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Developmental biology of the innate immune response: implications for neonatal and infant vaccine development

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Abstract

Molecular characterization of mechanisms by which human pattern recognition receptors (PRRs) detect danger signals has greatly expanded our understanding of the innate immune system. PRRs include Toll-like receptors (TLRs), nucleotide oligomerization domain-like receptors (NLRs), retinoic acid inducible gene-like receptors (RLRs) and C-type lectin receptors (CLRs). Characterization of the developmental expression of these systems in the fetus, newborn and infant is incomplete but has yielded important insights into neonatal susceptibility to infection. Activation of PRRs on antigen-presenting cells enhances co-stimulatory function, and thus PRRs agonists are potential vaccine adjuvants, some of which are already in clinical use. Thus study of PRRs has also revealed how previously mysterious immunomodulators are able to mediate their actions, including the vaccine adjuvant aluminum hydroxide (Alum) whose adjuvant activity depends on its ability to activate a cytosolic protein complex known as the Nucleotide Binding Domain Leucine-Rich Repeat and PYD-Containing Protein 3 (NALP3) inflammasome leading to IL-1 β production. Progress in characterizing PRRs is thus informing and expanding the design of improved adjuvants. This review summarizes recent developments in the field of innate immunity with special emphasis on developmental expression in the fetus, newborn and infant and its implications for the design of more effective neonatal and infant vaccines.

Newborns and infants are at high risk of infection: Need for effective neonatal and infant vaccines

On a global basis, infections result in ~2 million deaths per year in those less than 6 months of age (WHO) (1). Common pathogens in neonates and/or infants include Gram-positive bacteria (e.g., Group B Streptococcus, *S. pneumoniae*), Gram-negative bacteria (such as *E. coli*, *B. pertussis*), herpes simplex virus (HSV), respiratory syncytial virus (RSV) and rotavirus. This susceptibility highlights the medical need for effective vaccines for newborns and infants and the functional immunodeficiencies that must be overcome in designing vaccines that adequately protect the very young. As birth is the most reliable point of healthcare contact worldwide, vaccines that are active at birth are a major global health imperative (2). Design and development of such vaccines will require understanding of the developmental expression of innate immune pathways whose activation enhances the adaptive immune response.

Distinct aspects of neonatal innate immunity that pose challenges to effective vaccination early in life

The fetal immune system is heavily Th2-biased, presumably to avoid pro-inflammatory Th1-type alloimmune responses to maternal tissues that may trigger preterm birth or spontaneous abortion.

Birth triggers a dramatic shift in environment that places further demands on the function of the neonatal immune system, mediating the transition from a normally sterile intrauterine compartment to a foreign antigen (Ag)-rich outside world, including the first colonization of skin and gastrointestinal tract. In contrast to low levels of Th1-type cytokines (e.g. TNF, IL-12p70, IFN- γ), human neonatal plasma contains high levels of the Th2-type cytokine IL-6 *in vivo* at both birth and throughout the first week of life (3). IL-6 induces an acute phase response at birth that may serve to shield against bacterial infections and clear microbial products and/or PRR agonists.

The distinct polarization of fetal and early neonatal immune responses presents obstacles to effective neonatal immunization, including impaired antigen-presenting cell (APC) responses (e.g., impaired IFN γ production) to many (but not all) stimuli, a Th2 bias to immune responses, and impaired antibody (Ab) affinity maturation (4), as well as the potential inhibitory effect of maternal Abs (5).

Quantitative and qualitative differences exist between neonatal and adult APCs. Qualitative differences are evident in human monocytes *in utero* as assessed by flow cytometry indicating reduced expression levels of MHC class II molecules (6). Several mechanisms have been implicated in skewing neonatal APCs towards Th2-type responses, including: a) the production by placental tissues of TGF- β , progesterone and prostaglandin E2 that enhance Th2 cytokine production (7), and b) the presence in neonatal blood plasma of relatively high concentrations (~300 nM) of adenosine, an immunosuppressive endogenous purine metabolite (8,⁹). The patterns of neonatal cytokine production appear to be relevant *in vivo*, in that after birth, during the first week of life, human neonatal peripheral serum levels of TNF remain low (relative to human adult serum) whereas levels of IL-6, a Th2-polarizing cytokine with anti-inflammatory properties, rise.

Multiple studies document that human neonatal monocytes and APCs function suboptimally when tested *in vitro* with respect to co-stimulatory responses to most stimuli (10). Studies of murine neonatal dendritic cells (DCs) have demonstrated that Agpresentation increases during ontogeny, correlating with increased co-stimulatory molecule expression and increased responses to protein-conjugated, T cell-dependent polysaccharide Ags (11). However, C57BL/6 neonatal mice (1-7 days of age) demonstrated stronger LPS-induced inflammatory cytokine production by splenocytes in the presence of T cells *in vitro* and after i.p. injection *in vivo*, ascribed to neonatal quantitative deficiency of CD4+ and CD8+ T-cells (12). Thus, inflammatory responses in neonates appear to be dependent on the species (noting that innate immune genes are hypervariable between species, including between humans and mice (13, ¹⁴), experimental model (*in vitro* versus *in vivo*), extracellular culture medium (autologous 100% (vol/vol) adenosine-rich neonatal plasma/serum (15) vs. low concentrations of heat-inactivated fetal calf serum that are used in many studies) and particular stimulus studied.

In contrast to neonatal APCs, neonatal CD4+CD25(IL2-Ra)+ T reg cells (Treg) are fully functional and found at high abundance in human fetal lymphoid tissues (16) and newborn cord blood (17,¹⁸). Neonatal Treg cells suppress both T responder cell proliferation and Th1-cytokine (e.g., IFN- γ) production, induced by self-Ags, to limit adaptive immune responses. Treg mediate their effects by both cell contact-dependent and -independent mechanisms,

including secretion of IL-10, CD39 and CD73 (ectonucleotidase)-mediated generation of extracellular adenosine, and adenosine A2A receptor-mediated enhancement of cAMP concentrations in target T responder cells (19,²⁰). Neonatal inhibition of auto-immunity via Treg suppression has clear advantages as neonates first encounter the foreign-Ag rich world, however these effects may be detrimental to neonatal immunity to infection (21) and to neonatal vaccine responses (22,²³).

Innate immune activation enhances adaptive immune responses

Activation of Pattern Recognition Receptors (PRRs) on APCs such as macrophages and DCs enhances antigen-presenting activity and adaptive immune responses via direct and indirect mechanisms (24). PRR signaling is influenced by cross signals mediated via diverse PRR classes, tyrosine kinases, tyrosine phosphatases, ubiquitinating systems, and glucocorticoids, and therefore varies between different cell-types (25). Upon activation, DCs efficiently process and present Ags in the context of MHC, increase production of Th1-polarizing cytokines such as IL-12p70, and up-regulate co-stimulatory molecules (e.g., CD40, CD80, CD86) and chemokine receptors mediating cell migration from the tissues into the draining lymph nodes. Once inside the lymph nodes, DCs interact with naïve T and B cells inducing their differentiation into effector cells, thereby triggering an acquired immune response.

In addition to their direct effect on APCs, PRR agonists also enhance the transition from innate to adaptive immune responses via indirect mechanisms. For example, Tolllike receptor (TLR)-mediated DC activation induces IL-6 that renders T responder cells refractory to inhibition by suppressive Treg (26). Tissue-derived signals may also influence APCs and control the type of effector class generated (27). TLR-mediated activation of DCs enhances lymph node function via triggering DC migration to lymph nodes, expression of vascular endothelial growth factor, increasing high endothelial venule proliferation, remodeling primary feed arterioles, and increasing nodal blood flow and recruitment of rare Ag-specific lymphocytes (28,²⁹).

The impressive progress in defining the potential roles of PRRs has provided an opportunity to better understand the development of innate immune responses after birth, with potential implications for the optimization of vaccine development. Below, we review the current state of knowledge regarding the developmental expression of key PRRs, highlighting both recent progress and gaps in our knowledge.

Developmental expression of PRRs

Responses to microbial infection are initiated through an innate immune system that features diverse PRRs poised for activation in extracellular and intracellular locations (Figure 1).

TLRs—TLRs are type I transmembrane proteins with an extracellular amino-terminus and an intracellular carboxy-terminus. They are composed of various domains including, extracellular leucine rich repeat (LRR) with one or two cysteine-rich regions and an intracellular toll/interleukin-1 receptor (TIR) domain, named after its homology with the interleukin 1 receptor. Humans express ten TLRs: a) surface expressed TLRs include TLR2 (bacterial lipopeptides), TLR4 (lipopolysaccharide) and TLR5 (flagellin), b) endosomal TLRs include TLR7 and 8 (single stranded RNA, (30,³¹)) and TLR9 (unmethylated CpG DNA). Engagement of TLRs activates intracellular signalling cascades via Myeloid differentiation factor 88 (MyD88)-dependent and MyD88-independent pathways (32), including IL-1 receptor-associated kinase-4 (IRAK-4) recruitment, culminating in NF-κB activation and expression of pro-inflammatory cytokines, such as TNF, IL-6, and pro-IL1β (note that processing to mature IL-1β requires Nacht Domain Leucine-Rich Repeat and PYD-Containing Protein 3 (NALP3) inflammasome (INFL) action). TLR stimulation can also lead to the activation of several other

intracellular signalling pathways such as those involving Jun N-terminal kinase, mitogen activated protein kinase (MAPK) (33,³⁴), interferon regulatory factor (IRFs), and the FAS-associated death domain (FADD)-induced apoptosis pathway (32).

The importance of the TLR pathway for host defense in newborns and infants is apparent in the clinical consequences of TLR pathway defects. Defects of signaling molecules downstream of TLRs, including IRAK4 and MyD88-deficiency result in selective susceptibility to pyogenic infections (often streptococcal and staphylococcal) during childhood with improvement later in life (35-³⁷).

Cord blood monocytes of full-term human newborns express normal amounts of TLRs, yet upon TLR-mediated stimulation in whole cord blood (i.e., 100% vol/vol autologous, adenosine-rich plasma), agonists of TLRs 1-7 demonstrate a 1-3 log impairment in TNF α production relative to adult peripheral blood monocytes (15). One of the mechanisms accounting for this impairment is that during the hypoxia and stress accompanying the birth process, plasma concentrations of adenosine, an endogenous immunosuppressive purine metabolite, rise. Adenosine can act via A3 adenosine receptors on neonatal mononuclear cells to induce production of cAMP, a key second messenger that inhibits TLR-mediated production of Th1-polarizing cytokines (8,⁹). Other studies have demonstrated that LPS-induced responses of newborn mononuclear cells are diminished at birth due to reduced expression of the TLR adaptor molecule MyD88 (38) and by failure of nucleosome remodeling at the IL-12 promoter (39). Impaired TLR agonist-induced production of type I IFNs from human neonatal plasmacytoid DCs (pDCs) and neonatal monocyte-derived DCs (moDC) has also been described (40,⁴¹).

In contrast to agonists of TLRs 1-7, TLR8 agonists, including TLR7/8 agonists, are able to induce robust immune responses by human neonatal APC, comparable with those of healthy adult controls (42). Exposure of human neonatal cord blood-derived monocytes and APCs, including myeloid dendritic cells (mDCs) and moDCs, to TLR8 agonists induces robust (i.e., adult-level) phosphorylation of p38 MAP kinase, NF- κ B activation, pro-inflammatory cytokine production (TNF, IL-12), and up-regulation of costimulatory molecules (e.g., CD40).

RLRs—During the infection cycle of some viruses, double stranded (ds) RNA is produced which can be detected in the cell cytosol by retinoic acid inducible gene-like receptors (RLRs), such as retinoic acid inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA-5). These cytoplasmic proteins are composed of amino terminal caspase-recruitment domains (CARDs) and a carboxy-terminal helicase domain (43,⁴⁴). RLR activation induces CARD domain interaction with the CARD domain-containing adaptor protein, IFN- β promoter-stimulator (IPS)-1 (also known as mitochondrial antiviral signaling protein (MAVS), virus-induced signaling adaptor (VISA) and CARD adaptor inducing IFN- β (CARDIF)) leading to IRF3 and NF- κ B activation. Little is known regarding the developmental expression of RLRs at birth and in the neonate, an important area of future study.

NLRs—Nucleotide oligomerization domain-like receptors (NLRs) detect bacterial components and include members of the nucleotide-binding oligomerization domain (NOD) subfamily as well as NLRs associated with the INFL. The NOD proteins in humans are a family of over 20 cytosolic proteins. Structurally they are composed of: a) a variable N-terminal effector-binding domain, usually consisting of a pyrin domain (PYD) or CARD, cable of regulating homotypic and heterotypic binding; b) a centrally located NOD domain; c) a C-terminal ligand-recognition domain, which can be composed of LRRs. As with the RLRs, little is known regarding the developmental expression of NODs at birth and in the neonate.

The INFL is a cytosolic multi-component protein complex, including caspase-1, which upon activation cleaves pro-IL-1 β to the potent pro-inflammatory cytokine IL-1 β . IL-1 β has a central role in the onset of parturition, particularly in the context of intrauterine infection/inflammation. A recent study, found that: a) caspase-1 concentration in the amniotic fluid increases as a function of gestational age; b) women with spontaneous term labor had a higher median caspase-1 amniotic fluid concentration than women at term without labor, suggesting that the INFL may be activated in spontaneous parturition at term, and c) higher caspase-1 levels were associated with infection/inflammation (45). Human neonatal monocytes, in particular those of preterm newborns, demonstrate impaired IL-1 β production to LPS and to lipoteichoic acids. A search of Pubmed did not yield any publications relating to INFL function at birth.

CLRs—C-type lectins receptors (CLRs) can be produced as secreted soluble proteins, including mannose binding lectin (MBL), lung surfactant protein A (SP-A), or as transmembrane proteins such as selectins, mannose receptor and the DC-specific ICAM-3 grabbing non-integrin (DC-SIGN). MBL is an acute phase plasma protein whose expression in the liver is up-regulated during inflammation. MBL recognizes a variety of carbohydrate patterns found on infectious microorganisms including, bacteria (e.g. *Staphylococcus aureus*), fungi (e.g. *Saccharomyces cerevisiae*), viruses (e.g. HIV), and on altered self-glycoproteins (e.g. aberrant glycosylation of cancer cells). MBL binding to carbohydrate targets triggers activation of MBL-associated serine protease, cleaving complement proteins and promoting opsonisation/membrane attack complex formation (46). MBL can also act directly as an opsonin by binding to receptors which promote phagocytosis (46), and can modulate cytokine production *in vitro* and *in vivo* (47) thereby playing important roles in neonatal infection. Plasma MBL concentrations in both premature and full term neonates are lower than those of adults (48), but steadily rise during the first weeks of life, possibly as a consequence of the skew of neonatal cytokine responses towards IL-6, which induces the acute phase response (3,⁹). MBL deficiency is associated with an increased risk of bacterial sepsis (49).

From the standpoint of innate immune modulation of vaccine responses, MBL deficiency enhances mouse Ag-specific IgG production following immunization with tetanus toxoid-conjugated Group B streptococcus polysaccharide vaccine or tetanus toxoid alone (50). These data suggest that under certain circumstances MBL can inhibit Ab production. Further characterization of the MBL pathway may inform design of vaccines that minimize such inhibitory MBL effects.

Developmental expression of Antimicrobial proteins and peptides: APPs expressed by leukocytes and epithelial cells are ancient components of innate immune defense that are best known for their ability to kill microorganisms and neutralize their surface components. However, some APPs also demonstrate activity in modulating adaptive immune responses. For example, bactericidal/permeability-increasing protein (BPI), a neutrophil-derived primary granule protein with endotoxin binding activity, was recently shown to enhance APC function. Neonates are deficient in BPI expression, raising the possibility that may also impair delivery of naturally shed Gram-negative bacterial LPS outer membrane blebs to APCs. Defensin peptides can enhance production of anti-viral IFNs, that are important to Th1 polarization and crosspresentation (51). Cathelicidin peptides, found in neutrophil secondary granules, enhance adaptive immune responses *in vivo*. Indeed there is age-dependent maturation in the expression in human neonatal blood plasma of a broad range of APPs, and thus to the extent that these molecules contribute to modulating adaptive immune responses.

The recent progress in defining PRRs and APPs involved in triggering innate immune responses and thereby modulating adaptive immunity provides new opportunities to understand that function of adjuvants in currently administered vaccines, as outlined below.

Engagement of innate immunity by currently approved neonatal and infant vaccines

It is increasingly appreciated that an important determinant of vaccine efficacy is the ability of a given vaccine to activate the innate immune system to enhance APC function and Th1-polarizing adaptive responses (52). Currently, the two vaccines that are regularly given at birth in humans are the bacillus Calmette-Guerin (BCG) vaccine and the hepatitis B (HepB) vaccine (Table 1). BCG is capable of inducing a strong Th1-type immune responses in human neonatal cells *in vitro* (53) and *in vivo* (54), which is in part due its ability to activate multiple TLRs expressed by APCs (55). The BCG vaccine demonstrates that under certain conditions, the human neonatal immune system is capable of mounting a protective Th1-type response (56), but the underlying mechanisms of its efficacy at birth is not fully understood. Recent work has indicated that it is very likely that activation of multiple PRRs by BCG plays important roles in its efficacy. The BCG vaccine is a live bacterium and able to activate TLR2 (57,⁵⁸) and TLR4 (57,⁵⁸) by virtue of its cell wall that consists of peptidoglycan, arabinogalactan, and mycolic acids (59), and TLR9 through its CpG-rich DNA (60). More recently, the possibility that BCG also engages TLR8 has also been raised based upon associations of TLR8 polymorphisms with susceptibility to pulmonary TB, increased susceptibility in males (TLR8 is encoded on the X chromosome), and the ability of BCG to induce macrophage TLR8 expression (61).

Hepatitis B virus (HBV) is a global public health threat that has chronically infected over 350 million people worldwide (62). The HepB vaccine is composed of a virus-like particle containing the viral envelope protein hepatitis B surface antigen (HBsAg), prepared using recombinant DNA technology. HepB vaccine is composed of an aluminum-containing adjuvant, but is still incompletely immunogenic as ~10% of vaccinated populations fail to mount immune responses to HBsAg after immunization (Table 1).

Haemophilus influenzae type b (Hib) conjugate vaccines are initially administered at 6 to 8 weeks of age. Hib activates transfected HEK293 cells in a TLR2- and TLR4-dependent manner, likely reflecting expression of bacterial lipopeptides (TLR2) and lipopolysaccharide (TLR4) (63) (64) (Table 1). Indeed, the outer membrane protein complex (OMPC) found within the Hib-OMPC glycoconjugate vaccine is TLR2 and MyD88-dependant, and in the absence of TLR2, the immunogenicity of the Hib-OMPC vaccine is significantly reduced (64).

In addition to TLR-induced vaccine responses (65), activation of RLRs (RIG-I, MDA-5 (66)) has been reported for the wild type measles virus, suggesting that the attenuated strains used for vaccination may also engage this innate immune pathway (Table 1). To date, the US Food and Drug Administration (FDA) have approved a limited number of human vaccine adjuvants, chief among which is aluminium hydroxide, aluminum phosphate (typically referred to as 'Alum'). Alum is a commonly used adjuvant present in human and animal vaccines world-wide (HepB, DTaP, Hib, PCV7, HepA; Table 1) and is known for its ability to induce protective Th2-type responses. It was recently demonstrated that the key to Alum's adjuvant activity is its ability to activate the NALP3/INFL (67,⁶⁸) (Fig. 1). These conclusions were based on the observations that IL-1 β and IL-18 production by macrophages in response to Alum *in vitro* requires intact INFL signaling. Moreover, mice deficient in NALP3, apoptosis-associated speck-like protein containing a CARD (ASC) or caspase-1 failed to mount a significant Ab response to an Ag administered with Alum, whereas the response to complete Freund's adjuvant

remained intact. These data highlight the crucial role of the NALP3/INFL in Alum's adjuvant activity.

MF59 is an oil-in water squalene emulsion and the precise mechanism of its adjuvant effects is still largely unknown (69). Studies using fluorescently-labeled MF59 injected intramuscularly then observed 2 days later indicated partial localization in T-cell areas of the draining lymph node (70). MF59 has also been documented to induce a significant influx of macrophages to the site of infection in mice, which is chemokine receptor 2 (CCR2) dependant (71). Of interest, the oil emulsion incomplete Freund's adjuvant (not licensed in humans due to its toxicity) induces optimal IgG1 and IgG2c in a NOD2-dependant manner (72).

Translational research towards improved adjuvants for newborns and infants

Given that newborns and young infants are susceptible to multiple bacterial and viral pathogens, neonatal immunization is of particular importance because: (a) birth represents a likely point of contact of the newborn with health care providers, (b) early life immunization is associated with a substantially higher rate of vaccination coverage than immunization given at later time-points (2,⁷³), (c) vaccines that require multiple dosing regimens throughout infancy increase cost and reduce compliance, and (d) strategies involving immunization of the mother pose substantial logistical and medicolegal challenges. There is therefore an unmet medical need to develop new approaches are needed to develop vaccines that would be more effective very early in life or that would require fewer doses to generate durable and protective immune responses.

A novel approach to neonatal vaccination has recently been described which employs an attenuated strain of the intracellular pathogenic bacterium *Listeria monocytogenes* to deliver Ag to the cytoplasm of APC (74). Vaccinated neonatal mice induced strong CD8⁺ and CD4⁺ T cell responses and were protected following wild type challenge. Of note, *Listeria monocytogenes* is able to activate several PRRs including multiple NODs (IPAF, NALP3) (75) and can induce the expression of RIG-I (76), possibly accounting for its immunostimulatory properties. Therefore this approach could potentially overcome preexisting hurdles of maternal immune response interfering with neonatal vaccine responses (77). This live attenuated vaccine appeared safe in this murine model in that there was no associated mortality and no bacteria were recovered from the spleens or liver of immunized newborn mice 7 days post-vaccination.

Among the TLR agonists, TLR7/8 agonists hold particular promise as potential adjuvants for use in neonatal and infant vaccines as they induce robust production of the Th1-polarizing cytokines TNF α and IL-12 from neonatal (and adult) APCs that substantially exceeds responses induced by agonists of TLR-2, -4, or -7 (alone) (42,⁷⁸). Therefore TLR7/8 agonists have potential as novel neonatal vaccine adjuvants, due to their ability to activate both TLR-dependant and independent pathways (NALP3/INFL) mediating Th1-type responses from APC (78). In addition, human Treg express TLR8 and mediate reversal of Treg function upon exposure to TLR8 agonists (79) (Figure 2). Indeed TLR7/8 agonists, such as the synthetic low-molecular weight (< 400 Da) antiviral imidazoquinoline compounds imiquimod and resiquimod (R848), have already been used as immunomodulators for many years against specific viral infections. The U.S. Federal Drug Administration (FDA) approved imiquimod for the treatment of external genital and peri-anal warts caused by certain strains of human papilloma virus (HPV)⁽⁸⁰⁾, and it may also have activity against molluscum contagiosum (molluscipox virus) (81-⁸³). One of the main cellular targets of imiquimod is plasmacytoid DCs (IFN- α producing cells) which express high amounts of TLR7, whose engagement induced IFN- α in a MyD88-dependent manner (80,⁸⁴).

R848 is an effective adjuvant for HBsAg vaccination in mice, increasing humoral and cellular immune responses. R848 used in combination with the TLR9 agonists CpG ODN further strengthened the immune response and long-lasting HBsAg-specific T cells displaying effector memory phenotype were detected in mice (85). R848 or topical application of imiquimod administered with *Leishmania* Ag induced protective Th1 immune responses in mice, compared with *Leishmania* Ag alone. In addition, subcutaneous vaccination also induced protective immunity whereas intramuscular vaccination did not (86). Indeed, conjugation of the TLR7/8 agonist to the HIV Gag protein improved the magnitude of Th1 and CD8+ responses in adult *Rhesus macaques* (87). A combined TLR7/8 agonist compared with a pure TLR8 agonist can also induce greater Th1 responses and IFN α production from pDCs, which express TLR7 but not TLR8. IFN α is a key cytokine within a vaccine adjuvant setting, inducing Th1 differentiation (80,⁸⁸), induction of cytotoxic T lymphocytes, enhancement of cross presentation and of primary antibody responses and DC activation (89). Novel TLR7 and TLR8 agonists referred to as stabilized immune modulatory RNA (SIMRA) compounds have recently developed and have distinct pharmacodynamic characteristics (90). SIMRA compounds demonstrate greater stability in human sera compared with linear RNA, which is rapidly degraded by ubiquitous RNases. In addition, SIMRA compounds are able to activate TLR7 or TLR8 in HEK293 cells without the need for lipid carriers.

TLR9 agonists are also undergoing biopharmaceutical development for multiple indications, including as vaccine adjuvants for HBV by linking an immunostimulatory DNA sequence to the recombinant HBsAg. This vaccine formulation, which has currently completed phase III trials, may help drive Th1-type responses and reduce Th2 responses (91).

A murine study demonstrated that although the IPS-1 signaling pathway appears to be important for initial type I IFN responses, the TLR7/MyD88 pathway is needed for induction of protective immune responses to influenza A infection. Inactivated influenza virus vaccine failed to confer protection against lethal challenge with live influenza virus in TLR7- and MyD88-deficient mice. Thus protective adaptive immune responses to live attenuated influenza A virus strains are likely dependent on the TLR7-MyD88 pathway (92).

Priorities For Future Studies

In summary, infectious diseases account for over > 2 million deaths per year in newborns and infants less than 6 months of age. Early life vaccination offers several advantages, particularly for developing countries in which birth maybe the only point of contact with the healthcare system. There is thus an unmet medical need for improved neonatal vaccine adjuvants, which are able to improve the level, speed and longevity of protection, particularly by inducing Th1-polarizing cell-mediated immunity. In this context, neonatal vaccination is a feasible approach that is likely to be expanded in future years, an effort that will require the development of new adjuvants capable activating specific PRRs. The recent expansion in knowledge of PRRs in the neonate as described above provides new opportunities for developing novel delivery systems (e.g., intracellular/cytosolic) and/or adjuvants. As always, safety considerations will be paramount, but the possibility of vaccinating this vulnerable population provides a strong motivation to pursue effective vaccines for neonates and infants. Although neonatal vaccination offers many advantages safety concerns will be paramount and have recently been reviewed (93). There are concerns that neonatal vaccination could trigger autoimmunity through molecular mimicry or antigen immune system polarization (94,⁹⁵), however self-reactive neonatal T and B cells are eliminated by peripheral tolerance and there is substantial evidence that multiple pediatric vaccines, including BCG, are not linked to allergy or autoimmunity (96). Nevertheless, as with any new drugs, all novel adjuvant/Ag combinations, must undergo rigorous safety analysis. The rapidly expanding menu of antigen-adjuvant

combinations will require federal authorities to update and enhance the pathways to establishing vaccine safety and efficacy.

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Abbreviations

APC	antigen-presenting cell
BCG	Bacille Calmette-Guerin
MyD88	Myeloid differentiation factor 88
NALP3	Nacht Domain Leucine-Rich Repeat and PYD-Containing Protein 3
RLRs	retinoic acid inducible gene-like receptors
TLR	Toll-like receptor
Treg	T-regulatory cell

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Figure 1. Mechanisms of innate immune activation induced by vaccine adjuvants

TLR agonists found in multiple neonatal vaccines (Table 1) activate either cell associated or intracellularly located TLRs or NODs. These PRRs then interact with specific adaptor molecules culminating in NF-κB or IRF activation. Viral-derived products (dsRNA or ssRNA) can also activate endosomal TLRs, along with RLRs (such as RIG-I), which induce type-I IFN production. The vaccine adjuvant Alum activates the cytosolic NALP3/inflammasome leading to proIL-1β cleavage into bioactive IL-1β.

Figure 2. TLR8 agonists activate human APCs and reverse human Treg function

TLR8 agonists, such as R848, ssRNA and stabilized immune modulatory RNA (SIMRA) strongly activate human APCs via TLR8-dependent and TLR8-independent mechanisms including activation of the NALP3 inflammasome inducing IL-1 β production. Exposure of human neonatal APCs to TLR8 agonists induces robust phosphorylation of p38 MAP kinase and profound/prolonged disappearance of I κ B- κ , resulting in robust induction of protective Th1-type immune responses, including production of IL-12 and up-regulation of the co-stimulatory molecule CD40. TLR8 agonists also reverse suppression mediated by human Treg cells, via both direct action on Treg as well as by induction of APC production of IL-6, a cytokine that renders Trespender cells refractory to Treg-mediated inhibition.

Table 1

Activation of PRRs by approved pediatric vaccines (0-2 years of age)

Vaccine	Age	Adjuvant	PRR	Ref
Hepatitis B (Hep B) vaccine	Birth	Alum	NALP3/INFL	(67,68)
Bacille Calmette-Guerin (BCG)	Birth (not in USA)	CWS [†]	TLR2	(57-60)
		CWS [†]	TLR4	(57-59)
		ssRNA	TLR8*	(61)
		CpG DNA	TLR9*	(60)
			NODs,	(97)
			CTLs*	
Rotavirus vaccine	2 months	?	?	
Diphtheria, Tetanus, acellular Pertussis (DTaP) vaccine	2 months	Alum	NALP3/INFL	(67,68)
Haemophilus influenzae type b conjugate vaccine (Hib)	6 weeks	Alum	NALP3/INFL	(67,68)
		OMPC	TLR2	(64)
			TLR4*	(63)
Pneumococcal conjugate vaccine (PCV7)	6 weeks	Alum	NALP3/INFL	(67,68)
Trivalent inactivated Influenza vaccine (TIV)	6 months	dsRNA	TLR3*	(98)
		ssRNA	TLR7	(30,99)
		ssRNA	TLR8*	(31)
		dsRNA	RIG-1*	(100)
Measles, Mumps, Rubella (MMR) vaccine	12 months	hemagglutinin protein (Measles)	TLR2*	(65)
		dsRNA (Measles)	MDA-5*	(66)
Varicella vaccine	12 months	?	TLR2*	(101)
Hepatitis A (HepA) vaccine	12 months	Alum	NALP3/INFL	(67,68)
Meningococcal conjugate vaccine (MCV4)	2 years	bacterial porins	TLR2*	(102)

Note: The minimum age is given for vaccination (103).

Abbreviations: Outer membrane protein complex (OMPC), Inflammasome (INFL), Alum (Aluminium hydroxide), Melanoma differentiation-associated gene 5 (MDA-5), C-type lectin receptor (CTLs), Cell wall skeleton (CWS), single stranded (ss).

* PRR activation inferred based on published engagement of PRRs by wild type strains.

[†] CWS consists of a purified noninfectious material consisting of peptidoglycan, arabinogalactan and mycolic acids.