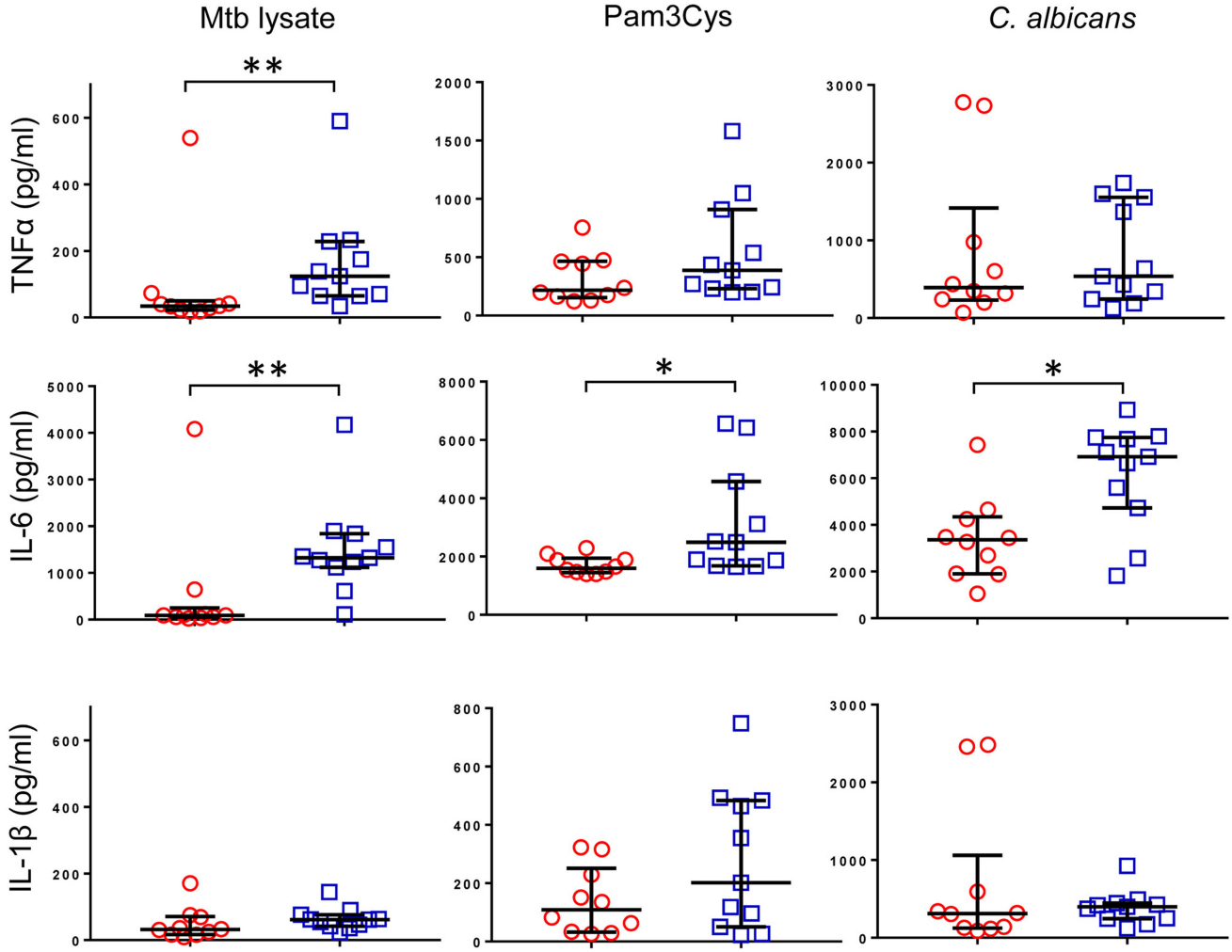

FIGURE 1 | Cytokine profile of bacillus Calmette–Guérin (BCG)-induced trained immunity in infants does not include TNFα or IL-1β. TNFα, IL-6, and IL-1β concentrations were measured in tissue culture supernatants after diluted whole blood from BCG-vaccinated (□, $n = 11$) and unvaccinated (○, $n = 10$) infants was cultured for 48 h with the indicated stimulants. Data shown is background subtracted (unstimulated samples). Wide bars indicate median response; narrow bars indicate 25th and 75th percentiles. Responses in vaccinated and unvaccinated groups were compared using the Mann–Whitney *U* test: $^{**}p < 0.01$; $^{*}p < 0.05$.

whereas *C. albicans* stimulation revealed BCG-associated increases in four analytes: EGF, IL-6, PDGF-AB/BB, MCP-3, but decreases in four further analytes: IL-2, IL-13, IL-17, IP-10. Despite distinct response profiles for each non-specific stimulant, we observed a common signature of increases in EGF, IL-6, and PDGF-AB/BB in response to three different stimulants: Pam3Cys, *C. albicans*, and *S. aureus*.

These data led us to conclude that BCG vaccination of infants, in this setting, induces a complex reprogramming of the innate immune system to respond mainly in an enhanced fashion when exposed *in vitro* to a panel of heterogeneous, non-specific stimuli. For a few cytokines, downregulation of production was observed after vaccination. Overall, this effect is consistent with trained immunity which is known to be mediated by cells of the innate immune system.

NK Cell CD69 Expression Is Increased in BCG-Vaccinated Infants following Pam3Cys Re-Stimulation *In Vitro*

To determine which innate immune cells were associated with altered cytokine and chemokine responses to non-specific stimuli in BCG-vaccinated infants, we stained PBLs recovered from 48 h diluted whole blood assays for markers of monocyte and NK cell phenotype and activation.

Consistent with BCG-associated increases in a broad array of cytokine and chemokine responses following *Mtb* lystae restimulation, we also detected concomitant increases in expression of CD11b and CD206 on monocytes (**Figure 3A**). There were no significant, BCG vaccination-associated changes to monocyte activation markers in response to non-specific stimuli. In addition

to its effect on monocytes, *Mtb* lystae stimulation also induced increased surface expression of the activation marker CD69 on NK cells (**Figure 3B**). Although there were no significant changes to monocyte activation markers in response to non-specific stimuli, BCG-vaccinated infants displayed a significant increase in NK cell CD69 expression in response to Pam3Cys (**Figure 3B**).

Based on these data, we conclude that, accompanying the cytokine/chemokine profile of BCG-induced trained immunity in infants, there is a role for activated NK cells but that their involvement depends upon the nature of the restimulating non-specific ligand.

Pam3Cys-Induced NK Cell Activation Correlates with the Secretion of IL-12p40 and IL-10

Pam3Cys is a ligand for TLR2 which is found on the surface of NK cells. We speculated that enhanced Pam3Cys-induced NK cell activation in BCG-vaccinated infants could be a result of changes to the cytokine milieu that this ligand induces (**Figure 2**) or due to intrinsic changes to NK cells following BCG vaccination that alter their responsiveness to TLR2-mediated Pam3Cys stimulation or a combination of the two. Although the scope of this study did

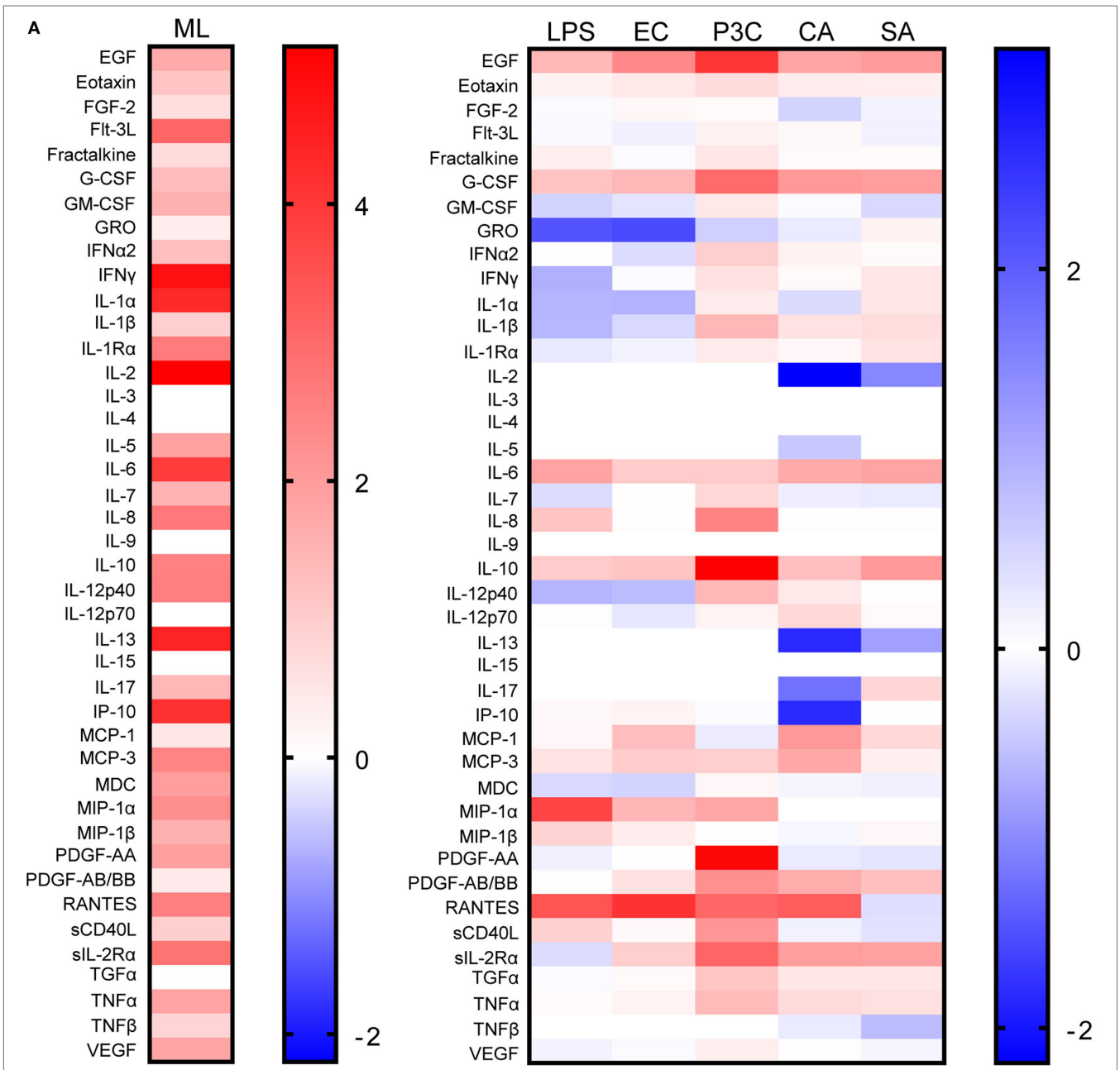

FIGURE 2 | Continued

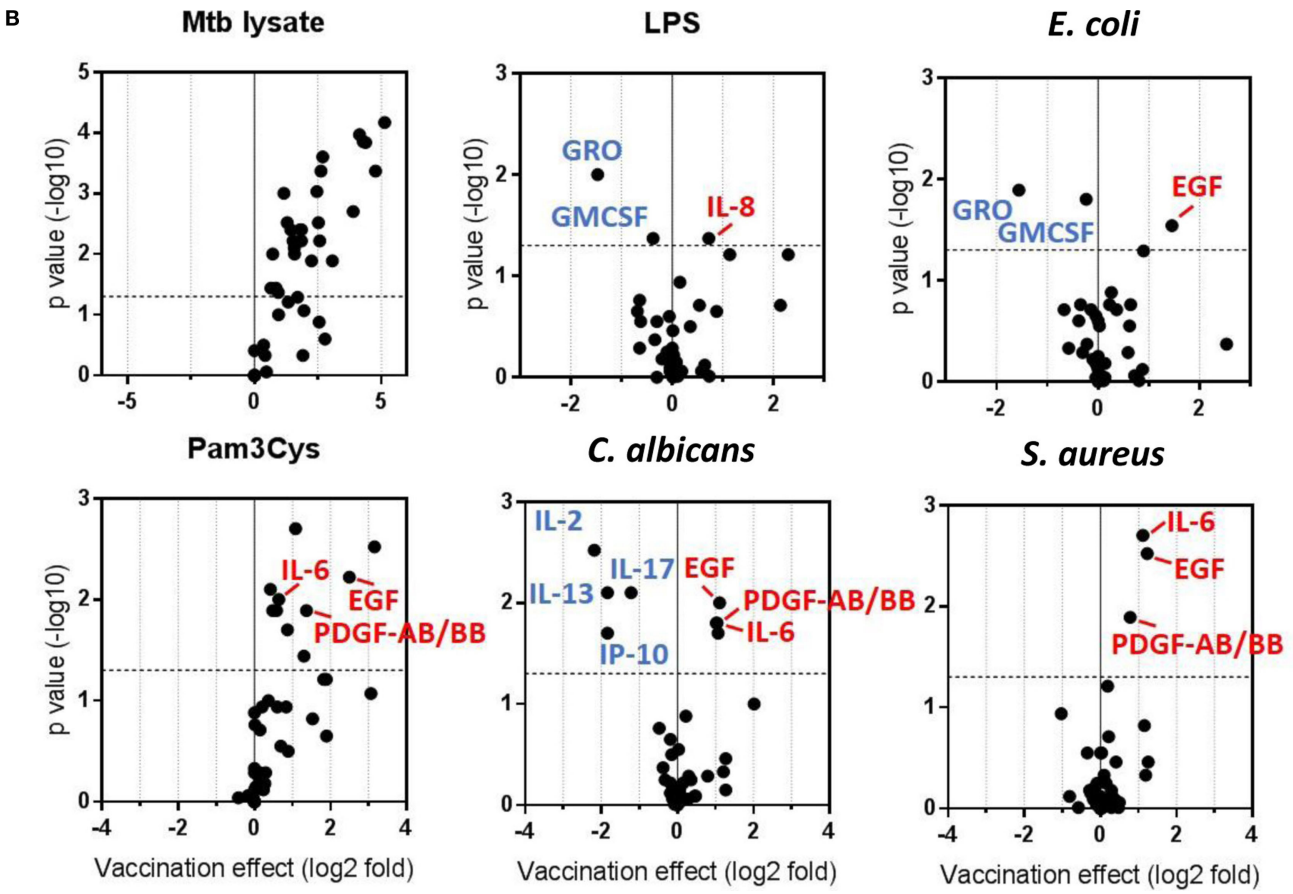

FIGURE 2 | Distinct patterns of cytokine and chemokine secretion characterize bacillus Calmette–Guérin (BCG)-induced trained immunity in response to different, non-specific stimuli. The concentrations of 42 cytokines and chemokines were measured in 48 h, diluted whole blood assay supernatants using multiplex bead array. **(A)** Heat maps represent the vaccination effect [the log2 median fold change in analyte concentration in the BCG-vaccinated infants ($n = 11$) compared to unvaccinated infants ($n = 10$)] on the indicated cytokine/chemokine responses to different stimuli: ML, *Mtb* lysate; LPS, lipopolysaccharide; EC, *E. coli*; P3C, Pam3Cys; CA, *C. albicans*; SA, *S. aureus*. **(B)** Volcano plots represent the vaccination effect on cytokine/chemokine responses to different indicated stimuli which are plotted on the *x*-axis against the –log10 *p* value on the *y*-axis (calculated by Mann–Whitney *U* test comparisons of responses in BCG-vaccinated and unvaccinated groups). Data points represent individual cytokines and chemokines. Significant responses of interest are labeled where clarity permits. Horizontal dotted lines represent a *p* value of 0.05.

TABLE 1 | BCG vaccination-associated cytokine and chemokine responses induced by specific and non-specific stimuli in 48 h diluted whole blood assays.

Stimulation	Overexpressed in BCG-vaccinated infants	Median fold change	Under-expressed in BCG-vaccinated infants	Median fold change
Mtb lysate	IL-2, IFNγ, IL-13, IL-1α, IP-10, IL-6	>10		
	Flt-3L, IL-8, IL-1Rα, IL-12p40, IL-10, MCP-3	5–10		
	MIP-1α, IL-5, TNFα, VEGF, MIP-1β, GM-CSF, IL-7, IL-17, IFNα2, eotaxin	2–5		
	CD40L, TNFβ, fractalkine, FGF- 2, TGFα	1–2		
Pam3Cys	IL-10, epidermal growth factor (EGF)	5–10		
	Platelet-derived growth factor (PDGF)-AB/BB, sCD40L, MIP-1α	2–5		
	IL-12p40, IL-6, MCP-3, IL-7, eotaxin	1–2		
Candida albicans	EGF, IL-6, PDGF-AB/BB, MCP-3	2–5	IL-2, IL-13, IL-17, IP-10	0.2–0.5
Staphylococcus aureus	EGF, IL-6	2–5		
	PDGF-AB/BB	1.7		
Escherichia coli	EGF	2.8	GM-CSF, GRO	0.2–1.0
Lipopolysaccharide	IL-8	1.7	GM-CSF, GRO	0.2–1.0

not allow us to address the intrinsic changes hypothesis, we were able to look at associations between the magnitude of Pam3Cys-mediated NK cell activation and the changes in cytokine and chemokine release in response to the same stimulant. Of the 10 analytes that were significantly upregulated in BCG-vaccinated infants in response to Pam3Cys, IL-12p40 and IL-10 secretion

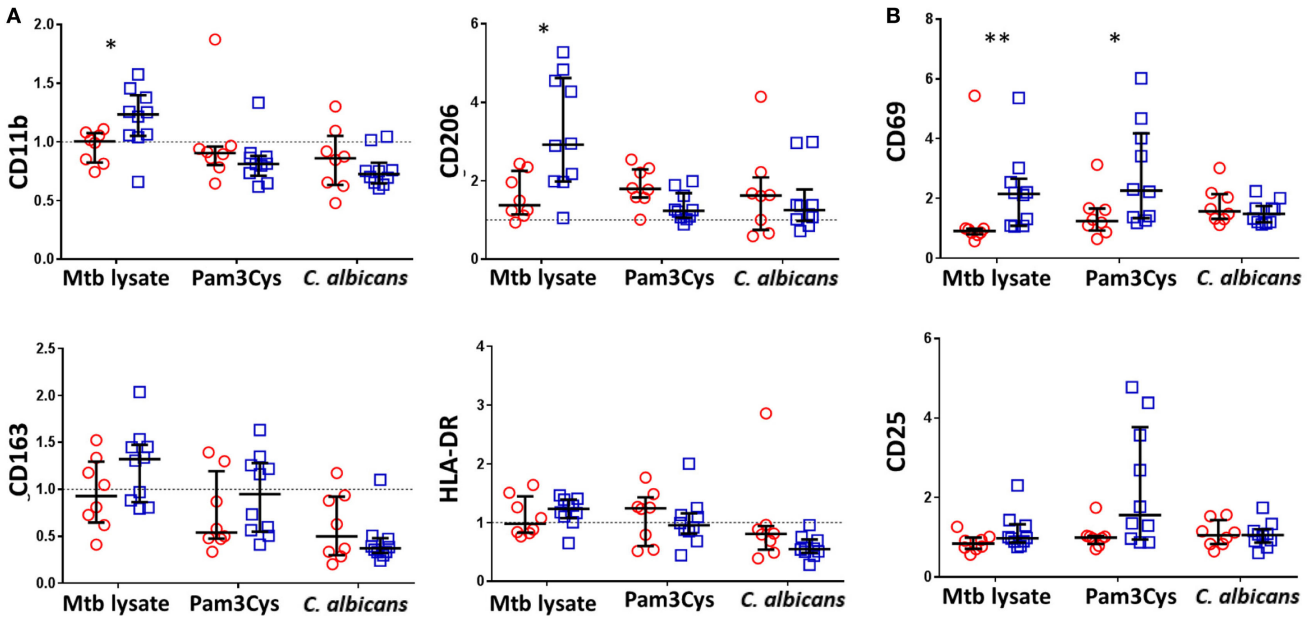

FIGURE 3 | Bacillus Calmette–Guérin (BCG) vaccination of infants is associated with enhanced natural killer (NK) cell activation in response to Pam3Cys. Following 48 h, diluted whole blood assay stimulation of samples from BCG-vaccinated (□, $n = 10$) and unvaccinated (○, $n = 8$) infants, cell pellets were cryopreserved and later analyzed by flow cytometry. **(A)** Monocytes were identified based on high CD14 and HLA-DR expression as well as on size and granularity (see Figure S1A in Supplementary Material) and stained for the indicated markers of monocyte activation and differentiation. Data points represent the fold change in MFI for samples incubated with the indicated stimulants compared to unstimulated samples. **(B)** CD56+ CD3− NK cells were gated (see Figure S1B in Supplementary Material) and the expression of the activation markers CD69 and CD25 analyzed. Data points represent the fold change in percent of activation marker expressing NK cells for samples incubated with the indicated stimulants compared to unstimulated samples. Responses in vaccinated and unvaccinated groups were compared using the Mann–Whitney *U* test: $^{*}p < 0.05$; $^{**}p < 0.01$.

demonstrated significant correlation with the extent of NK cell CD69 expression (**Figure 4**).

From these data, we conclude that cytokine secretion, most notably IL-12, may account at least in part for the TLR2-mediated activation of NK cells in BCG-vaccinated infants.

DISCUSSION

Trained innate immunity has, to date, been demonstrated and characterized largely in adults. Isolated PBMC released more TNFα, IL-6, and IL-1β when exposed to non-specific innate stimuli 3 months after BCG vaccination in adults, and monocytes displayed higher levels of activation markers (24). Mechanisms of trained immunity such as underlying epigenetic regulation and shifts in the metabolic pathways used have similarly been revealed in vaccinated adults and in adult cellular models *in vitro* (22, 32). If trained innate immunity is to provide a causal link between BCG vaccination of infants and non-specific protection against mortality due to infectious diseases other than those with a mycobacterial etiology (15, 16), then a detailed description of the phenomenon in infants and infant cells is needed.

In this paper, we have profiled the infant whole blood cytokine and chemokine secretome of trained immunity following BCG vaccination using a panel of non-specific innate stimuli and reveal a differentially expressed signature. A total of 11 analytes were overexpressed and 6 under-expressed in vaccinated infants, with different innate stimuli inducing distinct combinations of response elements: for example, the Pam3Cys-induced response included the overexpression of 10 analytes, while *S. aureus* only induced three analytes. Four analytes were overexpressed and four under-expressed in response to *C. albicans*; LPS and *E. coli* each induced one overexpressed and two under-expressed analytes. These distinctions most likely reflect the different characteristics of the stimuli involved. Two of them, LPS and Pam3Cys, are defined biochemical compounds that interact with specific pattern recognition receptors: TLR4 and TLR2/1 heterodimer, respectively. *C. albicans*, *S. aureus*, and *E. coli* are all HK preparations of microorganisms containing complex mixtures of pathogen-associated molecular patterns that will interact with an array of different pathogen recognition receptors or other innate antigen receptors. It has been suggested that different microorganisms act on multiple, distinct TLRs simultaneously in a manner likened to the playing of a unique "chord" on a "molecular piano" (17) and the different response profiles generated by different stimuli described here may be an illustration of that effect.

The pathways by which BCG mediates its training effect *in vivo* are yet to be fully elucidated, although a role for the mycobacterial cell wall component muramyl dipeptide, acting *via* the NOD2 receptor, has been described (24). β-glucan, another activator of trained immunity mediates its effects *via* a dectin-1/Raf1-dependant pathway (33), yet the role of dectin-1 in recognizing mycobacteria is probably minor at most. The emerging picture is one in which the recall of a trained immune response may be restimulated *via* different receptors to those that prime that same

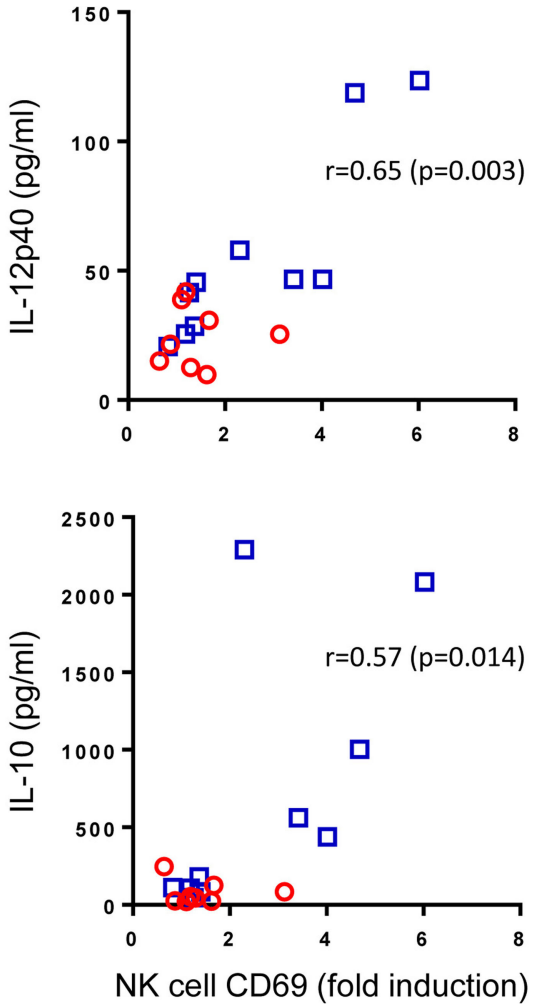

FIGURE 4 | Correlation between Pam3Cys (P3C)-induced natural killer (NK) cell CD69 expression and IL-12p40 and IL-10 secretion. P3C-specific, NK cell CD69 expression in samples from bacillus Calmette–Guérin (BCG)-vaccinated (□, $n = 10$) and unvaccinated (○, $n = 8$) infants were plotted against P3C-specific secretion of IL-12p40 and IL-10 in the same samples. The degree of correlation was measured using Spearman's rank correlation coefficient.

response and that the cytokine/chemokine signature of that recall is determined by the receptors involved. Although little is known at this time about the mechanisms responsible, this should be a focus of future studies.

Despite the differences described above, we described here a biosignature of BCG-induced trained innate immunity in infants comprising increases in EGF, IL-6, and PDGF-AB/BB, all of which were apparent in responses to three different innate stimuli. This is different to the characteristic signature of trained innate immunity in adults, which includes TNFα and IL-1β as well as IL-6. It must be however underlined that in adults not all the cytokines reported here were measured, and some of them may be upregulated as well in older individuals. The absence of canonical innate cytokines such as TNFα in this infant biosignature may reflect the maturation of the infant innate immune response, whereby the ability to make certain cytokines develop over the first 9 months of life (34). Despite the most extensive response profile, *Mtb* lysate stimulation did not upregulate EGF or PDGF-AB/BB. This may reflect the involvement of a broader array of pattern recognition receptors in response to *Mtb* lysate with downstream effects that manifest themselves as a distinct pattern of upregulated and downregulated markers. These observations illustrate the importance of an unbiased, multiplex approach to analyzing these responses. A more directed study focused on TNFα, IL-6, and IL-1β would have underestimated the effect of trained immunity in infants and would have missed the previously unreported markers EGF and PDGF-AB/BB, as well as the finding that *C. albicans*-induced trained immunity recall in BCG-vaccinated infants involves reduced IL-2, IL-13, IL-17, and IP-10 responses. It is interesting that IL-17 responses to *C. albicans* are reduced in vaccinated infants. Th-17 cells are known to be important in immune responses to *C. albicans* (35). BCG vaccination has been shown to enhance non-specific (innate-mediated) protection against candidiasis in SCID mice (24) and adult BCG vaccination activates heterologous Th-17 responses specific for non-mycobacterial ligands including *C. albicans* (31). Our data suggest that BCG is exerting a different influence on the infant immune response, steering it away from a bias toward Th-17 development that is known to exist in early life (36). Detailed analyses of epigenetic and transcriptional programs of training or tolerance induction have revealed upregulation and downregulation of genes that characterize these responses hence it is unsurprising that the profiles we report here involve both upregulation and downregulation of different cytokines and chemokines associated with both T-cell and inflammatory monocyte responses to stimuli such as *C. albicans* as well as to *E. coli* and LPS which have both been associated with the induction of tolerance (37, 38).

In contrast to our findings here, another report of infant BCG-associated, non-specific cytokine responses described increases in TNFα and IL-1β as well as in IL-6 in response to Pam3Cys (39). Differences between study designs that might explain this difference are that Jensen et al. carried out their study in a low-income country and in low birth weight infants. In addition, the genetic backgrounds of the populations studied were different. Their sampling time point of 4 weeks was earlier than that used here and there were some methodological differences in their whole blood assay. An interesting possible explanation is that, unlike Jensen et al., infants in our study had received DTP vaccination through the national vaccination program by the time of sampling which is thought to negatively affect the positive non-specific effects of BCG (40). Finally, compared to Jensen et al., our study was relatively small and exploratory and was possibly underpowered to detect small differences in cytokine responses. It should be noted that in agreement with our findings and those of others (7), Jensen et al. found no BCG-associated increase in TNFα in response to non-specific LPS stimulation.

The involvement of monocytes in adult trained immunity has been demonstrated using stimulating ligands that interact with receptors known to be found on monocytes and more directly by the demonstration that markers of monocyte activation and differentiation are upregulated following training (24). It is

also possible to isolate adult blood monocytes and train them directly with BCG and β-glucan *in vitro* (32). Although the assay described in this paper was designed primarily to look at secreted cytokines and chemokines, it was possible to harvest PBLs and to examine monocytes and NK cells for evidence of BCG vaccination-associated trained innate activation. We examined four markers of monocyte activation, but found no significant differences in their expression following non-specific stimulation of samples from vaccinated and unvaccinated infants. Stimulation with *Mtb* lystae, which also induced increases in 27 cytokines and chemokines in vaccinated infants, did increase levels of CD11b and CD207 on the surface of monocytes. Unlike responses induced by non-specific ligands in these experiments, it is likely that antigen-specific T-cell help is involved in these changes in monocyte activation. It is interesting that monocytes from BCG-vaccinated infants show increased expression of CD207 (mannose receptor), as this is usually found only on certain populations of differentiated macrophages and dendritic cells (41).

Natural killer cells from BCG-vaccinated infants displayed increased levels of the activation marker CD69 in response to Pam3Cys, as well as to *Mtb* lystae. Previous work has described long-lived mycobacteria-specific NK cells following infant BCG as well as a role for NK cells in BCG-induced trained immunity in adults and in BCG-vaccinated SCID mice which show increased resistance to *C. albicans* infection (25, 42). However, this is the first time their involvement in trained immunity to heterologous stimuli in infants has been described. NK cells express the TLR2 receptor *via* which they are reported to interact with mycobacteria (43, 44). NK cells are also activated in response to type 1 cytokines (45). Although we cannot determine the pathway of NK cell activation from the current data, we did observe a correlation between CD69 expression, IL-12p40 secretion, and IL-10 secretion in response to Pam3Cys. IL-12 is known to be a soluble activator of NK cells, while a role for IL-10 is less obvious. A limitation of this study was the small volume of blood available from infants which meant it was not possible to identify the cell type producing the cytokines and chemokines described. Clearly it would be interesting to determine whether the NK responses described above or other populations were a source, either by depletion studies or intracellular cytokine staining and future studies should address this. Additionally, as well as stimulation of the TLR2/1 heterodimer using Pam3Cys, it would be useful in future to determine more precisely the role of TLR2 either in homodimeric form or as a heterodimer with TLR6 also using specific ligands for these receptors.

This exploratory study was not designed to investigate the heterologous effects of infant BCG vaccination on clinical outcomes; however, if the non-specific immune responses we describe are to provide a mechanism for these effects, a causal relationship will need to be demonstrated in a larger study. The setting of such a study will need to be carefully considered as to date, the results of clinical trials have been mixed. Heterologous, BCG-induced protection against clinical outcomes related to infectious disease has been observed in a low-income setting in West Africa (15, 46) but was not recapitulated in another trial in a high income, European setting (47, 48).

In conclusion, we have described a novel whole blood signature of BCG-induced, trained innate immunity in infants that includes secretion of EGF, IL-6, and PDGF-AB/BB and involves activated NK cells. We also show distinct patterns of cytokine and chemokine release in response to different innate ligands. The data show different patterns to previously published descriptions of BCG-induced trained innate immunity in adults and low birth weight infants and suggest that more, larger scale studies of this effect in different populations are required for a more complete understanding.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the National Research Ethics Service Committee London-East and the ethics committee of the London School of Hygiene and Tropical Medicine with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the National Research Ethics Service Committee London-East and the ethics committee of the London School of Hygiene and Tropical Medicine.

AUTHOR CONTRIBUTIONS

SS performed the experiments, was responsible for the analysis and interpretation of data, and wrote the manuscript. JK and MN produced the panel of stimuli used in experiments. SS, JK, MN, and HD all contributed to the conception and design of the work, critically read and contributed to revisions of the manuscript, approved the final version of the manuscript, and agreed to be accountable for all aspects of the work including its accuracy and integrity.

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SUPPLEMENTARY MATERIAL

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HYPOTHESIS AND THEORY
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Neonatal Group B Streptococcal Disease in Otherwise Healthy Infants: Failure of Specific Neonatal Immune Responses

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Only a small proportion of newborn infants exposed to a pathogenic microorganism develop overt infection. Susceptibility to infection in preterm infants and infants with known comorbidities has a likely multifactorial origin and can be often attributed to the concurrence of iatrogenic factors, environmental determinants, underlying pathogenic processes, and probably genetic predisposition. Conversely, infection occurring in otherwise healthy full-term newborn infants is unexplained in most cases. Microbial virulence factors and the unique characteristics of the neonatal immune system only partially account for the interindividual variability in the neonatal immune responses to pathogens. We here suggest that neonatal infection occurring in otherwise healthy infants is caused by a failure of the specific protective immunity to the microorganism. To explain infection in term and preterm infants, we propose an extension of the previously proposed model of the genetic architecture of infectious diseases in humans. We then focus on group B streptococcus (GBS) disease, the best characterized neonatal infection, and outline the potential molecular mechanisms underlying the selective failure of the immune responses against GBS. In light of the recent discoveries of pathogen-specific primary immunodeficiencies and of the role of anticytokine autoantibodies in increasing susceptibility to specific infections, we hypothesize that GBS disease occurring in otherwise healthy infants could reflect an immunodeficiency caused either by rare genetic defects in the infant or by transmitted maternal neutralizing antibodies. These hypotheses are consistent with available epidemiological data, with clinical and epidemiological observations, and with the state of the art of neonatal physiology and disease. Studies should now be designed to comprehensively search for genetic or immunological factors involved in susceptibility to severe neonatal infections.

Keywords: newborn infant, life-threatening, primary immunodeficiency, genetic predisposition to disease, Mendelian diseases, monogenic, infection, group B streptococcus

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INTRODUCTION

Neonates are commonly thought to be vulnerable to pathogens because of neonatal immaturity, immune tolerance, or immune deviation, a developmentally regulated transitional state (1–4). These concepts, while useful to describe the highest incidence of infection during the neonatal age at the population level, do not take into account interindividual variability. Even if the highest incidence of

infection is observed during the first 28 days of life, the majority of newborn infants are resistant to common pathogens, and only a small proportion of infants exposed to a given microorganism develop overt disease.

It is very clear from epidemiological studies that multiple risk factors contribute to the individual risk of developing neonatal infections. Based on them, neonates can be classified into high- and low-risk groups; individual risks can be estimated; and preventive protocols can be designed for infants who are at high risk of suffering from severe infections (5–7).

Despite their proven clinical utility, most preventive protocols are only partially effective. This can be explained in part by incomplete adherence by healthcare practitioners and missed opportunities (8–10). However, another critical limitation is the inability of current protocols to accurately predict susceptibility to severe infection at the individual level. Furthermore, infections that occur in the absence of any recognizable factors are currently unpredictable.

Many research groups are focusing on the mechanisms of host susceptibility and resistance to pediatric and adult infections (11, 12). Conversely, neonatal infections have been much less studied from a host susceptibility perspective. Several layers of complexity have indeed prevented researchers from fully understanding the neonatal-specific protective immunity, beyond maternal protection of the neonate through transplacentally transmitted antibodies (Abs). Considering the global burden of neonatal infectious diseases, this looks like a missed opportunity to address a critical public health problem.

The study of neonatal infections raises several practical and ethical issues and is challenging from a scientific perspective. First, the neonatal immune system is a rapidly evolving entity, as is every other organ and system soon after birth (13). Second, and possibly more importantly, there is a complex immune interplay between the mother and the child. The maternal environment (the maternal immune system and microbiome) is intertwined with physiological and pathological processes occurring in the fetal and neonatal tissues (e.g., the maturation of fetal and neonatal immune responses, the composition of the neonatal microbiome) (13–17).

To date, little is known about the mechanisms leading to individual vulnerability and resistance to specific pathogens in the neonatal age. We here propose novel, testable hypotheses that could explain the interindividual differences in pathogen susceptibility and help dissect the molecular and cellular bases of severe neonatal infections.

EPIDEMIOLOGY OF NEONATAL INFECTIONS

The *Global Burden of Disease Study 2015* reports that "sepsis and other neonatal infections" account for 336,300 neonatal deaths each year worldwide (18).

The distribution of infecting microorganisms varies between term and preterm infants and is different in the neonatal period compared to other age groups. Early-onset and late-onset infections are defined as infection occurring during and after the first 6 days of life, respectively. According to other definitions, 48–96 h of life could be used as cutoff (19).

Group B streptococcus (GBS), or *Streptococcus agalactiae*, is one of the leading pathogens in neonatal infections occurring in full-term newborn infants during the first week of life (9). It is also the most frequent cause of sepsis and meningitis in young infants after the first week of life (20, 21). Recent reports show an increase in the proportion of *Escherichia coli* infection, mostly associated with urinary tract infection, in previously healthy, full-term infants aged 1 week to 3 months (22, 23). Other pathogens responsible for invasive infection in full-term infants include Gram-positive (*Staphylococcus aureus*, *Streptococcus* spp., *Enterococcus* spp., and, less frequently, *Listeria monocytogenes*) and Gram-negative microorganisms (*Klebsiella* spp., *Citrobacter* spp., *Serratia marcescens*, *Salmonella* spp., *Haemophilus influenzae*) (22, 23). Deep organ infections by *Candida* spp. and other fungal microorganisms are exceedingly rare in full-term infants.

In very low-birth-weight (VLBW; <1,500 g at birth) infants, Gram-negative pathogens are the most frequently isolated microorganism in early-onset infections, while Gram-positive bacteria are the most frequently isolated pathogens in late-onset infections in the neonatal intensive care units (NICUs), followed by Gram-negative bacteria and fungal organisms (24–29).

SUSCEPTIBILITY TO NEONATAL INFECTIONS

Maturation of the Neonatal Immune System

The neonatal immune responses differ in many aspects from immune responses in other age groups. A fine-tuning is required to balance the need for tolerance to beneficial antigens (microbial flora and nutrients) and the need for defense against harmful microorganisms.

The cord blood is enriched in CD4+CD25+ T regulatory cells with potent suppressor activity (30, 31) and other immunosuppressive cell populations including some B cell populations and CD71+ erythroid cells (32, 33).

Despite this strong immunosuppressive component, the neonatal immune system has been demonstrated to be able to mount pro-inflammatory responses that are appropriate for the protection against common pathogens in most infants (34). The two main components of the adaptive immune system, the T and B cell compartments, undergo maturation during human fetal life, with progressive and regulated acquisition of B and T cell repertoire diversity and complexity (35). In addition, the human cord blood possesses several pro-inflammatory cell populations, including newborn-specific interleukin (IL)8-producing T cells (36) and a population of CD4+ T cells with a memory-like phenotype and a variety of effector functions (37).

Cells of the neonatal adaptive immune system are capable of mounting a wide range of responses, from poor or "deviant" T helper 2 (Th2)-skewed antiinflammatory responses to balanced Th1/Th2 responses, and even strong adult-like pro-inflammatory responses (2–4, 38). A series of elegant experiments have shown

that neonatal T cells, unlike adult cells, are able to produce large amounts of the Th2 cytokines, IL4 and IL13, upon polyclonal stimulation (39, 40). This phenomenon is linked to extensive epigenetic modifications at the Th2 locus (*IL5*, *IL13*, and *IL4* genes) and in particular to hypomethylation of the conserved non-coding sequence 1 locus, an enhancer and coordinate regulator of Th2 cytokine production (38, 41). Despite this Th2 bias, neonatal adaptive immune responses can shift toward a dominant Th1 and pro-inflammatory response depending on the type of innate responses and the conditions of antigen exposure (38, 42).

Adaptive immune responses require, however, several days to take place. Neonates cannot rely on preexistent immunological memory because exposure to foreign antigens and pathogens is limited during intrauterine life (34, 42). Furthermore, humoral immunity largely depends on maternally transmitted antimicrobial IgG Abs during the first days of life. The rapid decline of maternal IgG in the neonatal plasma after birth (with a half-life of 21–30 days) is accompanied by a relatively slow maturation of both T-dependent and T-independent B-cell responses throughout the first months of life (13, 43).

Infections occurring in the neonatal period are, by definition, primary infections, and neonates mostly rely on the innate immune responses that provide a first line of defense against invading pathogenic microorganisms (34, 44, 45).

A number of studies demonstrated that the neonatal innate immune responses are characterized by dampened Th1-polarizing and pro-inflammatory responses [low amounts of tumor necrosis factor (TNF) upon toll-like receptor (TLR) stimulation] and by increased production of Th2-polarizing and antiinflammatory cytokines (higher IL6/TNF ratio compared to adult responses) (45–48). Furthermore, decreased phosphorylation of signal transducer and activation of transcription 1 in response to interferon gamma (IFN-γ) (49) and developmental maturation of specific dendritic cell subsets (50) contribute to the neonatal-specific Th2-polarizing innate immunity.

Interestingly, full-term healthy newborn infants do not appear specifically vulnerable to deep infection by microorganisms typically causing disease in immunodeficient patients, most notably *Nocardia* and fungi-like *Aspergillus*, *Candida*, *Cryptococcus*, *Pneumocystis*, and other opportunistic pathogens, suggesting a substantial maturation of the specific antifungal protective responses in most full-term newborn infants.

Altogether, the characterization of the neonatal immune responses over the past two decades has shown profound differences with adult immunity that in part explain the overall increased susceptibility to life-threatening infection of newborn infants. However, little is known so far about the interindividual differences in the immune protection against pathogens in the neonatal age.

Heritability of Neonatal Sepsis

There is controversy over the heritability of susceptibility to neonatal sepsis. In one study, comparing the concordance of late-onset sepsis in same-sex vs. unlike-sex twin pairs, no evidence was found of a genetic component of susceptibility to late-onset sepsis among VLBW infants (51). Conversely, another study compared sepsis concordance rates between monozygotic and dizygotic twins; the authors found that 49% of the variance in liability to late-onset sepsis could be explained by genetic factors alone and 51% by residual environmental factors (52). Both studies focused on cohorts of very preterm/VLBW infants. No study so far addressed the question of heritability of neonatal sepsis in late-preterm and full-term newborn infants.

The question of the role of the genetic background on neonatal host susceptibility to infection has been addressed by several studies. All published studies, included in a recent meta-analysis, used a candidate gene approach on cohorts of preterm infants (53). One genome-wide association study (GWAS) is ongoing (54). Such studies are useful to investigate the contribution of host genetics in the setting of a likely multifactorial pathogenesis, as it is probably the case for most infections occurring in preterm infants. Different approaches are needed to find the genetic determinants of susceptibility to life-threatening infections occurring in full-term infants with no underlying medical conditions in which susceptibility to infection is largely unexplained.

Lessons from Inborn Errors of Immunity in Pediatric Infections

Inborn errors of immunity or primary immunodeficiencies (PIDs) are a group of genetic disorders characterized by increased susceptibility to infection. Historically, the so-called conventional PIDs have been the first PIDs described and dissected from a molecular perspective (55). They are typically Mendelian diseases, caused by highly penetrant single-gene defects. They often occur in families or in the presence of consanguinity and are characterized by a profound defect in one or more arms of the immune system leading to susceptibility to recurrent infections by a broad range of microorganisms (56).

Over the past two decades, it has become clear that infectious diseases previously thought to be due to the sole virulence of the pathogen may be the expression of a monogenic disorder underlying a PID. Inborn errors of immunity resulting from single-gene defects have been shown to underlie multiple bacterial infections [myeloid differentiation primary response 88 (MYD88) and interleukin 1 receptor-associated kinase 4 (IRAK4) deficiency], monogenic susceptibility to mycobacterial disease (deficiency of genes in the IL12/IFN-γ loop), herpes simplex encephalitis (defect in TLR3-dependent immune responses), and severe primary *Influenza* virus infection (interferon regulatory factor 7 deficiency) (56–60).

These "non-conventional" PIDs are distinguished from conventional PIDs as they often occur in sporadic cases without any family history of severe infection. Individuals affected by non-conventional PIDs are often otherwise healthy. The immunological phenotype is not detectable with first-line immunological studies, and the disease might manifest as a single episode of severe and potentially lethal infection caused by a common or opportunistic pathogen, mostly during primary infection (56, 58, 59).

The discovery of non-conventional PIDs suggested that monogenic conditions might underlie infectious diseases of infancy and childhood more frequently than previously thought

(11). The model of the genetic architecture of human infectious diseases that has been proposed based on these observations suggests that infections occurring early in life are more likely to be caused by single-gene disorders (61).

PIDs in Neonatal Infections

The proportion of neonatal infections that can be explained by known PIDs is unknown. However, there is evidence from case reports or small case studies that life-threatening infections occurring early in life may represent the first phenotypic manifestation of an inborn error of immunity.

The role of conventional PIDs in conferring susceptibility to infection in the neonatal age has been recently reviewed by Walkovich and Connelly (62). It is important here to remember that a high index of suspicion is required, given that the infectious and potential extraimmunological phenotypes may be only partially expressed during the neonatal period.

Non-conventional PIDs have also been shown to underlie life-threatening neonatal infections. Pyogenic infections occurring during the first few weeks of life have been described as the first phenotypic manifestation of IRAK4 and MYD88 deficiencies (63–65). *Klebsiella pneumoniae* infection often striking in neonatal units as a fulminant and fatal disease, has been linked in some pediatric patients to IL12 receptor subunit beta 1 deficiency (66).

Loss-of-function mutations in interferon induced with helicase c domain 1 (*IFIH1*), a cytosolic sensor of the viral RNA, have been implicated as causative factors in lower respiratory tract infections (pneumonitis, bronchiolitis) caused by RNA viruses (67). Interestingly, the phenotype of IFIH1 deficiency is narrow (restricted to few related RNA viruses), transient (recurrence was found in one of eight patients), and organ specific (only affects the lungs).

Variants in single Ig And TIR domain containing (*SIGIRR*) have been implicated as a possible causative or facilitating factor of necrotizing enterocolitis (NEC) (68), but fulminant and infection-associated NEC (69) in infants with no other identifiable facilitating iatrogenic factor or medical condition has not been linked yet to a genetic condition.

Spectrum of Neonatal Infections

From a clinical perspective, newborn infants suffering from life-threatening infections may be divided in two major groups:

(1) Newborn infants with a known medical condition. This group includes all infants admitted to a NICU (therefore exposed to nosocomial pathogens) and specifically very preterm (<32 weeks gestational age) and extremely preterm (<28 weeks gestational age) infants, infants undergoing surgery, infants with organ disease (e.g., urinary tract malformations, neurological conditions), and infants receiving medical procedures or treatments that are *per se* sufficient to explain an increased vulnerability to colonizing microorganisms. Infections in this group are multifactorial or linked to one specific known factor of vulnerability, and only a small proportion of the risk is probably explained by individual genetic variation.

(2) Otherwise healthy, full-term, or late-preterm newborn infants with no identifiable medical conditions. Severe infections in these infants occur without any apparent risk or facilitating factor and, from a host perspective, can be considered idiopathic diseases.

Most of these infections occur as isolated events (the spectrum of susceptibility is extremely narrow, in most cases restricted to a single microorganism) and rarely recur.

Some infections are almost never observed in healthy children after the first year of life or in adults. These include neonatal GBS disease, viral bronchiolitis, and rare cases of infection-related NEC in late preterm and full-term infants. Conversely, other infections are not age specific, but may occur with particular frequency and severity in the neonatal period and infancy. These include infections by *E. coli*, *Klebsiella* spp., *Listeria monocytogenes*, and other Gram-negative and Gram-positive pathogens.

The biological underpinnings of the interindividual differences in resistance and vulnerability to specific pathogens in otherwise healthy infants are currently unknown.

Hypothetical General Model for Neonatal Infections

A general model to explain susceptibility to neonatal infections in full-term and preterm infants is lacking.

Single factors with high effect size explain some of the most severe diseases occurring in infants without known comorbidities. A prime example in neonatal medicine is the rare occurrence of rapidly progressive neonatal jaundice and kernicterus in otherwise healthy, full-term babies, which is due to neonatal hemolysis resulting from either monogenic defects (e.g., spherocytosis, G6PD deficiency) or alloimmune maternal Abs (anti-Rh, anti-ABO) (70). Conversely, hemolysis leading to kernicterus in extremely preterm infants is more likely to be multifactorial, depending on the combined contribution of common genetic polymorphisms, underlying medical conditions, iatrogenic factors, and other environmental determinants (71).

As a general observation, single-gene or single-factor disorders are more likely to underlie severe neonatal disease phenotypes in otherwise healthy full-term infants, while a multifactorial pathogenesis is more likely to explain mild-to-severe neonatal disease in the presence of comorbidities or iatrogenic factors, with severity depending on the underlying pathogenic process (**Table 1**).

Along the same lines, we here suggest that single factors with high effect size may underlie life-threatening infections in otherwise healthy, full-term, or late-preterm babies, while a polygenic/multifactorial model may better explain the occurrence and severity of infections in very and extremely preterm infants.

Accordingly, we propose an extension of the model of the genetic architecture of infectious diseases proposed by Alcais et al. (61) to include full-term and preterm infants (**Figure 1**).

Additional host factors (maternal antimicrobial protective Abs, vaginal and breast microbiome, epigenetic, and maturational changes in the immune system) and determinants of microbial virulence may also modulate disease severity (17).

TABLE 1 | Mechanisms of disease in term and preterm infants.

Involved tissue/ organ	Disease phenotype	Single-factor disorders	Multifactorial conditions
Red cells, liver	Neonatal jaundice with/without bilirubin encephalopathy	**Monogenic disorders** (spherocytosis, elliptocytosis, G6PDH deficiency, Lucey–Driscoll and Crigler–Najjar syndromes) **Maternal Abs** (ABO alloimmunization, Rh alloimmunization)	Prematurity, metabolic or respiratory acidosis, alterations of blood–brain barrier, hypoproteinemia, liver immaturity, polycythemia
Megakaryocytic lineage	Neonatal thrombocytopenia	**Monogenic disorders** (genetic thrombocytopenias) **Maternal Abs** (auto- or alloimmune thrombocytopenia)	Mild thrombocytopenia in small-for-gestational-age infants, infants with perinatal asphyxia; thrombocytopenia in infants with bacterial and viral infections and/or intravascular disseminated coagulation
Thyroid	Neonatal hypothyroidism	**Monogenic disorders** (genetic thyroid dysgenesis and dyshormonogenesis) **Maternal Abs** (anti-TPO, anti-TSHr, anti-TG)	Maternal exposure to iodopovidone, iodopovidone use in term and preterm infants (Wolff–Chaikoff effect due to iodine transdermal resorption)
Immune system	Neonatal infection	• Urinary tract malformation • Mendelian predisposition to life-threatening infection? • Maternal anti-cytokine Abs?	Infections in infants with underlying medical conditions facilitating exposure and translocation of the pathogens to the bloodstream

This table reports examples of hematological and non-hematological neonatal disease phenotypes that can be explained by either monofactorial or multifactorial conditions. The list is non-comprehensive, and other conditions explained by the same mechanisms include neonatal hyperthyroidism, arrhythmias and neuromuscular disorders. Monofactorial conditions, that include monogenic disorders and pathogenic maternal Abs are, in general, severe, often explain disease in full-term infants but can also underlie disease in preterm infants. Disease phenotypes linked to multifactorial conditions can be mild to severe and are generally found in infants with co-morbidities.
Abs, antibodies; GSPDH, glucose-6-phosphate dehydrogenase; TPO, thyroperoxidase; TSHr, thyroid-stimulating hormone receptor; TG, thyreoglobulin.

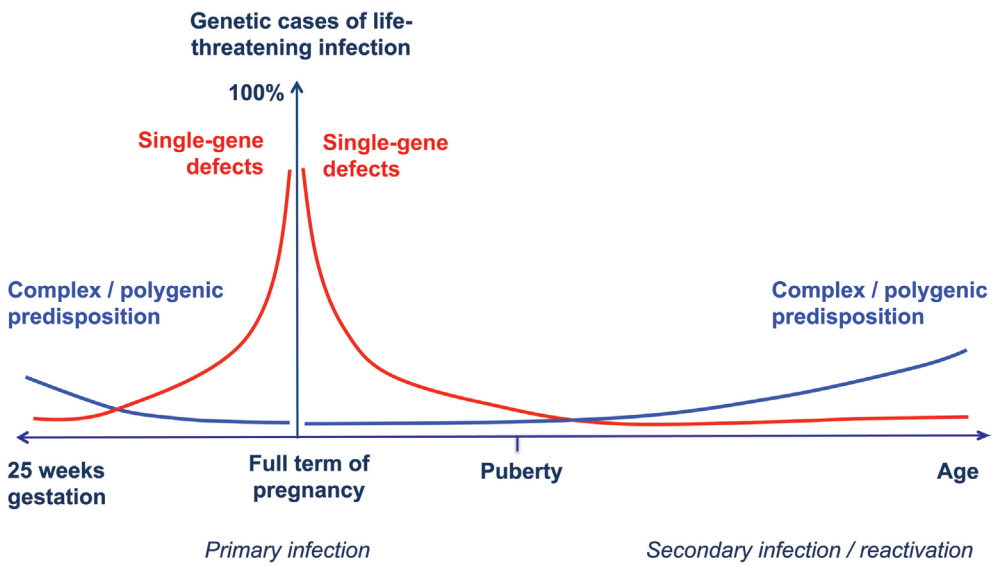

FIGURE 1 | Human genetic architecture of infections. Modified from Alcais et al. (61). The contribution of Mendelian genetic defects (red lines) to life-threatening infectious diseases is mostly observed during childhood, while complex interactions between environmental influences and polygenic susceptibility (blue lines) play a more important role for infections occurring later in life. We propose a specular trend for the contribution of human genetic variants to infection susceptibility with decreasing gestational age. In newborn infants at extremely low gestational ages, exogenous factors play a major role, while host genetic defects are more likely to explain life-threatening infection in full-term, otherwise healthy babies.

Current Evidence Supporting the Model

In preterm infants, multiple factors are well known to contribute to both the occurrence and the severity of infections. Colonization of deep mucosal tissues by hospital-acquired microorganisms and translocation to the bloodstream is facilitated by several factors: biomedical devices (endotracheal or nasogastric tubes), invasive procedures, thin skin and mucosal layers, central catheters, total parenteral nutrition, drugs (histamine type 2 receptor-antagonists, steroids, antibiotics), delayed initiation of enteral nutrition with formula milk, associated diseases, male gender, an incomplete maturation of the preterm immune system (6, 35, 72–74), and, possibly, a weak polygenic predisposition (52).

In full-term infants, supporting evidence for a role of single host factors in determining susceptibility to infection is provided

by the example of urinary tract malformation as one single, high effect-size factor, in determining susceptibility to urosepsis (75) independently of other protective or risk factors. In infants with urinary tract malformation, the effect of the alterations in urinary flow on the facilitation of urosepsis exceeds by far the effect of other potentially modulating factors.

Where no apparent determinant of higher susceptibility to infection is identified, a failure of the individual specific protective innate immune responses can be hypothesized. The failure of specific arms of the immune system that are non-redundant in the neonatal defense against a given microorganism would exceed in effect size the modulating potential of other protective factors.

The view of single-gene defects contributing to the burden of neonatal infections in otherwise healthy infants is supported by the growing body of evidence in the literature describing neonatal infections as the first phenotypic manifestation of a known conventional or non-conventional PID (62–65, 67, 76). However, the great majority of neonatal infections still need to be characterized from a host molecular perspective.

NEONATAL GBS DISEASE

In the past years, given its predominant role among neonatal infections, neonatal GBS disease has been extensively characterized from an epidemiological standpoint. The elucidation of the mechanisms underlying neonatal vulnerability to GBS may serve as a model to understand the pathogenesis of other neonatal infectious diseases. In the following paragraphs, we discuss the unique susceptibility to GBS infection of some young infants and propose that it could be due to genetic or immune factors.

Epidemiology and Clinical Characteristics

Group B streptococcus is a Gram-positive, β-hemolytic bacterium frequently colonizing the human gastrointestinal and genitourinary tracts. Invasive GBS disease is extremely rare in healthy adults, with a reported incidence of 10/100,000 non-pregnant individuals (20, 21). Young infants, pregnant and post-partum women, and older adults with underlying medical conditions display higher rates of invasive disease (77).

The global incidence of neonatal GBS disease is estimated to be as high as 0.53/1,000 live births (78). The incidence is highest in infants during the first 3 months of life and dramatically declines afterward (7). Early-onset GBS disease (EOD, onset during the first 6 days of life) occurs after vertical transmission of the bacterium through ascending infection or during delivery through a GBS-colonized birth canal. Risk of EOD can be reduced by administration of antibiotics to the mother during labor. Late-onset GBS disease (LOD) (onset between 7 and 89 days of life) is thought to result from horizontal transmission in most cases. The source of GBS can been identified in some cases. Potential routes of transmission include persistent mucous membrane and skin colonization from acquisition of GBS at birth or after birth from mothers with vaginal colonization; gut colonization through ingestion of infected breast milk from mothers with or without mastitis; or the community or hospital environment (15, 79–81). No preventive strategy exists for LOD. After the introduction of *intrapartum* antibiotic prophylaxis in clinical practice, the incidence of EOD has dropped in the United States from 1.7/1,000 live births in 1993 to ~0.3/1,000 live births, but the incidence of LOD remained stable (7, 82). Clinically, neonatal GBS disease has the features of a severe, life-threatening bacterial infection with systemic disease (sepsis), often associated with organ involvement (meningitis, osteoarthritis, NEC), requiring admission to a NICU. Untreated, it is almost always fatal with multiorgan failure due to septic shock and disseminated intravascular coagulopathy. Case-fatality ratio was as high as 50% in the 1970s (7) and has now dropped to <10% (78, 82), thanks to improvements in neonatal intensive care techniques and the prompt detection of clinical signs of infection and immediate initiation of antibiotic treatment.

Established Risk Factors for Human Neonatal GBS Disease and Gaps in Knowledge

Approximately 50% of infants born to GBS-colonized mothers (10–30% of all pregnancies) are in turn colonized. Of these, only 1–2% develops overt EOD (7). Data on the proportion of GBS-exposed infants developing LOD are lacking, but it is probably low, given a likely increase in the cumulative exposure/colonization rate with age and a concurrent decline in the incidence of GBS disease.

During the past decades, epidemiological studies led to the identification of several risk factors for EOD, including maternal colonization with GBS and bacteriuria, prematurity, chorioamnionitis, and/or *intrapartum* fever, prolonged (>18 h) premature rupture of membranes (PROM), low maternal anticapsular polysaccharide GBS Abs (29, 83–88), and GBS disease in an older sibling (89). Established risk factors for LOD include prematurity and gut colonization by the pathogen (80, 90).

In many cases, invasive GBS infection develops in otherwise healthy, full-term newborn infants, with as many as 42% of early-onset cases (91) and most late-onset cases occurring in the absence of any established risk factor. Known risk factors are therefore unable to reliably predict the occurrence of GBS disease at the individual level. Rather, they identify groups of infants enriched for determinants of susceptibility, but the nature of such determinants has remained elusive.

GBS Microbial Load and Virulence Factors

Fetal and neonatal exposure to the microorganism is the *sine qua non-for* neonatal colonization and subsequent infection. Heavy maternal vaginal colonization has since long been recognized as a risk factor for EOD, possibly due to greater bacterial inoculum to the lungs (7). High bacterial load in maternal milk has been linked to neonatal gut colonization and subsequent invasive LOD (80). The determinants of maternal carriage and the maternal bacterial overgrowth are poorly understood. Mild maternal disease may accompany heavy maternal colonization: maternal GBS urinary tract infection in pregnancy is considered a sign of heavy colonization (7), and maternal mastitis may be responsible for high bacterial load in maternal milk (80).

Additional microbial factors, beyond bacterial load, contribute to the development of invasive disease. Ten different GBS serotypes have been described (Ia, Ib, II–IX), based on the capsular polysaccharide antigen. Serotypes Ia, Ib, II, III, and V are most frequently found in EOD; serotype III is the most frequently isolated serotype in LOD and meningitis, but all serotypes can cause neonatal infection (19, 82, 92, 93). The capsular polysaccharide is thought to contribute to the virulence of the microorganism by aiding to escape the host immune responses. Deeper investigation on GBS isolates through multilocus sequence typing and grouping of genetically related sequence types (STs) into clonal complexes (CCs) has shown that most human isolates belong to few CCs (CC1, CC10, CC17, CC19, CC23, and CC26) (94–98) (http://pubmlst.org/sagalactiae/). The hypervirulent CC17 strains (including the hypervirulent ST-17 strain) are newborn specific. They possess the adhesin HvgA and other surface proteins conferring the ability to invade the neonatal central nervous system and are responsible for most LOD with meningitis, but are usually not responsible for adult disease (99). Strains belonging to all the six CCs have been reported in EOD (82, 98, 99).

The neonatal-specific hypervirulence of some bacterial strains and the bacterial load may explain in part the occurrence of neonatal disease. Nonetheless, individual susceptibility is not fully explained by bacterial virulence, especially in cases in which infection is caused by non-hypervirulent strains.

Protective Immunity to GBS

One fundamental and yet-unanswered question in the field is which are the non-redundant pathways of the innate immune system conferring neonatal protection to GBS.

Several different methodologies in *in vitro* and animal models have been used to attempt to answer this question.

Both knockout mouse and *in vitro* models of GBS infection identified a critical role for TLR and IL1 receptor signaling and/or signaling through MYD88 in bacterial clearance, TNF-mediated inflammation, septic shock, and microglia activation and neurodegeneration (100–109). Specifically, TLR2 and IL1R signaling have been shown to be both beneficial and harmful, depending on the experimental conditions (101, 110–112). A role for IL6, IL10, IL12, and IL18 has been demonstrated in mouse models of GBS infection through administration of anticytokine specific Abs (113–116).

The relevance of the studied pathways in the experimental settings may largely depend on the experimental conditions. Conversely, the non-redundant role of the studied signaling pathways in the human model in natural (as opposed to experimental) conditions still needs to be elucidated (117).

One human study suggested that a null polymorphism in sialic acid-binding immunoglobulin-like lectin 14 (*SIGLEC14*) influences human inflammatory responses to GBS in neutrophils and amniotic membranes and is possibly correlated with GBS-related preterm birth (118), but no data are available on the possible role of SIGLEC proteins in the pathogenesis of GBS infection.

HYPOTHESIS

Despite advances in the understanding of both the host and the microbial sides of neonatal GBS infection, currently available data are not able to fully explain neonatal susceptibility to infection at the individual level.

We hypothesize that susceptibility to neonatal GBS disease in otherwise healthy infants is due to a failure of the specific neonatal protective innate immune responses to GBS. This neonatal immunodeficiency could be either intrinsic (genetic defect in the infant) or extrinsic/environmental (interference of maternal Abs). In the next paragraphs, we present the genetic and the "maternal antibody" hypotheses of GBS disease and explain how these fit with current evidence.

The Genetic Hypothesis of GBS Disease

Several reports, recently reviewed (76), demonstrate that adult and neonatal GBS infection may be a phenotypic expression of both conventional (Kostmann disease, transient hypogammaglobulinemia of infancy, chronic granulomatous disease, activated phosphatidylinositol 3-kinase δ syndrome—like immunodeficiency, C2 and IgG4 subclass deficiency, and isolated congenital asplenia) and non-conventional (IRAK4 and MYD88 deficiency) PIDs. Even when occurring in the context of a non-conventional PID, neonatal GBS infection may be one of the several manifestations of a broader phenotype that, for MYD88 and IRAK4 deficiency, includes susceptibility to multiple pyogenic bacteria. Conversely, most cases of neonatal GBS disease occur as an isolated infection, indicating that the susceptibility to GBS is pathogen specific and not linked to a more general state of immunosuppression.

We hypothesize that inborn errors of the primary innate immune responses to GBS, i.e., monogenic susceptibility to GBS disease, underlie some cases of isolated neonatal GBS infection occurring in otherwise healthy neonates. The clinical and immunological phenotypes of isolated neonatal GBS disease may indeed be consistent with those of non-conventional PIDs (57): (i) GBS disease is a potentially lethal infection striking early in life; (ii) the infecting strain/serotype and its virulence factors, while accounting for some variability in the occurrence and severity of infection (92, 119, 120), are not sufficient to explain susceptibility and resistance at the individual level; (iii) the spectrum of susceptibility is extremely narrow, restricted to GBS; and (iv) in most cases, there are no immunological defects at first-line immunological studies that would be consistent with conventional PIDs. In addition, GBS infection usually strikes once in life and only rarely recurs (~1% of cases) (121). This observation is consistent with a low recurrence rate in the subset of non-conventional PIDs characterized by immunodeficiency of the protective immunity to primary infections (57).

The highest incidence of GBS disease during the first 3 months of life would be explained by the high likelihood of being exposed to GBS in the perinatal period and/or by the full penetrance of the genetic defects in this age group.

Recurrence of GBS infection concerns only a small percentage of cases, both singletons and twins, and has been linked to re-exposure to GBS through maternal milk or other sources, to inappropriate treatment, or to persistence of GBS on skin and mucosal surfaces after the first infectious episode (122–125). A genetic explanation for recurrence is also plausible. In some PIDs of protective immunity to primary infection, the genotype has

been shown to influence the recurrence rate (126). Therefore, recurrence of neonatal GBS disease may indicate a more severe phenotype or represent the phenotypic manifestation of a specific genetic defect.

The occurrence of GBS disease in siblings (89), as well as the recurrence described in a consanguineous family (76), suggests that the genetic hypothesis may be a plausible explanation for some cases. Infection by poorly virulent strains, the presence of other cases with overlapping phenotypes in the family, consanguinity in the parents, recurrence and severity of the clinical signs, and slow or absent response to antimicrobials despite appropriate treatment strengthen (although their absence does not exclude) the hypothesis of a PID underlying GBS infection.

The "Maternal Antibody" Hypothesis of GBS Disease

The highest incidence of GBS disease is registered during the first 3 months of life, with most cases (77–78%) occurring during the first week of life (20, 21). This observation, together with a known role of GBS in prenatal disease, both prematurely and at full term of pregnancy (GBS-related stillbirth, term or preterm PROM, chorioaminionitis), suggest that a maternal factor might be particularly important for perinatal infection.

Recently, neutralizing anticytokine auto-Abs have been found in adult and pediatric patients suffering from life-threatening infections, revealing novel mechanisms of unusual susceptibility to specific pathogens (127–130). Auto-Abs against IL17 and/or IL22 have been associated with chronic mucocutaneous candidiasis; anti-IFN-γ auto-Abs with adult-onset immunodeficiency; anti-IL6 auto-Abs with recurrent skin infection; and auto-Abs against GM-CSF with pulmonary alveolar proteinosis (131).

The clinical phenotypes resulting from anticytokine auto-Abs partially (anti-IFN-γ, anti-IL6) or completely (anti-IL17, anti-GM-CSF) overlap with known monogenic conditions affecting the same pathways, demonstrating that Ab-mediated diseases may be immunophenocopies of monogenic immune disorders.

In neonates, autoimmunity is an exceedingly rare condition, but Ab-mediated disease due to transplacental crossing of maternal auto- or allo-Abs is a well-recognized and relatively frequent mechanism of organ dysfunction. This has been shown in the thyroid (congenital hypothyroidism), the blood (fetal and neonatal hemolytic disease and fetal and neonatal auto- and alloimmune thrombocytopenia), the neuromuscular junction (transient neonatal myasthenia gravis), the heart (congenital heart block due to SSA/Ro Abs), and other organs and tissues (132–137). We therefore hypothesize that neonatal GBS disease may be caused by yet-undiscovered neutralizing maternal auto-Abs or allo-Abs against components of the fetal and neonatal immune system that are non-redundant in conferring neonatal protection against GBS. The progressive decay of circulating maternal Abs in the infant plasma might then explain the decreasing incidence of infection over the first 3 months of life. Furthermore, the presence of pathogenic circulating Abs in the maternal blood would be consistent with the occurrence of mild disease in the mother (GBS-related urinary tract infection or mastitis) that is often associated with neonatal GBS disease, as well as with the well-documented higher risk of GBS-EOD in infants with a previous sibling with GBS disease (7). Finally the removal, with exchange transfusion, of pathogenic Abs from neonatal plasma could be an additional explanation to the efficacy of the procedure in infants with septic shock (138).

The proposed mechanism could in part explain neonatal GBS disease in full-term infants. Despite transplacental transfer of Abs is reduced at low gestational ages, allo- or autoimmune pathogenic maternal Abs have been demonstrated to be able to cause disease in the preterm infant or during gestation (71, 134). Therefore, the "maternal antibody" hypothesis could also explain some cases of neonatal GBS disease occurring in preterm infants.

Testing the Hypotheses—Possible Study Methodologies

Previous studies that addressed the role of genetics in the susceptibility to neonatal infection focused on the associations between selected common single-nucleotide polymorphisms and infectious outcomes (139). A more integrated approach including genomics, transcriptomics, proteomics, and functional studies is required to uncover the precise molecular determinants of susceptibility to specific pathogens causing neonatal infections.

Ad hoc studies should be designed depending on the phenotype under investigation.

Multicenter GWASs may provide some insight into the pathogenesis of suspected multifactorial infections as, for instance, those occurring in preterm infants. GWAS are currently ongoing on neonatal cohorts (54).

Exome or genome sequencing studies have potential to uncover the cause of suspected monogenic disorders. Cases should be prioritized based on the clinical profile most suggestive of a monogenic etiology, including extreme severity, consanguinity, recurrence of infection, and familial presentation. Depending on the design of the study, analysis of the trio (proband and parents) and of the family or cohort studies should be carried out to uncover the individual, rare (<1% in the general population), and functionally deleterious genetic variants that best fit the most likely genetic model (*de novo*, autosomal dominant with complete or incomplete penetrance, autosomal recessive with mono- or biallelic mutations). This approach could shed light on the pathways that are non-redundant in neonatal protection against GBS.

Functional follow-up will be required to validate candidate variants and confirm their causative role. These studies should be designed to assess the integrity of the molecular pathways affected by the mutations and determine how they are relevant to the neonatal immune responses in primary cells and/or immortalized cell lines.

Laboratory experiments will be also needed to investigate the possible interfering effect of maternal plasma on the neonatal immune responses. The laboratory tests could include cytokine production assays, detailed analyses of RNA (transcriptome analysis) and protein expression in *ex vivo* samples (blood collected during sepsis), and *in vitro* experiments (stimulation of patient and control cells with different ligands, cell differentiation

TABLE 2 | Comparison of neonatal hemolytic disease and neonatal group B streptococcus (GBS) disease.

	Neonatal hemolytic disease	Neonatal GBS disease
Physiological condition	Mild jaundice (~50% newborn infants)	GBS colonization (~10% of infants at birth; probably higher cumulative colonization rate during the first 3 months of life)
Disease	Life-threatening jaundice/ kernicterus	Life-threatening infection
Incidence of disease in the absence of prevention	Estimated ~1/1,000	EOD: 1.8/1,000 LOD: 0.3/1,000
Incidence after prevention	0.4–2.7/100,000	EOD: 0.3/1,000 LOD: 0.3/1,000
Prenatal disease	Facultative: fetal anemia/ erythroblastosis	Facultative: term/preterm premature rupture of membranes, chorioamnionitis, GBS-related stillbirth
Screening/early diagnosis	Highly effective: direct and indirect Coombs test/ serial plasma bilirubin	Partially effective: universal screening of pregnant women for GBS/C-reactive protein, blood count, cultures after onset of infection
Prevention of life-threatening disease	Phototherapy	*Intrapartum* antibiotic prophylaxis
Treatment	Phototherapy; blood exchange	Antibiotics; intensive care; blood exchange
Molecular mechanisms	Known (red cells genetic defects, maternal AB0/Rh alloimmunization)	Unknown

EOD, early-onset GBS disease; LOD, late-onset GBS disease.
Incidence is expressed as number per 1,000 (or 100,000) live births.

assays) in the presence of maternal or control plasma. Specific assays should be used for the detection of specific Abs in the maternal and in the perinatal plasma.

Ultimately, these experiments should aim at demonstrating a causative link between the molecular findings, the observed cellular phenotypes, and the patient's clinical phenotype.

CONCLUSION

Transient susceptibility to a narrow range of infections during the neonatal age may be explained by inborn errors of immunity, in the context of a relatively immature, non-redundant immune system. The early recognition of a PID as an essential contributing factor to a severe neonatal infection is clinically very relevant, as it may change the management and allow the referral of the patient to the clinical immunologist for specific follow-up and family counseling.

In parallel fields, the discovery of concurrent genetic and auto-/alloimmune mechanisms for several neonatal diseases has dramatically changed practice, as exemplified by the development of highly effective screening and diagnostic procedures for neonatal hemolysis, which reduced the incidence of fetal erythroblastosis and neonatal bilirubin encephalopathy by two orders of magnitude, from ~1/1,000 to ~1/100,000 live births (**Table 2**) (140). Similar observations can be made for congenital hypothyroidism and other common and rare neonatal diseases (**Table 1**).

Current prevention efforts, although invaluable for neonatal health, only had a limited impact on the global incidence of neonatal infections (9, 141) (**Table 2**). A more complete understanding of the mechanisms underlying the interindividual variability in the neonatal innate immune responses to pathogens is required to develop highly effective, pathogen-specific and individual-tailored preventive protocols.

AUTHOR CONTRIBUTIONS

AB conceived the manuscript; conducted the literature search; and drafted, edited, and approved the final version of the paper. MS participated in the discussion of ideas, helped with the writing, revised critically, and approved the final version of the manuscript. JF participated in the discussion of ideas, edited, revised critically, and approved the final version of the manuscript.

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