CHARACTERIZATION OF ALPHAVIRUS REPLICON PARTICLE BASED VACCINES AND ADJUVANTS IN A NEONATAL MURINE MODEL

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ABSTRACT

Syed Muaz Khalil: Characterization of alphavirus replicon based vaccines and adjuvants in a neonatal murine model
(Under the direction of Laura J. White)

Infectious diseases are the leading cause of childhood morbidity and mortality worldwide. Not only is there a lack of vaccines for many deadly diseases, early life immunization with the available vaccines is frequently ineffective and requires multiple doses to induce protective immune response. Additionally, the neonatal immune responses to infection and vaccination are biased towards T_H2 at the cost of pro-inflammatory T_H1 responses needed to combat intracellular pathogens. However, upon appropriate stimulation, the neonatal immune system can induce adult-like T_H1 responses. We hypothesized that non-propagating Venezuelan equine encephalitis virus replicon (VEE)-based vaccine platforms (VRP) are good candidates for safe early life immunization, based on their ability to (1) target dendritic cells (DC) in the draining lymph node and (2) induction of robust innate immune response through intracellular amplification of replicon RNA.

In the VRP expression vector, the same replicon particle functions to deliver the antigen and to serve as innate immune-stimulant. On the other hand, in the VRP as adjuvant (GVI3000), the replicon particle only provides the danger signal function, while the antigen is not expressed from the replicon particle, but co-delivered as purified protein or inactivated virions. By separating the antigen delivery function from the adjuvant function in the GVI3000, we were

able to demonstrate the role of the adjuvant function in the VRP, and to start to understand how it mediates the changes in the quality and magnitude of the immune response in the neonate.

In this thesis, using a neonatal mouse model, we showed that both platforms induced effective and protective immunity against two different infectious agents, influenza virus and dengue virus. Furthermore, VRP replicon particles served as useful tools to better understand the induction of the neonatal immune response.

In summary, the VRP expression vector vaccines and adjuvants induced an adult-like adjuvant effect against dengue influenza antigens, and have the potential to improve the immunogenicity and protective efficacy of new and existing neonatal vaccines.

I dedicate this work to my parents, Mozaffar and Farzana, my wife, Sarah, my brothers, Musab and Muasir, my sister-in-laws, Sumaiya and Aiysha, my kids, Ahmed Zakareya and Rayhana, my nieces and nephew, Zara, Isra, and Yusuf, and last but not the least, my father- and mother-in-law, John and Nancy.

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LIST OF COMMONLY USED ABBREVIATIONS

Ab	Antibody
Ad	Adenovirus
APC	Antigen presenting cell
ASC	Antibody-secreting cell
AST	Average survival time
BMDC	Bone marrow dendritic cell
cDC	Conventional dendritic cell
CFA	Complete Freund's adjuvant
CHIK	Chikungunya virus
CNS	Central nervous system
CRBC	Chicken red blood cell
CT	Cholera toxin
CTL	Cytotoxic T lymphocyte
CSE	Conserved sequence element
DC	Dendritic cell
DENV	Dengue virus
DHF	Dengue Hemorrhagic Fever
DLN	Draining lymph node
DNA	Deoxyribonucleic acid
DSS	Dengue Shock Syndrome
EID	Egg infectious dose
ELISA	Enzyme-linked immunosorbant assay

ELISPOT	Enzyme-linked immunospot assay
ER	Endoplasmic reticulum
FDA	Food and Drug Administration
GALT	Gut-associated lymphoid tissue
GFP	Green fluorescent protein
GVI3000	VRP adjuvant with V3000 envelope
GVI3A	VRP adjuvant with mutations in the nt3 of 5' UTR
HA	Hemagglutinin
HAI	Hemagglutinin inhibition assay
HEV	High endothelial venules
Hib	Haemophilus influenzae type b
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
hpi	Hours post infection
HS	Heparin sulfate
iDC	Inflammatory dendritic cell
iDENV	Inactivated dengue virus
iFlu	Inactivated influenza virus
IFA	Incomplete Freund's adjuvant
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
i.c.	Intracranial

IPV	Inactivated polio vaccine
IRF	Interferon regulatory factor
ISCOM	Immune stimulating complex
ISG	Interferon stimulated gene
IU	Infectious units
kb	Kilobase
KO	Knockout
LAV	Live-attenuated vaccine
LC	Langerhans cell
LOD	Limit of detection
LRT	Lower respiratory tract
MAdCAM-1	Mucosal addressin cell adhesion molecule-1
MALT	Mucosal-associated lymphoid tissue
MHC I	Major histocompatibility complex I
MHC II	Major histocompatibility complex II
MLN	Mesenteric lymph node
MV	Monovalent
MVA	Modified vaccinia Ankara
NAb	Neutralizing antibody
NHP	Non-human primate
NK cells	Natural Killer cells
NRAMP	Natural resistance-associated macrophage protein
nsP	Nonstructural protein

nt	Nucleotide
NTR	Non-translated region
ONN	O'nyong-nyong virus
OPV	Oral polio vaccine
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cell
PRR	Pattern recognition receptor
RKO	Receptor knockout
RNA	Ribonucleic acid
RRV	Ross River virus
RT	Room temperature
SEM	Standard error of the mean
SFV	Semliki Forest virus
SHIV	Simian human immunodeficiency virus
SIgA	Secretory IgA
SIN	Sindbis virus
SIV	Simian immunodeficiency virus
TB	Mycobacterium tuberculosis
TCID	Tissue culture infectious dose
TGF	Transforming growth factor

T_H1	T-helper 1
T_H2	T-helper 2
TNF	Tumor necrosis factor
TLR	Toll-like receptor
TV	Tetravalent
URT	Upper respiratory tract
VEE	Venezuelan equine encephalitis virus
VRP	VEE replicon particles
WHO	World Health Organization
YFV	Yellow fever virus

CHAPTER 1: INTRODUCTION

1.1. Vaccination and Immunology

1.1.1. Brief History

The earliest recorded use of vaccination can be dated back to 1000 *A.D.* in ancient China, where individuals who were naïve to smallpox vaccination were inoculated with the content from smallpox vesicules, pustules or scabs (1, 2). Later records show this practice to be widespread in parts of Asia and North Africa (1, 3). The introduction of vaccination in England is credited to Lady Mary Wortley Montagu—the wife of English ambassador to the Ottoman Empire—who observed its practice in Constantinople in early eighteenth century (1). During that period, smallpox was the cause of 8-20% of all deaths in both rural and urban areas (4). However, it was well-known at the time that milkmaids who had shown signs of pox-like infection acquired from cows they milked, were well-protected from the worst symptoms of smallpox disease (1, 5). Edward Jenner, a rural physician in the late eighteenth century, used this knowledge to draw a link between the mild cowpox infection and protection from smallpox infection.

In 1796, Jenner inoculated an 8-year old boy with cowpox virus taken from an infected milkmaid and challenged the child "some months afterwards" with smallpox (1, 4). The boy was protected and showed no sign of severe smallpox disease (4). The work of Edward Jenner was honored by Louis Pasteur—one of the most influential vaccinologist—by coining the term

vaccine (1). The work started by Jenner has led to smallpox being the only communicable disease to be completely eradicated from its natural environment (5).

Following the example of Jenner, in 1881, Louis Pasteur observed that culture plates with *Pasteurella septica*, which causes a lethal form of cholera in chicken, left on the lab bench for two weeks, did not lead to illness in chickens (2, 3). In fact, the inoculated chickens were protected after infection with fresh *P. septica* cultures. This observation led to Pasteur's conclusion that external insults can cause changes in bacteria leading to its attenuation but not any loss of immunogenicity (2, 3). Following these results, Pasteur replicated his success with vaccination against anthrax and rabies (1-3). In the twentieth century several pathogens were attenuated for vaccination, including *Mycobacterium bovis* by Calmette and Guerin in 1921 (6) and yellow fever virus by Max Theiler (7). It is a remarkable feat achieved by the pioneers of vaccination to have developed several highly effective live, attenuated vaccines given that the knowledge of pathogen and host immunity was so limited.

1.1.2. Immune Response to Vaccination

The pace of vaccine development increased exponentially in the twentieth century resulting in vaccines against several lethal diseases such as diphtheria (1923), pertussis (1926), tetanus (1927), Influenza A (1936), paralytic poliomyelitis (1955), measles (1963), mumps (1967), rubella (1969), Haemophilus influenza type b (Hib) (1988), and Hepatitis A (1991) and B (1981) (2, 8). This could be partially attributed to the development of virus propagation in cell cultures by Hugh and Mary Maitland in 1928 (2). This and other advances have continued the pace of vaccine research unabated to this day. Widespread use of vaccines has been credited with increasing the human life expectancy in the 21st century (9, 10). The benefit of immunization is

especially evident in early life as it is estimated that early life immunization is saving 3 million children a year worldwide (4). It is estimated that approximately 80% of children were immunized at birth in 1990, up from 5% estimate in 1974 (4). However, many more lives could be saved if existing immunization programs were implemented properly (4).

Immune response to an effective vaccine is multi-faceted and complex. Our knowledge of how vaccines work is growing but still limited. Vaccines that are currently available were, for the most part, developed empirically with little understanding of the host immune response (11). The primary effector of vaccine-mediated immune response is antigen-specific antibody. However, several factors are important during an antibody response, including the quality, location, persistence etc. of antibody response. Persistence of antibody response underscores the important role B-cells play in effective immunity. Furthermore, T-cells also play an important role in the induction of high-affinity antibodies and immune memory, in addition to being direct effectors themselves.

1.1.2.1. Antibody-response

Vaccine-induced antibodies can mediate a protective immune response in the serum by minimizing or eliminating the spread of the pathogen systemically. IgG antibodies in the serum are primarily responsible for reducing the threat of viremia or bacteremia (12). The important role played by vaccine-induced systemic antibodies is evidenced by several examples. Hepatitis A virus infection has been shown to be prevented after passive administration of immune serum IgG in humans (13). In fact, if IgG is administerd during the incubation period of hepatitis A, it can reduce the severity of the disease and potentially convert clinical infections into subclinical incidents (14). Studies in non-human primates show that serum antibodies can protect from

paralytic poliomyelitis virus. Sera from a hyperimmune NHP was able to prevent virus spread to the CNS and reduce viremia in passively immunized animals, even though viral replication in the gut was no different from control animals (15). Several other studies also demonstrate the protective effect of systemic serum IgG including to pertussis (12), yellow fever virus infection (16), rabies virus infection (17), meningococcal polysaccharides (18), pneumococcal polysaccharides (19), and typhoid polysaccharides (20). Serum IgG against bacterial toxins, such as diphtheria and tetanus, have also been demonstrated to provide protection (21, 22).

Antibodies can also have a pathogenic effect as seen after dengue infection. Sub-neutralizing antibody concentrations against an infecting dengue virus serotype can enhance the risk of severe dengue disease, such as dengue hemorrhagic fever or dengue shock syndrome (23-25). Therefore, any live-attenuated vaccine design has to overcome this challenge by inducing protective antibody response against all four dengue serotypes simultaneously. Issues related to dengue vaccines are addressed in more detail below.

1.1.2.2. Mucosal response

Mucosal surface includes all of body's mucus-covered epithelia that is exposed to the external world, such as those of respiratory, gastrointestinal, and urogenital tract as well as the exposed cornea/conjunctiva (26). These surfaces play a crucial role in preventing infections while simultaneously facilitating in the uptake of nutritional components and respiratory exchanges (27). In terms of surface area, the combined mucosal surface is the largest immunological organ of the human body and as such 90% of all infections initiate at the mucosal surfaces (26). In general, parenteral route of immunization does not lead to efficient mucosal immunity, even if the immunization leads to a systemic immune response (3). The two most

practical and effective ways of mucosal immunization are through oral and nasal routes (28). An example of an effective oral vaccine is OPV, which replicates in the gut and delivers antigen to the gut-associated lymphoid tissues (GALT), inducing a potent intestinal immune response. Immunization through nasal route has also been efficient, such as for cold-adapted influenza virus vaccine (29).

Although all antibody isotypes are present at the mucosal surfaces, IgA antibodies are the predominant isotypes in the mucosal secretion (30-32). The adaptive immune response at the mucosa initiates at the mucosal-associated lymphoid tissues (MALT), including the production of IgA-secreting B-lymphocytes (27). Furthermore, antigen transport to the MALT also starts the process of lymphocyte activation with effector functions at the mucosal surface (27).

1.1.2.3. T-cell response

Most current vaccines mediate protection through highly specific serum IgG antibodies (11). However, for some diseases such as tuberculosis, a passive antibody-mediated immune response is inefficient and T-cell induction by BCG vaccination is the primary effector of protection (33). There is evidence in other cases where vaccine induced T-cells contribute in mediating protection. Long-term protection in children immunized in infancy with pertussis vaccination seems to be from CD4 T-cells, after the antigen-specific antibodies have waned (34-37). Measles immunization of infants is another example where antigen-specific antibody response is inefficient either due to immune immaturity of infant immune system or maternal antibody interference. Immunized children remain susceptible to measles infection, but are protected against severe disease, which is attributed to the induction of IFNγ producing CD4 T-cells (38-40). These examples show that while prevention of infection can only be achieved by

antigen-specific antibodies, T-cells can prevent complications and reduce the severity of disease in their absence. Other examples where both vaccine-induced antigen-specific antibodies and T-cells influence the outcome of infection are rotaviruses (41-43) and influenza viruses (44-46).

CD8 T-cells play a very important role in the clearance of virus- and bacterial-infected cells in chronic infections (47, 48). There are several examples where these cell-mediated immune responses can provide protection after primary infection or after reactivation from latency, such as varicella-zoster virus (49), herpes simplex virus type 1 (50), herpes simplex virus type 2 (51), Epstein-Barr virus (52), cytomegalovirus (53), and hepatitis C virus (54).

1.1.2.4. Innate immune response

The innate immune system plays a crucial first step in initiating the anti-pathogen immune response. Although it does not have the specificity or the memory of the T- and B-cells antigen receptors of the adaptive immune response, pattern recognition receptors (PRRs) of the innate immune response permit detection of a very wide range of potential pathogens (55). Both T- and B-cells employ multiple PRRs and utilize the cells and molecules of the innate immune system for their effector functions (55). PRRs, such as toll-like receptors (TLRs) in the endosome or RIG-I/MDA5 in the cytosol, control many DC functions and activate signals that initiate the adaptive immune response (56-58). In the context of vaccination, activating the appropriate PRRs and innate immune signaling is crucial in determining the strength, duration, and type of adaptive immune response. Live-attenuated vaccine approaches are usually the best inducers of humoral, cellular, and mucosal immune responses because they, like wild-type organism, can stimulate the innate immune response. However, for other gene-based vaccines

approaches, such as non-replicating antigens, an adjuvant is required for the stimulation of the innate immune system.

1.1.2.5. Herd immunity

Herd immunity is the common term applied to the concept that not all individuals in a given population have to be immune to a certain disease to protect all members (59, 60). However, there is an important distinction that should be made between *herd immunity* and *herd protection* (61). Herd immunity implies a secondary spread of attenuated pathogen to unimmunized individuals from those that have been immunized, as was observed with OPV (62). On the other hand, herd protection would mean that unimmunized individuals are protected or have a lesser probability of infection—due to immunized individuals in the population—without actually developing immunity against the pathogen (61). Regardless of the term used, herd immunity (or protection) is an important factor in any vaccine development and immunization program. Three factors that should be taken into account to develop herd immunity in a population prior to immunization are: the infectivity of the disease agent (highly infectious pathogens requires higher immunization rates), the vulnerability of the population (urban, crowded areas will require higher immunization coverage), and environmental factors (disease such as meningitis is more prevalent during winter months and hence require better protection during those months) (59).

1.1.3. Early Life Immunology and Challenges

The early life (<5 years) is an extremely sensitive period of human life in terms of mortality due to pathogenic infections (63-65). It is estimated that in 2012 approximately two-

third of the mortality in children under the age of 5 years was due to infectious diseases (66). The burden of infectious diseases in newborns is especially high. The main underlying reason that makes newborns vulnerable to infections is their unique immune system which is adapted for postnatal life (67). Immunologically, humans are born with an immune system that favors tolerance at the cost of weak immune responses to infections, especially during the first month of life (neonatal) (68-70). The neonatal immune system gradually matures towards an adult-like immune system during infancy (<1 year of age). The susceptibility during neonatal and infant period could be due to the lack of immunological memory in the newborns. Research also shows that—compared to adults—newborns have: smaller number of immune cells in peripheral lymphoid tissues, qualitatively distinct immune cells, different proportion and phenotypes of immune cells and their subtypes, as well as deficiencies or "immune deviations" among APCs, T-cells, and B-cells (reviewed in reference (68)). When challenged with immune stimuli, children under the age of 2 months show T_H2 cell polarization, weak T_H1 polarization, and low innate antiviral type I IFN responses (71). Despite the unique nature of neonatal immune system, it is now believed that—given appropriate stimuli—neonatal immune system can mount at least a T-cell response which is comparable to an adult *in vivo* (72-74).

1.1.3.1. Adaptive immunity

Research in both neonatal mice (<7 days) and neonatal humans (<28 days) have shown that the T-cell-dependent and –independent antibody responses during this period is different from adults (reviewed in references (69, 70)). The antibody response in neonatal period is reported to be delayed, of lower magnitude, short-lived, qualitatively different (IgG2a in mice and IgG2 in humans are reduced indicating a T_H2 bias), have lower average affinity, and reduced

heterogeneity (68). Maternal antibodies in human infants can further reduce the antibody response, most probably by removing the immunizing antigens (75, 76).

The differences in antibody response between an adult and neonate can be attributed to differences in either the B-cells or inadequate response by T_H cells. B-cell population in neonatal spleen has been shown to be primarily immature, which fails to upregulate co-stimulatory markers such as CD86 and MHCII (69, 77). However, other studies have shown the neonatal B-cells in the lymph nodes and the periphery tend to be more 'mature' and show less of the defects displayed by splenic B-cells (68, 78). Vaccines that target the B-cells in lymph nodes might induce adult-like antibody response in neonates (79).

There have been other explanations for the poor antibody response in neonates. In case of T-cell independent antigens, B-cells in the marginal zone are the primary responders in adults (80). However, marginal-zone B-cells are rare in neonatal spleens and appear at 1-2 weeks in mice and 1-2 years in humans, coinciding temporally with the acquisition of ability to mount antibody response to polysaccharide antigens (81-83). On the other hand, for T-cell dependent antigens, adult-like antibody response in neonates is possibly hampered by the absence—at birth—of three crucial splenic microarchitectural structures: lymphoid follicles, follicular-dendritic-cell (FDC) networks, and germinal centers (84). These structures start developing after birth in a complex interplay of cytokines and lymphocytes with germinal centers appearing by the end of 3 weeks in mice, coinciding with the time when mice are capable of mounting adult-like antibody response (85). In human infants, the germinal center appears around 4 months post-birth (86). It can be concluded that the development process of microarchitectural structures, rather than any innate deficiency in the B-cells, might be the reason for deficient antibody response in neonates.

Regardless of the pace of neonatal immune system development post-birth, it has been reported that antigen-specific memory B-cells can be induced in early life. Human infants when immunized with oral poliomyelitis virus (OPV) vaccine at three days old had very weak primary antibody response, which was significantly enhanced after a second dose two months later compared to unprimed infants (87). Similarly, antibody response to hepatitis B immunization showed memory response after the administration of the second dose (88). In contrast, tetanus toxoid immunization in human neonates did not show any antibody enhancement after booster shots during infancy indicating no memory response (89). Therefore, the development of memory B-cell response might be dependent on the antigen, the delivery system, or the adjuvant.

It is well established that the T-cell response in neonatal mice is biased towards T_H2 cell lineage (68). However, well known promoter of a strong T_H1 response, such as certain DNA vaccines and CPG motifs, have been shown to induce T_H1 responses in neonatal mice (reviewed in references (90-92)). The bias towards T_H2 immune response in human infants is not as clear-cut as in neonatal mice (68). However, just as in neonatal mice, human infants are capable of inducing adult-like T_H1-cell response to certain vaccines, such as for BCG (93, 94). Several reasons have been suggested for the apparent weak T_H1 response and the T_H2 bias of neonates. First, it has been shown that neonatal mice have at least 1-2 log reduction in total number of T-cells compared to adult animals (72, 73, 91), which might reduce their protective capacity. Second, antigen load can also determine the T_H response as reducing the antigen load of mouse leukemia virus from high to low switched the response from T_H2 to T_H1 (74). Third, the T-cell repertoire in neonates might affect the T_H response as significant numbers of peripheral T-cells in neonates in the first few weeks of life are generated by hematopoietic cells of fetal origin, which typically mount a T_H2 response (95-97). Similar results have been reported in human

neonates, where immune responses that develop to allergens *in utero* are T_H2 (98-100). Finally, it has been suggested that upon re-exposure to antigens, T_H1 cells from neonates undergo apoptosis skewing the T_H response to T_H2 (101).

For a long time, nenoates were thought to be deficient in CD8+ cytotoxic T lymphocyte (CTL) function (68). However, several studies have indicated that CD8+ CTL primary and memory response can be induced after a primary immunization in neonates: live replicating vaccines (74, 102-104), adult DCs (73), DNA vaccines (105-107), and oligonucleotides containing CpG motifs (108, 109). Human infants, congenitally infected with cytomegalovirus (110) or Trypanosoma cruzii (111), induce significant level of CTLs indicating that even in fetus, mature CTL activity is possible.

1.1.3.2. Innate immunity

Due to the absence of any immunological memory, neonatal innate immune system plays a crucial first step in initiating an immune response to pathogens or a vaccine. However, there are several differences between a neonatal and an adult innate immune system with important implications for the generation of an effective adaptive immune response. The predominant T_H2 microenvironment of the fetus influences the neonatal T_H1/ T_H2 profile by skewing the predominant response to T_H2 (112, 113). In contrast to T_H1 cytokines, The T_H2 cytokines during pregnancy at the maternal-fetal interface are important for the success of pregnancy (114). The primary mechanism by which neonatal T-cells differentiate into T_H2 cells is through secretion of high levels of IL-4 and low levels of IFNγ by T_H1 cells (68, 115-118). IL-4, in both humans and animals, inhibits the expression of IL-12Rβ2 in naïve T-lymphocytes, which leads to the inhibition of T_H1 response and the development of a T_H2 response (117, 119).

The role played by the innate immune cells in shaping the immune response is critical and is summarized next. Neutrophils are the most numerous polymorphonuclear leukocytes and are one of the first cells to target and eliminate pathogens (120). Neutrophils are qualitatively and quantitatively deficient in early life compared to adults in the following ways: impaired neutrophil recruitment due to high levels of anti-inflammatory T_H2 cytokine IL-6 in neonates (121), reduced phagocytic activity (122), reduced killing mechanism such as production of reactive oxygen species and antimicrobial proteins and peptides (123, 124), and diminished neutrophil rolling and adhesion, especially in preterm infants (125).

NK cell subsets of the cellular immune response are another line of defense against pathogens, especially in viral infections (126). Functionally, NK cells are important for cytolytic action and IFNγ production. However, NK cell functions—degranulation and release of lytic factors—are reduced in early life (127). It has been proposed that increased level of TGFβ, through inhibition of transcriptional control factors T-bet and GATA-3 and promoting differentiation of Treg cells, is responsible for the suppression of NK cell function and enhanced risk of infection in early life (128, 129).

It has been recently shown that immunosuppressive $CD71^+$ erythroid precursor cells can impair neonatal host defense against infection (130). The authors demonstrated that the ablation of $CD71^+$ erythroid cells in neonatal mice or their natural postnatal decline correlated positively with loss of immunosuppression, enhanced production of TNF upon stimuli by phagocytes and APCs, and increased resistance to pathogens, such as *L. monocytogenes* and *E. coli*.

APCs are at the interface between innate and adaptive immune response. However, studies have shown that there are several differences between neonatal and adult APCs. Human cord blood cells show significantly reduced LPS-induced TNF secretion compared to adult

PBMCs (131). The early life APC cytokine response is distinct from adults in that there is a high-level expression of anti-inflammatory IL-6 and IL-10 as well as macrophage production of immunosuppressive IL-27 cytokine (132). As professional APCs, DCs are essential in directing the adaptive immune response (133). Studies primarily performed in murine models demonstrate qualitative and quantitative differences between adults and neonates (134). *Ex vivo* culture of neonatal mice splenic cDCs show lower expression of MHC class II, CD86 and cytokines such as IL-12p70, IFNγ, and antigen processing and presentation ability (135). While pDCs in human cord blood and adult peripheral blood are comparable in quantity, the amount of cDCs is significantly reduced (136). As was observed in murine models, neonatal cDCs in the human cord blood show impaired TLR-mediated IL-12p70 production (137, 138), while both cDCs and pDCs have reduced TLR-mediated multifunctional response (136).

1.2. Need for Early Life Vaccines

One of the eight goals of the United Nations' Millennium Development Goals calls for reduction of mortality rate by two-thirds in children <5 years of age (65). Diarrheal and respiratory diseases caused by viral and bacterial pathogens are the common causes of mortality in early life (139). Additionally, malaria, tuberculosis, and HIV are also important childhood pathogens (140). Therefore, development of safe vaccines that can induce effective immunity to various infectious diseases soon after birth is a great public health challenge.

1.2.1. Influenza vaccines

Influenza is the most common cause of lower respiratory tract infections with approximately 48 million annual infection and 40,000 deaths (141). Influenza type A is the more

prevalent cause of morbidity and mortality, while influenza type B can cause regional epidemics of lesser severity than type A (142). Influenza virus initiates infection at ciliated columnar epithelial cells after breaching the mucous lining of the respiratory tract (143). The HA precursor protein cleaves into HA1 and HA2 by post-translational processing before membrane fusion (144). Although influenza infection is primarily targeted towards the trachea and upper respiratory tract, lung lesions can occur during a pandemic (145). The amount of virus produced is directly correlated to the extent and the severity of disease (142).

Immunity generated after a natural infection is primarily driven by serum antibodies as well as antibodies at the mucosal surfaces of the respiratory tract (142). Additionally, influenza specific T-cells play a role in the recovery (142). Finally, innate immune cytokines, such as interferon and other antiviral proteins also mediate protection from infection (142).

Licensed influenza vaccines are primarily of two kinds: live-attenuated and inactivated (146). Use of live-attenuated vaccine allows for a mimicking of a natural infection and the development of immunity against an attenuated viral strain, which minimizes the risk of a full blown infection (142). Inactivated vaccines are produced by inactivation of influenza virus with formalin or β-propiolactone followed by several purification steps to remove impurities (147). The effectiveness of influenza vaccines in preventing laboratory-confirmed symptomatic influenza varies form 50%-80% and depends on the age, immune status, and the relative similarity with the circulating strain (148). However, none of the currently available influenza vaccines are recommended for use in infants <6 months old (146). Additionally, although live-attenuated have been shown to be more effective in children 6-11 months old compared to inactivated vaccines, they have also been associated with safety concerns, such as increased rate

of wheezing and hospitalization (149, 150). There are currently no adjuvants included in licensed inactivated influenza vaccine formulations that can improve immune responses (147).

1.2.2. Dengue vaccines

Dengue virus (serotypes 1-4) is the cause of the leading mosquito-borne viral disease in the tropics and sub-tropics, with an annual estimate of approximately 390 million infections (151). Up to half a million people are hospitalized every year with severe dengue disease (Dengue Hemorrhagic Fever/Dengue Shock Syndrome; DHF/DSS), including a large proportion of children (152). Each dengue serotype can cause mild to severe disease. Additionally, infection with one serotype induces life-long immunity to that dengue serotype but short-lived immunity against other serotypes. As the immunity declines, the presence of non-neutralizing dengue antibodies becomes a risk factor for DHF/DSS. DHF/DSS occurs after primary infection in infants born to dengue-immune mothers (24) or upon a secondary heterotypic infection in older individuals (25). The leading hypothesis to explain DHF is the presence of sub-neutralizing antibody levels, which leads to an enhancement of dengue virus infection of Fc-receptor-bearing cells (also known as antibody-dependent enhancement) (23).

Neutralizing antibodies are thought to be the primary correlate of protection through prevention of host cell binding and target cell infection (153). However, a recent study reported that CD8+ T-cells correlated positively with protection in an HLA-specific manner (154). There are other considerations in designing and administering an effective dengue vaccine. Firstly, any effective dengue vaccine must be tetravalent and induce equivalent neutralizing antibody response to all four serotypes because of theoretical enhanced risk of severe disease if incomplete immunity is induced to one of the four components. Secondly, tetravalent vaccines

should avoid serotype interference whereby a dominant serotype in a live attenuated vaccine prevents other serotypes from inducing an effective immunity. Reports have described such serotype interference resulting in an unbalanced antibody response and need for additional immunizations to achieve a tetravalent response (155). Finally, vaccines that are targeted towards children <1 year old should also address the issue of maternal antibody interference that can occur if live attenuated vaccines are administered in children born to dengue-immune mothers and presumably have passively transferred antibodies.

Currently, there are no licensed dengue vaccines. However, several live attenuated vaccines are in various stages of clinical trial [concisely reviewed in (156)]. A point to note is that none of the vaccines in clinical trials are being promoted for use in children <1 year due to the various issues addressed above.

1.3. Current Vaccine Technologies

Most licensed vaccines fall into three categories: live attenuated (LAV), inactivated pathogen, and subunit/protein derived from infectious organisms (Table 1.1). Although some of them, like polio or smallpox and others, have been extremely efficacious, these vaccine technologies have not completely eliminated the threat of morbidity and mortality from infectious diseases. It is estimated that currently infectious diseases account for approximately 25% of all mortality (157). Therefore, there is a need for the development of vaccines against other pathogens and advancement of gene-based vaccines by improving DNA vaccines, vectored vaccines, and adjuvants for non-replicating antigens.

Table 1.1 - Current vaccine platforms

Vaccine platform	Benefits	Limitations	Examples used in humans
Live attenuated	Low cost No adjuvant needed Highest immunogenicity Humoral, cellular and mucosal Long-lasting immunity	Poor stability Possible reversion to pathogenic May be harmful in immune-compromised	Smallpox, oral polio, measles, mumps, rubella, hepatitis A, yellow fever, rotavirus, influenza
Inactivated	Better stability Safer, absent reversion	Higher cost No cellular or mucosal immunity Adjuvant needed Short lasting immunity Booster required	Influenza, polio, Japanese encephalitis, Tick-borne encephalitis, rabies
Subunit and Virus-like particles	Safe Multimeric antigen	Higher cost Adjuvant needed	Hepatitis B, human papillomavirus
Live recombinant vectors: Poxvirus Adenovirus Alphavirus Others	Very immunogenic Humoral, cellular and mucosal Safe (non-propagating vectors)	Ineffective in the presence of pre-existing immunity	In clinical trials
DNA-based	Antigens presented in the context of MHCI	Limited immunogenicity In humans Integration into the host genome is a potential risk	Not licensed for human use

1.3.1. Live-attenuated vaccines

LAVs are essentially a weakened form of the pathogenic bacteria or viruses generated in the lab. Although the LAVs were originally designed through serial *in vitro* passage or random chemical mutagenesis without knowing its effects or risk of reversion, more recent designs includes removing one or more genes that encode virulence or metabolic factors (158). Their design reduces the risk of symptomatic disease, while mimicking a natural infection. Therefore, they elicit a long-lasting potent humoral, cellular, and mucosal immune response, often after one or two dose(s). Despite its advantages, LAVs have poor stability, ability to revert to the pathogenic strain, and may cause complications in immune-compromised individuals. Some of the infectious diseases that have been successfully immunized against using LAVs are smallpox, oral poliomyelitis, mumps, rubella, influenza, etc.

1.3.2. Inactivated vaccines

Inactivated vaccines are produced after the pathogen is killed with chemicals (such as formalin), heat, or radiation. Because the pathogen is killed, there is no chance of reversion in inactivated vaccines. Additionally, these vaccines are very stable and can easily be stored. However, unlike LAVs, inactivated vaccines are not very immunogenic and require adjuvants to boost the immune response. Furthermore, immunization with inactivated vaccines may require multiple doses to maintain immunity. Inactivated vaccines that have been used in humans include influenza, polio, rabies, etc.

1.3.3. Subunit vaccines

Another class of vaccines is termed subunit vaccines, as they only contain antigens derived from a pathogen that best stimulate the immune system and not the entire pathogen. Therefore, subunit vaccines have even lower risk of adverse reaction. However, the process of determining the most immunogenic antigens and epitopes of a pathogen is a very time consuming and expensive process. Furthermore, subunit vaccines require adjuvant for efficient priming and induction of immune response. Hepatitis B is an example of subunit vaccines that have been approved for human use. Human papillomavirus vaccines (Gardasil and Cervarix) can also be categorized with subunit vaccines as they only contain the surface glycoproteins that interact to form virus-like particles.

1.3.4. DNA vaccines

Although they have been a relatively recent development, DNA vaccines have shown to be relatively safe and able to induce both humoral and cellular immunity in small rodents and

non-human primates (159, 160). Studies in humans have shown that DNA vaccines can induce a potent CTL response against malaria (161). DNA vaccines are also considered relatively stable and more cost-effective during manufacturing and storage (162). Other advantages that a DNA vaccine has includes the ability to immunize against multiple antigens using a single plasmid (162) and the stimulation of the innate immune system through the activation of TLR9 by unmethylated CpG motifs present on bacterial plasmid DNA (163). Finally, DNA vaccines can be advantageous in neonatal immunizations where passively transferred antibodies of maternal origin can interfere with vaccines, if the maternal antibodies recognize presented antigen (164). However, there are some limitations to DNA vaccines. DNA vaccines require a large dose to be effective in inducing immune response (162). Additionally, DNA vaccines are usually administered intramuscularly, which are effective in small animal models; however, they have been relatively inefficient in the induction of antibody response in humans and non-human primates (162).

In addition to the current vaccine technologies described above, viral expression vector vaccines have been extensively studied. Below we will describe in detail the use and effect of these potential vaccine candidates.

1.3.5. Viral expression vectors

One of the primary interests in vaccinology has been to understand the induction of immune response after a natural infection and mimic those after immunization. Towards that end, vaccinologists have used viruses as a tool for probing the immune system as well as for immunization. The advantage of using viruses for immunization is based on the fact that viruses have evolved to develop structures and mechanism that are highly effective in infecting cells,

expressing viral proteins, and sometimes replicating to high titers. These viral vector vaccines are generated using molecular genetic approaches and use their efficient cell entry and replication mechanism to express heterologous genes, which then act as the immunizing antigen [reviewed in (165-167)]. Vaccines that use bacterial genome as expression vectors have been reviewed elsewhere (168, 169). There are many examples of viral expression systems. Below we will focus on three of the most studied systems: poxviruses, adenoviruses, and alphaviruses.

1.3.5.1. Poxviruses

Poxviruses are linear, double-stranded DNA viruses that are relatively large with genome size of approximately 200,000 base pairs (170). As virus expression vectors, poxviruses have several advantages. First, poxvirus vaccine vectors are very stable and retain immunogenicity upon lyophilization, which overcomes an important hurdle in immunization, especially in the poor, developing world (171). Second, poxviruses can be easily genetically manipulated and can incorporate large amount of multiple foreign DNA, making them cost-effective (170, 172). And, thirdly, poxvirus vaccine vectors are quite versatile as they can induce effective immunity through multiple routes of immunization (170).

Preexisting immunity is a concern for any viral vector vaccine and so it is true for poxvirus vectors. However, in the case of vaccinia virus, individuals under the age of 30 are generally vaccinia-naïve, due to the cessation of smallpox vaccination in the early 1970s (170). Additionally, systemic immunity to poxvirus can be overcome through the mucosal delivery as the mucosal and systemic immune systems are distinct (171, 173, 174). As with most viral vaccine vectors, one of the most common ways to minimize the effect of preexisting immunity has been to implement a heterologous prime-boost strategy, and hence has been suggested for

poxvirus immunization as well. There are several poxvirus vaccine vectors, but the two most well known are modified vaccinia Ankara (MVA) (175) and NYVAC (based on the Copenhagen strain) (176).

Poxvirus vaccine vectors have been shown to be effective inducer of humoral and cellular immunity, including upon mucosal delivery (177). In several studies, poxvirus vaccine vectors induced significant immunity to several infectious diseases, including influenza (178), measles (179), SHIV (180). The potential of poxvirus vaccine vectors should be further pursued.

1.3.5.2. Adenoviruses

The adenoviruses (Ad) are non-enveloped icosahedral virus with linear, double stranded DNA genome of 36 kb length encoding more than 30 proteins (171, 172, 181). Ad have a variety of attractive features that make them attractive candidates for vaccination: stability (because they are naked, non-enveloped), ease of manipulation, growth to very high titers (purified up to 10^{12} particles per ml), simple purification, broad tissue tropism, and they do not integrate into the host genome while existing extra-chromosomally (171, 181). Similar to poxviruses, Ad retain immunogenicity after lyophilization and can be delivered through all routes of immunization (171). While replication-competent Ad vectors are useful where viral replication is desired for high expression of immunogen using low doses, replication-incompetent Ad vectors can also achieve robust immune response albeit with high dose vaccination (181). Replication-incompetent Ad vectors are designed with either lack of structural genes in the genome or mutations in the structural components (182).

Currently, there are 51 serotypes with six species described for Ad, with Ad2 and Ad5 causing approximately 5-10% of all upper respiratory tract infections reported in children (172).

Because they are so prevalent, pre-existing immunity could be a concern for Ad vector immunization. Additionally, Ad vectors, especially replication-competent, have limited capacity to carry heterologous antigens (165). Inflammation in the host due to induction of multiple signaling pathways can lead to dose-dependent toxicity (181). Also, at high multiplicity of infection replication-defective Ad vector can self-replicate, leading to further inflammation and tissue damage (183). Researchers have suggested use of different Ad strains to circumvent pre-existing immunity as well as oral inoculation to minimize the potential of a systemic hyper-inflammation (165, 171). Ad vectors have been demonstrated to be effective in rodent and NHP models against several pathogens, such as malaria (184), Ebola (185), SHIV (186), measles (187), and certain cancers (188). Due to the potential that Ad vector vaccines hold in the treatment of both infectious diseases and cancers, further studies should continue towards developing new candidate vaccines.

1.3.5.3. Alphaviruses

Alphaviruses have a small genome (approximately 12 kb in length), and are single stranded, positive sense, RNA viruses belonging to the Togoviridae family. Due to their relatively small size and icosahedral nucleocapsid, the heterologous gene sequence that can be added to alphavirus vectors is limited, with heterologous genes of 1 kb length are stable upon viral passage (189). Three alphaviruses, Sindbis virus (SIN), Semliki forest virus (SFV), and Venezuelan equine encephalitis (VEE) have shown potential as expression vector vaccines against infectious disease and cancer (reviewed in (190-196)). Both propagation competent and propagation defective alphavirus vectors have been designed and studied. In the propagation defective alphavirus vectors, the replicon genome with structural genes substituted by an

immunizing gene, is packaged into virions which can infect cells but not produce new virus particles (190).

Alphaviruses vectors are highly immunogenic as they express high levels of heterologous antigens and by targeting DCs (197, 198) induce potent humoral, cellular, and mucosal immunity (190-196). Additional advantages of Alphaviruse vectors are their ability to induce innate immune signaling and relative absence of preexisting immunity in the general population (167, 199). Some of the protective immune response that have been demonstrated include against influenza virus (200-202), Ebola virus (203), and SIV (204, 205). A detailed discussion of one of the alphavirus replicon vector, VEE, is presented below in detail.

1.3.6. Adjuvants

The first instance of adjuvant use was done by Ramon in 1925, when it was demonstrated that it was possible to increase antigen-specific levels of diphtheria or tetanus anti-toxin levels by adding bread crumbs, tapioca, agar, starch oil, lecithin, or saponin [reviewed in (206)]. The few adjuvants that are currently in use, such as aluminum salts and oil-in-water emulsions, were developed empirically and there is still much to be discovered about their mechanism of action (207). However, effects of numerous adjuvant candidates have been described in humans: improved vaccine efficacy, increase in humoral and T cell response, longer duration of protective effect, increased cross-protection within a pathogenic strain or between strains, antigen dose sparing, reduced number of vaccine doses to reach protective threshold, and modulating the antibody isotype response as well as T-cell (T_H1, T_H2, T_H17, etc.) responses (208). Adjuvants can be subdivided into several general categories: mineral salts, oil-emulsion, plant derivatives, microbial derivatives, particulate, endogenous immunostimulant, and synthetic (Table 1.2). We

introduce VEE replicon-based adjuvant as a new category of bio-adjuvant. Below, we will briefly introduce a few examples of some of the above listed adjuvant categories.

Table 1.2 - Current adjuvant technologies

Type of Adjuvant	General Examples	Specific Examples (brand name)	Mechanism of action
Mineral salts	Aluminum hydroxide Aluminum phosphate	Alhydrogel (Alum) Adju-Phos	Depot
Emulsion – Oil in Water	Freund's Incomplete adjuvant	Montanide ISA 51	Depot
Emulsion – Water in Oil	Saponins	QS-21	Depot
Microbial derivatives	Bacterial DNA Bacterial exotoxins Endotoxin-based adjuvants	CpG oligonucleotides [TLR9] Cholera toxin (CT) Monophosphoryl lipid A (MPL) [TLR4]	Immunostimulatory
Particulate	Immunostimulatory complex (ISCOMs) Virosomes, Liposomes, Proteosomes	ISCOMATRIX	Immunostimulatory
Endogenous immunostimulant	Cytokines	IL-2, IL-12, IFNγ	Immunostimulatory
Synthetic Pathogen-Specific Molecular Pattern based Adjuvants	Imidazoquinoline-like molecules Synthetic polynucleotides	Imiquimod [TLR7], Resiquimod [TLR7/8] Poly A:U, Poly I:C [TLR3]	Immunostimulatory
Bio-adjuvants	VEE replicon particles (?)		Mimics early events of natural infection

1.3.6.1. Mineral salts (Alum)

Aluminum-based mineral salts, or alum, have been in use for almost a century and are the only adjuvants approved for use in licensed human vaccines by the FDA in the U.S. (206). The mechanism of action of alum is still in debate. However, it has been suggested that alum mediates its effect through "depot" effect, whereby antigens are retained in alum-induced (together with clotting agent fibrinogen) depot at the injection site and released slowly (209, 210). However, it has also been recently demonstrated that upon vaccination in fibrinogen-deficient mice, although alum depots were not formed, the antibody response was comparable to wild-type mice (210). This study shows that alum depots are dispensable for the adjuvant effect of alum, although it is still possible that alum can form "antigen depots" in the draining lymph nodes following transport from the site of immunization (211). The primary effect of alum is to regulate T_H1/ T_H2 environment (212) by promoting T_H2 cytokines, in particular IL-4, and

improving T_H2 IgG subtype, which is IgG1 in mice (213, 214). It has been recently shown that the mechanism of alum innate immune activation goes through NLRP3 inflammasome activation (215, 216). Further investigations demonstrated that the initial activation of the T_H2 immune response by alum occurs through PI3 kinase activation and subsequent inhibition of IL12p70 secretion by DCs, a key cytokine that drives naïve T_H response towards T_H1 (217). Although there has been tremendous amount of discovery in alum adjuvanticity, further research is needed to understand the immunomodulatory effects of alum compounds and the protective antibody response it elicits.

1.3.6.2. Emulsion (Freund's Adjuvants)

Freund's adjuvant—incomplete (IFA) and complete (CFA)—were discovered over 50 years ago and have been experimentally used in various studies (218). However, much remains unknown about its mode of immune activation. While IFA is composed of a paraffin oil surfactant mixture that forms a water-in-oil emulsion when mixed with an antigen, CFA additionally contains heat-killed Mycobacterium preparation, which further increases its immunogenicity (218). Both IFA and CFA induce potent humoral response, but only CFA induces cell-mediated immune response, suggesting that the Mycobacterium preparation drives the that response (218). Furthermore, the cytokine profile in CFA is more T_H1 than IFA (219).

There are some serious concerns with regard to the safety of both IFA and CFA as they have been associated with moderate to extreme local inflammation upon immunization in animal models and human subjects (218, 220). They have also been associated with the formation of granulomas or tubercles, which was dependent on the induction of TNF by the adjuvant (218,

221, 222). Although potent adjuvants, safety concern should be further addressed before Freund's adjuvants can be a candidate for human vaccination.

Saponins are triterpene glycosides obtained from the bark of *Quillaja saponaria* Molina tree, which are native to South America (223). Quil A is a partially purified saponin used as adjuvants in veterinary vaccines (224). Quil A has also been used as a component of immune-stimulating complexes (ISCOMs), which are small (40nm), cage-like particles additionally containing cholesterol and phospholipids (225). Although ISCOMs are very potent adjuvants, there are some safety concerns due to toxicity of Quil A in mice (225). Nonetheless less toxic saponins as part of ISCOMs are in development for human use (226).

1.3.6.3. Microbial derivative (CpG) and other TLR ligands

CpG motifs are considered pathogens associated molecular patterns (PAMPs) because they are ubiquitous in microbial genome but rare in vertebrates (227). They are very potent adjuvants of innate immune system inducing protection in both rodents and primates (163, 228). CpG are TLR agonists that activate TLR9. Just as any other TLR agonist (except for TLR3), TLR ligation initiates a complex signaling pathway involving MyD88, resulting in transcription factor NF-kB activation and the release of pro-inflammatory cytokines IL-6, IL-12, and TNF-α (229, 230). Immune effect of CpG includes increased cellular migration towards draining lymph nodes (231), higher expression of DC co-stimulatory markers (232), higher cytokine production (233), production of IgG2a antibodies (234), and enhanced cell-mediated immune response (206). In summary, CpG are a promising candidate as adjuvants due to their potent immune response.

Other TLR ligands have been unwittingly used in vaccines for decades, evidenced by the

fact that vaccines containing bacterial cell walls, bacterial DNA, or viral RNA, can engage distinct TLRs and activate immune cells (235). Specific microbial molecules have been defined as ligands for these TLRs: lipopolysaccharides (LPS) (recognized by TLR4), flagellin (TLR5), and single (TLR7/8) or double-stranded RNA (TLR3) (236).

1.4. Venezuelan Equine Encephalitis Virus (VEE)

Venezuelan equine encephalitis virus (VEE) is a single stranded positive-sense RNA virus of the genus *alphavirus* and family *Togoviridae*. While alphaviruses are mosquito-borne infections, VEE is maintained in nature in an enzootic cycle between mostly *Culex* mosquitoes and small rodents (171). Alphaviruses are divided into two groups, Old World and New World viruses depending on their geographic origin. VEE is considered a member of New World alphavirus as it is endemic in Central America and the northern regions of South America and represents a significant burden of disease in these regions. The Old World alphaviruses, such as Sindbis virus (SIN), Semliki Forest virus (SFV), Ross River virus (RRV), Chikungunya virus (CHIK), and O'nyong-nyong virus (ONN) cause a somewhat different disease with arthritic and/or arthralgic symptoms, as well as fever and rash (237).

VEE was first isolated from infected equine brains in 1938 with the first documented case of human infection reported in the 1950s (238). VEE has caused sporadic outbreaks of febrile and neurological disease in both human and equid population throughout the past century (238). While the case mortality in humans is 0.5-1%, in equid populations, the case mortality can be as high as 50% (239). Early attempts to prepare vaccines through formalin inactivation of VEE were unsuccessful until the development of live-attenuated TC-83 vaccine in 1961 (240). TC-83 was prepared by passaging the virulent IAB strain 83 times in cell culture of guinea-pig heart

cells (240). TC-83 and its inactivated version C-84 have been used to vaccinate equines and humans with TC-83 proving to be extremely immunogenic (238). Due to the potential of outbreaks and its use as an agent of biological weapon (238), VEE biology has been extensively studied.

1.4.1. VEE Genome and Replication

VEE is a single stranded, positive-sense RNA virus with a genome of approximately 11.5 kb length capped with a 5' terminal 7-methylguanosine and 3' polyadenylated tail (241). The genome is divided into two regions: 5' two-third contains the viral non-structural proteins (nsp1-4) in a single open reading frame, while the 3' one-third contains the structural components (capsid, E2, 6K, and E1 proteins) on a second open reading frame expressed from a subgenomic 26S promoter (242). There are four conserved sequence elements (CSE), important for replication, spread throughout the genome: two near the 5' end, one between non-structural and structural gene cassettes, and one immediately upstream of poly-A tail (243). Additionally, one short non-translated region (NTR) at each 5' and 3' terminal ends are also important for replication (242). VEE genome is encapsidated in an icosahedral nucleocapsid surrounded by envelope containing spikes formed from trimers of glycoproteins, E1 and E2, heterodimers (242). While the molecular mechanism of VEE replication is relatively understudied, other alphaviruses, especially SIN and SFV, have been extensively studied. The review below summarizes the findings in other alphaviruses with VEE mentioned where appropriate.

The receptor for VEE attachment is currently unknown with c-type lectins (244), laminin receptor (245), and heparin receptor (HS) proteoglycans (246) proposed as candidates. Recently, natural resistance-associated macrophage protein (NRAMP) has been suggested as the cellular

receptor for SIN in both mammalian and insect cells (247). However, the authors also reported that RRV infection was NRAMP-independent, indicating that alphaviruses might use diverse set of receptors. Studies have indicated that E2 protein mediates attachment followed by entry through virus-cell membrane fusion in the early endosome. The fusion is mediated at low pH via a hydrophobic amino acid sequence in the E1-protein. Viral nucleocapsid release into the host cytoplasm occurs immediately after the receptor-mediated endocytosis (242). A viral fusion-independent entry mechanism has also been suggested for SIN, whereby following attachment the virus directly releases its genome into the plasma membrane without inducing membrane fusion or disassembling the viral protein shell (248).

Since alphavirus genomic RNA acts as a messenger RNA, immediately after release into the cytoplasm, the host translation machinery translates the viral genome into the precursor P123 or P1234 (249). The predominant species produced is P123, while 10-20% readthrough of the opaI codon at the nsp3-nsp4 junction produces P1234 polyprotein in SIN (242). The P1234 polyprotein is rapidly cleaved at the nsp3/4 junction by the *cis*-acting domains in nsp2, resulting in P123 and nsp4 production (242). Minus sense RNA strand is produced from a complex of P123 polyprotein and free nsp4, the viral RNA-dependent RNA polymerase, initiating at the 3' CSE of the parental genome within 3-4 hours of infection (242). The production of minus strand is rather limited, due to the presence of few parental genomes and limited nsp4 quantity (250). Subsequently, P123 polyprotein is cleaved at nsp1/2 and nsp2/3 junctions yielding nsp1, nsp2, and nsp3 (251). Production of the nsp1-3, together with nsp4, irreversibly shifts the viral machinery to start the production of positive-sense genome (252). This leads to the production of two RNA species using two different promoters: 49S located in the 3' NTR of the minus strand produces full-length genomic RNA and the 26S located in between nonstructural and structural

gene cassette yields subgenomic RNA (242). The subgenomic RNA is produced in a 5-10 fold molar excess of the genomic RNA ensuring adequate production of structural components for assembly of new virions (242).

The translation of subgenomic RNA produces structural polyprotein capsid-PE2-6K-E1 followed by immediate cleavage of the capsid protein. The capsid protein binds the genomic RNA at the encapsidation signal (253). One molecule of genomic RNA interacts with 240 copies of the capsid protein in the cytoplasm to form the nucleocapsid (238).The rest of structural polyprotein (PE2-6K-E1) then translocates to the ER (254). After folding and conformational changes, the viral E1 and E2 are glycosylated (255). Subsequently, the viral glycoproteins egress from the ER into the Golgi network followed by passage into trans-Golgi network, where furin cleavage of PE2 yields mature E2 glycoproteins (256). Initiation of virus budding occurs when nucleocapsids, which diffuse throughout the cytoplasm, encounter and bind the E2-E1 complex at the plasma membrane (257). Overall, the alphavirus infection process leads to inhibition of host RNA production, protein synthesis, and eventual apoptosis (237, 258).

1.4.2. VEE pathogenesis

VEE infection in equines and humans leads to a range of symptoms, from asymptomatic to acute and fatal encephalitis (238). Some of the factors that affect disease severity are patient age, immune status, and the virus serotype (259). The incubation of VEE can last from 2-5 days with symptomatic infections resulting in some of the following symptoms: fever, chills, malaise, myalgia in the thigh and back, nausea, vomiting, and severe retro-orbital or occipital pain. These symptoms subside within a week in the majority of cases; however, severe infections can result

in convulsions, stupor, and coma. Death occurs in <1% of cases accompanied by diffuse congestion and edema with hemorrhage in the brain, lungs, and GI tract (260).

Infection of rodents with VEE has yielded many similarities between the observed pathogenesis to those observed in humans and equines (261, 262). Because of the ease of handling mouse as well as the availability of molecularly cloned VEE and non-propagating VEE replicon particles (VRP), mice have been widely used to understand VEE pathogenesis (198, 263-266). VEE exhibits a biphasic disease in mice, similar to the pattern in equines, with an initial lymphotropic phase replication in the peripheral lymph nodes followed by infection of the CNS (neurotropic phase), which leads to lethal encephalitis by day 6-8 (261). Biphasic infection of VEE using both virulent and molecularly cloned mutants has been unequivocally demonstrated in mice (263, 266, 267).

The early steps in the pathogenesis of VEE and induction of innate immune responses by VEE were discovered using VRPs, which are single-replication cycle, propagation-incompetent virus particles. Using VRP expressing GFP (GFP-VRP), initial target cells were identified as Langerhan cells, as they stained positively for DEC-205 and MHCII (198). Due to the morphological similarity of the infected cells to DCs, the authors concluded that DCs appear to be the primary target of VEE infection. Furthermore, Tonkin et al. demonstrated that DCs were sufficient for the immune activity of VRP, with monocyte-derived inflammatory DCs (iDC) most frequently targeted and most dramatically recruited in the draining lymph nodes of mice (268). VRP infection of DCs has been demonstrated to occur at the site of injection followed by rapid transfer (approximately 4 hours) of the infected DCs to the DLN and replication in the DLN as early as 6 hpi (267). It is suggested that VRP can also travel through passive lymphatic drainage to the DLN and infect recruited inflammatory DCs (268). Early replication of VEE in

the DLN leads to viremia, which peaks at around 12 hpi with multiple organs infected by 18 hpi, including the spleen, heart, lung, and kidney (267). Peripheral titers peak between 24-48 hpi followed by CNS infection through the olfactory neuroepithelium and the trigeminal nerve (264, 266, 267).

Innate and adaptive immune responses have been important in host response to VEE infection. Induction of robust systemic IFN-α/β occurs within 6 hpi, although its exact mechanism is not known (269-271). The average survival time of IFN-α/β-receptor deficient mice infected with VEE was reduced to 30 hours from 7.7 days in wild-type control, possibly due to an inability to control viral replication and dissemination into the periphery (271). The complement system also plays an important part in the clearance of VEE from the periphery and prevents CNS invasion in mice (272). Antibodies play an important protective role in anti-VEE immunity, with both VEE-specific IgM and IgG able to clear virus form the periphery (269). However, antibody response initiated during the primary immune response was not sufficient to prevent invasion of the CNS and lethal outcome in mice (237). Finally, the anti-VEE T-cell response has been shown to be crucial in the recovery from VEE-induced encephalomyelitis with IFNγ producing CD4+ T-cells more effective than CD8+ T-cells (273).

1.4.3. VEE-based vaccine vectors

Many insights about VEE-host interactions have been gained after the development of a full-length infectious cDNA from viral genomic RNA isolated from the Trinidad Donkey strain (201, 274, 275). This cDNA was cloned into a plasmid, pV3000, which can be *in vitro* transcribed to obtain full length, infectious RNA. The RNA can then be used to produce virus (V3000), which should be genetically identical to TD VEE strain, through electroporation into

permissive cells. This easy-to-use, reverse genetics system can be further used to generate VEE vectors to deliver heterologous antigens, including two vectors that have been described. The first is termed double promoter vector and contains a second 26S subgenomic promoter downstream of the original 26S subgenomic promoter with structural genes (200). These propagation-competent vectors allow for the expression of a foreign antigen from the second 26S promoter and induce strong humoral and cellular immune responses in the infected cell (200). A second VEE vector utilizes the reverse genetic system to replace the viral structural genes with a heterologous antigen and is called VRP. The VRP vectors are capable of infecting target cells, undergo a single round of replication, and do not propagate beyond the first infected cell as they can't produce new virions due to the lack of structural genes in their RNA genome (201). It has been shown that VRP immunization induces protective immunity in rodent models of anthrax, botulism, Whipple's disease, Lassa fever, Ebola, influenza, and human papillomavirus (202, 203, 276-281). Additionally, in non-human primate models, VRP immunization generated protective immunity against Marburg, Ebola, smallpox, dengue virus, and SIV (156, 204, 282-285).

VRP vectors are packaged using two helper RNAs supplied *in trans*, expressing the capsid and the glycoproteins and lacking the packaging signaling, together with the replicon transcript. While the replicon genome allows for the reproduction of the replicon RNA itself as well as the helper RNAs, the helper RNAs produce the structural proteins necessary for virus packaging. Since only the replicon genome has the packaging signal, released VRPs contain only the replicon RNA and not the helper RNAs. Additionally, the highly expressed transgene in the packaging cells are excluded from VRP as its assembly only includes the alphavirus structural proteins with the virus-specific packaging signal.

1.4.4. VEE-based adjuvants

VEE-based adjuvants are related to the VRP vectors described above and are generated in a similar way. GVI3000 (previously known as nVRP) is a virus like particle that is generated by complementation of replicon RNA, which has the 26S subgenomic promoter and the structural genes deleted, together with the two helper RNAs (286). Just like the VRP vectors, GVI3000 particles do not propagate beyond the first infected cell and hence function as a first-in-class biological adjuvant. GVI3000 functions by inducing a strong natural and self-regulated innate immune response in the draining lymph node, which leads to an induction of immune response against the co-delivered killed virus antigen or the soluble protein antigen (287). The ensuing adaptive immune response towards the co-delivered antigen is comprehensive (humoral, cellular, and mucosal) and long lasting. Adjuvant studies using GVI3000 in mouse model induced robust immune response to co-delivered soluble antigens, (KLH, OVA, *P. falciparum* CelTOS, and soluble RSV), virus-like particles (norovirus), and inactivated viruses (dengue, influenza, and Sabin polio strains) [(286-290) and personal communication P. Jorquera, D. Tonkin, L. White, and R. Johnston]. More specifically, the immune responses observed using GVI3000 adjuvant included (1) enhanced antibody response, (2) balanced T_H1/T_H2 response, (3) antigen-specific T-cell response, (4) mucosal IgA, IgG, and T-cell response and (5) protection. While most of the studies looking at the adjuvanticity of GVI3000 were performed in mice, some studies have been successfully performed in rats, ferrets, and non-human primates (R. Johnston, personal communication).

Another VEE based adjuvant is GVI3A, which contains a mutation in the GVI3000 genome at the nt3 of the 5' NTR. The nt3 mutation is a major attenuating mutation described in the TC-83 vaccine strain (271, 291) making it possible for the adjuvant production in BSL-2

conditions. While much remains to be understood, GVI3A adjuvant induced protective immune response in neonatally immunized mice challenged after a single immunization (unpublished observation).

CHAPTER 2: A TETRAVALENT ALPHAVIRUS-VECTOR BASED DENGUE VACCINE PROVIDES EFFECTIVE IMMUNITY IN AN EARLY LIFE MOUSE MODEL#

2.1. OVERVIEW

Dengue viruses (DENV1-4) cause 390 million clinical infections every year, several hundred thousand of which progress to severe hemorrhagic and shock syndromes. Preexisting immunity resulting from a previous DENV infection is the major risk factor for severe dengue during secondary heterologous infections. During primary infections in infants, maternal antibodies pose an analogous risk. At the same time, maternal antibodies are likely to prevent induction of endogenous anti-DENV antibodies in response to current live, attenuated virus (LAV) vaccine candidates. Any effective early life dengue vaccine has to overcome maternal antibody interference (leading to ineffective vaccination) and poor induction of antibody responses (increasing the risk of severe dengue disease upon primary infection). In a previous study, we demonstrated that a non-propagating Venezuelan equine encephalitis virus replicon expression vector (VRP), expressing the ectodomain of DENV E protein (E85), overcomes maternal interference in a BALB/c mouse model. We report here that a single immunization with a tetravalent VRP vaccine induced NAb and T-cell responses to each serotype at a level equivalent to the monovalent vaccine components, suggesting that this vaccine modality can overcome

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#This chapter previously appeared as an article in the journal Vaccine. The original citation is as follows: Syed Muaz Khalil, Daniel R. Tonkin, Melissa D. Mattocks, Andrew T. Snead, Robert E. Johnston, Laura J. White. “A tetravalent alphavirus-vector based dengue vaccine provides effective immunity in an early life mouse model,” *Vaccine*, 2014 Jul 7;32(32):4068-74.

serotype interference. Furthermore, neonatal immunization was durable and could be boosted later in life to further increase NAb and T-cell responses. Although the neonatal immune response was lower in magnitude than responses in adult BALB/c mice, we demonstrate that VRP vaccines generated protective immunity from a lethal challenge after a single neonatal immunization. In summary, VRP vaccines expressing DENV antigens were immunogenic and protective in neonates, and hence are promising candidates for safe and effective vaccination in early life.

2.2. INTRODUCTION

The four serotypes of dengue virus (DENV) are the leading cause of the most important mosquito-borne viral disease worldwide, with annual estimates of approximately 390 million infections (151). The World Health Organization also estimates that up to half a million people are hospitalized with severe dengue disease (Dengue Hemorrhagic Fever/Dengue Shock Syndrome; DHF/DSS), and among them a large proportion are children (152). Children and adults are at increased risk of severe dengue upon a secondary infection with a different serotype. In addition, infants born to dengue immune mothers are at an increased risk of DHF/DSS during a primary infection, and account for more than 5% of all DHF cases (292, 293). This increased risk in infants seems to correlate with maternal antibody titers dropping to sub-neutralizing levels, and becoming potentially enhancing (292, 293). At present, there are no licensed dengue vaccines available, and the ones in development may not be effective in infants.

In addition to the challenges inherent to immunizing early in life, when the immune system is suboptimal, additional unique challenges are encountered in the development of dengue vaccines. (A) A dengue vaccine must be tetravalent (TV) and induce equivalent and

durable neutralizing antibodies (NAbs) against all 4 serotypes simultaneously, due to the theoretical enhanced risk of severe disease if incomplete immunity is induced. (B) Serotype interference has been described among the components of some TV LAV vaccines in development. The dominant serotype prevents other serotype(s) from inducing adequate responses, resulting in incomplete immunity and the need for additional vaccinations over a one year period to achieve a tetravalent response (294). (C) In dengue endemic areas, most children are born with maternal antibodies (Abs) to DENV. These Abs protect in the first months, but also have the potential to interfere with and reduce the efficacy of LAV. Therefore, there is a need for early life vaccines that can induce balanced NAb responses after a single immunization given early in life, and that are not subject to maternal antibody interference.

Venezuelan equine encephalitis virus replicon particles (VRP) are non-propagating viral vectors that can express high levels of an antigen protein after a single round of replication. VRP-based vaccines expressing various antigens induced protective immunity in rodent models (202, 203, 276-281), and in non-human primates (NHP) (283, 285). A VRP-based dengue vaccine candidate is immunogenic and protective in adult mice and NHP (156, 295). Furthermore, VRP expressing DENV2 prME was immunogenic in weanling mice even in the presence of maternal antibodies that prevented immunization with live virus (295).

Here we hypothesize that the VRP vectors are well suited as an effective early life vaccine platform for dengue. First, VRP are propagation incompetent, and therefore safe. Second, VRP immunization is not dependent on vector propagation and amplification. Therefore, serotype interference is minimized as indicated by balanced responses to the 4 dengue serotypes in adult mice and non-human primates. Third, VRP induce strong Th1 immune responses, potentially overcoming one of the deficiencies in the neonatal immune response. And

finally, since VRP contain no DENV antigens, maternal antibodies are less likely to interfere with the vaccine, as was demonstrated previously (295).

2.3. MATERIALS & METHODS

Cells and Viruses

Vero81, BHK-21, and C6/36 cells were obtained from the American Type Culture Collection (ATCC) and appropriately maintained as described previously (156). WHO reference DENV strains were used in the neutralization assays: DENV1 WP, DENV2 S-16803, DENV3 CH53489, and DENV4 TVP-360 (provided by R. Putnak from WRAIR). The viruses were propagated no more than three times in C6/36 cells, titrated on Vero cells, and stored at -80°C. The mouse-adapted, neurovirulent New Guinea C (NGC) strain of DENV2 used for challenge studies (provided by the late Robert Shope, UTMB, Galveston, TX) was amplified no more than twice in C6/36 cells and stored at -80°C at a concentration of 1×10^7PFU/ml.

DV1-4 VRP Vaccines

The construction and production of VRP vectors expressing a C-terminal truncated protein representing 85% of the E protein (E85) from DENV1-4 as well as their source have been described in detail elsewhere (156).

Mice and immunizations

Pregnant and adult BALB/c mice were purchased from Charles River and were housed at the Global Vaccines Inc. or at the University of North Carolina. The animals were housed and cared for in accordance with the protocols approved by each facility's Institutional Animal Care

and Use Committees. Pregnant mice were closely observed for date and approximate time of delivery. Adult mice (6-8 weeks old) or neonatal mice (7 days old) were anesthetized (only adults) and injected in both rear footpads with vaccine or diluent (5μl in each rear footpad.

Neutralization assay

Neutralizing antibody titers specific for DENV1-4 were quantified using a flow cytometry-based neutralization assay on Vero cells, as previously described (156). MAb 2H2 (binds prM protein) was used for intracellular staining of dengue-infected cells.

Surface and intracellular staining (ICS)

Mouse spleens were harvested, homogenized, and strained through 40μm cell strainers to get single cell suspensions. The CD8/CD4 T cell responses were elicited after splenocytes were cultured in RPMI-10 media containing brefeldin A (GolgiPlug, BD Biosciences) with the appropriate peptide pool (2 μg/ml) (see figure legends for detail) for 18 h at 37°C. Cells were stained for CD8 and CD4 (eBioscience) in PBS at 4°C, fixed and permeabilized in Cytofix/Cytoperm (BD). For ICS, fixed cells were stained with antibody specific for IFN-γ (eBioscience). Cells were analyzed on an Accuri™ C6 Flow Cytometer (BD). The final results were obtained after normalizing the value in each sample by subtracting the value obtained in mock-stimulated cells. Spleens harvested from GFP-VRP immunized mice were also included as control.

IFN-γ enzyme-linked immunospot (ELISPOT) assay

An IFN-γ ELISPOT assay was performed to measure DENV3 E-specific IFN-γ-secreting

cells in the popliteal draining lymph nodes of neonatally immunized mice. Mouse IFN-γ capture antibody (R&D Systems) was incubated on nitrocellulose membrane plates (96 well; Millipore) overnight at 4°C. Single cell suspensions in RPMI-10 (2.5×10^5 cells per well) from popliteal DLN were added after washing the plates. Peptides (see figure legends for detail) were added at final concentration of 2 μg/ml for 36 h. IFN-γ spots were developed according to manufacturer's protocol (R&D Systems) and the plates scanned by Cellular Technologies Ltd. [Shaker Heights, OH].

Challenge

Survival after DENV challenge was studied in mice three weeks after MV and TV neonatal immunization. Immunized and PBS control mice were anesthetized and challenged by intracranial injection with 5×10^4 PFU of mouse-adapted strain DENV2 NGC virus. Mice were observed for 25 days for weight change, morbidity, and mortality. Mice were euthanized if their weight dropped below 80% of their initial weight.

Statistical analysis

Neutralization titers and immune cell values were evaluated for statistically significant differences by either the ANOVA or Mann–Whitney tests (GraphPad Prism). Statistical significance is reported as follows (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

2.4. RESULTS

VRP expressing monovalent dengue E85 protein induces a sustained neutralizing antibody response after a single immunization

The kinetics of the Nab response after one immunization with DENV3 E85-VRP was compared in adult and neonatal mice. Adult and neonatal mice were given a single immunization with 1×10^6 IU of DV3-VRP. Mice were bled at 3, 6, and 13 (15 in neonates) weeks post immunization (wpi) (**Figures 2.1A & 2.1B**). A single immunization in both adult and neonatal mice induced a NAb response in all animals. In neonates, the NAb titers against DENV3 remained below the limit of detection at 3 wpi, increased and peaked at around 6 wpi and remained relatively stable for up to 15 wpi (last time-point tested). While induction of NAbs in adult mice showed similar kinetics, titers were significantly higher than those in neonates. A VRP vaccine expressing E85 from a serotype 2 (DV2-VRP) yielded serotype-specific NAbs in neonates with similar kinetics to DV3-VRP (data not shown).

Neutralizing antibody titers induced by monovalent (MV) and tetravalent (TV) vaccination

To determine whether each serotype VRP vaccine was immunogenic, and whether there was serotype interference among the 4 components in the TV formulation, adult and neonatal mice were immunized with each MV component separately or in the TV mix, and NAbs were measured. In adult mice, two immunizations with TV-VRP induced comparable NAb titers to each dengue serotype (**Figure 2.2A-D**). In addition, the NAb titers induced by each serotype when administered as MV vs. TV vaccine were similar ($p>0.2$). In neonates, a single immunization with TV vaccine induced NAbs to all 4 serotypes in every animal. Like in adult mice, NAb titers for serotypes 1, 2, and 3 (serotype 4 was lost to analysis) were not significantly different between the TV and MV formulations, (**Figure 2.2E-H**), suggesting that serotype interference was minimized when using the VRP vaccine approach.

VRP induces T cell responses to DENV3 E protein in mice immunized with monovalent or tetravalent vaccine

To determine whether DENV envelope-specific T-cells are induced upon VRP immunization, adult mice were immunized with either 10^6 IU of DV3-VRP or a TV formulation with 10^6 IU each of the four serotypes. All mice were boosted with the homologous vaccine before harvesting spleens 8 days post-boost for ICS. In cells stimulated with a pool of DV3 E peptides, we observed IFNγ+ CD8+ T-cell responses against DENV3 envelope for both MV and TV immunizations, and these did not differ significantly, p=0.49 **(Figure 2.3A)**. Furthermore, DENV3 E-specific IFNγ+ CD4+ T cells were induced and were not significantly different between MV and TV vaccines (p=0.07). In a separate experiment, a similar result was observed (data not shown).

Neonatal mice received a single immunization with 10^6 IU DV3-VRP (**Figure 2.3B**). DLN and spleens were harvested on 8 dpi. Due to the limited number of immune cells present in neonatal DLN, an IFNγ-ELISPOT assay was performed instead of ICS. We observed that both MV and TV immunizations induced similar levels of IFNγ-secreting cells in the neonatal DLN (p=0.93). Antigen-specific IFNγ-secreting cell numbers in the spleen were reduced; however, they were not significantly different for MV and TV immunized mice (p=0.94). Since the harvested immune cells were stimulated with a panel of pooled DENV3 envelope peptides, we infer that most, if not all, of the IFNγ secreting cells were antigen-specific T-cells.

Neonatal priming with VRP improves immune responses upon a later adult immunization

Three groups of mice were immunized on d7 post-birth, on d7 & d35, or only on d35. All mice received 10^6 IU DV3-VRP (**Figure 2.4A**). On d56, serum was collected for neutralization

assays. Significantly higher $Neut_{50}$ titers were observed in mice that had been primed as neonates and boosted as adults (GMT = 601) compared to immunization on d7 only (GMT = 95; $p < 0.05$) The increase after the booster immunization was mostly due to the prime, mice immunized on d35 only had reduced NAb titers (GMT = 121; $p=0.0519$). A parallel experiment with DV2-VRP was set up to investigate the CD8 T-cell responses, in which the spleens were harvested on day 43. Using ICS, single cell suspensions of splenocytes were evaluated for the presence of IFNγ-secreting CD8 T-cells following stimulation with a panel of pooled DENV2 E-protein peptides. As seen for the NAb response, there was a significantly higher T-cell response in mice receiving two immunizations compared to those that only received immunization on d35 only or day 7 only (**Figure 2.4B**).

A single neonatal immunization with the VRP-based vaccine confers protection from dengue challenge

Protection was determined using an intracranial DENV2 challenge model. Neonatal mice immunized with TV-VRP, DV2-VRP or PBS were challenged 3 weeks later with a mouse brain-adapted DENV2 strain. While all the PBS immunized mice succumbed to challenge by day 17, mice receiving either the MV DV2-VRP or the TV vaccine had significantly better protection with 5/6 ($p=0.0024$) and 4/6 ($p=0.0018$) mice surviving the challenge, respectively (**Figure 2.5**). There was no significant difference between the MV and TV immunized groups. In a separate experiment, weanling BALB/c mice (21 days old) vaccinated with 10^6 IU DV2-VRP were protected (8 out of 9 animals) from a lethal challenge at three weeks post-immunization. The control mice immunized with 10^6 IU HA-VRP (expressing an irrelevant influenza HA antigen) did not confer any protection (0/8) (supplementary figure 2.1).

2.5. DISCUSSION

There is currently no licensed vaccine for dengue, and the vaccine candidates in advanced clinical trials do not target children <12-15 months of age. Developing a dengue vaccine that can be administered during the first year of life is important for 2 reasons. First, in dengue endemic areas, most children are born with dengue maternal antibodies, which are protective during the first few months of life. However, these maternal antibodies become a risk factor for severe disease via ADE when they wane to sub-neutralizing titers (292, 293). An early life vaccine would protect these infants at risk of DHF/DSS. Second, vaccinating early in life in endemic regions would have the advantage of vaccinating a population more likely to be uniformly DENV-naïve, avoiding having to vaccinate older subjects with differences in pre-existing immunity, which may affect vaccine-induced immunity in unknown ways.

Our study is the first to address the challenges of immunizing against dengue early in life, and to demonstrate that the VRP vaccine platform is an effective early life dengue vaccine candidate. The present study demonstrates that VRP vaccines can induce NAbs in neonatal mice after a single immunization and that the titers remain stable until at least 15wpi. Although these titers were reduced at all time-points post-immunization compared to those in adult mice, this finding was not surprising, as the neonatal immune response is known to be weaker (68, 70, 101, 296), resulting in lower serum antibody titers (36, 297) of shorter duration (298, 299).

Dengue vaccines in development seek to induce robust NAbs to all 4 serotypes (300). However, results from the first dengue phase IIb efficacy trial suggest that cell-mediated immunity may be needed in a dengue vaccine (301), and a recent study suggests that the DENV-specific CD8+ T-cell response in humans correlates with protection in an HLA-specific manner

(302). VRP vaccines induce robust CD8+ CTL responses in adult mice to other antigens (303). Even though neonates induce significantly reduced CTL responses (70), our studies showed dengue-specific IFNγ-secreting cells in the DLN of neonatal mice, which we infer to be predominantly CD8+ (**Figure 2.3B**). This is based on observations in adult mice, which showed an approximately 10-fold higher quantity of dengue-reactive CD8+ cells compared to CD4+ cells (**Figure 2.3A**). Direct comparison between neonates and adults in the current study is difficult, because there was only a single neonatal immunization, while adult mice were immunized twice. However, this is the first study to our knowledge that shows induction of a DENV-specific T-cell response in neonatal mice after a single immunization. We predict that addition to the immunization cocktail of VRP expressing DENV NS3 and/or NS5, major targets of T-cell responses, will result in a stronger antigen-specific CD8+ T-cell response.

The induction of equivalent NAb responses has been a major challenge for vaccines based on replicating antigens, where different rates of replication or immunodominance result in unbalanced responses (294, 304). In a TV, non-propagating VRP vaccine, the 4 components presumably have similar replication rates, reducing the chances of serotype interference. Indeed, we show here a VRP-based TV vaccine induced equivalent titers to each serotype after a single dose in neonates, and the NAbs induced were comparable to those induced by each individual component when tested as a MV vaccine.

It is possible that the serotype-specific NAbs measured 3 weeks after TV vaccination could be attributed to cross-reactive NAbs induced by the dominant serotypes. This is unlikely for two reasons. First, studies in adult mice indicate that MV E85-VRP vaccines predominantly induce homologous NAbs with very low amounts of cross-reactive NAbs (data not shown). Second, since cross-reactive NAbs induced by one serotype are short-lived (305, 306),

measuring NAbs to all 4 serotypes long after immunization reflects a true TV response. This was indeed demonstrated 73-weeks after immunization of adult mice with two doses of TV VRP vaccine expressing DENV1-4 prME (supplementary figure 2.2).

A previous study demonstrated that a DENV-VRP vaccine was not subject to otherwise interfering maternal antibodies when it was used to immunize weanling mice born to DENV-immune dams (295). Maternal antibody interference is one of the most important obstacles to developing an early life dengue vaccine, especially because LAV are likely to be ineffective at this age due to maternal antibodies interfering with the replication of the vaccine strains.

Reducing the risk of DHF in early life after a single immunization will allow opportunities for a second immunization to further improve the immune response. Anti-vector immunity is undetectable-to-low after a single VRP immunization in NHP, and does not prevent a second VRP immunization from having a booster effect on dengue-specific NAbs (156). This suggested that infants primed with a VRP vaccine could be safely and effectively boosted with a second dose of a VRP vaccine. Neonatal mice primed on d7 appear to produce more NAb after a boost on d35 than animals that received a single inoculation on d35 (p=0.0519). Similarly, the CD8+ T-cell response is significantly higher when the mice were first primed as neonates (p=0.0498). Although these p-values hover around the limit of significance, we feel that immunizing neonatal animals not only will have a salutary effect on protection of neonates as maternal NAb wanes, but also will prime an improved boost response going forward. The second immunization as an adult could utilize a homologous or heterologous vaccine platform to broaden and strengthen the immune response. Although anti-vector immunity is a concern that remains to be addressed in humans, studies in adult mice (unpublished data) and macaques (156), have shown anti-VEE NAbs do not have any significant effect on the induction of NAbs to

vaccine antigens. Another potential concern is the chance of *in vivo* recombination if vaccination occurs at the same time as infection with wild type virus. This event is highly unlikely to result in recombinant virus of more virulence than the wild-type infecting virus itself.

Figure 2.1

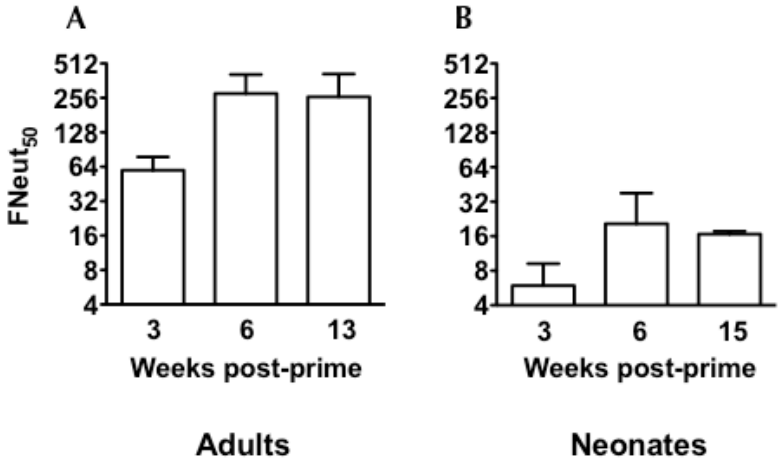


Figure 2.1. DENV3-VRP vaccine induces neutralizing antibody response after a single immunization in neonates and adult mice.

Adult mice were anesthetized before immunization by intraperitoneal (i.p.) injection with a 4/1 mixture (vol/vol) of ketamine (50 μg/g body weight) and xylazine (15 μg/g body weight). **(A)** Six adult BALB/c mice (6 weeks old) were immunized with 1×10^6 IU of monovalent DV3 -VRP and neutralizing antibody titers were measured in the sera at 3, 6, and 13 weeks post-immunization. **(B)** Six neonatal mice were immunized on day 7 after birth with 1×10^6 IU of monovalent DV3 -VRP. Three, 6, and 15 weeks after the immunization, neutralization antibody titers were measured in the sera of the immunized mice. Note: There were only five samples for 6 weeks time-point. Data are presented as 50% neutralization titers ($Neut_{50)}$ (GMT +/- 95% CI). The limit of detection for this assay was 10.

Figure 2.2

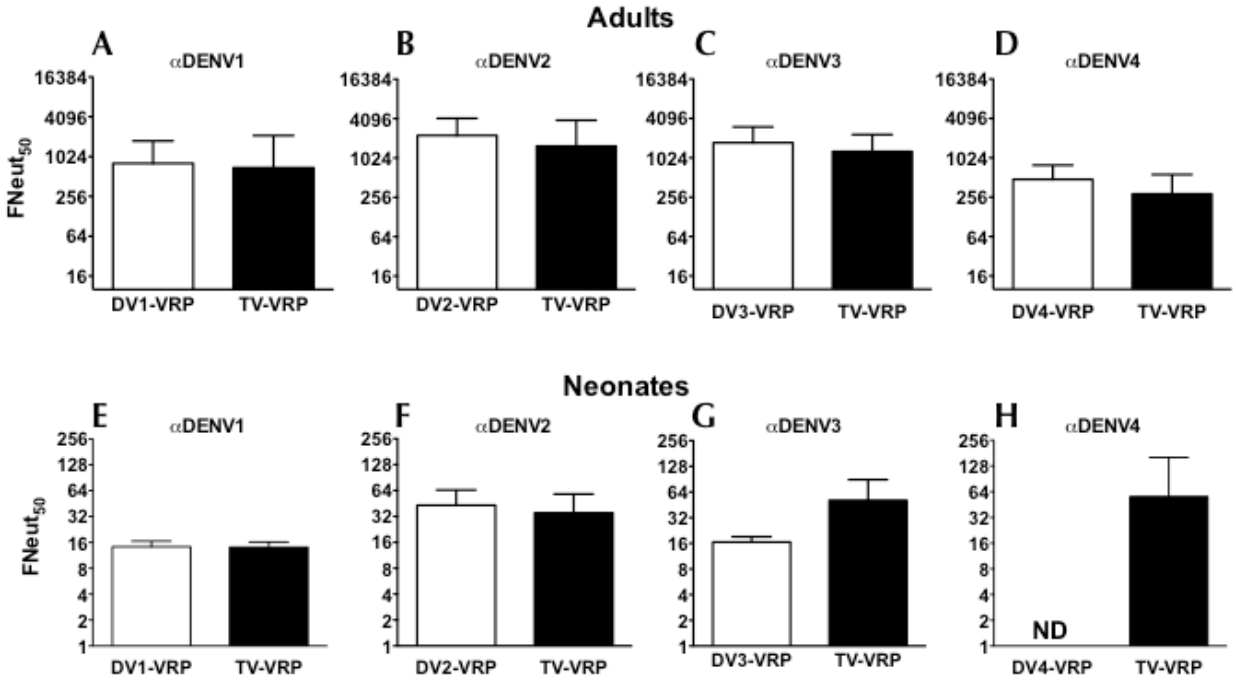


Figure 2.2: Serotype-specific Nab titers induced by each MV and TV vaccine in adult and neonatal mice. (A-D)

Eight adult BALB/c mice per group were immunized and boosted (under anesthesia) 4 weeks later with 2×10^6 IU of each monovalent vaccine, DV1-VRP, DV2-VRP, DV3-VRP, or DV4-VRP or with a tetravalent cocktail of the above four VRP vaccines at 10^6 IU each. NAbs were determined at 3 weeks post boost. Graphs A-D represent $Neut_{50}$ titers against serotypes 1-4 respectively. Each graph includes NAbs induced by the monovalent (white bars) or the tetravalent (black bars) vaccine. Data are presented as GMT +/- 95% CI. The limit of detection for this assay was 10. (*p* values: DENV1 = 0.59; DENV2 = 0.24; DENV3 = 0.24; DENV4 = 0.26). **(E-H)** Neonatal mice were immunized on day 7 after birth with 1×10^6 IU of either monovalent DV1-VRP (N=7), DV2-VRP (N=9) or DV3-VRP (N=6). A tetravalent immunization with 1×10^6 IU of each DV1-, DV2, DV3, and DV4-VRP expression vectors were

administered to a group of 6 neonatal mice. $Neut_{50}$ titers were determined at 15 weeks post immunization. (*p* values: DENV1 = 0.95; DENV2 = 0.52; DENV3 = 0.01). Monovalent DV4-VRP immunization data was lost to analysis (Figure 2H).

Figure 2.3

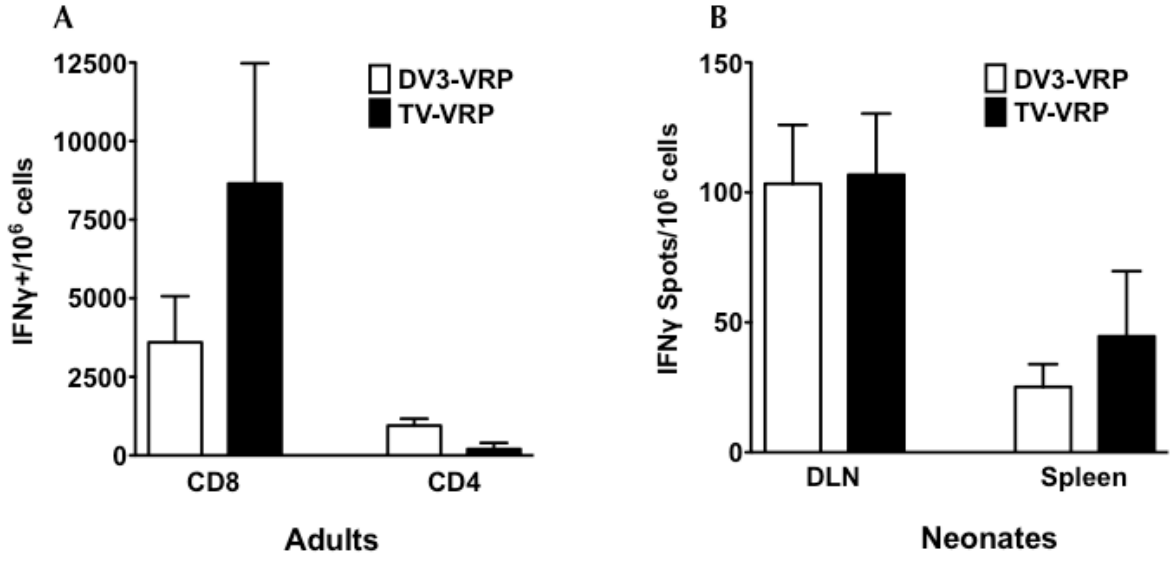


Figure 2.3: Induction of DENV E-specific cell-mediated immunity (CMI) after monovalent and tetravalent VRP immunization in adult and neonatal mice.

(A) CD4+ and CD8+ T cell responses in adult mice. Adult BALB/c mice (4 per group) were immunized and boosted (under anesthesia) 4 weeks later with 10^6 IU of either monovalent DV3-VRP or a tetravalent cocktail of 10^6 IU each of DV1-VRP, DV2-VRP, DV3-VRP, and DV4-VRP (TV-VRP). Splenocytes were obtained 8 days after booster immunization and stimulated with peptide *in vitro*. Overlapping peptides derived from DENV3 E protein were used (68 DENV3 E peptides, BEI NR-9228), which were resuspended in DMSO and pooled. Cells were stained by ICS for IFNγ after surface staining of CD4 and CD8. Data were normalized to mock-stimulated splenocytes of each individual mouse. **(B)** CMI after a single immunization in neonatal mice. Six neonatal mice per group (five in tetravalent DLN group) were immunized with 10^6 IU of either monovalent DV3-VRP or a tetravalent cocktail of 10^6 IU each of DV1-VRP, DV2-VRP, DV3-VRP, and DV4-VRP (TV-VRP). At 8 days post immunization spleens and DLN were harvested. The two popliteal DLNs from each mouse were combined. For

immune cell stimulation, 68 DENV3 E peptides obtained from BEI (NR-9228) were individually resuspended in DMSO, and pooled in three panels: A (peptides 1 to 23), B (peptides 24 to 46), and C (peptides 47 to 68). Unpublished observations in adult mice showed the highest immune response to E was in peptide pool B (includes amino acid sequence from 174 to 343), DLN immune cells were stimulated with E peptide pool B. IFNγ-producing cells were quantified by ELISPOT assay. Data were normalized to mock-stimulated DLN cells of each individual mouse. All results were graphed as mean +/- SEM.

Figure 2.4

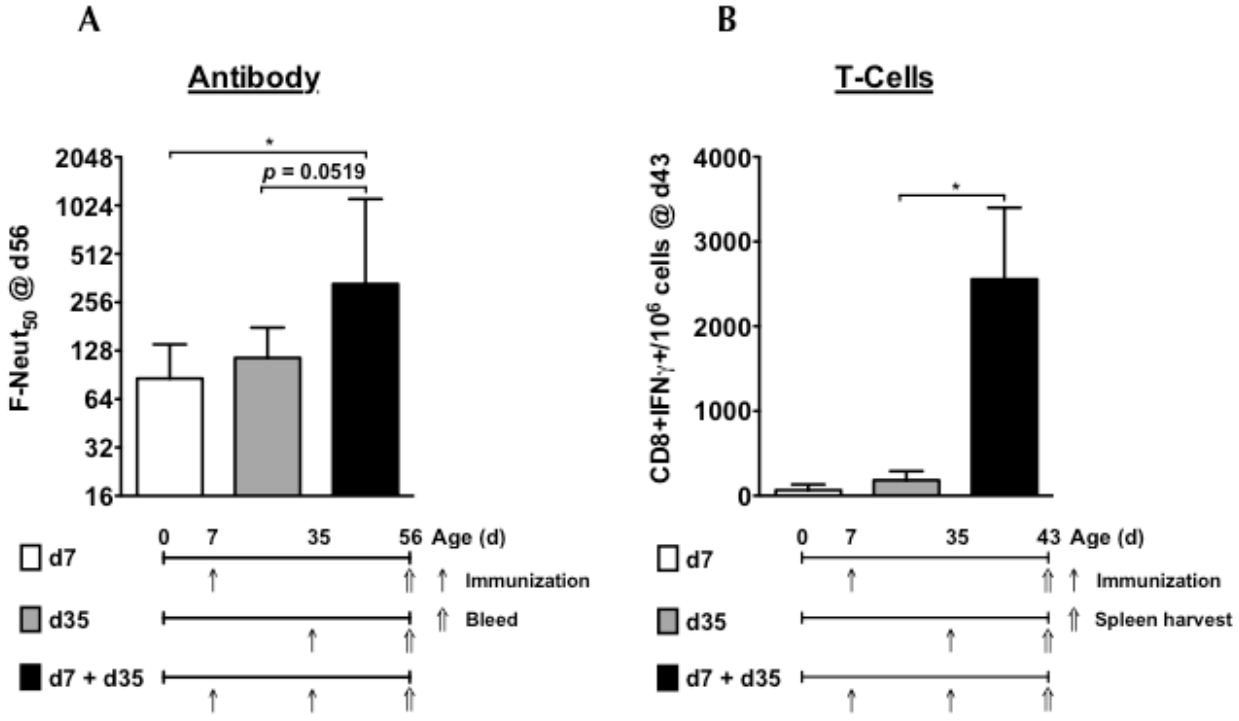


Figure 2.4: Neonatal immunization with VRP vaccine primes robust neutralizing antibody and T-cell responses.

(A) Neutralizing antibody responses – Three groups of mice were immunized with 10^6 IU of DV3-VRP as follows: on day 7 after birth, groups 1 (n=6, white bars) and group 2 (n=6, black bars) received VRP vaccine, and group 3 (n=5, gray bars) received diluent (PBS + 0.1%HSA). On day 35, groups 2 and 3 received VRP vaccine, and group 1 received diluent (all under anesthesia). On day 56, all groups were bled and Neut50 titers vs. DENV2 were determined. Bars represent GMT +/- 95% CI. **(B)** T-cell responses – Three groups of mice were immunized with 10^6 IU of DV2-VRP as described in figure 4A. Groups 1 and 2 had 3 mice each and group 3 had 4 mice. On day 43 (8 days post boost) splenocytes from all mice were harvested followed by stimulation with DENV2 E-protein peptide cocktail (67 peptides, BEI NR-507), surface staining

for CD8, and intracellular cytokine staining for IFNγ. Data were normalized to mock-stimulated splenocytes of each individual mouse, and expressed as number of CD8+ T cells per 10^6 cells. Results were graphed as mean +/- SEM. * indicates $p<0.05$.

Figure 2.5

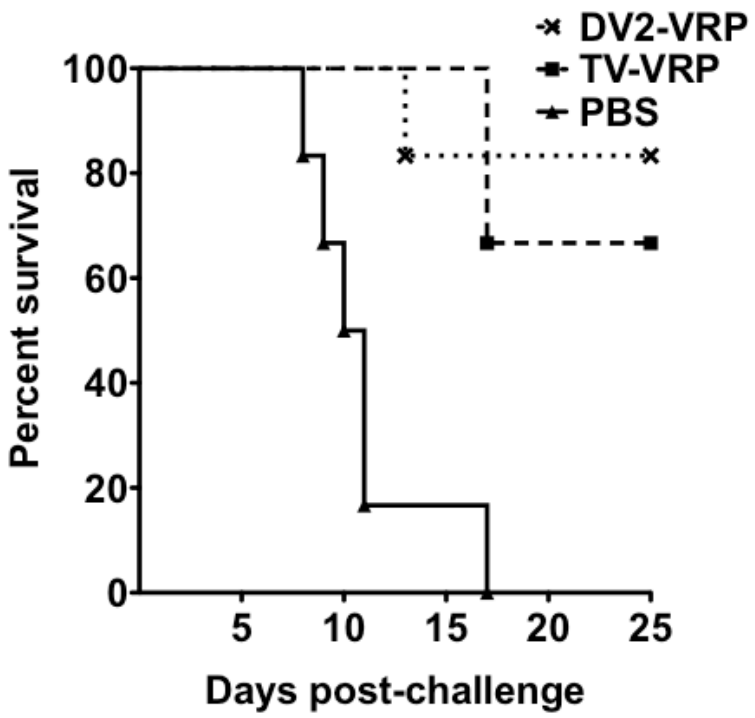
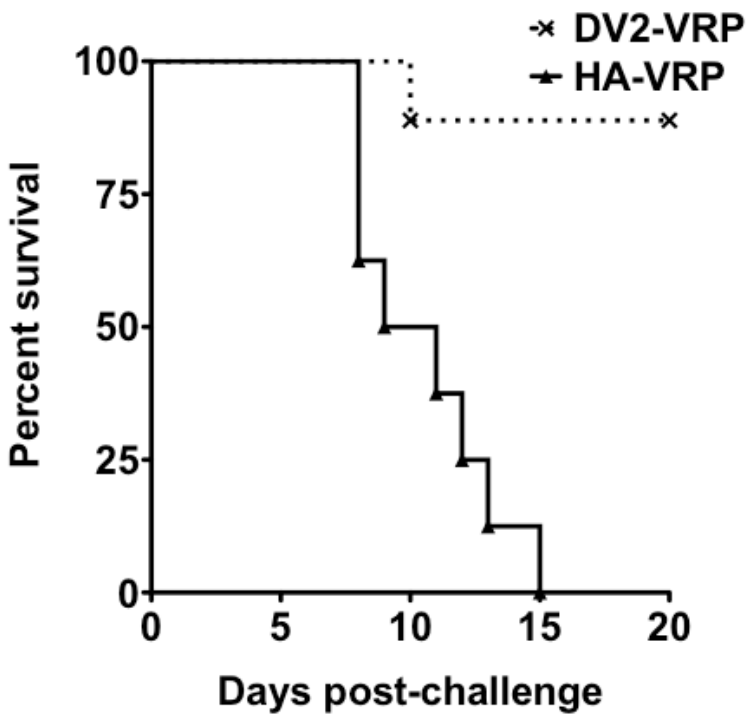


Figure 2.5: VRP expression vector immunization is protective in mice after a single immunization on day 7.

Six 7-day-old neonatal BALB/c mice were immunized with 10^6 IU of either monovalent DV2-VRP or a tetravalent cocktail of 10^6 IU each of DV1-VRP, DV2-VRP, DV3-VRP, and DV4-VRP (TV-VRP). Six control neonatal mice were immunized with diluent. Three weeks after immunization, all mice were challenged intracranially (under anesthesia) with 5×10^4 PFU of a mouse-adapted, neurovirulent New Guinea C strain of dengue virus serotype 2. The mice were observed daily for 25 days after challenge. Results are presented as percent survival.

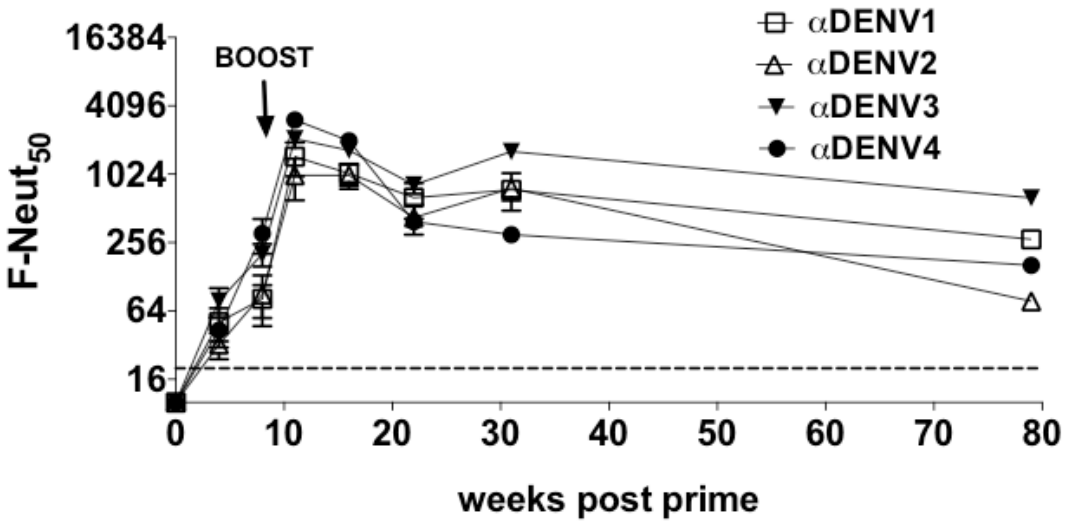
Supplementary Figure 2.1



Supplementary Figure 2.1: Monovalent DV2-VRP immunization is protective in mice after a single immunization on day 21.

Nine 21-day-old weanling BALB/c mice were immunized with 10^6 IU of monovalent DV2-VRP. Eight control weanling mice were immunized with VRP expressing influenza hemagglutinin (VRP-HA). Three weeks after immunization, all mice were challenged intracranially (under anesthesia) with 1×10^4 PFU of a mouse-adapted, neurovirulent New Guinea C strain of dengue virus serotype 2. The mice were observed daily for 20 days after challenge. Results are presented as percent survival.

Supplementary Figure 2.2



Supplementary Figure 2.2: TV-prME-VRP vaccine induces long-term neutralizing antibody response to each component of the vaccine in adult mice.

All mice were anesthetized before immunization by intraperitoneal (i.p.) injection with a 4/1 mixture (vol/vol) of ketamine (50 μg/g body weight) and xylazine (15 μg/g body weight). Eight adult BALB/c mice (6 weeks old) were immunized (prime) with 1.8×10^5 IU of each VRP expressing the full-length prME cassette (DV1-, DV2-, DV3-, and DV4-prME-VRP) in a cocktail and boosted at 8 weeks post-prime with a cocktail containing 2×10^5 IU of each prME-VRP. Neutralizing antibody titers were measured in the sera at 0, 4, 8, 11, 16, 22, 31, and 79 weeks post-prime immunization. Sera from week 0 (pre-immunization) are assigned a value of 1:10, which is one dilution below the limit of detection (LOD) of this assay (1:20). Dotted line represents the LOD. Data are presented as 50% neutralization titers ($Neut_{50}$) (GMT +/- 95% CI).

CHAPTER 3: AN ALPHAVIRUS-BASED ADJUVANT ENHANCES SERUM AND MUCOSAL ANTIBODIES, T CELLS AND PROTECTIVE IMMUNITY TO INFLUENZA VIRUS IN NEONATAL MICE∗

3.1. OVERVIEW

Neonatal immune responses to infection and vaccination are biased towards T_H2 at the cost of pro-inflammatory T_H1 responses needed to combat intracellular pathogens. However, upon appropriate stimulation, the neonatal immune system can induce adult-like T_H1 responses. Here we report that a new class of vaccine adjuvant is especially well suited to enhance early life immunity. The GVI3000 adjuvant is a safe, non-propagating, truncated derivative of Venezuelan equine encephalitis virus that targets dendritic cells (DC) in the draining lymph node (DLN) and produces intracellular viral RNA without propagating to other cells. RNA synthesis strongly activates the innate immune response so that in adult animals, co-delivery of soluble protein antigens induces robust humoral, cellular and mucosal responses. The adjuvant properties of GVI3000 were tested in a neonatal BALB/c mouse model using inactivated influenza virus antigen (iFlu). After a single immunization, mice immunized with GVI3000-adjuvanted iFlu had significantly higher and sustained Flu-specific IgG antibodies, mainly IgG2a (T_H1), compared to the antigen-only group. GVI3000 significantly increased antigen-specific CD4+ and CD8+ T-cells, primed mucosal immune responses, and enhanced protection from lethal challenge. As

∗ This chapter previously appeared as an article in the The Journal of Virology. The original citation is as follows: Syed Muaz Khalil, Daniel R. Tonkin, Andrew T. Snead, Griffith D. Parks, Robert E. Johnston, Laura J. White. "An Alphavirus-based Adjuvant Enhances Serum and Mucosal Antibodies, T cells and Protective Immunity to Influenza Virus in Neonatal Mice," *The Journal of Virology*, 2014 Jun 4 pii: JVI.00327-14. [Epub ahead of print].

seen in adult mice, the GVI3000 adjuvant increased the DC population in the DLN, caused activation and maturation of DC, and induced proinflammatory cytokines and chemokines in the DLN soon after immunization, including IFN-gamma, TNF-alpha, G-CSF and IL-6. In summary, the GVI3000 adjuvant induced an adult-like adjuvant effect with an influenza vaccine, and has the potential to improve the immunogenicity and protective efficacy of new and existing neonatal vaccines.

3.2. INTRODUCTION

The World Health Organization (WHO) estimates approximately 2 million deaths in neonatal and infant humans (<1 year of age) every year worldwide due to acute infections caused by a limited number of pathogens (157). The availability of effective early life vaccines against those agents would have a significant impact on disease burden in neonates and infants, who are especially vulnerable to infectious diseases, and in whom the immune responses generated by most currently available early life vaccines are suboptimal. The need for effective early life vaccines is especially important in resource-poor countries, where the period immediately after childbirth is often the only point of contact with the healthcare system.

A major obstacle in the development of early life vaccines is that the neonatal immune system is geared more towards a T_H2 response at the cost of more pro-inflammatory T_H1 responses needed to combat intracellular pathogens (68, 307). The neonatal antibody response to conventional subunit and live attenuated vaccine antigens is of limited magnitude and duration and CD8+ T cell responses also are reduced compared to adults (70, 134, 308). The predisposition of the neonatal immune system towards a T_H2 response is caused by the suboptimal innate immune response, with delayed maturation of neonatal dendritic cells (DCs)

and limited production of inflammatory cytokines, which leads to inefficient antigen presentation and stimulation of naïve T-cells (65, 309-311). Therefore, many vaccines that are effective in adults are poorly immunogenic in early life, hence requiring multiple booster immunizations (70).

Influenza viruses cause millions of annual infections worldwide, with up to 40,000 deaths reported in the U.S. alone (141). Newborns and infants are at higher risk for influenza-related mortality because of their immature immune systems, which can often lead to severe viral pneumonitis or bacterial super-infection (312). Protection from influenza, like most respiratory viruses, is optimally conferred through virus-specific antibodies, such as IgG and IgA. However, antibody-mediated protection does not fully protect against heterologous strain infection due to the variability in the surface glycoproteins (313). On the other hand, T-cell epitopes are highly conserved across influenza strains and robust T-cell response can induce a broader protection (314). Currently available influenza vaccines—tetravalent inactivated (TIV) or live attenuated (LAIV)—are not recommended for use in infants <6 months (146). While the LAIV immunization has been more effective in young children (6-11 months) compared to TIV, it has been associated with safety concerns, such as increased rate of wheezing and hospitalization (149, 150). Therefore, the development of inactivated influenza vaccines, with improved efficacy in infants less than 6 months of age is urgently needed. In this study, we chose inactivated influenza virus as a model antigen.

Studies have shown that upon appropriate stimulation, the otherwise "immature" neonatal immune system has the capacity to induce adult-like T_H1 immune responses, and protect against infections (68). The best example of a neonatal T_H1 vaccine is the Bacille Calmette Guerin (BCG) vaccine against tuberculosis, which induces strong, adult-like IFNγ responses and a

protective T_H1 response in newborns. The WHO has recommended BCG for immunization immediately after birth (315). An increased interest in developing and testing T_H1-inducing adjuvants for early life immunizations has resulted in a number of studies in human cord blood cells and in neonatal animal models, using cytokines and ligands of Toll-like receptors (TLR). Stimulation of CD4+ T-cells from human umbilical cord blood with recombinant IL-12 resulted in the induction of adult-like IFNγ responses (316). TLR7/8 agonists, R848, or TLR8 agonist VTX-294 showed T_H1-responses, indicated by production of cytokines TNFα, IL-1β, and IL-12 secreted by APCs from newborn cord blood (317, 318). CpG, aTLR9 agonist, can induce adult-like DC and T cell activation, while failing to induce adult-like Ab responses (109). IC31 (Intercell AG), a two-component adjuvant consisting of an antibacterial peptide and the TLR9 agonist ODN1a, when combined with a Pneumococcal conjugate vaccine, enhances protective immunity in neonatal mice (319). A synthetic water-in-oil emulsion based adjuvant, CRL-8941 (116), and Complete Freund's Adjuvant, CFA (72), induced adult-like T_H1 immune responses in neonatal mice, indicated by induction of antigen-specific IgG2a antibodies (an indicator of a T_H1 response in BALB/c mice) and/or IFNγ-secreting splenocytes. However, CRL-8941 was reported by the authors to cause "significant local toxicity" in neonatal mice while CFA is universally toxic. Therefore, there is a continued need for safe and effective adjuvants that can promote strong, balanced and protective newborn immune responses to new and existing vaccines. The two adjuvants currently approved as components of human vaccines, alum and MF59, are both inducers of T_H2 polarized immune responses (320). Therefore, an adjuvant promoting a T_H1 response might be useful in inducing a balanced vaccine response by the already T_H2 biased neonatal immune system.

In this study, we investigated the effectiveness of a new class of adjuvants in early life vaccination. The novel adjuvant GVI3000 is derived from an alphavirus genome. Venezuelan equine encephalitis virus (VEE) is a positive-sense, single stranded RNA virus representative of the alphavirus genus. Its ~11.5 Kb RNA genome encodes four non-structural proteins and 3 structural proteins, the latter expressed from a 26S subgenomic promoter. Although the adjuvant properties of attenuated VEE virus have been suggested for some time (321, 322), our group has developed and characterized propagation incompetent VEE replicon particles with strong adjuvant activity (286), and analogous adjuvants have been derived from Semliki Forest virus (323) and Sindbis (Mikkelsen and Johnston, unpublished). VEE adjuvant particles have been referred to as nVRP (286, 287) or GVI3000 (290), and contain a truncated VEE genome that includes the 5' and 3' UTRs and the nonstructural genes, but lack the 26S subgenomic promoter and the structural protein genes. When inoculated into adult mice by subcutaneous, intradermal or intramuscular routes with a number of soluble (OVA, KLH) or inactivated particulate antigens (noro-, polio-, or influenza viruses), these adjuvant particles can enhance humoral, cellular, and mucosal immune responses (286, 287, 289, 290). Additionally, enhanced protection against influenza and dengue viruses was demonstrated in a macaque model (156, 324). Upon subcutaneous immunization of mice, GVI3000 particles enter Langerhans cells (LC) at the site of inoculation where viral RNA replication and LC activation presumably occurs (198). These infected immune cells then rapidly migrate to the draining lymphoid node(s) (DLN). Alternatively, GVI3000 particles can migrate directly to DLN, where they preferentially target DCs (198, 325). In both human- and murine-derived DCs, *in vitro* studies have shown an upregulation of co-stimulatory molecules as well as secretion of proinflammatory cytokines, such as type I IFN, IL-6, and TNF after infection (303, 325, 326). In adult mice, cytokine

secretion was observed systemically and in the DLN as soon as 6 hours after GVI3000 inoculation (unpublished observation). Tonkin et al (268) demonstrated that DCs infected *ex vivo* with GVI3000, when inoculated into adult BALB/c mice, were sufficient to enhance *in vivo* systemic, mucosal, and cellular responses. Furthermore, the immune cells most frequently targeted and recruited to the draining lymph node were monocyte-derived inflammatory DCs.

Based on the properties of the GVI3000 adjuvant, we predicted that it might be well suited as a T_H1-polarizing vaccine adjuvant to enhance neonatal immunization. We hypothesize that GVI3000 can induce a sufficiently strong DC activation to overcome the neonatal limitation to induce a Th1 response, hence promoting an "adult-like immune response," shifting the neonatal immune system to a more balanced T_H1- T_H2 response, inducing mucosal and T cell responses, and improving vaccine efficacy in neonates.

In this study we used a 7-day old BALB/c neonatal mouse model, which has been reported to more closely resemble the stage of immune maturation and immune-function limitations in human neonates (70). We immunized 7-day old and adult mice with inactivated influenza virions (iFlu) in the presence of GVI3000 adjuvant and compared the effect of the adjuvant on induced adaptive and innate immune responses after a single immunization of the two age groups. Antigen-specific antibody responses were significantly enhanced by the adjuvant and lasted for at least several months. GVI3000-adjuvanted iFlu also was able to induce antigen-specific T-cells in the DLN of immunized neonates, and primed a mucosal IgA response. The addition of the GVI3000 adjuvant to the iFlu vaccine resulted in a significant increase in protection from a lethal challenge. Furthermore, as observed in adults, inflammatory DCs were the most frequently recruited and targeted immune cells in the DLN. The pro-inflammatory innate immune response profile was similar to that in adult immunized mice, indicating that

GVI3000 exploits similar pathways in both neonates and adults and can overcome any shortcomings in the neonatal immune system. These findings support the conclusion that co-delivery of GVI3000 and an inactivated vaccine can significantly improve immune responses and protection against challenge in neonatal mice.

3.3. MATERIALS & METHODS

Cells

Vero81 cells were obtained from the American Type Culture Collection (ATCC). The Vero81 cells were maintained at 37°C in Dulbecco's modified Eagle's medium (DMEM)/F12 medium supplemented with 10% fetal calf serum, penicillin/streptomycin (100 U/ml), and streptomycin (100 μg/ml) in the presence of 5% CO_2. L929 mouse fibroblast cells (ATCC CCL-1) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin/streptomycin (100 U/ml), and 0.29 mg/ml of L-glutamine. Chicken red blood cells (CRBCs) were obtained from Charles River Laboratories (Material No. 10100767), stored at 4°C for hemagglutination inhibition assays.

Viruses and vaccine antigens

Live Influenza A/PR/8/34 virus (Material No. 10100374) and formalin-inactivated Influenza virus A/PR/8/34 (iFlu) (Material No. 10100782) were obtained from Charles River Laboratories and stored at -80°C until use.

Adjuvant VEE Replicon Particles (GVI3000)

The production of GVI3000 adjuvant particles, also known as nVRP, has been described

previously (201, 204, 287). Briefly, *in vitro* transcribed VEE replicon RNA genome, together with two helper RNAs—expressing viral structural genes in trans—were co-electroporated into Vero81 cells. GVI3000 particles were collected in the culture media 24 h after electroporation. These particles contain only replicon RNA and exclude helper RNAs, which lack the viral-specific packaging signal, rendering these particles propagation incompetent. Cytopathic effect testing was performed on each production batch to confirm the absence of detectable propagation-competent virus that could arise as a contaminant due to RNA recombination. The adjuvant particles were concentrated by ultracentrifugation through a 20% sucrose cushion, suspended in PBS containing 1% human serum albumin, and stored at -80°C until use. GVI3000 infectious particles were then titered by infection of Vero cells as measured by immunofluorescent staining of VEE non-structural proteins, using polyclonal sera from mice immunized with VEE non-structural protein 2.

Two modalities of VEE replicon RNA were packaged in this study: (i) GVI3000 replicon RNA, which encodes the 5' and 3' untranslated regions (UTR) flanking the viral nonstructural genes, but lacks the sequence between the nsP4 stop codon (5 nts before the 26S mRNA transcription start site) and the beginning of the 118-nt 3' UTR (287). (ii) GVI3000-GFP replicon RNA, which encodes the viral nonstructural protein genes and also the sequence for GFP under the control of the viral 26S subgenomic promoter. GVI3000-GFP was used as reporter particles to identify GVI3000-infected cells. All known *cis*-acting signals for RNA replication were included in the GVI3000 genomes. All replicon particles were packaged using wild-type VEE (V3000) envelope sequences (274).

Mice and immunizations

Pregnant and adult BALB/c mice were purchased from Charles River and were housed at the Global Vaccines Inc. animal facility. All studies were carried out in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals. All animal protocols were approved by the Global Vaccines, Inc. Institutional Animal Care and Use Committees (IACUC) prior to their performance. Pregnant mice were closely monitored for date and approximate time of delivery.

Seven days after birth, neonatal mice were injected in both rear footpads with PBS or one of the following formulations: iFlu alone, iFlu mixed with alum (Alhydrogel 2%; Invivogen) or iFlu mixed with GVI3000. The immunization volume was 10μl total (5μl in each rear footpad). Mice were immunized once unless otherwise stated. Adult control mice (6-8 weeks old) were immunized in both rear footpads (10ul/footpad) after being anesthetized by i.p. injection with a 4/1 mixture (vol/vol) of ketamine (50 μg/g body weight) and xylazine (15 μg/g body weight). Mice were observed daily for a week for any adverse reaction to the immunization.

Flu challenge

The mouse flu challenge model used here has been described elsewhere (200). Three weeks after neonatal immunization, mice were challenged with 10^6 EID_{50} (50% egg infectious dose) of influenza virus strain PR/8/34 (Charles River, MA). The challenge dose represented the lowest dose that was 100% lethal in control mice immunized with diluent, and was optimized in a separate experiment. The virus was diluted in a total volume of 50μl, and 25μl was introduced into each nare under light isoflurane anesthesia. Mice were observed for 11 days for weight, morbidity, and mortality. The animals were monitored for clinical signs of disease including

ruffling, hunching, signs of dehydration (concave dorsal surface), and reduced spontaneous mobility. To assess protection from post-challenge influenza virus replication in the nose, mice were neonatally immunized, and five weeks later, mice were challenged intranasally with 10^6 EID_{50} of influenza virus strain PR/8/34. At 48 h post challenge, mice were euthanized and nasal tissues were harvested from the tip of the nose to the anterior of the eye sockets, as described before (286, 327). Nasal tissue was collected in 10% FBS media and centrifuged, followed by storage in Trizol at -80°C until RNA isolation.

RNA Isolation & Real-time PCR

RNA extraction from nasal tissues was performed using the PureLink™ RNA Mini Kit (Ambion) according to the manufacturer's protocol. RNA samples were then treated with DNase using the TURBO DNA-*free*™ kit (Ambion), following the manufacturer's protocol. Quantification of PR8 HA negative strand RNA levels in the nasal tissues was performed by RT-PCR as has been described previously (328). Briefly, cDNAs were prepared using reverse-transcription with oligo(dT). RT-PCR was performed using TaqMan gene expression assays (Applied Biosystems) and gene-specific primers & probes (Life Technologies). The samples were then run on an ABI Prism 7000 real-time PCR system. During each reaction, a cycle threshold (C_T) value for the target gene of interest was generated, which was then normalized to the C_T value of the housekeeping gene (glyceraldehyde-3-phosphate dehydrogenase; GAPDH) yielding a $\Delta\mathbf{C_T}$ value. The $\Delta\mathbf{C_T}$ value of the nVRP-infected sample was then subtracted from the $\Delta\mathbf{C_T}$ value of the antigen-only sample, resulting in a $\Delta\Delta\mathbf{C_T}$ value. Fold increase in mRNA expression in nVRP group relative to the expression level in antigen-only group were determined by using the formula $2^{\Delta\Delta\mathbf{CT}}$.

The HA-specific primers (forward: ACTGGACCTTGCTAAAACCC [starting at nt772]; reverse: CATTGATGC GTTTGAGGTGATG) & probes (/56-FAM/CCCAAAGCC/ZEN/TCTACTCAGTGCGAA A/3IABkFQ/) for RT-PCR were designed and obtained from Integrated DNA Technologies (IDT). Additionally, PR8 HA negative strand RNA standards were prepared from the influenza virus stock used for infection and were run on each sample plate. For the analysis, C_T value from each sample was plotted on the standard curve to obtain PR8 viral titer. All C_T values were normalized to the housekeeping gene 18S.

Analysis of antigen-specific IgG in serum and IgA in fecal extracts by ELISA

Mouse sera was collected at 3, 6, 9, and 15 weeks after immunization and stored at -20°C. For preparation of fecal extracts, fecal pellets collected 10-14 days after boost were suspended at 0.2 g/ml and disrupted by vortex mixing at 4°C in PBS containing 10% goat serum and 1X protease inhibitors (Roche; product number 11873580001). Samples were centrifuged, and supernatants were filtered through 0.22μm filters. Antigen-specific IgG and IgA antibodies were detected by ELISA on 96-well high binding plates (Thermo Scientific) coated with 4μg/ml iFlu in PBS. Sera and fecal extracts were added to plates in serial dilutions. Antigen-specific antibodies were detected with horseradish peroxidase conjugated antibodies specific for mouse IgG (Southern Biotech) or mouse-IgA (Southern Biotech) followed by addition of SureBlue (TMB Microwell Peroxidase Substrate; KPL) for 30 min. Endpoint titers were determined as the last sample dilution that generated an OD450 reading of greater than 0.2. For determination of total IgA levels in fecal extracts, 96-well plates were coated with 0.4μg/ml rabbit anti-mouse-IgA (Invitrogen), ELISAs were performed as above, and a standard curve generated from dilutions of purified murine IgA (Sigma). This standard curve was used to determine the

concentration of both antigen-specific and total IgA in fecal extracts.

Hemagglutination inhibition assay

Flu-specific Hemagglutination inhibition assay (HAI) has been described elsewhere (*Current Protocols in Immunology* (2001) 19.11.1-19.11.32). Complement was inactivated by heating the serum sample at 56°C for 30 min. Mouse serum was then pre-adsorbed with 1% suspension of chicken red blood cells (CRBCs) resulting in a 1:5 dilution. Briefly, four hemagglutination units (HAU) of influenza virus strain PR8 in 50μl were mixed with 2-fold serial dilutions of pre-adsorbed mouse serum (50μl per dilution) and incubated at room temperature for 30 min (starting dilution 1:10). Fifty microliters of a 1% suspension of CRBCs was then added to each well. In serum dilutions with anti-HA antibody, virus was bound by antibody and was not available to agglutinate the CRBCs. The maximal reciprocal serum dilution where agglutination was completely inhibited is reported as the HAI titer.

IFNαβ bioassay

The levels of biologically active type I IFN in mouse sera were determined using an interferon bioassay as previously described (329). Briefly, L929 mouse fibroblasts cells were seeded in 96 well plates. Mouse serum samples, including the standards, were acidified to a pH of 2.0 overnight, then neutralized to pH of 7, and added to cells in serial two-fold dilutions. After 24 hr incubation, 10^5 PFU of encephalomyocarditis virus (EMCV) was added to each well. Twenty-four hours post-infection, cell viability was determined using 3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma), and the absorbance was read on a microplate reader at 570 nm. To obtain type I IFN value in international units (IU/ml), the

absorbance from each sample was compared to a dilution series of IFNα standard (BEI; NR-3076) present in each plate.

Surface staining of cells from Draining Lymph Nodes (DLN)

At the indicated time points, both draining popliteal lymph nodes from each animal were harvested, combined and homogenized through 40μm cell strainers. Cells were washed, counted by hemocytometer and stained at 4°C for desired surface receptors with a selection of the following fluorochrome-conjugated antibodies specific for CD3, CD4, CD8, CD11b, CD11c, CD40, CD80, CD86, MHCII (eBioscience), Ly-6G and Ly-6C (BD Bioscience) in 1% BSA/PBS. After staining, cells were washed and then fixed in 2% paraformaldehyde in PBS for 15 min at room temperature. Cells were analyzed on an Accuri™ C6 Flow Cytometer (BD).

IFN-γ enzyme-linked immunospot (ELISPOT) assay

IFN-γ ELISPOT assay was performed to measure influenza PR8 HA-specific IFN-γ-secreting CD4 and CD8 cells in the popliteal draining lymph node and spleen of immunized mice. Mouse IFN-γ capture antibody (R&D Systems) was incubated on nitrocellulose membrane plates (96 well; Millipore) overnight at 4°C. Before the addition of cells, the plates were washed and blocked for at least 2 h with complete RPMI-10 (10% fetal bovine serum) at 37°C. Single cell suspensions in RPMI-10 (2.5×10^5 or 5×10^5 cells per well) from either popliteal draining lymph node or spleen were then added to the plates. Additionally, feeder cells (2.5×10^5 cells per well) from a naïve adult BALB/c mouse spleen were added to all wells. Finally, CD4 (HA 110-120; SFERFEIFPKE) and CD8 (HA 518-526; IYSTVASSL) immunodominant HA peptides from AnaSpec were added to the appropriate cells for 36 hrs. Cells were removed from plates

and washed, a biotinylated mouse IFN-γ detection antibody (R&D Systems) was added to the plates, and the plates were incubated for another 36 h at 4°C. Membranes were again washed, incubated with a streptavidin–alkaline phosphatase conjugate for 2 h at RT. Plates were washed, and spots were developed following addition of BCIP/NBT substrate. Plates were scanned by Cellular Technologies Ltd. [Shaker Heights, OH]. Assay background values were obtained from sample wells with no peptide/protein stimulation and were subtracted from the values obtained with the influenza HA-peptide. Data are normalized to the number of antigen-specific IFN-γ-secreting-cells per 10^6 cells.

Tissue Culture Infectious Dose 50 ($TCID_{50}$) Assay

Virus titers in the lungs were determined by $TCID_{50}$ assay on MDCK cells. Lungs collected on day 3 post-challenge were homogenized, serially diluted 10-fold, and then added in quadruplicate to cells in a 96-well plate. After 48 h incubation, an influenza hemagglutination assay (HA) using 5% chicken red blood cells was performed on the media from each well. $TCID_{50}$ titers were determined using the Reed and Muench calculator.

Multiplex analysis for cytokine expression in the DLN

To determine cytokine levels in the DLN of mice immunized with iFlu alone or with nVRP, the draining popliteal lymph nodes from each mouse were harvested, combined and placed in 100μl of PBS containing 1X protease inhibitors (Roche; product number 11873580001). The lymph nodes were mechanically homogenized with a pestle, followed by centrifugation at 4°C. Supernatant was transferred to another tube and frozen on dry ice. Cytokine levels in the samples were determined by Luminex-based multiplex assay. For one

experiment, levels of 18 cytokines were tested using the Milliplex MAP Mouse Cytokine/Chemokine Magnetic Bead Panel (EMD Millipore, Billerica, MA). The assay was performed by the Center for Gastrointestinal Biology and Disease's Immunotechnologies Core at the University of North Carolina at Chapel Hill. Multianalyte profiling was performed on a Bio-Plex 200 system with an XY platform and high-throughput fluidics (Bio-Rad Laboratories, Hercules, CA). Calibration and validation microspheres for classification and reporter readings, as well as optics and fluidics verification, were also obtained from Bio-Rad. The instrument's sheath fluid was prepared from concentrate obtained from Luminex (Luminex Corporation, Austin, TX). Fluorescence data was acquired using Bio-Plex Manager 6.0 software, and all cytokines were successfully detected. Data analysis was performed using the same software, and a best-fit standard curve was obtained using either four- or five-parameter regression, from which the sample concentrations were derived. Cytokines that were undetectable were assigned a value of half of the lowest limit of detection as listed in the product manual.

Statistical analysis

Antibody titers and cytokine values were evaluated for statistically significant differences by either the ANOVA or Mann–Whitney test (GraphPad Prism). Statistical significance is reported as follows (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$)

3.4. RESULTS

GVI3000 safely enhances humoral immune responses to inactivated flu (iFlu) in neonatal mice

To determine the adjuvant effect of GVI3000 on humoral responses to iFlu in neonatal mice, we assessed the magnitude, duration and quality of serum antibodies as well as induction of fecal antibody responses by iFlu alone or formulated with the GVI3000 adjuvant. Seven-day old BALB/c mice from one litter received a single immunization with 2μg of iFlu mixed with GVI3000 (10^5IU) while mice from a second litter were immunized with 2μg of iFlu only. Mice were monitored daily for local reactogenicity, morbidity and mortality. No adverse effects of immunization with iFlu alone or iFlu+GVI3000 were observed. Over the past several years approximately 2,000 adult mice and over 350 neonatal mice have been immunized with GVI3000 adjuvant without any observable signs of adverse reaction (268, 286, 287, 290, 327, 330, 331)(Daniel Tonkin, Benjamic Steil, and Patricia Jorquera, personal communications). Flu-specific IgG ELISA antibody titers in the iFlu-only group were modest and did not increase between 6 and 15 weeks post immunization **(Figure 3.1A)**. In the GVI3000-adjuvanted group, the titers were significantly higher for all time-points tested compared to the iFlu-only group. From week 6 to week 15, the titers in GVI3000-adjuvanted mice continued to increase significantly ($p<0.05$) compared to week 3 (**Figure 3.1A**). Additionally, week 15 titers were significantly higher compared to week 6. The flu-specific IgG2a titers (indicator of T_H1 response) in the GVI3000-adjuvanted immunization group were significantly higher at 3, 6 and 15 weeks **(Figure 3.1B)**, while the IgG1 (indicator of Th2 response) titers were comparable between the groups **(Figure 3.1C),** suggesting that the GVI3000 adjuvant-mediated enhanced IgG response was predominantly in the IgG2a subclass. We performed our studies in BALB/c strain of mice, although previous results from our lab has shown that adult C57BL/6, 129 Sv/Ev, and other strains of mice produce a similar T_H1-biased response upon GVI3000 immunization.

Previous studies have suggested the important role mucosal immunity plays in protecting against influenza virus, which primarily infects and provokes inflammation at respiratory mucosal sites (332). A unique feature of the GVI3000 adjuvant is its ability to induce—in adult mice—an antigen-specific mucosal IgA antibody response after a non-mucosal route of immunization (286, 327). We chose to measure the IgA response in the fecal pellets of immunized mice as IgA detection is more reliable and unambiguous measure of mucosal response compared to nasal or tracheal lavages. Additionally, it is known that mice have a common mucosal system, in which responses at one mucosal tissue likely mirrors those in other mucosal sites (333). Published studies in adult BALB/c mice have shown that induction of antigen-specific IgA in GVI3000-adjuvanted mice was significantly enhanced in fecal extracts similar to the increase in the nasal epithelium (286, 330). To address whether GVI3000 can prime the induction of antigen-specific mucosal IgA in early life immunizations, neonatal mice were primed with iFlu alone or iFlu + GVI3000, and ten weeks later, they were boosted with iFlu antigen only. The amount (ng of flu-specific IgA / μg of total IgA) of flu-specific IgA antibody found in fecal materials 10 days after the boost was measured by ELISA **(Figure 3.1D)**. Presenting the IgA data as (ng of flu-specific IgA / μg of total IgA) allows for normalization of the fecal pellet size, which can vary between mice groups, genders, and within the same group sampled at different times, making it a reliable and accurate measure (334). While the signal for iFlu-only induction of IgA was at the background level, a significantly higher amount of flu-specific IgA was observed in mice that had GVI3000 adjuvant in the prime. Importantly, the amount of flu-specific IgA in the adjuvanted group was not significantly lower than that found in adult mice (1.59 ng in neonates compared to 1.74 ng flu-specific IgA per μg total IgA in adults), when mice from both age groups were compared in the same experiment (data not shown).

To further assess the quality of the flu-specific antibodies being induced, we measured the ability of the antibodies in the sera to inhibit hemagglutination of chicken red blood cells using an HA binding inhibition assay (HAI). We included an experimental group adjuvanted with alum (Alhydrogel, 1:5), a well-characterized benchmark adjuvant, formulated as indicated by the manufacturers. Six weeks after immunization, HAI titers in the GVI3000 adjuvanted group (GMT = 24) were significantly higher than those in the iFlu-only group (GMT = 10). The HAI titer in the alum-adjuvanted group (GMT = 16) was not significantly different from either GVI3000 or no adjuvant groups **(Figure 3.1E)**. At other time points (3 and 15 weeks), we did not observe any difference in HAI titers between the immunization groups (data not shown).

In summary, GVI3000 adjuvant significantly enhanced antibody responses to iFlu in neonatally immunized mice. Enhanced serum antibodies were mostly of the IgG2a class and had HAI activity. While antibody titers continued to increase during the 15 weeks of study, these titers were of much lower magnitude compare to those induced by the same vaccine regime in adult mice (unpublished observation). While this was true for serum antibodies after one dose, the ability of the adjuvant to prime a mucosal response seemed as effective in neonates as in adults.

GVI3000 adjuvant induces antigen-specific CD4 and CD8 T-cells after a single immunization in neonatal mice

While the contribution of T-cell immunity to protection against influenza remains a matter of debate, the flu model was utilized to determine whether GVI3000 can enhance the induction of antigen-specific T-cells in neonatal mice. We chose to understand the T-cell responses in the DLN (popliteal) as opposed to the more distal LN (such as mediastinal). T-cell

responses are present in distal LNs at reduced but still detectable levels, relative to DLN responses. In these initial neonatal studies of immunity induced by GVI3000 it was prudent to first evaluate responses in the DLN where we anticipate the most robust response, with the expectation that immunity detected in one tissue will correlate with immune memory throughout the animal. Groups of three 7-day old mice were immunized in both rear footpads with iFlu alone (2μg), or iFlu mixed with GVI3000 (10^5IU). In this experiment, a group adjuvanted with alum also was included. Eight days after immunization, animals were sacrificed and single cell suspensions were prepared from the pooled (2 per mouse) popliteal DLN. Cells were evaluated for the presence of IFNγ-secreting CD8 and CD4 T-cells following stimulation with immunodominant HA peptides. We chose to detect HA-specific T-cells instead of other T-cells described in literature (such as nucleoprotein, NP) as we were not sure which proteins, other than HA, were intact in the inactivated influenza antigen. Furthermore, based on our experience, adult BALB/c mice are capable of inducing T-cells specific for multiple antigens after a multivalent immunization, indicating that the T-cell response will most likely be not limited to HA antigen only. Animals receiving iFlu alone produced undetectable to very low levels of flu-specific $CD4^+$ and $CD8^+$ T-cells. However, iFlu formulated with GVI3000 adjuvant resulted in a significant increase of both $CD4^+$ and $CD8^+$ T cells **(Figure 3.2)**. Although the alum group had low but detectable amounts of $CD4^+$ T-cells and $CD8^+$ T-cells, they were significantly lower than the GVI3000 immunized group. Taken together, these results strongly suggest that GVI3000 adjuvant can promote the induction of a CD8+ T cell response in the T_H2-polarized neonatal immune system.

Neonatal mice immunized with GVI3000-adjuvanted iFlu vaccine have reduced morbidity and mortality and reduced early post challenge virus titers in nose and lungs

Two independent flu challenge experiments were performed. In the first experiment, four litters of mice were immunized on day 7 post birth with either PBS (N=8), iFlu-only (N=5), iFlu with GVI3000 (10^5IU; N=5) or iFlu formulated with alum (1:5; N=7). Three weeks after immunization, the mice received a lethal challenge by intranasal inoculation with 10^6 EID_{50} of Influenza A/PR/8/34. The mice were monitored daily for 11 days for weight loss, morbidity, and mortality. **Figure 3.3A** shows the survival curves. All the PBS-immunized control mice succumbed to the challenge. All mice that received a single GVI3000-adjuvated immunization were protected. Three out of 7 mice that received alum-adjuvanted iFlu antigen survived, while 2 out of 5 mice in the iFlu-only immunized group survived. A repeat challenge experiment using a different stock of live influenza virus was done with three immunization groups: PBS control (N=6), iFlu only (N=11) and iFlu with GVI3000 (N=11). In this experiment mice were monitored for 25 days. No protection was observed in PBS control mice or iFlu-only immunized mice, while 7 out of 11 mice survived in the GVI3000-adjuvanted group **(Figure 3.3B)**. Taken together, the two experiments demonstrate that the GVI3000-adjuvanted vaccine outperforms vaccine alone, but that the vaccine adjuvanted with alum was no more protective than vaccine alone. Post-challenge weight change relative to the starting weight is shown in **Figures 3.3C-F** for the first challenge experiment. In the GVI3000 adjuvant group, one mouse showed no weight loss, although the remaining animals lost ~12% of their starting weight on average before recovering completely; none of the mice showed any overt clinical signs of disease. In the alum group, the three mice that survived the challenge showed ruffling and hunching. Taken together, these results demonstrate that GVI3000-adjuvanted vaccine mediated significant protection from

influenza induced morbidity and mortality in neonates.

The effect of neonatal immunization on replication of challenge virus in the nasal tissue was detected at 48 h post-challenge by real-time PCR. There was a significant decrease in virus titer in the GVI3000- and the alum-adjuvanted groups compared to the iFlu-only group when mice were challenged 5 weeks post neonatal immunization **(Figure 3.4A)**. Additionally, we measured virus titers in the lung on day 3 post-challenge. Mice that received the GVI3000-adjuvanted vaccine showed no detectable $TCID_{50}$ titers (level of detection = 43.1 $TCID_{50}$/ml), which was a reduction of over two orders of magnitude compared to titers from PBS ($p<0.01$), iFlu only ($p<0.01$) and iFlu+alum groups ($p<0.05$) **(Figure 3.4B)**. Although titers in the alum-adjuvanted group were significantly higher compared to GVI3000-adjuvanted mice, they were significantly lower compared to iFlu ($p<0.05$).

These results are consistent with the survival data shown in Figure 3, and suggest that although alum provides comparable protection early in the nose, GVI3000 adjuvant is better at preventing replication in the lungs as well as preventing morbidity and mortality.

GVI3000 primarily targets DCs and promotes recruitment of multiple immune cell types into the DLNs of neonatal mice

GVI3000 adjuvant induces recruitment and infection of APCs in the DLN of adult mice. Although DCs are the primary targets of GVI3000, other immune cells are also targeted and it is likely that the targeting of DCs and other APCs play a role in the adjuvant effect in adult mice (268, 327). Further investigation led to the findings that inflammatory DCs, although rare in the DLNs during steady state, were rapidly recruited to the site of inflammation and were the subsets that had the largest fold-increase in immune cell population (268).

Given that the neonatal immune response is limited in part by a reduced numbers of immune cells, one important question was to determine the targets of GVI3000 in neonatal mice. Groups of five mice were immunized in both footpads with PBS, iFlu (1μg) alone, or iFlu together with GVI3000 expressing GFP (GVI3000-GFP; 10^5IU). GVI3000-GFP is identical to GVI3000 except that a subgenomic promoter driving the expression of GFP has been added downstream of NSP4. As GVI3000 cannot propagate to surrounding cells, and as infection of and RNA replication in the target cells must occur to allow GFP expression, cells containing GFP unequivocally identify GVI3000 target cells that support RNA replication (286). At 12hpi, both popliteal DLNs were harvested from each neonatal mouse, combined and homogenized. First, we measured the cellularity of the DLN, or total number of cells per lymph node by flow cytometry, using the volume analyzed by Accuri™ C6 Flow Cytometer (BD) and back-calculating the number of cells in the starting sample. Neonates immunized with iFlu mixed with GVI3000-GFP had significantly higher DLN cellularity compared to mice immunized with iFlu alone **(Figure 3.5A)**. Previously published results in adult BALB/c mice showed a similar 2-3 fold increase in DLN cellularity in the presence of GVI3000 (268). Infiltrating immune cells were identified by antibody staining for the following surface markers: conventional DCs (cDCs; CD11c+, CD11b-), inflammatory DCs (iDCs; CD11c+, Ly6C-hi) **(Figure 3.5B)**, macrophages (CD11b+, CD11c-), and neutrophils (Ly6G+). Except for the cDC population, which did not differ significantly between the groups, all immune cell types measured (iDCs, neutrophils, and macrophages) showed significant population increases at 12hpi **(Figure 3.5C)**. The same was true for immune cell populations (B- and T-cells) at 24 hpi (data not shown). Inflammatory DCs showed the most significant increase (11× fold increase; $p = 0.0079$), and a similar increase was observed at 12, 18 and 24 hpi (data not shown).

To determine which cell populations were targeted by GVI3000 in the neonates, GFP positive cells were quantified in the DLN at 18 hpi. Although GFP expression was observed in several different immune cell types, iDCs were the predominant target of GVI3000 replication **(Figure 3.5D)**. Up to two-thirds of all cells expressing GFP were iDCs. Conventional DCs, macrophages, and neutrophils each represented 6-8% of all infected cells. Studies in adult BALB/c mice showed a similar infection pattern of cell populations (268). Approximately 11% of the GFP positive cells were unidentified. Previous reports and unpublished observations in adult mice suggest that these unidentified cells may be T-cells, B-cells, and NK cells (268).

GVI3000 adjuvant activates neonatal dendritic cells

The activation and maturation of infected and bystander DCs was identified by measuring the early *in vivo* expression of MHCII and the co-stimulatory molecules CD40, CD80, and CD86. Three groups of four neonatal mice were immunized with: PBS, iFlu (1μg) alone, or iFlu with GVI3000-GFP (10^5IU). GVI3000-GFP served as an adjuvant to iFlu and as an expression vector for GFP. This marker was used to distinguish the activation state of infected vs. bystander DCs in the GVI3000 adjuvanted group. Both DLNs were harvested from each mouse, homogenized, and stained at 18hpi. After gating for inflammatory DCs, the cells were further gated for GFP expression and analyzed for MHCII, CD40, CD80, and CD86 (Figure 6). The expression level of all activation/co-stimulatory markers (indicated by Mean Fluorescence Intensity, MFI) observed in the iFlu-only group did not differ from the PBS immunized group. There were no differences in the MFI of activation/co-stimulatory markers between the iDCs of the antigen-only group and the GFP-negative iDCs in GVI3000-GFP immunized group **(Figure 3.6A-D)**. However, iDCs that were GFP-positive (i.e. GVI3000 infected) had significantly higher

MHCII, CD40, CD80, and CD86 MFIs. The trend was similar for cDCs, with MHCII, CD80, and CD86 showing significantly higher GFP-positive cells compared to GFP-negative cells and cDCs in the antigen-only immunized group (data not shown). We believe that the GFP-positive cells detected in this experiment were due to direct infection by GVI3000-GFP and not due to phagocytosis of infected cells. It has been previously demonstrated in adult BALB/c mice that GVI3000-GFP infection leads to uniform GFP signaling in the cytoplasm instead of punctate GFP signaling, which would have indicated phagocytosis (198) (Laura White, unpublished observations).

In summary, as seen in adult mice, GVI3000 targets and activates neonatal iDCs and cDCs. Bystander iDCs were not activated at 18 h in the DLN of neonates receiving GVI3000 adjuvant. This seems to be different to what was reported *ex vivo* in bone marrow-derived DCs from adult mice , where bystander DCs were also activated (303).

GVI3000 induces several inflammatory cytokines in neonatal mice early after immunization

Studies in adult mice have shown that the GVI3000 adjuvant can significantly enhance the inflammatory cytokine/chemokine response after immunization (287). These effector molecules appear within a few hours and return to basal levels within 24 to 36 hours after exposure to GVI3000. It has been suggested that human neonatal plasmacytoid DCs are incapable of inducing Type I IFNs similar to adult levels (335). Systemic type I IFN was induced to a high level in serum by 6 hpi in neonatal mice immunized with GVI3000-adjuvanted flu vaccine **(Figure 3.7)**. Furthermore, the response remains significantly higher compared to antigen-only immunized mice at least until 24hpi. By 36 hpi, the level of type I IFN had ebbed to

near basal levels. The kinetics of type I IFN induction is similar for the two antigens tested (iFlu and Fluzone® 2011 vaccine). The levels and kinetics of these IFN responses were similar to those observed in control adult BALB/c mice (data not shown), indicating that 7-day old mice are capable of adult-like type I IFN innate immune responses to GVI3000-adjuvanted antigens, including both rapid induction and return to basal levels.

To measure local induction of other inflammatory cytokines in immunized neonatal mice, an 18-plex assay was performed measuring levels of cytokine/chemokine proteins in the popliteal DLN 12 hrs after footpad immunization **(Table 3.1)**. Several cytokines had 20 to 1,500 fold increases in the GVI3000 adjuvanted group compared to antigen-only, including IFNγ, IP-10, MCP-1, and MIG. Another group of cytokines, including G-CSF, MIP-1β, and RANTES also were higher by 5 to 15 fold at 12 hpi. Adult DLNs analyzed at the same time showed a very similar trend, qualitatively and quantitatively, with GM-CSF, IL-1β, and MIP-1β also showing >5-fold increases.

3.5. DISCUSSION

The data presented here demonstrates the feasibility of a new class of adjuvants for early life vaccination. The non-propagating alphavirus replicon particle adjuvant, GVI3000, was safe in neonatal mice, and when co-delivered with iFlu antigen, enhanced multiple effector functions of the neonatal immune system including antigen-specific IgG2a antibodies, mucosal IgA, and CD4+ and CD8+ T cells, indicating a shift from a T_H2-polarized to a balanced T_H1/T_H2 immune response that potentiated a significant increase in protection from a lethal influenza challenge. Many of the innate immune response cytokines and chemokines induced by GVI3000 in adult mice also were induced in neonatal animals. As in adults, neonatal DCs were targeted and

activated by the adjuvant particles, and a high proportion of inflammatory cells were recruited to the DLN. The relevance of these findings are several fold: First, it is the first report, to our knowledge, to show a protective immune response to influenza in a neonatal animal model, suggesting that the alphavirus adjuvants presumptively could be used in infants. Second, a single immunization with the GVI3000-adjuvanted vaccine was sufficient to protect in this model, which represents a significant improvement over existing early life vaccines that require multiple immunizations, including current vaccines for influenza. This is especially relevant in poor countries, where birth and early life are often the only opportunities for patient contact with healthcare providers. Third, GVI3000 activates neonatal innate immunity and enhances all arms of the adaptive immune system, including the humoral, cellular and mucosal compartments.

It is well established from studies in animal models and humans that the immune response to vaccination in neonates is weaker than that of adults (68, 70, 101, 296). It is characterized by **i)** serum antibody titers that are lower in magnitude (36, 297) and of shorter duration (298, 299), **ii)** APCs that show reduced upregulation of co-stimulatory molecules (69), **iii)** T-cells that exhibit a T_H2 bias, secreting high levels of IL-4 and low amounts of IFNγ (68, 115-118), and **iv)** significantly limited CTL responses to vaccines (reviewed in reference (70)). In this study, we confirmed the age-related deficiencies in neonatal mice relative to adults in different areas of the adaptive and innate immune responses. After a single immunization IgG Ab titers in neonates were significantly lower compared to those induced in adult mice, and HAI titers, while induced in adults, were undetectable in the neonatal mice. In addition, upon vaccination with antigen only, neonatal mice showed approximately 20 fold fewer cells in the popliteal DLN compared to adults, including reduced numbers of iDCs, cDCs, neutrophils and macrophages. Furthermore, the neonatal response to antigen alone was characterized by lower

expression of most cytokines in a panel of 18 inflammatory cytokines and chemokines examined. Other deficiencies observed in the neonatal responses to iFlu, such as minimal to undetectable induction of CD4+ or CD8+ T cells and no mucosal IgA priming, were also present in the adult responses, and therefore were in part determined by the nature of the antigen.

Although the overall magnitude of the serum Ab titers in the GVI3000-adjuvanted groups were still lower in neonates compared to adults, the GVI3000 adjuvant was responsible for a significant increase in flu-specific IgG antibodies, especially IgG2a, at 3, 6, and 15 weeks, and HAI Abs at 6 weeks after a single immunization. Additional experiment using an inactivated dengue virus antigen (serotype 4; iDENV4) also showed significantly higher dengue-specific total IgG and IgG2a antibodies early after immunization (Supplementary Figure 1). Comparing with alum-adjuvanted iDENV4, we observed that while both adjuvanted neonatal mice groups had similar total IgG antibodies, GVI3000-adjuvanted mice primarily had increase in IgG2a subclass, while alum had increase in IgG1 subclass. In the mucosal compartment, antigen-specific IgA was induced after two immunizations only when GVI3000 was present in the priming inoculation, with titers that were similar to those induced in mice primed as adults. Since we have previously demonstrated that unadjuvanted iFlu in adult mice does not induce mucosal IgA after one or two immunizations (Thompson and Johnston, unpublished), our data suggests that the induction of mucosal IgA in neonatally primed mice is due to the primary immunization and not to the antigen-only boost. The present study assumes that the fecal IgA was produced at the mucosal surface (336), as has been observed with iFlu in adults (290, 327). Therefore, the GVI3000 adjuvant may be of particular benefit in neonatal vaccines against mucosal pathogens. In the neonatal T_H2-polarized immune response, antigen-specific, IFNg-producing T cells are poorly induced by immunization with non-replicating antigens (70). GVI3000 was able to

overcome this deficiency after one immunization.

The presence of GVI3000 also resulted in improvement of protection in the intranasal lethal challenge model. However, in the second challenge experiment (Figure 3B), not only the GVI3000-adjuvanted group, but also the control unadjuvanted group had a higher number of lethal infections compared to the first challenge experiment (Figure 3A), implying that the second challenge was more stringent than the first one. This can be explained as the two independent challenge experiments were performed with different challenge stocks (live influenza PR8) obtained separately from the same commercial vendor (Charles River, MA), and that it is not unexpected that minor differences in virus titers may result in differences in virulence. Our results show that while GVI3000-adjuvanted neonatal mice had significantly improved protection compared to mice immunized with iFlu only, the use of alum as an adjuvant did not improve protection, despite similar functional HAI antibody titers at 3 weeks post-immunization. This implies that the improved protection mediated by GVI3000 may be attributed to immune effectors other than HAI antibodies. It has been shown previously that antibodies are effective in protection from homologous influenza virus challenge in adult mice (337-339). It is likely that in neonates, due to the weaker antibody response to iFlu (even with adjuvant) those antibody levels may not be sufficient for complete protection. Under these circumstances, and as suggested by our data, cellular and mucosal immune responses, if induced, could play a significant role in early life protection.

One of the reported deficiencies of the neonatal immune system is the limited quantity of immune cells available as compared to adults (68, 91, 135, 340-342). Consistent with this, our observations show that the cellularity of the popliteal DLN of an unimmunized 7-day old BALB/c mouse or an age-matched animal immunized with antigen-only was lower than a 6-

week old adult mouse. In GVI3000-adjuvant inoculated neonatal mice we observed a significant 2-3 fold increase of DLN cellularity, which was similar to the percentage increase in cellularity induced by GVI3000 in adult mice reported by Tonkin et al (268). Additionally, we observed that the total number of iDCs in GVI3000-adjuvanted neonatal DLN accounted for approximately 8% of the total number of cells in the DLN, while it was <2% in the adult DLN (268). The robust recruitment and targeting of iDCs suggests an important role for these cells in the mediation of the GVI3000 adjuvant effect. DCs have been shown to be sufficient to mediate the GVI3000 adjuvant effect (268), but it is possible that other cell types complement the role of iDCs. We therefore cannot definitively point to a specific cell type that mediates the immune enhancement driven by GVI3000, but we predict that the ability of GVI3000 to trigger cell recruitment in neonates is an important component of this outcome.

We hypothesized that, as in adult mice, the adjuvant effect of GVI3000 in neonates was associated with the ability to **i)** target and activate neonatal DCs, and **ii)** induce a robust T_H1 innate immune response. Previous *in vitro* studies using VEE replicon particles (VRP) expressing GFP to infect adult human (303) and murine BMDCs (325) showed an upregulation in co-stimulatory molecules CD40, CD80, and CD86. Additional studies using VRP-transduced human DCs showed DC maturation, secretion of proinflammatory cytokines, and significant expansion of antigen-specific T-cells (303). In our *in vivo* system, we observed that both conventional and inflammatory DCs had significantly higher expression of MHCII, CD40, CD80, and CD86 after administration of GVI3000. However, this increase was observed only in DCs that were GVI3000-positive (GFP positive), but not in the "bystander DCs". These results indicate that the infected DCs are the primary driver of the adjuvant effect of GVI3000 in the neonates. This finding is in contrast to what was described in *in vitro* studies of adult DCs, where

both GVI3000 infected and bystander DCs showed up-regulation of those markers (303). We can speculate that the absence of activation of by-stander DCs in neonates at the time points examined may contribute to the reduced magnitude of the immune responses in neonates compared to adults. On the other hand, we cannot rule out that the "by-stander" DCs would show a significant increase in expression of maturation and costimulatory molecules if examined at later times, after 18 h, as was observed *in vitro* at 24 h post-infection of human DCs (303). Further studies are needed to better understand the kinetics of DC maturation and expression of costimulatory molecules *in vivo* and its effect on T-cell response.

Our second hypothesis was that the significantly improved humoral and cellular GVI3000-adjuvant effect was associated with a robust T_H1 cytokine response initiated early after immunization. Consistent with this hypothesis a rapid, strong, and self-limited innate immune response was observed locally and systemically upon GVI3000 immunization, based on the rapid cytokine induction. The innate immune responses were consistent even when other antigens were used (data not shown), indicating that the GVI3000 adjuvant was primarily responsible for the enhanced response.

Our results indicate that GVI3000 can induce adult-like systemic and local innate immune responses in neonates. Although type I IFN function is not required for the adjuvant activity of GVI3000 in adult mice (330), it is an indicator of GVI3000 RNA replication and of a robust innate immune response. The observation that neonatal mice had adult-level type I IFN responses to GVI3000 indicates that the adjuvant may overcome the impairment in type I IFN induction observed in newborn cord blood (343, 344). Additionally, the type I IFN response was systemic, peaking at 12 h post immunization and falling to background level by 36 hrs. This demonstrates that the GVI3000-mediated inflammatory response is transient, reducing any

chance of prolonged inflammation or toxicity. One of the reasons for immunizing neonatal mice through the footpad was the ability to investigate the process of inflammation in a single DLN. Cytokine expression analysis in the DLN further supported our second hypothesis in that the enhanced cellular immune response is preceded by a T_H1-promoting innate immune response. As with systemic Type I IFN expression, a similar pattern of peak expression of most cytokines at 12 hpi was observed with gradual reduction to background level by 36 hpi (data not shown).

IFNγ is a hallmark of a T_H1 response; however, its production is impaired in newborns as evidenced by lack of IFNγ induction in neonatal mice after subcutaneous injection of LPS (345). BCG vaccination is one of the few examples of vaccines that can induce adult-like IFNγ as has been demonstrated in CD4 T-cells of two-month old human newborns—immunized at birth—after stimulation with a mycobacterial purified protein derivative (94). IFNγ protein expression was rapidly upregulated in neonatal mice after GVI3000 inoculation, which induced an adult-like response (adult data not shown). The IFNγ-responsive chemokine IP-10 (CXCL10) (346) was significantly upregulated in GVI3000 adjuvanted neonatal mice to a level similar to that in adult animals. As observed in adult mice, GVI3000 did not induce IL-12 protein expression, even though this cytokine is strongly associated with induction of IFNγ and a T_H1 response. Therefore, further investigation is needed to elucidate the role of IL-12, or lack thereof, in the GVI3000 adjuvant mode of action. Other cytokines that were upregulated, but do not have a clearly defined role in T_H1 responses, were MCP-1, MIG, and MIP-1β. Overall, the cytokine expression pattern between neonates and adults was quite similar, indicating **i)** neonates are capable of mounting an adult-like innate immune response, and **ii)** the mechanism whereby the GVI3000 adjuvant induces innate immune response may be similar in neonates and adults.

Improved technologies are urgently needed for vaccines against several infectious

diseases, especially for children in developing countries. This study demonstrates the potential of GVI3000 as an effective adjuvant for early life vaccination combined with currently available and future vaccines.

Figure 3.1

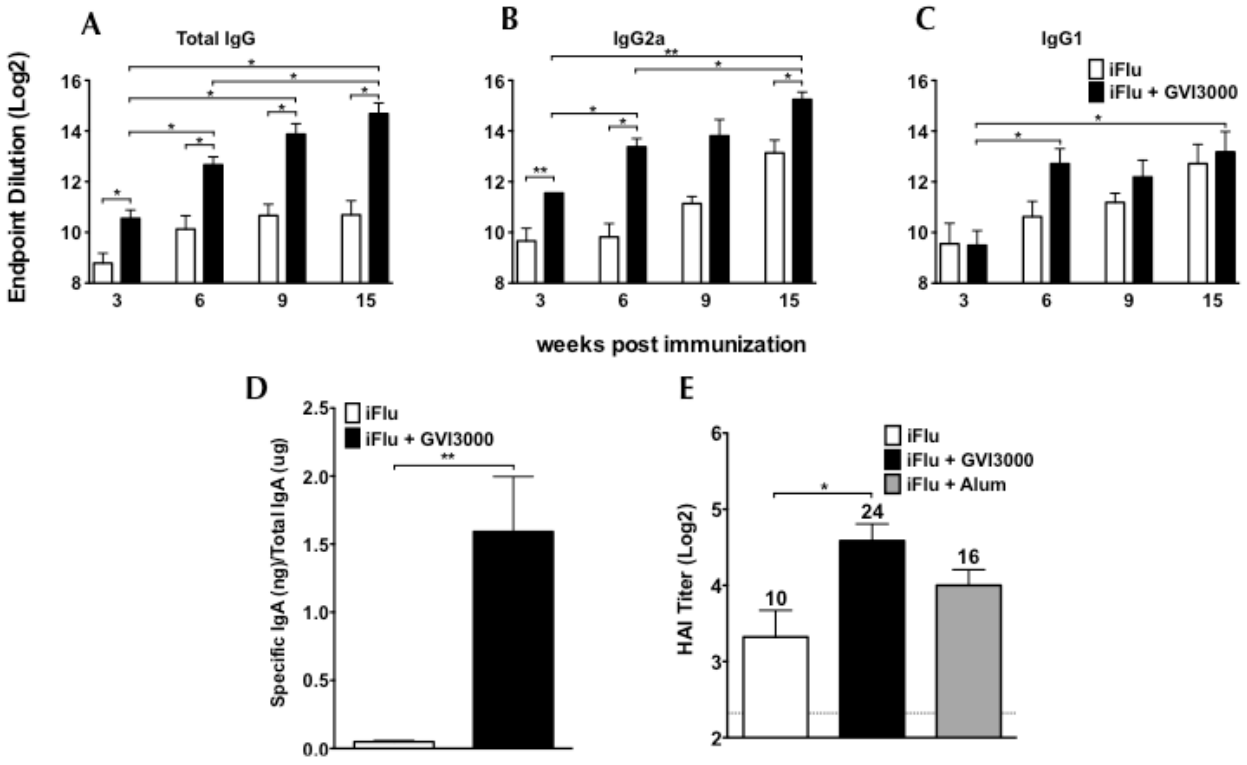


Figure 3.1: GVI3000 is an effective adjuvant to iFlu after a single dose in mice immunized as neonates.

Three to five 7-day old BALB/c mice per experimental group were primed through f.p. injection with iFlu (2μg) in the presence (gray bars) or absence (empty bars) of 10^5 IU of GVI3000. Three, 6, 9, and 15 weeks after the immunization, flu-specific **(A)** IgG and, **(B)** IgG1 and IgG2a antibodies were measured in sera by ELISA. **(C)** GVI3000 induces mucosal immune response in neonatally primed mice. Five or six 7-day old BALB/c mice were primed through f.p. injection with iFlu (1μg) only (empty bars) or with 10^5 IU of GVI3000 (gray bars). Ten week post-prime all groups were boosted with iFlu (1μg) alone. Ten days post-boost, antigen-specific IgA were measured in fecal extracts by ELISA. IgA amount produced is presented as ng of flu-specific IgA / μg of total IgA. **(D)** Antibodies with Influenza hemagglutination inhibition activity were

measured by Hemagglutination Inhibition Assay (HAI) at 6 weeks after the immunization. All data are presented as the mean +/- SEM. *, $p<0.05$; **, $p<0.01$ compared with iFlu alone as determined by Mann-Whitney.

Figure 3.2

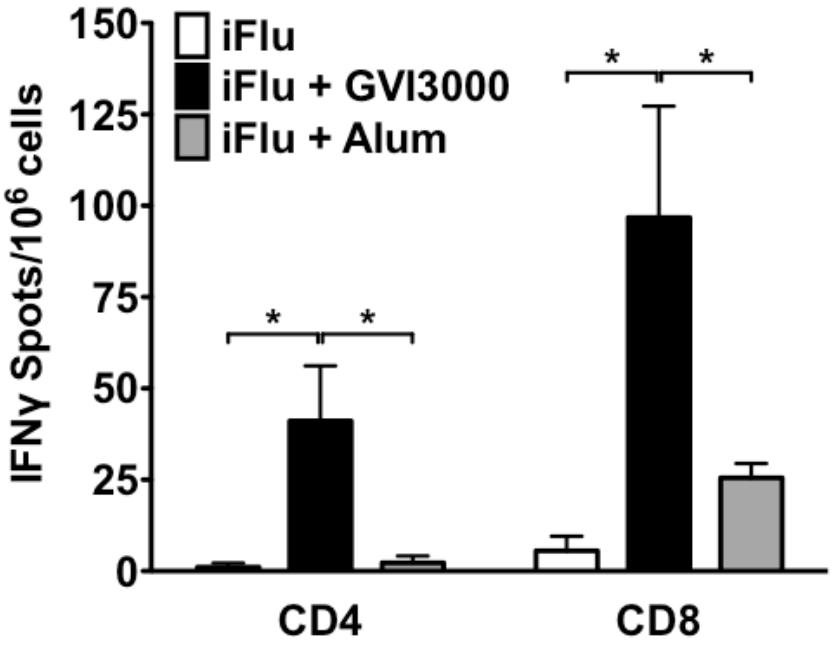


Figure 3.2: GVI3000 adjuvant promotes flu-specific cellular immune response in neonatal mice DLN after a single neonatal immunization.

Three 7-day old BALB/c mice were primed through both rear f.p. with iFlu (2μg) in the presence (gray bars) or absence (empty bars) of 10^5 IU of GVI3000. As a control adjuvant, three mice from one litter were immunized with alum (1:2 by volume; black bars). Eight days after immunization, both popliteal draining lymph nodes from each mouse were harvested and pooled. DLN cells from each experimental group were stimulated with immuonodominant CD4 and CD8 HA peptides followed by IFNγ release ELISPOT assay. Data was normalized to the mock-stimulated positive spots for each experimental group and graphed as number IFNγ positive spots per 10^6 DLN cells. Data are presented as the mean +/- SEM. *, $p<0.05$; **, $p<0.01$ compared with iFlu alone as determined by Mann-Whitney.

Figure 3.3

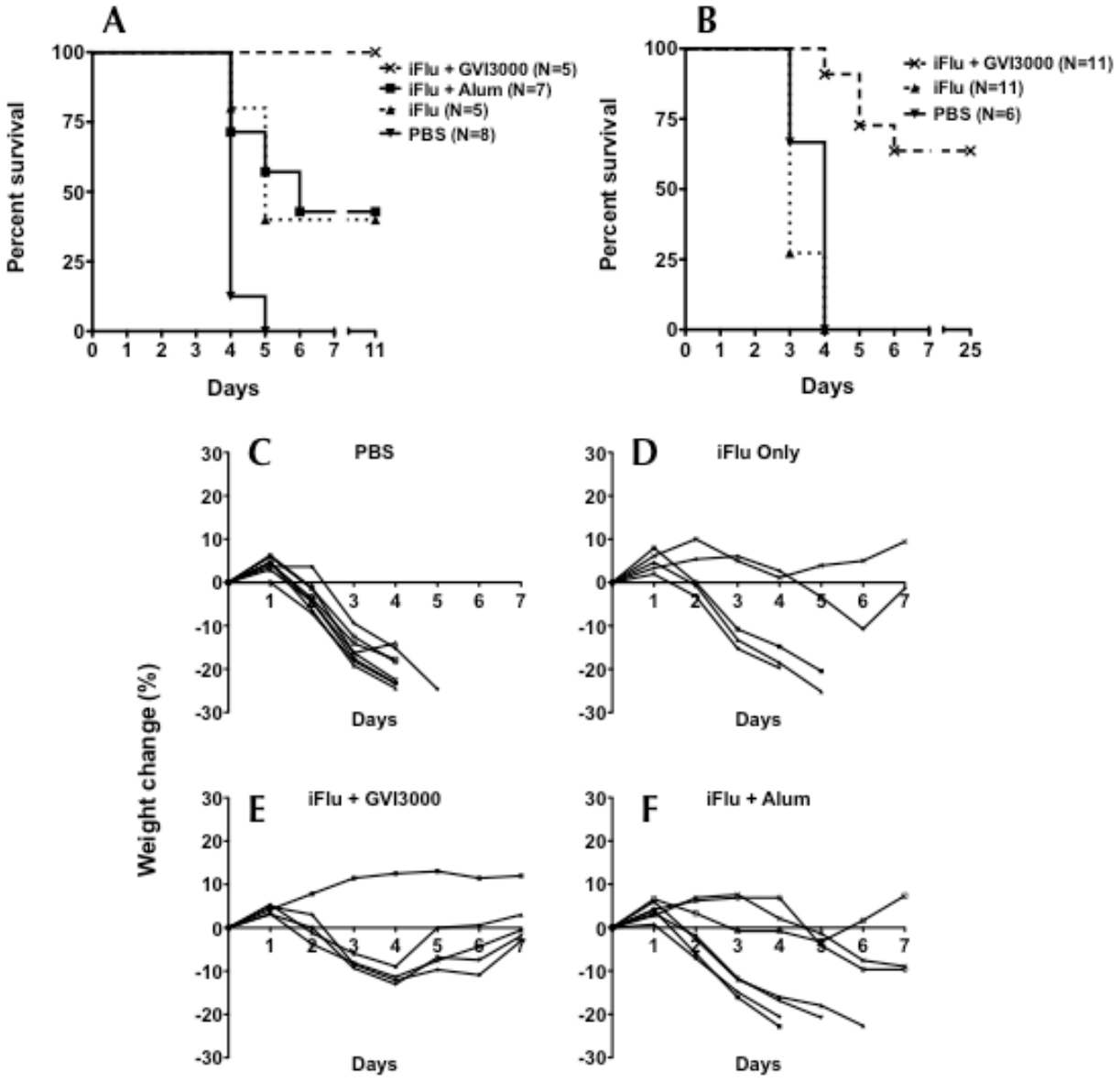


Figure 3.3: GVI3000-adjuvanted iFlu protects neonatally immunized mice from a lethal influenza challenge.

(A) Neonatal mice were immunized with iFlu only (N=5), or with either GVI3000 (10^5IU; N=5) or alum (1:5; N=7). Included in the challenge were PBS immunized mice (N=8). All mice were intranasally challenged with a lethal dose of 10^6 EID_{50} of Influenza A/PR/8/34 virus at 3 weeks after immunization. Mice were monitored for **(A)** mortality and **(C-F)** weight for 7 days after the

challenge. **(B)** Neonatal mice were immunized with iFlu only (N=11), or with GVI3000 (10^5IU; N=11). Included in the challenge were PBS immunized mice (N=6). All mice were intranasally challenged with a lethal dose of 10^6 EID_{50} of Influenza A/PR/8/34 virus at 3 weeks after immunization and monitored for survival.

Figure 3.4

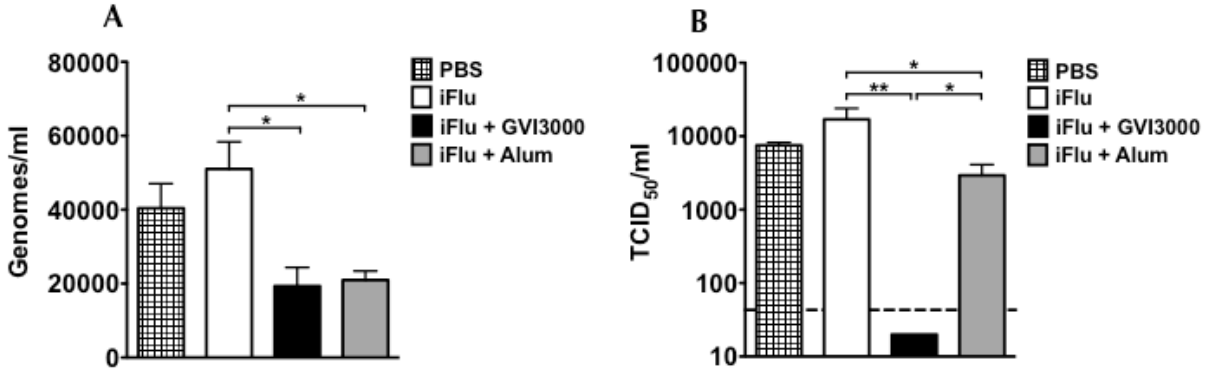


Figure 3.4: Viral titers in nasal tissues and lungs of challenged mice.

Groups of 5 seven-day old mice were immunized with either iFlu (1μg) alone or with an adjuvant: GVI3000 (10^5IU) or alum (Alhydrogel 1:5). An additional PBS immunized group was included. Five weeks after the single immunization, mice were challenged intranasally with 10^6 EID_{50} of Influenza A/PR/8/34. **(A)** Mice were euthanized 48 h post-challenge and nasal tissues collected in Trizol. Following RNA extraction, real-time PCR was done with PR8 HA primer/probes. C_T values were plotted on PR8 HA standard curve to calculate virus titer. **(B)** Mice were euthanized 3 days post-challenge and their lung harvested. After homogenization of lung tissues, $TCID_{50}$ assay was performed on MDCK cells. Cell supernatants were collected after 48 h of incubation and hemagglutination assay was performed with 5% chicken red blood cells. $TCID_{50}$/mL values were calculated using the Reed-Muench method. All data are presented as the mean +/- SEM. * $p<0.05$, ** $p<0.01$ as determined by Mann-Whitney.

Figure 3.5

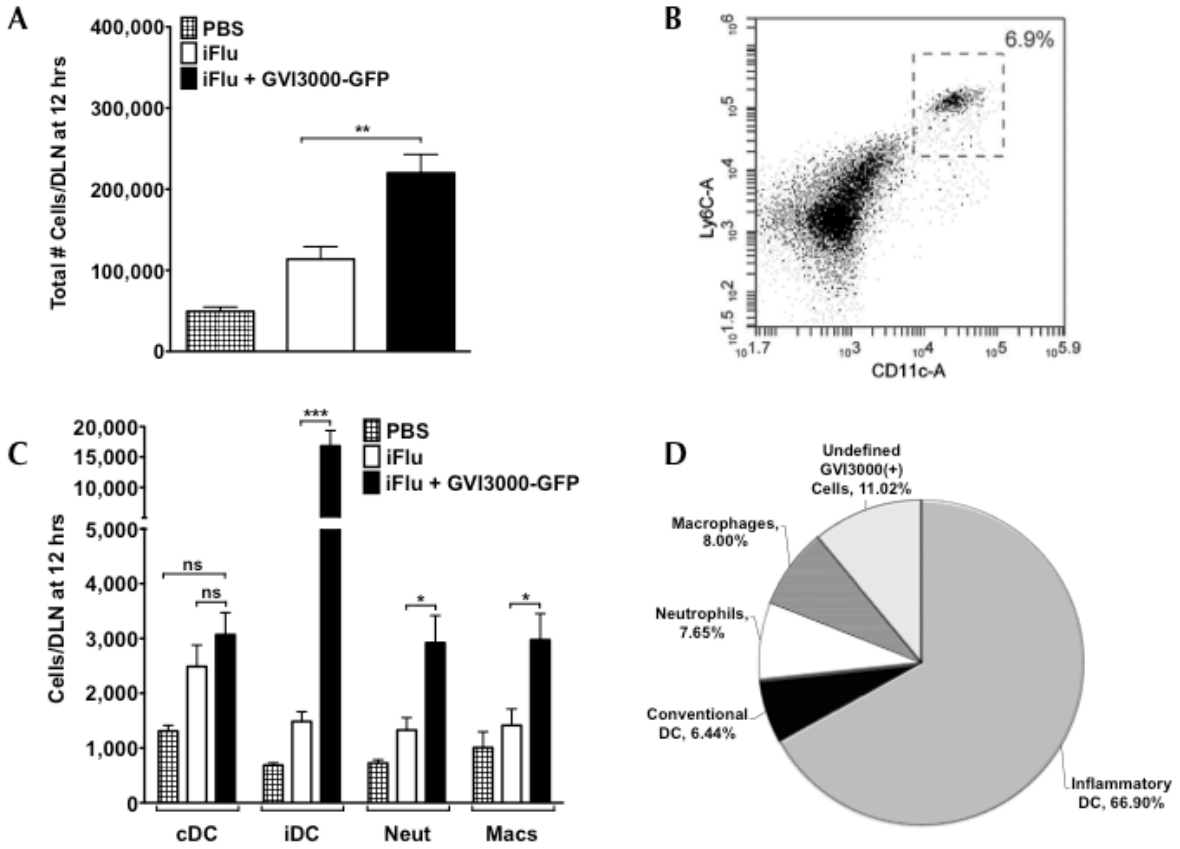


Figure 3.5: GVI3000 enhances inflammation in the DLNs of neonatal mice.

Five 7-day old BALB/c mice were immunized in both f.p. with PBS only (empty bars), and iFlu (1µg) in the presence (gray bars) or absence (black bars) of 10^5 IU of VRP-GFP. At 12hpi, both popliteal DLNs from each neonatal mouse were harvested, combined, and manually disrupted into single cell suspension. **(A)** DLN cellularity was assessed by using the volume analyzed by Accuri™ C6 Flow Cytometer (BD) and back-calculating the starting sample amount. **(B)** A representative histogram for inflammatory Dendritic cell gating, **(C)** Using fluorescent antibody staining, immune cells were stained for DCs (CD11c+), Inflammatory DCs (CD11c+, Ly6C-hi), Neutrophils (Ly6G+), and macrophages (CD11c-, CD11b+). Graph represents the total number

of each immune cell present in the DLN sample. **(D)** Cells that were positive for GFP expression were then gated for different immune cells' surface markers. Data are presented as the mean +/- SEM. * $p<0.05$, ** $p<0.01$, and *** $p<0.001$ as determined by Mann-Whitney.

Figure 3.6

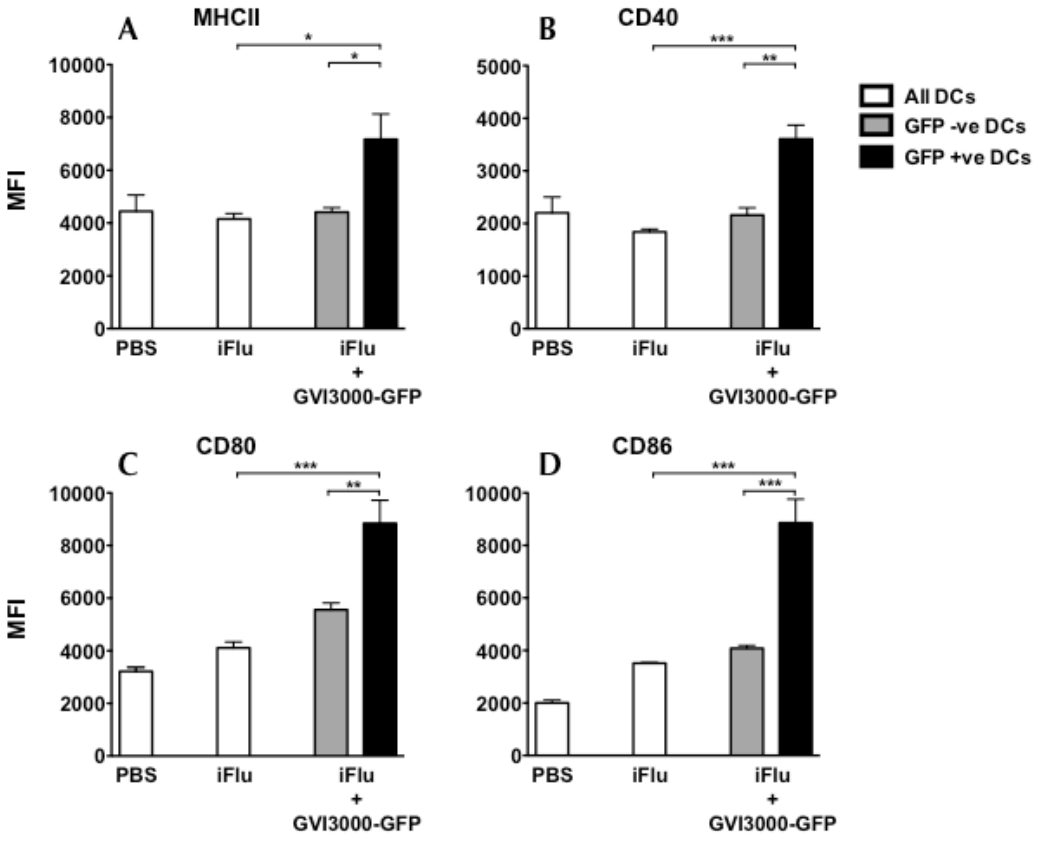


Figure 3.6: Dendritic cells are activated in VRP-GFP adjuvanted mice.

Three groups of four mice each were immunized with PBS, iFlu (2μg) only, or iFlu (2μg) with VRP-GFP (10^5IU). Immune cells from the DLN (two per mice per pool) were harvested and stained at 18 hpi for inflammatory DCs (CD11c+, Ly6C-hi). VRP-GFP adjuvanted group had both GFP-positive (black bars) and -negative (empty bars) DCs. Inflammatory DCs were gated for **(A)** MHCII, **(B)** CD40, **(C)** CD80, and **(D)** CD86 surface markers. Data are presented as the mean +/- SEM. * p<0.05, ** p<0.01, and *** p<0.001 as determined by Mann-Whitney.

Figure 3.7

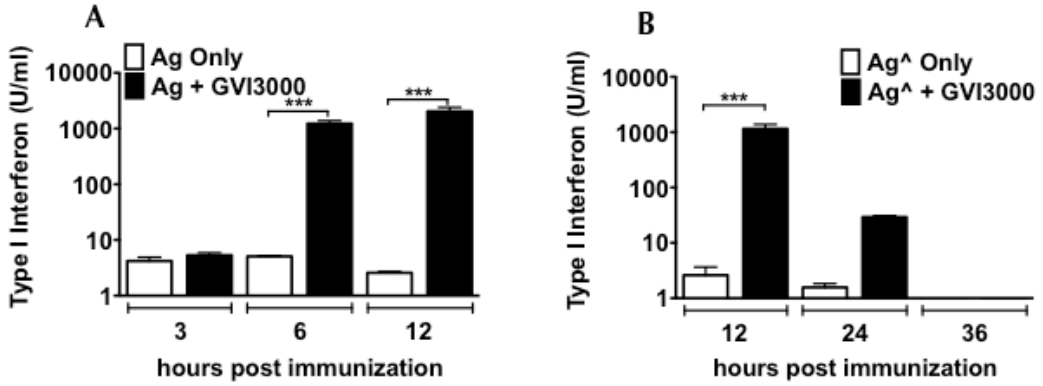


Figure 3.7: GVI3000 induces systemic Type I interferon immune response neonatal mice.

At least four 7-day old BALB/c mice were immunized through f.p. with Ag (iFlu; 1μg) or Ag^ (Fluzone; 1μg) by itself (empty bars) or with 10^5 IU of GVI3000 (gray bars). At 3, 6, 12, 24, and 36hpi mice were terminally bled. Type I IFN response was measured by IFN bioassay. Data are presented as the mean +/- SEM. *** $p<0.001$ as determined by Mann-Whitney.

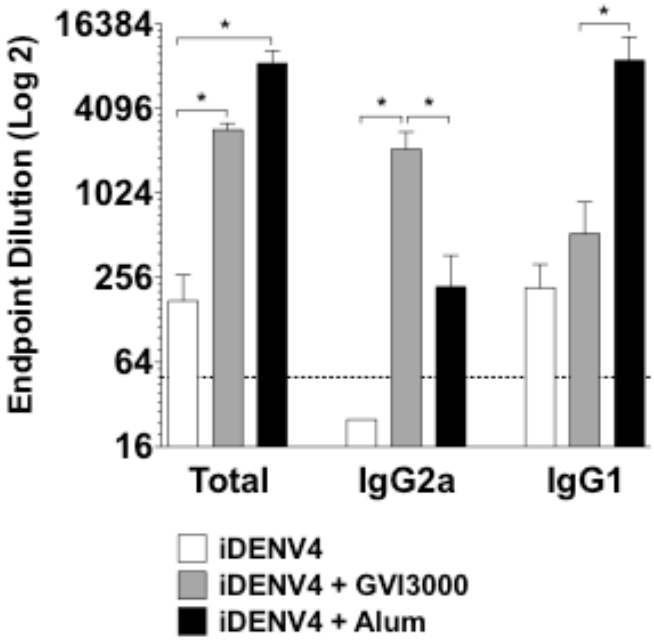
Table 3.1

Cytokine levels in the DLNs of neonatal mice at 12hpi (pg/mL±SEM).

	PBS	iFlu	iFlu + GVI3000	Fold Increase over iFlu
G-CSF	28.84 ± 1.55	29.92 ± 1.92	173.23 ± 7.66	5.79
GM-CSF	6.90 ± 0.57	11.21 ± 3.72	36.02 ± 1.64	3.21
IFNγ	0.60 ± 0.00	0.60 ± 0.00	917.16 ± 102.16	1,528.60*
IL-1β	4.10 ± 0.51	11.99 ± 1.72	25.78 ± 0.96	2.15
IL-2	28.11 ± 1.71	30.44 ± 0.47	33.25 ± 1.18	1.09
IL-4	0.26 ± 0.06	0.41 ± 0.10	1.54 ± 0.13	3.76
IL-5	1.02 ± 0.52	4.94 ± 1.83	18.37 ± 0.91	3.72
IL-6	32.21 ± 11.14	51.72 ± 2.81	148.99 ± 4.67	2.88
IL-10	7.63 ± 4.20	9.60 ± 3.15	20.48 ± 2.20	2.13
IL-12p70	2.40 ± 0.00	2.40 ± 0.00	2.40 ± 0.00	1.00
IP-10	79.55 ± 7.78	99.66 ± 11.41	2731.05 ± 69.26	27.40*
MCP-1	8.07 ± 2.87	11.02 ± 3.55	230.22 ± 18.52	20.89*
MIG	122.99 ± 30.75	73.29 ± 5.66	7338.89 ± 227.30	100.13*
MIP-1α	11.77 ± 0.94	18.13 ± 0.98	55.51 ± 4.89	3.06
MIP-1β	7.02 ± 0.95	8.63 ± 0.90	134.45 ± 12.81	15.58
MIP-2	75.43 ± 1.71	70.57 ± 2.70	96.66 ± 3.63	1.37
RANTES	7.02 ± 0.93	5.63 ± 1.05	39.78 ± 3.14	7.07
TNFα	1.60 ± 0.00	1.60 ± 0.00	5.24 ± 0.66	3.28

Footpad immunization of 7-day old mice. * indicates a significant difference of at least $p < 0.05$.

Supplementary Figure 3.1



Supplementary Figure 3.1: GVI3000 is an effective adjuvant to inactivated Dengue (serotype 4; iDENV4) after a single dose in mice immunized as neonates.

Three to six 7-day old BALB/c mice per experimental group were primed through f.p. injection with iDENV4 (2μg) in the presence (gray bars) or absence (empty bars) of 10^5 IU of GVI3000 adjuvant. A third group of mice were immunized with iDENV4 (2μg) adjuvanted with alum (1:2 volume). Three weeks after the immunization, DENV4-specific Total IgG, IgG2a, and IgG1 antibodies were measured in sera by ELISA. The limit of detection (LOD) for this assay was 1:50 as indicated by the dashed line. Data below the LOD was assigned a value of 1:25, which is one dilution below the LOD. All data are presented as the mean +/- SEM. *, $p<0.05$ compared with iFlu alone as determined by Mann-Whitney.

CHAPTER 4: DISCUSSION & FUTURE DIRECTIONS

4.1. Contributions and impact to the field

It started with a crude but effective smallpox vaccine in 1796, but the field of vaccinology has grown tremendously in the past two centuries. Vaccines remain one of the most important modern medical achievements. One disease, smallpox, has been eliminated, while others have been very successfully contained. The continued discoveries in understanding the pathogens, hosts, and their interaction has led to the development of novel treatment and prevention techniques for many diseases. To put a number on the lives saved and disease prevented (or their severity reduced) due to effective vaccination would, in my view, be underestimating the success. Probably due to the success of smallpox vaccines and others, in the late 1960s, the Surgeon General of the U.S., Dr. William H. Stewart, was (in)famously quoted as saying: "*It is time to close the book on infectious diseases, and declare the war against pestilence won*" (347). However, the fight against infectious diseases is still not over (and it probably never will). Although we have had success with traditional vaccination methods, continued development of safe, effective, and novel vaccine technologies will be important to combat infectious diseases. It is of further importance that the new vaccines provide protection in the most vulnerable of our population, including the very young.

The challenges of immunization in early life are greater in many ways than in an adult. Researchers in the field are beginning to better understand the differences between neonates and adults in terms of how the innate and adaptive immune responses are induced and regulated.

Those differences favor tolerance, but leave the very young less equipped to defend against pathogens. It has now become clear that the neonatal immune system is capable of inducing adult-like responses if the appropriate pathways are stimulated (68, 309).

The work presented in this thesis further supports those findings and contributes towards our understanding of how the induction of the innate and adaptive immune responses in neonatal mice compare to those in adult mice, and identifies pathways of the innate immune response that, when stimulated in the neonate, change the quality of the adaptive response.

Two different alphavirus-based vaccine platforms were used to show not only the induction of effective and protective immunity against two different infectious agents, influenza and dengue, but also as tools to better understand the induction of the neonatal immune response. In the VRP expression vector, the same replicon particle functions to deliver the antigen and to serve as innate immune-stimulant. On the other hand, in the VRP as adjuvant, the replicon particle only provides the danger signal function, while the antigen is not expressed from the replicon particle, but co-delivered as purified protein or inactivated virions. By separating the antigen delivery function from the adjuvant function in the VRP as adjuvant particles (GVI3000), we were able to demonstrate the role of the adjuvant function in the VRP, and start to understand how it mediates the changes in the quality and magnitude of the immune response in the neonate. We propose a model for how the adjuvant works in neonates in figure 4.1.

The work in this thesis suggests that a viral expression vector that operates by inducing a robust innate immune response would be able to overcome to some extent the lack of sufficient and protective immunity seen in neonates. This work should serve as a foundation for further research into the development of early life human vaccines for multiple pathogenic agents.

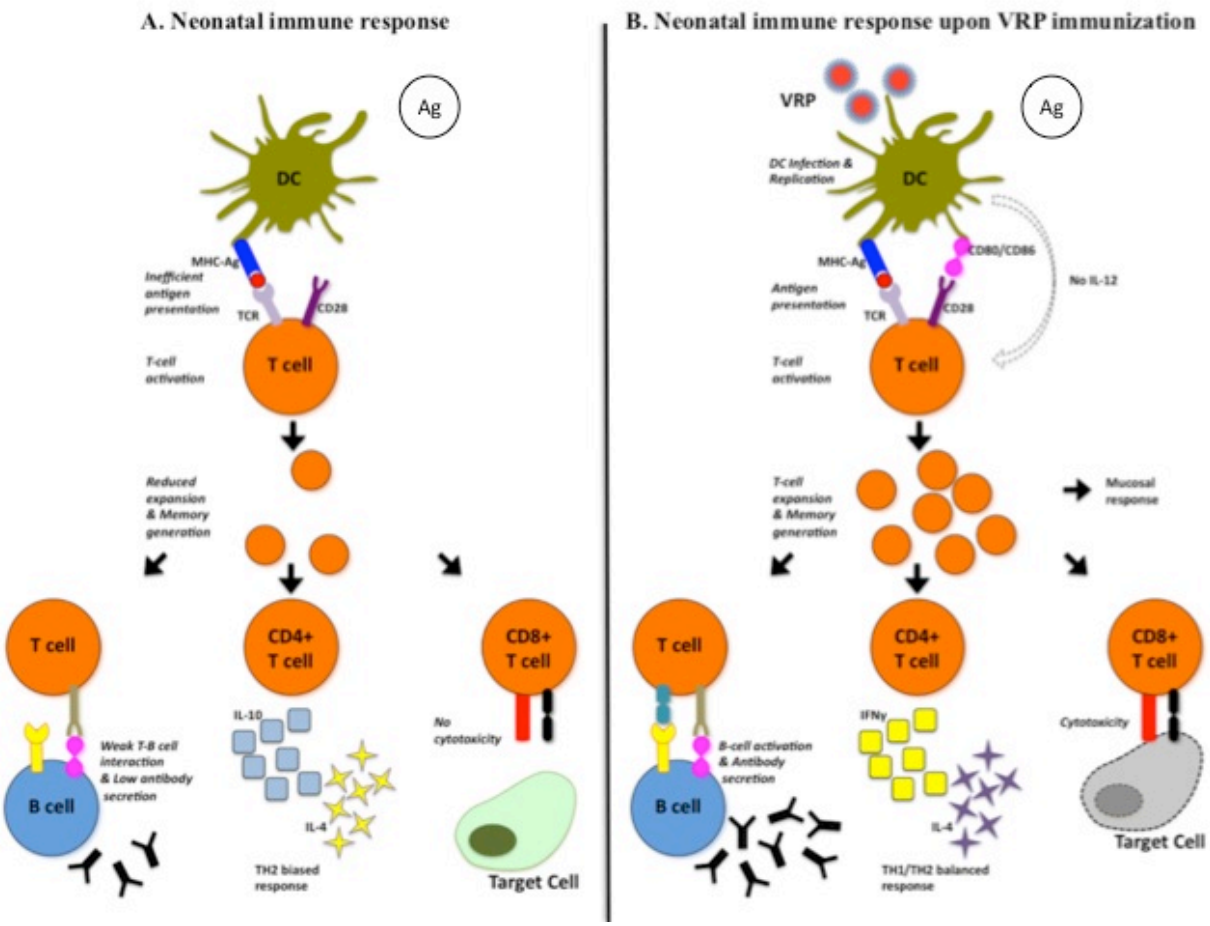


Figure 4.1. VRP expression vector and adjuvant particle can overcome impairments in the neonatal immune response.

(A) Neonatal immune response to vaccines leads to inefficient antigen presentation and low expression of co-stimulatory molecules resulting in reduced expansion of T-cells, low antibody response, T_H2-biased immunity, and insignificant cytotoxicity. (B) VRP as expression vector or adjuvants activate DCs to enhance antigen presentation and co-stimulatory molecule expression, leading to T-cell expansion and memory generation. Furthermore, there is efficient T-B cell interaction, a balanced T_H1/T_H2 response, and induction of cytotoxic CD8+ T-cells. VRP replicon particles can also induce mucosal immunity. VRPs infection does not lead to the detectable production of IL-12.

4.2. Immune induction by alphavirus-based vaccines and adjuvants in neonatal mice

4.2.1. Humoral immune response

It has been shown that vaccine-induced antibodies can play a protective role by either minimizing the spread of pathogens or eliminating them completely in several infectious disease models (12, 16-20). However, one of the main challenges of neonatal immunization is the induction of a potent antibody response. More specifically, the antibody response is delayed, of lower magnitude, short-lived, qualitatively different, has lower average affinity, and reduced heterogeneity (68). Additionally, the antibody response in early life is geared more towards a T_H2 response (involved in allergic reactions and immune tolerance) at the cost of less pro-inflammatory T_H1 responses needed to combat intracellular pathogens (68, 307). Therefore, any vaccine-induced antibody response should be able to overcome these obstacles to induce a protective immune response in early life. In our studies, we observed that both VRP platforms induced an immune response in neonatal mice that was predominantly a T_H1 response. Although it was not possible to measure this T_H2 to T_H1 shift in VRP vector studies, by separating the antigen delivery from the adjuvant function we were able to ask how the adjuvant particles modified the immune response induced to the co-delivered antigen. The GVI3000 adjuvant study (chapter 3) allowed us to observe this shift mediated by the VRP adjuvant function. Very interestingly, the shift towards T_H1 response (measured with IgG2a antibody response) did not come at a cost of reduction in T_H2 response (IgG1 response). Comparing the GVI3000 adjuvant response to a well-known, approved-for-human-use adjuvant, alum, we demonstrated that the two adjuvants differed in their mechanisms of enhancing the antibody responses: the increase being in the antigen-specific total IgG due to IgG2a (T_H1) in GVI3000-adjuvanted antigen, while

it was IgG1 (T_H2) in alum-adjuvanted antigen. Our study reiterates the importance of measuring specific classes of antibody response in characterizing any vaccine. It also demonstrates that neonates are capable of inducing a T_H1 response upon appropriate stimulation.

One interesting and somehow unexpected finding was that a single immunization could achieve an antibody response that remained stable until at least 15 weeks post immunization (last time point tested). This was unexpected based on what is known of the kinetics of antibody responses after a prime immunization, and raises some interesting questions about the immune response maturation after vaccination with a VRP vaccine. The mechanism of how such a durable immunity is achieved from a single immunization in the neonate remains unknown. One testable possible explanation is that the VRP-induced antibody-secreting plasma cells remain in circulation in significant quantity over an extended period. Therefore, future studies should look into the induction, duration, and establishment (in the bone marrow) of plasma cells upon VRP immunization.

Our studies confirmed some of the previous reports in the literature that the antibody responses to vaccination in neonatal mice were of lower magnitude compared to adults. We also confirmed previous reports that showed induction of a memory response in early life. Upon a second immunization with VRP vector, we observed an amplification of the neutralizing antibody response indicating generation of immune memory. However, we did not measure some of the other reported defects in neonatal antibody response, such as shorter life span, lower average affinity, and reduced heterogeneity. Therefore, more studies are needed to understand the quality and characteristics of the antibody response in VRP immunization. These experiments should include adult controls to interpret the results in context of age-related deficiencies.

4.2.2. Cell-mediated immune response

It has been reported that the T-cell response in neonates is weak due to the reduced quantity and delayed maturation of T-cells in the spleen compared to the lymph nodes (348). Our results confirmed the low number of T-cells in the spleen after a single immunization compared to the draining lymph nodes. Additionally, our results suggest that antigen-specific T-cells can be elicited earlier than 10 days post-immunization, as has been reported (349, 350) to as early as 8 days post single immunization. We also demonstrated that, as observed with the antibody response upon a boost, the antigen-specific CD8+ T-cell response was also boosted significantly upon a second VRP immunization. This experiment provides evidence that neonatal immunity is not only capable of inducing memory B-cell responses but also memory T-cell responses. Future studies can determine the duration of the memory response in VRP-immunized neonates compared to the memory response in adults. It is possible that there will be differences in the longevity and strength of the VRP induced immunity.

4.2.3. Innate immune responses

The primary characteristic of the VRP-based vaccines' and adjuvants' effectiveness is its ability to induce strong proinflammatory T_H1 cytokine response as well as DC activation and maturation (198, 268, 287, 330, 331). Further research is needed to establish a definite link between strong innate immune response and effective adaptive immune response in VRP immunization. However, it has been suggested that a weak, T_H2-biased neonatal immune system can be modulated to induce a strong adaptive immune response with the use of an appropriate T_H1 adjuvant (309). We have characterized the local and systemic innate immune response

showing that the VRP is capable of inducing rapid and strong T_H1 cytokines/chemokines in neonatal mice. In contrast to reports that human neonatal pDCs were incapable of producing type I IFNs [reviewed in (64)], we observed induction of a systemic antiviral Type I IFN response in neonatal mice, which was comparable to adult mice. Whether VRPs will be able to induce type I IFN in human neonatal pDCs is to be determined. Further reports have suggested deficiency in the production of cytokines such as IFNγ and TNF in the neonates (345, 351). The results from a comprehensive multiplex analysis demonstrated that the induction of innate immune cytokines upon VRP immunization showed a mixed pattern. While IFNγ was highly upregulated, comparable to adult levels, other cytokines, such as IL-6 and TNF, were marginally upregulated. However, studies in adult mice have shown that VRPs were able to significantly induce both TNF and IL-6. The overall trend indicated that the neonatal cytokine/chemokine response was slightly reduced in the draining lymph node. Further studies will need to determine whether this reduction is associated with certain immune cells and whether it is a deficiency in "global" neonatal innate immune response.

One question that has eluded the present and the past research into VRP immunization is the role of IL-12. IL-12 plays an important role in the induction of a proinflammatory T-cell dependent adaptive immune response as well as IFNγ expression. In the case of APCs from neonatal cord blood, decreased expression of IL-12 has been reported (137). However, in our mouse studies, we did not observe any changes in the draining lymph node IL-12 protein and mRNA levels after VRP immunization of neonatal or adult mice. This observation was true for several time-points for up to 72 hours post immunization. Very little information is available in the literature on IL-12-independent proinflammatory immune response (352). Consequently,

whether VRP immunization induces the proinflammatory immune response through an IL-12-independent mechanism require further investigations.

In vitro studies using adult DCs show that VRPs can upregulate activation and maturation molecules CD40, CD80, and CD86. In our *in vivo* studies, we were able to demonstrate that neonatal DCs were also capable of undergoing activation and maturation. Data in the literature is mixed with some reports suggesting deficient DC maturation and response (353, 354), while others show functionally mature properties of DCs under certain activating conditions (340, 342). Our observations, therefore, indicates that VRPs belong to the class of T_H1 activating vaccines/adjuvants that can strongly activate DCs. Additionally, we demonstrated that DCs, specifically iDCs, were the primary cells recruited to the draining lymph node and targeted by VRPs. The precise role of iDCs in the adjuvant effect of VRPs is currently not understood. However, our results showed that a greater proportion (in relation to total immune cell recruitment to the DLNs) of iDCs are recruited in neonates than adults DLNs—8% in neonates as opposed to <2% in adults. Whether this differential recruitment of iDCs is characteristic of neonatal immune response should be further investigated.

4.2.4. Mucosal immune response

Mucosal immunity provides protection against various pathogens and its induction is an important consideration in the design of a vaccine. However, vaccines administered parenterally usually don't induce effective mucosal immunity, even when potent systemic immunity is induced (3). VRPs have been described as having an additional ability to induce mucosal immunity after a subcutaneous immunization (286, 327). The proposed mechanism suggested for VRP-induced mucosal immunity is through the up-regulation of $\alpha_4\beta_7$ integrin expression on B-

cells in the DLN as well as the expression of mucosal addressin cell adhesion molecule 1 (MAdCAM-1) on the DLN high endothelia venules (327). Our work attempted to understand whether antigen-specific mucosal IgA are induced in neonatal mice. After antigen-only boost immunization, significantly higher amount of IgA was measured in mice that were neonatally primed with VRP adjuvant compared to mice that only had antigen-only neonatal immunization. This result suggested that mucosal memory is induced in neonatal mice. Whether the VRP can induce mucosal IgA after a prime immunization, in adults or in neonates remains to be demonstrated. More sensitive assays measuring antibody secreting cells in the mucosal sites will need to be performed to address this question. Future experiments will attempt to understand whether the neonatal immune system is capable of expressing markers of mucosal response, $\alpha_4\beta_7$ integrin or MAdCAM-1 and whether VRPs can enhance their expression.

4.3. Current challenges and future directions

4.3.1. Autoimmunity

A critical consideration in designing a safe early life vaccine against infectious diseases should be the "cost" of manipulating the neonatal immune response. The neonatal immune environment allows the neonates to learn tolerance towards alloantigens, including to maternally derived antigens that may be present. Tolerance has been demonstrated when primary immunization in the neonates generates a mixed T_H1/ T_H2 response; however, upon re-exposure with the same antigen, the response shifts to T_H2 (68, 307). The induction of this memory T_H2 response to self-antigens might be disrupted with a strong T_H1 inducing vaccine or adjuvant leading to development of autoimmunity. We do not completely understand how VRP vaccines affect the tolerance and autoimmune functions of the neonates. It is possible that the

proinflammatory response induced by VRP in neonates will generate a certain amount of immune response against self-antigens. Although many more studies need to be performed to understand autoimmunity in VRP immunization, our experiment characterizing the T_H1/ T_H2 response using IgG subclasses of antibodies demonstrated that the T_H1 response was boosted without a significant reduction in T_H2 response. This result is surprising considering the fact that T_H1 response can negatively regulate the T_H2 responses. One explanation could be the fact that VRP vaccines are non-propagating, single-replication, self-limited virus particles that do not chronically infect the immune cells. The transient nature of VRPs might therefore limit the negative regulation of T_H2 cells by responding T_H1 cells.

An issue related to autoimmunity is the presence and function of Treg cells. Treg cells are important for the control of autoimmunity and maintenance of tolerance. Treg cells are fully active and play an important role in the neonatal immune system (355, 356). However, the effect of early life vaccination on Treg cells is not well understood. We predict that fully functional Treg cells in the neonates might suppress VRP vaccine-induced T-cell response. However, based on our results, the T-cell responses were robust upon VRP immunization and were boosted upon a second booster immunization. Future studies can determine the role of Treg cells by suppressing their function in VRP immunized neonatal animals.

4.3.2. Anti-VRP immunity

One of the limitations to the use of modified viruses as vaccine vectors is that pre-existing immunity to those viruses may diminish the effectiveness of the vaccine. For example, most humans have antibodies to adenoviruses, and this has limited the use of adenovirus vectors (165, 172). In the case of the VRP vectors, the presence of anti-VEE immunity in VEE-endemic

areas of Central and South America may be a valid concern. However, the fraction of the population seropositive for VEEV in northern South America and Central America is relatively low and limited to small geographically defined regions. Additionally, the last VEEV epidemic was 2 decades ago, limiting the scope of this concern. Whether this issue will determine the efficacy of VRP vaccines in a significant number of people remains to be seen. Another theoretical concern is that VRPs (expression vectors and adjuvants) can induce anti-VEE immunity in addition to the induction of an immune response against the expressed transgene or co-delivered antigen. This has been shown in mice and non-human primates, where anti-VEE neutralizing antibodies are detectable in some animals after one, and in all animals after two, VRP immunizations [(156), D. Tonkin personal communication]. The anti-VEE immune response could then reduce the efficacy of subsequent VRP immunizations, whether to the same or a different antigen. Although this remains to be demonstrated in humans, studies in mice suggest that anti-VEE antibodies do not reduce the immunogenicity of further VRP vaccinations. In mice, a secondary VRP immunization expressing a different transgene from the primary immunization did not result in a weaker immune response to the secondary transgene compared to mice that were only immunized once with the VRP expressing the second transgene (N. Davis, personal communication). Furthermore, a recent study in adult non-human primates showed that anti-VEE immunity did not prevent a boost in the immune response upon a secondary homologous vaccination (156). Our work also demonstrated that a secondary homologous immunization induced a significantly better immune response than a single VRP immunization, although we did not measure the anti-VEE immune response in these mice. Future studies should attempt to understand the induction of anti-VEE immunity in neonatal mice not only upon VRP expression vector immunization but also after using VRPs as adjuvants.

One way vaccinologists have overcome the issue of anti-vector immunity is through the use of a heterologous prime-boost strategy. Using a different vector or even a live-attenuated vaccine as a secondary immunization to VRP prime immunization could avoid an anti-VEE immune response. Use of a live-attenuated vaccine after a neonatal VRP prime immunization could also potentially overcome the issue of maternal antibody interference (see below).

4.3.3. Maternal antibodies issues

Inhibition of vaccine response by maternal antibodies poses a major challenge in neonatal and infant immunizations to live-attenuated as well as non-live vaccines [reviewed in (70)]. This has been shown for the live-attenuated measles vaccine, which is not administered before 12 months of age due to maternal antibodies interfering with the vaccine. This results in unvaccinated susceptible infants acquiring measles, making the task of eradicating the disease very difficult (357).

In the case of dengue, the majority of children in dengue-endemic countries are born with anti-dengue antibodies passively transferred from the mother. The presence of maternal antibodies in these infants has two implications. First, these antibodies would interfere with a live-attenuated dengue vaccine if given during the first year of life. Previous studies have demonstrated that passively transferred maternal antibodies, which interfered with a live dengue immunization, did not reduce the immune response to VRPs expressing dengue antigen in weanling mice born to dengue immune dams (295). Therefore, a VRP expression vector vaccine can avoid inhibition by maternal antibodies, which can be explained in part by the fact that the dengue antigen is not presented on the VRP surface. The use of VRP adjuvants, however, does not necessarily circumvent the issue of maternal antibody interference against the co-delivered

antigen. Future studies should examine whether VRP adjuvants can improve immune response and overcome any inhibition of co-delivered antigen by maternal antibodies. As VRPs are efficient inducer of a T-cell response, which is unaffected by the presence of maternal antibodies (68, 70), we predict that the VRP, as expression vectors or adjuvants, can efficiently improve immune responses.

A second implication of the presence of maternal anti-dengue antibodies in the newborn is that, although those antibodies would protect from dengue infection during the first few months, as maternal antibody titers decline and reach sub-neutralizing titers, the risk of antibody-dependent enhancement (ADE) increases which can lead to severe dengue disease (DSS/DHF) with potentially fatal consequences (292, 293). A dengue vaccine that could be administered early in life would protect these infants from severe dengue. Based on the results presented in Chapter 2, we hypothesize that VRP immunization would induce an endogenous anti-DENV antibody response in the infant, preventing the antibodies from dropping to sub-neutralizing concentration and reducing the risk of ADE. In a recently developed mouse model for dengue ADE, it was demonstrated that adult mice immunized with a VRP expressing DENV2 E protein were protected from antibody-mediated enhanced infection (358). The above hypothesis remains to be tested in neonates with passively transferred maternal antibodies.

4.3.4. Further improvement in VRP vaccines

The sensitive nature of early life immunization and the short window of time available to induce protective immunity makes it imperative that vaccines provide maximum efficacy with minimum dose and fewer frequency of immunization. In that respect, effort is needed in improving VRP design, which ensures continued effective immune induction while further

enhancing the safety of the VRP vaccines. One such technology is GVI3A adjuvant, which is similar to GVI3000 with a mutation in the nt3 of 5' NTR. This is a major attenuating mutation described in the TC-83 vaccine strain of VEE (271, 291). Our unpublished results show that GVI3A provided protective immunity upon influenza challenge in neonatal mice after a single immunization. Further work in characterizing GVI3A adjuvant is ongoing.

4.3.5. NHP models and clinical trials

Although neonatal mouse models are useful in allowing us to understand the mechanism and efficacy of VRP vaccines, they do not completely predict the effect a vaccine will have in neonatal humans. The present study has laid the foundation for further testing of VRP vaccines in neonatal non-human primates, which should more closely resemble the response in human neonates. If VRP vaccines are considered safe, efficacious, and protective in neonatal NHPs—as they have been for adult NHPs (156, 204, 282-285)—clinical trials in neonatal humans should be conducted.

REFERENCES

1. **Levine MM, Lagos R.** 2004. Vaccines and Vaccination in Historical Perspective, New Generation Vaccines, 3rd ed. Marcel Dekker, New York.

2. **Plotkin SL, Plotkin SA.** 2008. A short history of vaccination, Vaccines, 4th ed. W. B. Saunders, Philadelphia.

3. **Plotkin SA.** 2005. Vaccines: past, present and future. Nature medicine **11:**S5-11.

4. **Andre FE.** 2003. Vaccinology: past achievements, present roadblocks and future promises. Vaccine **21:**593-595.

5. **Fenner F.** 1993. Smallpox: emergence, global spread, and eradication. History and philosophy of the life sciences **15:**397-420.

6. **Calmette A.** 1931. Preventive Vaccination Against Tuberculosis with BCG. Proceedings of the Royal Society of Medicine **24:**1481-1490.

7. **Theiler M, Smith HH.** 1937. The Use of Yellow Fever Virus Modified by in Vitro Cultivation for Human Immunization. The Journal of experimental medicine **65:**787-800.

8. **Plotkin SA.** 1999. Vaccination against the major infectious diseases. Comptes rendus de l'Academie des sciences. Serie III, Sciences de la vie **322:**943-951.

9. **Bonanni P.** 1999. Demographic impact of vaccination: a review. Vaccine **17 Suppl 3:**S120-125.

10. **Plotkin SA.** 2002. Vaccines in the 21st century. Hybridoma and hybridomics **21:**135-145.

11. **Siegrist CA.** 2004. Vaccine immunology, 4th ed. W. B. Saunders, Philadelphia.

12. **Plotkin SA.** 2001. Immunologic correlates of protection induced by vaccination. The Pediatric infectious disease journal **20:**63-75.

13. **Conrad ME, Lemon SM.** 1987. Prevention of endemic icteric viral hepatitis by administration of immune serum gamma globulin. The Journal of infectious diseases **156:**56-63.

14. **Stapleton JT.** 1992. Passive immunization against hepatitis A. Vaccine **10 Suppl 1:**S45-47.

15. **Bodian D.** 1953. Experimental studies on passive immunization against poliomyelitis. III. Passive-active immunization and pathogenesis after virus feeding in chimpanzees. American journal of hygiene **58:**81-100.

16. **Mason RA, Tauraso NM, Spertzel RO, Ginn RK.** 1973. Yellow fever vaccine: direct challenge of monkeys given graded doses of 17D vaccine. Applied microbiology **25:**539-544.

17. **Plotkin SA.** 2000. Rabies. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America **30:**4-12.

18. **Zangwill KM, Stout RW, Carlone GM, Pais L, Harekeh H, Mitchell S, Wolfe WH, Blackwood V, Plikaytis BD, Wenger JD.** 1994. Duration of antibody response after meningococcal polysaccharide vaccination in US Air Force personnel. The Journal of infectious diseases **169:**847-852.

19. **Konradsen HB, Henrichsen J.** 1991. The need for revaccination 10 years after primary pneumococcal vaccination in splenectomized adults. Scandinavian journal of infectious diseases **23:**397.

20. **Plotkin SA, Bouveret-Le Cam N.** 1995. A new typhoid vaccine composed of the Vi capsular polysaccharide. Archives of internal medicine **155:**2293-2299.

21. **McComb JA.** 1964. The Prophylactic Dose of Homologous Tetanus Antitoxin. The New England journal of medicine **270:**175-178.

22. **McComb JA, Dwyer RC.** 1963. Passive-Active Immunization with Tetanus Immune Globulin (Human). The New England journal of medicine **268:**857-862.

23. **Halstead SB.** 1988. Pathogenesis of dengue: challenges to molecular biology. Science **239:**476-481.

24. **Halstead SB, Nimmannitya S, Cohen SN.** 1970. Observations related to pathogenesis of dengue hemorrhagic fever. IV. Relation of disease severity to antibody response and virus recovered. The Yale journal of biology and medicine **42:**311-328.

25. **Sangkawibha N, Rojanasuphot S, Ahandrik S, Viriyapongse S, Jatanasen S, Salitul V, Phanthumachinda B, Halstead SB.** 1984. Risk factors in dengue shock syndrome: a prospective epidemiologic study in Rayong, Thailand. I. The 1980 outbreak. American journal of epidemiology **120:**653-669.

26. **McKenzie BS, Brady JL, Lew AM.** 2004. Mucosal immunity: overcoming the barrier for induction of proximal responses. Immunologic research **30:**35-71.

27. **Neutra MR, Pringault E, Kraehenbuhl JP.** 1996. Antigen sampling across epithelial barriers and induction of mucosal immune responses. Annual review of immunology **14:**275-300.

28. **Levine MM, Dougan G.** 1998. Optimism over vaccines administered via mucosal surfaces. Lancet **351:**1375-1376.

29. **Cox RJ, Brokstad KA, Ogra P.** 2004. Influenza virus: immunity and vaccination strategies. Comparison of the immune response to inactivated and live, attenuated influenza vaccines. Scandinavian journal of immunology **59:**1-15.

30. **Brandtzaeg P, Johansen FE.** 2005. Mucosal B cells: phenotypic characteristics, transcriptional regulation, and homing properties. Immunological reviews **206:**32-63.

31. **McGhee JR, Mestecky J, Dertzbaugh MT, Eldridge JH, Hirasawa M, Kiyono H.** 1992. The mucosal immune system: from fundamental concepts to vaccine development. Vaccine **10:**75-88.

32. **Mestecky J, Lue C, Russell MW.** 1991. Selective transport of IgA. Cellular and molecular aspects. Gastroenterology clinics of North America **20:**441-471.

33. **Hanekom WA.** 2005. The immune response to BCG vaccination of newborns. Ann N Y Acad Sci **1062:**69-78.

34. **Ausiello CM, Lande R, Urbani F, Di Carlo B, Stefanelli P, Salmaso S, Mastrantonio P, Cassone A.** 2000. Cell-mediated immunity and antibody responses to Bordetella

pertussis antigens in children with a history of pertussis infection and in recipients of an acellular pertussis vaccine. The Journal of infectious diseases **181:**1989-1995.

35. **Ausiello CM, Lande R, Urbani F, la Sala A, Stefanelli P, Salmaso S, Mastrantonio P, Cassone A.** 1999. Cell-mediated immune responses in four-year-old children after primary immunization with acellular pertussis vaccines. Infection and immunity **67:**4064-4071.

36. **Giuliano M, Mastrantonio P, Giammanco A, Piscitelli A, Salmaso S, Wassilak SG.** 1998. Antibody responses and persistence in the two years after immunization with two acellular vaccines and one whole-cell vaccine against pertussis. The Journal of pediatrics **132:**983-988.

37. **Salmaso S, Mastrantonio P, Tozzi AE, Stefanelli P, Anemona A, Ciofi degli Atti ML, Giammanco A, Stage IIIWG.** 2001. Sustained efficacy during the first 6 years of life of 3-component acellular pertussis vaccines administered in infancy: the Italian experience. Pediatrics **108:**E81.

38. **Gans HA, Arvin AM, Galinus J, Logan L, DeHovitz R, Maldonado Y.** 1998. Deficiency of the humoral immune response to measles vaccine in infants immunized at age 6 months. JAMA : the journal of the American Medical Association **280:**527-532.

39. **Gans HA, Maldonado Y, Yasukawa LL, Beeler J, Audet S, Rinki MM, DeHovitz R, Arvin AM.** 1999. IL-12, IFN-gamma, and T cell proliferation to measles in immunized infants. J Immunol **162:**5569-5575.

40. **Gans HA, Yasukawa LL, Alderson A, Rinki M, DeHovitz R, Beeler J, Audet S, Maldonado Y, Arvin AM.** 2004. Humoral and cell-mediated immune responses to an early 2-dose measles vaccination regimen in the United States. The Journal of infectious diseases **190:**83-90.

41. **Offit PA, Cunningham SL, Dudzik KI.** 1991. Memory and distribution of virus-specific cytotoxic T lymphocytes (CTLs) and CTL precursors after rotavirus infection. J Virol **65:**1318-1324.

42. **Offit PA, Dudzik KI.** 1989. Rotavirus-specific cytotoxic T lymphocytes appear at the intestinal mucosal surface after rotavirus infection. J Virol **63:**3507-3512.

43. **Offit PA, Svoboda YM.** 1989. Rotavirus-specific cytotoxic T lymphocyte response of mice after oral inoculation with candidate rotavirus vaccine strains RRV or WC3. The Journal of infectious diseases **160:**783-788.

44. **Blazevic V, Trubey CM, Shearer GM.** 2000. Comparison of in vitro immunostimulatory potential of live and inactivated influenza viruses. Human immunology **61:**845-849.

45. **Ghendon Y.** 1990. The immune response to influenza vaccines. Acta virologica **34:**295-304.

46. **Mbawuike IN, Piedra PA, Cate TR, Couch RB.** 1996. Cytotoxic T lymphocyte responses of infants after natural infection or immunization with live cold-recombinant or inactivated influenza A virus vaccine. Journal of medical virology **50:**105-111.

47. **Doherty PC, Topham DJ, Tripp RA, Cardin RD, Brooks JW, Stevenson PG.** 1997. Effector CD4+ and CD8+ T-cell mechanisms in the control of respiratory virus infections. Immunological reviews **159:**105-117.

48. **Lilic D.** 2002. New perspectives on the immunology of chronic mucocutaneous candidiasis. Curr Opin Infect Dis **15:**143-147.

49. **Arvin AM.** 1992. Cell-mediated immunity to varicella-zoster virus. The Journal of infectious diseases **166 Suppl 1:**S35-41.

50. **Liu T, Khanna KM, Chen X-P, Fink DJ, Hendricks RL.** 2000. CD8+ T Cells Can Block Herpes Simplex Virus Type 1 (HSV-1) Reactivation from Latency in Sensory Neurons. The Journal of experimental medicine **191:**1459-1466.

51. **Milligan GN, Bernstein DI, Bourne N.** 1998. T lymphocytes are required for protection of the vaginal mucosae and sensory ganglia of immune mice against reinfection with herpes simplex virus type 2. J Immunol **160:**6093-6100.

52. **Callan MF.** 2003. The evolution of antigen-specific CD8+ T cell responses after natural primary infection of humans with Epstein-Barr virus. Viral immunology **16:**3-16.

53. **Karrer U, Wagner M, Sierro S, Oxenius A, Hengel H, Dumrese T, Freigang S, Koszinowski UH, Phillips RE, Klenerman P.** 2004. Expansion of protective CD8+ T-cell responses driven by recombinant cytomegaloviruses. J Virol **78:**2255-2264.

54. **Quiroga JA, Llorente S, Castillo I, Rodriguez-Inigo E, Pardo M, Carreno V.** 2006. Cellular immune responses associated with occult hepatitis C virus infection of the liver. J Virol **80:**10972-10979.

55. **Coffman RL, Sher A, Seder RA.** 2010. Vaccine adjuvants: putting innate immunity to work. Immunity **33:**492-503.

56. **Fearon DT, Locksley RM.** 1996. The instructive role of innate immunity in the acquired immune response. Science **272:**50-53.

57. **Iwasaki A, Medzhitov R.** 2004. Toll-like receptor control of the adaptive immune responses. Nat Immunol **5:**987-995.

58. **Wu J, Chen ZJ.** 2014. Innate immune sensing and signaling of cytosolic nucleic acids. Annual review of immunology **32:**461-488.

59. **Berger A.** 1999. How does herd immunity work? Bmj **319:**1466-1467.

60. **Goncalves G.** 2008. Herd immunity: recent uses in vaccine assessment. Expert review of vaccines **7:**1493-1506.

61. **Paul Y.** 2004. Herd immunity and herd protection. Vaccine **22:**301-302.

62. **Iwai M, Nakayama T, Matsuura K, Hasegawa S, Ando S, Obara M, Nagai Y, Yoshida H, Horie H.** 2006. Assessment of efficacy of a live oral poliovirus vaccine for virulent Sabin-like poliovirus 1 strains in Japan. Acta virologica **50:**139-143.

63. **Bortolussi R, Henneke P, Kollmann T.** 2013. Host defense against common early life-threatening infections. Clinical & developmental immunology **2013:**350808.

64. **Levy O.** 2007. Innate immunity of the newborn: basic mechanisms and clinical correlates. Nat Rev Immunol **7:**379-390.

65. **PrabhuDas M, Adkins B, Gans H, King C, Levy O, Ramilo O, Siegrist CA.** 2011. Challenges in infant immunity: implications for responses to infection and vaccines. Nat Immunol **12:**189-194.

66. **Liu L, Johnson HL, Cousens S, Perin J, Scott S, Lawn JE, Rudan I, Campbell H, Cibulskis R, Li M, Mathers C, Black RE, Child Health Epidemiology Reference Group of WHO, Unicef.** 2012. Global, regional, and national causes of child mortality: an updated systematic analysis for 2010 with time trends since 2000. Lancet **379:**2151-2161.

67. **Hostetter MK.** 2012. What we don't see. The New England journal of medicine **366:**1328-1334.

68. **Adkins B, Leclerc C, Marshall-Clarke S.** 2004. Neonatal adaptive immunity comes of age. Nat Rev Immunol **4:**553-564.

69. **Marshall-Clarke S, Tasker L, Parkhouse RM.** 2000. Immature B lymphocytes from adult bone marrow exhibit a selective defect in induced hyperexpression of major histocompatibility complex class II and fail to show B7.2 induction. Immunology **100:**141-151.

70. **Siegrist CA.** 2001. Neonatal and early life vaccinology. Vaccine **19:**3331-3346.

71. **Kollmann TR, Levy O, Montgomery RR, Goriely S.** 2012. Innate immune function by Toll-like receptors: distinct responses in newborns and the elderly. Immunity **37:**771-783.

72. **Forsthuber T, Yip HC, Lehmann PV.** 1996. Induction of TH1 and TH2 immunity in neonatal mice. Science **271:**1728-1730.

73. **Ridge JP, Fuchs EJ, Matzinger P.** 1996. Neonatal tolerance revisited: turning on newborn T cells with dendritic cells. Science **271:**1723-1726.

74. **Sarzotti M, Robbins DS, Hoffman PM.** 1996. Induction of protective CTL responses in newborn mice by a murine retrovirus. Science **271:**1726-1728.

75. **Albrecht P, Ennis FA, Saltzman EJ, Krugman S.** 1977. Persistence of maternal antibody in infants beyond 12 months: mechanism of measles vaccine failure. The Journal of pediatrics **91:**715-718.

76. **Bjorkholm B, Granstrom M, Taranger J, Wahl M, Hagberg L.** 1995. Influence of high titers of maternal antibody on the serologic response of infants to diphtheria vaccination at three, five and twelve months of age. The Pediatric infectious disease journal **14:**846-850.

77. **Benschop RJ, Brandl E, Chan AC, Cambier JC.** 2001. Unique signaling properties of B cell antigen receptor in mature and immature B cells: implications for tolerance and activation. J Immunol **167:**4172-4179.

78. **Tasker L, Marshall-Clarke S.** 2003. Functional responses of human neonatal B lymphocytes to antigen receptor cross-linking and CpG DNA. Clinical and experimental immunology **134:**409-419.

79. **Jakobsen H, Bjarnarson S, Del Giudice G, Moreau M, Siegrist CA, Jonsdottir I.** 2002. Intranasal immunization with pneumococcal conjugate vaccines with LT-K63, a nontoxic mutant of heat-Labile enterotoxin, as adjuvant rapidly induces protective immunity against lethal pneumococcal infections in neonatal mice. Infection and immunity **70:**1443-1452.

80. **Martin F, Oliver AM, Kearney JF.** 2001. Marginal zone and B1 B cells unite in the early response against T-independent blood-borne particulate antigens. Immunity **14:**617-629.

81. **Mosier DE, Johnson BM.** 1975. Ontogeny of mouse lymphocyte function. II. Development of the ability to produce antibody is modulated by T lymphocytes. The Journal of experimental medicine **141:**216-226.

82. **Rijkers GT, Sanders EA, Breukels MA, Zegers BJ.** 1998. Infant B cell responses to polysaccharide determinants. Vaccine **16:**1396-1400.

83. **Timens W, Boes A, Rozeboom-Uiterwijk T, Poppema S.** 1989. Immaturity of the human splenic marginal zone in infancy. Possible contribution to the deficient infant immune response. J Immunol **143:**3200-3206.

84. **Ansel KM, Ngo VN, Hyman PL, Luther SA, Forster R, Sedgwick JD, Browning JL, Lipp M, Cyster JG.** 2000. A chemokine-driven positive feedback loop organizes lymphoid follicles. Nature **406:**309-314.

85. **Pihlgren M, Tougne C, Bozzotti P, Fulurija A, Duchosal MA, Lambert PH, Siegrist CA.** 2003. Unresponsiveness to lymphoid-mediated signals at the neonatal follicular dendritic cell precursor level contributes to delayed germinal center induction and limitations of neonatal antibody responses to T-dependent antigens. J Immunol **170:**2824-2832.

86. **Timens W, Rozeboom T, Poppema S.** 1987. Fetal and neonatal development of human spleen: an immunohistological study. Immunology **60:**603-609.

87. **Dong DX, Hu XM, Liu WJ, Li JS, Jin YC, Tan SG, Chen TQ, Fu JZ, Niu BY, Yu HM, et al.** 1986. Immunization of neonates with trivalent oral poliomyelitis vaccine (Sabin). Bulletin of the World Health Organization **64:**853-860.

88. **Andre FE, Zuckerman AJ.** 1994. Review: protective efficacy of hepatitis B vaccines in neonates. Journal of medical virology **44:**144-151.

89. **Dengrove J, Lee EJ, Heiner DC, St Geme JW, Jr., Leake R, Baraff LJ, Ward JI.** 1986. IgG and IgG subclass specific antibody responses to diphtheria and tetanus toxoids in newborns and infants given DTP immunization. Pediatr Res **20:**735-739.

90. **Adkins B.** 2000. Development of neonatal Th1/Th2 function. Int Rev Immunol **19:**157-171.

91. **Garcia AM, Fadel SA, Cao S, Sarzotti M.** 2000. T cell immunity in neonates. Immunologic research **22:**177-190.

92. **Siegrist CA.** 2000. Vaccination in the neonatal period and early infancy. Int Rev Immunol **19:**195-219.

93. **Hussey GD, Watkins ML, Goddard EA, Gottschalk S, Hughes EJ, Iloni K, Kibel MA, Ress SR.** 2002. Neonatal mycobacterial specific cytotoxic T-lymphocyte and cytokine profiles in response to distinct BCG vaccination strategies. Immunology **105:**314-324.

94. **Vekemans J, Amedei A, Ota MO, D'Elios MM, Goetghebuer T, Ismaili J, Newport MJ, Del Prete G, Goldman M, McAdam KP, Marchant A.** 2001. Neonatal bacillus Calmette-Guerin vaccination induces adult-like IFN-gamma production by CD4+ T lymphocytes. Eur J Immunol **31:**1531-1535.

95. **Douagi I, Andre I, Ferraz JC, Cumano A.** 2000. Characterization of T cell precursor activity in the murine fetal thymus: evidence for an input of T cell precursors between days 12 and 14 of gestation. Eur J Immunol **30:**2201-2210.

96. **Foss DL, Donskoy E, Goldschneider I.** 2001. The importation of hematogenous precursors by the thymus is a gated phenomenon in normal adult mice. The Journal of experimental medicine **193:**365-374.

97. **Jotereau F, Heuze F, Salomon-Vie V, Gascan H.** 1987. Cell kinetics in the fetal mouse thymus: precursor cell input, proliferation, and emigration. J Immunol **138:**1026-1030.

98. **Prescott SL, Macaubas C, Holt BJ, Smallacombe TB, Loh R, Sly PD, Holt PG.** 1998. Transplacental priming of the human immune system to environmental allergens: universal skewing of initial T cell responses toward the Th2 cytokine profile. J Immunol **160:**4730-4737.

99. **Prescott SL, Macaubas C, Smallacombe T, Holt BJ, Sly PD, Holt PG.** 1999. Development of allergen-specific T-cell memory in atopic and normal children. Lancet **353:**196-200.

100. **Prescott SL, Macaubes C, Yabuhara A, Venaille TJ, Holt BJ, Habre W, Loh R, Sly PD, Holt PG.** 1997. Developing patterns of T cell memory to environmental allergens in the first two years of life. International archives of allergy and immunology **113:**75-79.

101. **Li L, Lee HH, Bell JJ, Gregg RK, Ellis JS, Gessner A, Zaghouani H.** 2004. IL-4 utilizes an alternative receptor to drive apoptosis of Th1 cells and skews neonatal immunity toward Th2. Immunity **20:**429-440.

102. **Fadel SA, Ozaki DA, Sarzotti M.** 2002. Enhanced type 1 immunity after secondary viral challenge in mice primed as neonates. J Immunol **169:**3293-3300.

103. **Franchini M, Abril C, Schwerdel C, Ruedl C, Ackermann M, Suter M.** 2001. Protective T-cell-based immunity induced in neonatal mice by a single replicative cycle of herpes simplex virus. J Virol **75:**83-89.

104. **Kovarik J, Gaillard M, Martinez X, Bozzotti P, Lambert PH, Wild TF, Siegrist CA.** 2001. Induction of adult-like antibody, Th1, and CTL responses to measles hemagglutinin by early life murine immunization with an attenuated vaccinia-derived NYVAC(K1L) viral vector. Virology **285:**12-20.

105. **Bot A, Bot S, Bona C.** 1998. Enhanced protection against influenza virus of mice immunized as newborns with a mixture of plasmids expressing hemagglutinin and nucleoprotein. Vaccine **16:**1675-1682.

106. **Hassett DE, Zhang J, Slifka M, Whitton JL.** 2000. Immune responses following neonatal DNA vaccination are long-lived, abundant, and qualitatively similar to those induced by conventional immunization. J Virol **74:**2620-2627.

107. **Martinez X, Brandt C, Saddallah F, Tougne C, Barrios C, Wild F, Dougan G, Lambert PH, Siegrist CA.** 1997. DNA immunization circumvents deficient induction of T helper type 1 and cytotoxic T lymphocyte responses in neonates and during early life. Proc Natl Acad Sci U S A **94:**8726-8731.

108. **Brazolot Millan CL, Weeratna R, Krieg AM, Siegrist CA, Davis HL.** 1998. CpG DNA can induce strong Th1 humoral and cell-mediated immune responses against hepatitis B surface antigen in young mice. Proc Natl Acad Sci U S A **95:**15553-15558.

109. **Kovarik J, Bozzotti P, Love-Homan L, Pihlgren M, Davis HL, Lambert PH, Krieg AM, Siegrist CA.** 1999. CpG oligodeoxynucleotides can circumvent the Th2 polarization of neonatal responses to vaccines but may fail to fully redirect Th2 responses established by neonatal priming. J Immunol **162:**1611-1617.

110. **Marchant A, Appay V, Van Der Sande M, Dulphy N, Liesnard C, Kidd M, Kaye S, Ojuola O, Gillespie GM, Vargas Cuero AL, Cerundolo V, Callan M, McAdam KP, Rowland-Jones SL, Donner C, McMichael AJ, Whittle H.** 2003. Mature CD8(+) T lymphocyte response to viral infection during fetal life. The Journal of clinical investigation **111:**1747-1755.

111. **Hermann E, Truyens C, Alonso-Vega C, Even J, Rodriguez P, Berthe A, Gonzalez-Merino E, Torrico F, Carlier Y.** 2002. Human fetuses are able to mount an adultlike CD8 T-cell response. Blood **100:**2153-2158.

112. **Morein B, Abusugra I, Blomqvist G.** 2002. Immunity in neonates. Veterinary immunology and immunopathology **87:**207-213.

113. **Morein B, Blomqvist G, Hu K.** 2007. Immune responsiveness in the neonatal period. Journal of comparative pathology **137 Suppl 1:**S27-31.

114. **Wegmann TG, Lin H, Guilbert L, Mosmann TR.** 1993. Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon? Immunology today **14:**353-356.

115. **Adkins B.** 1999. T-cell function in newborn mice and humans. Immunology today **20:**330-335.

116. **Barrios C, Brandt C, Berney M, Lambert PH, Siegrist CA.** 1996. Partial correction of the TH2/TH1 imbalance in neonatal murine responses to vaccine antigens through selective adjuvant effects. Eur J Immunol **26:**2666-2670.

117. **Kovarik J, Siegrist CA.** 1998. Optimization of vaccine responses in early life: the role of delivery systems and immunomodulators. Immunology and cell biology **76:**222-236.

118. **Siegrist CA.** 2007. The challenges of vaccine responses in early life: selected examples. Journal of comparative pathology **137 Suppl 1:**S4-9.

119. **Adkins B, Bu Y, Guevara P.** 2001. The generation of Th memory in neonates versus adults: prolonged primary Th2 effector function and impaired development of Th1 memory effector function in murine neonates. J Immunol **166:**918-925.

120. **Kolaczkowska E, Kubes P.** 2013. Neutrophil recruitment and function in health and inflammation. Nat Rev Immunol **13:**159-175.

121. **Fielding CA, McLoughlin RM, Colmont CS, Kovaleva M, Harris DA, Rose-John S, Topley N, Jones SA.** 2005. Viral IL-6 blocks neutrophil infiltration during acute inflammation. J Immunol **175:**4024-4029.

122. **Filias A, Theodorou GL, Mouzopoulou S, Varvarigou AA, Mantagos S, Karakantza M.** 2011. Phagocytic ability of neutrophils and monocytes in neonates. BMC pediatrics **11:**29.

123. **Levy O.** 2002. Impaired innate immunity at birth: deficiency of bactericidal/permeability-increasing protein (BPI) in the neutrophils of newborns. Pediatr Res **51:**667-669.

124. **Strunk T, Doherty D, Richmond P, Simmer K, Charles A, Levy O, Liyanage K, Smith T, Currie A, Burgner D.** 2009. Reduced levels of antimicrobial proteins and peptides in human cord blood plasma. Archives of disease in childhood. Fetal and neonatal edition **94:**F230-231.

125. **Nussbaum C, Gloning A, Pruenster M, Frommhold D, Bierschenk S, Genzel-Boroviczeny O, von Andrian UH, Quackenbush E, Sperandio M.** 2013. Neutrophil and endothelial adhesive function during human fetal ontogeny. Journal of leukocyte biology **93:**175-184.

126. **Jost S, Altfeld M.** 2013. Control of human viral infections by natural killer cells. Annual review of immunology **31:**163-194.

127. **Guilmot A, Hermann E, Braud VM, Carlier Y, Truyens C.** 2011. Natural killer cell responses to infections in early life. Journal of innate immunity **3:**280-288.

128. **Marcoe JP, Lim JR, Schaubert KL, Fodil-Cornu N, Matka M, McCubbrey AL, Farr AR, Vidal SM, Laouar Y.** 2012. TGF-beta is responsible for NK cell immaturity during ontogeny and increased susceptibility to infection during mouse infancy. Nat Immunol **13:**843-850.

129. **Yang L, Pang Y, Moses HL.** 2010. TGF-beta and immune cells: an important regulatory axis in the tumor microenvironment and progression. Trends Immunol **31:**220-227.

130. **Elahi S, Ertelt JM, Kinder JM, Jiang TT, Zhang X, Xin L, Chaturvedi V, Strong BS, Qualls JE, Steinbrecher KA, Kalfa TA, Shaaban AF, Way SS.** 2013. Immunosuppressive CD71+ erythroid cells compromise neonatal host defence against infection. Nature **504:**158-162.

131. **Levy O, Zarember KA, Roy RM, Cywes C, Godowski PJ, Wessels MR.** 2004. Selective impairment of TLR-mediated innate immunity in human newborns: neonatal blood plasma reduces monocyte TNF-alpha induction by bacterial lipopeptides, lipopolysaccharide, and imiquimod, but preserves the response to R-848. J Immunol **173:**4627-4634.

132. **Kraft JD, Horzempa J, Davis C, Jung JY, Pena MM, Robinson CM.** 2013. Neonatal macrophages express elevated levels of interleukin-27 that oppose immune responses. Immunology **139:**484-493.

133. **Janeway CA, Jr., Medzhitov R.** 2002. Innate immune recognition. Annual review of immunology **20:**197-216.

134. **Willems F, Vollstedt S, Suter M.** 2009. Phenotype and function of neonatal DC. Eur J Immunol **39:**26-35.

135. **Dakic A, Shao QX, D'Amico A, O'Keeffe M, Chen WF, Shortman K, Wu L.** 2004. Development of the dendritic cell system during mouse ontogeny. J Immunol **172:**1018-1027.

136. **Kollmann TR, Crabtree J, Rein-Weston A, Blimkie D, Thommai F, Wang XY, Lavoie PM, Furlong J, Fortuno ES, 3rd, Hajjar AM, Hawkins NR, Self SG, Wilson CB.** 2009. Neonatal innate TLR-mediated responses are distinct from those of adults. J Immunol **183:**7150-7160.

137. **Goriely S, Van Lint C, Dadkhah R, Libin M, De Wit D, Demonte D, Willems F, Goldman M.** 2004. A defect in nucleosome remodeling prevents IL-12(p35) gene transcription in neonatal dendritic cells. The Journal of experimental medicine **199:**1011-1016.

138. **Vanden Eijnden S, Goriely S, De Wit D, Goldman M, Willems F.** 2006. Preferential production of the IL-12(p40)/IL-23(p19) heterodimer by dendritic cells from human newborns. Eur J Immunol **36:**21-26.

139. **Wright PF, Wright PF.** 1998. Infectious diseases in early life in industrialized countries. Vaccine **16:**1355-1359.

140. **Schellenberg D, Menendez C, Kahigwa E, Font F, Galindo C, Acosta C, Schellenberg JA, Aponte JJ, Kimario J, Urassa H, Mshinda H, Tanner M, Alonso P.** 1999. African children with malaria in an area of intense Plasmodium falciparum transmission: features on admission to the hospital and risk factors for death. Am J Trop Med Hyg **61:**431-438.

141. **Centers for Disease C, Prevention.** 2010. Estimates of deaths associated with seasonal influenza --- United States, 1976-2007. MMWR. Morbidity and mortality weekly report **59:**1057-1062.

142. **Belshe RB, Walker RE, Stoddard JJ, Kemble G, Maassab HF, Mendelman PM.** 2008. Influenza vaccine-live, p. 291-309, Vaccines, 4th ed. W. B. Saunders, Philadelphia.

143. **Klenk HD, Rott R.** 1988. The molecular biology of influenza virus pathogenicity. Advances in virus research **34:**247-281.

144. **Klenk HD, Rott R, Orlich M, Blodorn J.** 1975. Activation of influenza A viruses by trypsin treatment. Virology **68:**426-439.

145. **Shaw MW, Arden NH, Maassab HF.** 1992. New aspects of influenza viruses. Clinical microbiology reviews **5:**74-92.

146. **Labella AM, Merel SE.** 2013. Influenza. The Medical clinics of North America **97:**621-645, x.

147. **Bridges CB, Katz JM, Levandowski RA, Cox NJ.** 2008. Inactivated influenza vaccines, 4th ed. W. B. Saunders, Philadelphia.

148. **Nicholson KG, Wood JM, Zambon M.** 2003. Influenza. Lancet **362:**1733-1745.

149. **Belshe RB, Edwards KM, Vesikari T, Black SV, Walker RE, Hultquist M, Kemble G, Connor EM, Group C-TCES.** 2007. Live attenuated versus inactivated influenza vaccine in infants and young children. The New England journal of medicine **356:**685-696.

150. **Zangwill KM, Belshe RB.** 2004. Safety and efficacy of trivalent inactivated influenza vaccine in young children: a summary for the new era of routine vaccination. The Pediatric infectious disease journal **23:**189-197.

151. **Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyes CL, Drake JM, Brownstein JS, Hoen AG, Sankoh O, Myers MF, George DB, Jaenisch T, Wint GR, Simmons CP, Scott TW, Farrar JJ, Hay SI.** 2013. The global distribution and burden of dengue. Nature.

152. **WHO.** 2009. Dengue guidelines for diagnosis, treatment, prevention and control. http://www.who.int/tdr/publications/documents/dengue-diagnosis.pdf **World Health Organization, Geneva, Switzerland**.

153. **Thomas SJ, Endy TP.** 2013. Current issues in dengue vaccination. Curr Opin Infect Dis **26:**429-434.

154. **Weiskopf D, Angelo MA, de Azeredo EL, Sidney J, Greenbaum JA, Fernando AN, Broadwater A, Kolla RV, De Silva AD, de Silva AM, Mattia KA, Doranz BJ, Grey HM, Shresta S, Peters B, Sette A.** 2013. Comprehensive analysis of dengue virus-specific responses supports an HLA-linked protective role for CD8+ T cells. Proc Natl Acad Sci U S A **110:**E2046-2053.

155. **Guy B.** 2009. Immunogenicity of sanofi pasteur tetravalent dengue vaccine. Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology **46 Suppl 2:**S16-19.

156. **White LJ, Sariol CA, Mattocks MD, Wahala MPBW, Yingsiwaphat V, Collier ML, Whitley J, Mikkelsen R, Rodriguez IV, Martinez MI, de Silva A, Johnston RE.** 2013. An alphavirus vector-based tetravalent dengue vaccine induces a rapid and protective immune response in macaques that differs qualitatively from immunity induced by live virus infection. J Virol **87:**3409-3424.

157. **WHO.** 2014. World Health Statistics, http://apps.who.int/iris/bitstream/10665/112738/1/9789240692671_eng.pdf?ua=1. World Health Organization, Geneva, Switzerland.

158. **Kaper JB, Rappuoli R.** 2004. An Overview of Biotechnology in Vaccine Development, New Generation Vaccines, 3rd ed. Marcel Dekker, New York.

159. **Robinson HL, Hunt LA, Webster RG.** 1993. Protection against a lethal influenza virus challenge by immunization with a haemagglutinin-expressing plasmid DNA. Vaccine **11:**957-960.

160. **Ulmer JB, Deck RR, DeWitt CM, Friedman A, Donnelly JJ, Liu MA.** 1994. Protective immunity by intramuscular injection of low doses of influenza virus DNA vaccines. Vaccine **12:**1541-1544.

161. **Wang R, Doolan DL, Le TP, Hedstrom RC, Coonan KM, Charoenvit Y, Jones TR, Hobart P, Margalith M, Ng J, Weiss WR, Sedegah M, de Taisne C, Norman JA, Hoffman SL.** 1998. Induction of antigen-specific cytotoxic T lymphocytes in humans by a malaria DNA vaccine. Science **282:**476-480.

162. **Cui Z.** 2005. DNA Vaccine, p. 257-289. *In* Leaf Huang M-CH, Ernst W (ed.), Advances in Genetics, vol. Volume 54. Academic Press.

163. **Krieg AM.** 2002. CpG motifs in bacterial DNA and their immune effects. Annual review of immunology **20:**709-760.

164. **Siegrist CA, Barrios C, Martinez X, Brandt C, Berney M, Cordova M, Kovarik J, Lambert PH.** 1998. Influence of maternal antibodies on vaccine responses: inhibition of antibody but not T cell responses allows successful early prime-boost strategies in mice. Eur J Immunol **28:**4138-4148.

165. **Brave A, Ljungberg K, Wahren B, Liu MA.** 2007. Vaccine delivery methods using viral vectors. Molecular pharmaceutics **4:**18-32.

166. **Draper SJ, Heeney JL.** 2010. Viruses as vaccine vectors for infectious diseases and cancer. Nature reviews. Microbiology **8:**62-73.

167. **Dudek T, Knipe DM.** 2006. Replication-defective viruses as vaccines and vaccine vectors. Virology **344:**230-239.

168. **Dietrich G, Spreng S, Favre D, Viret JF, Guzman CA.** 2003. Live attenuated bacteria as vectors to deliver plasmid DNA vaccines. Current opinion in molecular therapeutics **5:**10-19.

169. **Garmory HS, Leary SE, Griffin KF, Williamson ED, Brown KA, Titball RW.** 2003. The use of live attenuated bacteria as a delivery system for heterologous antigens. Journal of drug targeting **11:**471-479.

170. **Moss B.** 2004. Vaccinia Virus and Other Poxviruses as Live Vectors, p. 313-323. *In* Levine MM, Kaper JB, Rappuoli R, Liu MA (ed.), New Generation Vaccines, 3rd ed. Marcel Dekker, New York.

171. **Souza AP, Haut L, Reyes-Sandoval A, Pinto AR.** 2005. Recombinant viruses as vaccines against viral diseases. Brazilian journal of medical and biological research = Revista brasileira de pesquisas medicas e biologicas / Sociedade Brasileira de Biofisica ... [et al.] **38:**509-522.

172. **Liniger M, Zuniga A, Naim HY.** 2007. Use of viral vectors for the development of vaccines. Expert review of vaccines **6:**255-266.

173. **Belyakov IM, Moss B, Strober W, Berzofsky JA.** 1999. Mucosal vaccination overcomes the barrier to recombinant vaccinia immunization caused by preexisting poxvirus immunity. Proc Natl Acad Sci U S A **96:**4512-4517.

174. **Hofer T, Muehlinghaus G, Moser K, Yoshida T, H EM, Hebel K, Hauser A, Hoyer B, E OL, Dorner T, Manz RA, Hiepe F, Radbruch A.** 2006. Adaptation of humoral memory. Immunological reviews **211:**295-302.

175. **Sutter G, Moss B.** 1992. Nonreplicating vaccinia vector efficiently expresses recombinant genes. Proc Natl Acad Sci U S A **89:**10847-10851.

176. **Tartaglia J, Perkus ME, Taylor J, Norton EK, Audonnet JC, Cox WI, Davis SW, van der Hoeven J, Meignier B, Riviere M, et al.** 1992. NYVAC: a highly attenuated strain of vaccinia virus. Virology **188:**217-232.

177. **Gherardi MM, Esteban M.** 2005. Recombinant poxviruses as mucosal vaccine vectors. The Journal of general virology **86:**2925-2936.

178. **Sutter G, Wyatt LS, Foley PL, Bennink JR, Moss B.** 1994. A recombinant vector derived from the host range-restricted and highly attenuated MVA strain of vaccinia virus stimulates protective immunity in mice to influenza virus. Vaccine **12:**1032-1040.

179. **Stittelaar KJ, Wyatt LS, de Swart RL, Vos HW, Groen J, van Amerongen G, van Binnendijk RS, Rozenblatt S, Moss B, Osterhaus AD.** 2000. Protective immunity in macaques vaccinated with a modified vaccinia virus Ankara-based measles virus vaccine in the presence of passively acquired antibodies. J Virol **74:**4236-4243.

180. **Amara RR, Villinger F, Staprans SI, Altman JD, Montefiori DC, Kozyr NL, Xu Y, Wyatt LS, Earl PL, Herndon JG, McClure HM, Moss B, Robinson HL.** 2002. Different patterns of immune responses but similar control of a simian-human immunodeficiency virus 89.6P mucosal challenge by modified vaccinia virus Ankara (MVA) and DNA/MVA vaccines. J Virol **76:**7625-7631.

181. **Patterson LJ, Peng B, Nan X, Robert-Guroff M.** 2004. Live Adenovirus Recombinants as Vaccine Vectors, p. 325-335. *In* Levine MM, Kaper JB, Rappuoli R, Liu MA (ed.), New Generation Vaccines, 3rd ed. Marcel Dekker, New York.

182. **Lai CM, Lai YK, Rakoczy PE.** 2002. Adenovirus and adeno-associated virus vectors. DNA and cell biology **21:**895-913.

183. **Russell WC.** 2000. Update on adenovirus and its vectors. The Journal of general virology **81:**2573-2604.

184. **Bruna-Romero O, Gonzalez-Aseguinolaza G, Hafalla JC, Tsuji M, Nussenzweig RS.** 2001. Complete, long-lasting protection against malaria of mice primed and boosted with two distinct viral vectors expressing the same plasmodial antigen. Proc Natl Acad Sci U S A **98:**11491-11496.

185. **Sullivan NJ, Sanchez A, Rollin PE, Yang ZY, Nabel GJ.** 2000. Development of a preventive vaccine for Ebola virus infection in primates. Nature **408:**605-609.

186. **Shiver JW, Fu TM, Chen L, Casimiro DR, Davies ME, Evans RK, Zhang ZQ, Simon AJ, Trigona WL, Dubey SA, Huang L, Harris VA, Long RS, Liang X, Handt L, Schleif WA, Zhu L, Freed DC, Persaud NV, Guan L, Punt KS, Tang A, Chen M, Wilson KA, Collins KB, Heidecker GJ, Fernandez VR, Perry HC, Joyce JG, Grimm KM, Cook JC, Keller PM, Kresock DS, Mach H, Troutman RD, Isopi LA, Williams DM, Xu Z, Bohannon KE, Volkin DB, Montefiori DC, Miura A, Krivulka GR, Lifton MA, Kuroda MJ, Schmitz JE, Letvin NL, Caulfield MJ, Bett AJ, Youil R, Kaslow DC, Emini EA.** 2002. Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity. Nature **415:**331-335.

187. **Schindler C, Fooks A, Stephenson J, Liebert UG.** 1994. Replication-incompetent adenoviruses as vectors for protective immunization against measles virus infection. Behring Institute Mitteilungen**:**109-115.

188. **Zhai Y, Yang JC, Kawakami Y, Spiess P, Wadsworth SC, Cardoza LM, Couture LA, Smith AE, Rosenberg SA.** 1996. Antigen-specific tumor vaccines. Development and characterization of recombinant adenoviruses encoding MART1 or gp100 for cancer therapy. J Immunol **156:**700-710.

189. **Caley IJ, Betts MR, Davis NL, Swanstrom R, Frelinger JA, Johnston RE.** 1999. Venezuelan equine encephalitis virus vectors expressing HIV-1 proteins: vector design strategies for improved vaccine efficacy. Vaccine **17:**3124-3135.

190. **Davis NL, Johnston RE.** 2004. RNA Virus Replicon Vaccines, p. 337-351. *In* Levine MM, Kaper JB, Rappuoli R, Liu MA (ed.), New Generation Vaccines, 3rd ed. Marcel Dekker, New York.

191. **Davis NL, West A, Reap E, MacDonald G, Collier M, Dryga S, Maughan M, Connell M, Walker C, McGrath K, Cecil C, Ping LH, Frelinger J, Olmsted R, Keith P, Swanstrom R, Williamson C, Johnson P, Montefiori D, Johnston RE.** 2002. Alphavirus replicon particles as candidate HIV vaccines. IUBMB life **53:**209-211.

192. **Lundstrom K.** 2002. Alphavirus-based vaccines. Current opinion in molecular therapeutics **4:**28-34.

193. **Lundstrom K.** 2003. Alphavirus vectors for vaccine production and gene therapy. Expert review of vaccines **2:**447-459.

194. **Polo JM, Gardner JP, Ji Y, Belli BA, Driver DA, Sherrill S, Perri S, Liu MA, Dubensky TW, Jr.** 2000. Alphavirus DNA and particle replicons for vaccines and gene therapy. Developments in biologicals **104:**181-185.

195. **Riezebos-Brilman A, de Mare A, Bungener L, Huckriede A, Wilschut J, Daemen T.** 2006. Recombinant alphaviruses as vectors for anti-tumour and anti-microbial immunotherapy. Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology **35:**233-243.

196. **Schlesinger S, Dubensky TW.** 1999. Alphavirus vectors for gene expression and vaccines. Current opinion in biotechnology **10:**434-439.

197. **Gardner JP, Frolov I, Perri S, Ji Y, MacKichan ML, zur Megede J, Chen M, Belli BA, Driver DA, Sherrill S, Greer CE, Otten GR, Barnett SW, Liu MA, Dubensky TW, Polo JM.** 2000. Infection of human dendritic cells by a sindbis virus replicon vector is determined by a single amino acid substitution in the E2 glycoprotein. J Virol **74:**11849-11857.

198. **MacDonald GH, Johnston RE.** 2000. Role of dendritic cell targeting in Venezuelan equine encephalitis virus pathogenesis. J Virol **74:**914-922.

199. **Saikh KU, Lee JS, Kissner TL, Dyas B, Ulrich RG.** 2003. Toll-like receptor and cytokine expression patterns of CD56+ T cells are similar to natural killer cells in response to infection with Venezuelan equine encephalitis virus replicons. The Journal of infectious diseases **188:**1562-1570.

200. **Davis NL, Brown KW, Johnston RE.** 1996. A viral vaccine vector that expresses foreign genes in lymph nodes and protects against mucosal challenge. J Virol **70:**3781-3787.

201. **Pushko P, Parker M, Ludwig GV, Davis NL, Johnston RE, Smith JF.** 1997. Replicon-helper systems from attenuated Venezuelan equine encephalitis virus: expression of heterologous genes in vitro and immunization against heterologous pathogens in vivo. Virology **239:**389-401.

202. **Schultz-Cherry S, Dybing JK, Davis NL, Williamson C, Suarez DL, Johnston R, Perdue ML.** 2000. Influenza virus (A/HK/156/97) hemagglutinin expressed by an alphavirus replicon system protects chickens against lethal infection with Hong Kong-origin H5N1 viruses. Virology **278:**55-59.

203. **Pushko P, Bray M, Ludwig GV, Parker M, Schmaljohn A, Sanchez A, Jahrling PB, Smith JF.** 2000. Recombinant RNA replicons derived from attenuated Venezuelan equine encephalitis virus protect guinea pigs and mice from Ebola hemorrhagic fever virus. Vaccine **19:**142-153.

204. **Davis NL, Caley IJ, Brown KW, Betts MR, Irlbeck DM, McGrath KM, Connell MJ, Montefiori DC, Frelinger JA, Swanstrom R, Johnson PR, Johnston RE.** 2000. Vaccination of macaques against pathogenic simian immunodeficiency virus with Venezuelan equine encephalitis virus replicon particles. J Virol **74:**371-378.

205. **Mossman SP, Bex F, Berglund P, Arthos J, O'Neil SP, Riley D, Maul DH, Bruck C, Momin P, Burny A, Fultz PN, Mullins JI, Liljestrom P, Hoover EA.** 1996. Protection against lethal simian immunodeficiency virus SIVsmmPBj14 disease by a recombinant Semliki Forest virus gp160 vaccine and by a gp120 subunit vaccine. J Virol **70:**1953-1960.

206. **Kenney RT, Edelman R.** 2004. Adjuvants for the Future, p. 213-223. *In* Levine MM, Kaper JB, Rappuoli R, Liu MA (ed.), New Generation Vaccines, 3rd ed. Marcel Dekker, New York.

207. **De Gregorio E, D'Oro U, Wack A.** 2009. Immunology of TLR-independent vaccine adjuvants. Current opinion in immunology **21:**339-345.

208. **Delany I, Rappuoli R, De Gregorio E.** 2014. Vaccines for the 21st century. EMBO molecular medicine **6:**708-720.

209. **Harrison WT.** 1935. Some Observations on the Use of Alum Precipitated Diphtheria Toxoid. American journal of public health and the nation's health **25:**298-300.

210. **Munks MW, McKee AS, Macleod MK, Powell RL, Degen JL, Reisdorph NA, Kappler JW, Marrack P.** 2010. Aluminum adjuvants elicit fibrin-dependent extracellular traps in vivo. Blood **116:**5191-5199.

211. **Oleszycka E, Lavelle EC.** 2014. Immunomodulatory properties of the vaccine adjuvant alum. Current opinion in immunology **28C:**1-5.

212. **Mosmann TR, Coffman RL.** 1989. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annual review of immunology **7:**145-173.

213. **Lindblad EB, Elhay MJ, Silva R, Appelberg R, Andersen P.** 1997. Adjuvant modulation of immune responses to tuberculosis subunit vaccines. Infection and immunity **65:**623-629.

214. **Ulanova M, Tarkowski A, Hahn-Zoric M, Hanson LA.** 2001. The Common vaccine adjuvant aluminum hydroxide up-regulates accessory properties of human monocytes via an interleukin-4-dependent mechanism. Infection and immunity **69:**1151-1159.

215. **Eisenbarth SC, Colegio OR, O'Connor W, Sutterwala FS, Flavell RA.** 2008. Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. Nature **453:**1122-1126.

216. **Franchi L, Nunez G.** 2008. The Nlrp3 inflammasome is critical for aluminium hydroxide-mediated IL-1beta secretion but dispensable for adjuvant activity. Eur J Immunol **38:**2085-2089.

217. **Mori A, Oleszycka E, Sharp FA, Coleman M, Ozasa Y, Singh M, O'Hagan DT, Tajber L, Corrigan OI, McNeela EA, Lavelle EC.** 2012. The vaccine adjuvant alum inhibits IL-12 by promoting PI3 kinase signaling while chitosan does not inhibit IL-12 and enhances Th1 and Th17 responses. Eur J Immunol **42:**2709-2719.

218. **Billiau A, Matthys P.** 2001. Modes of action of Freund's adjuvants in experimental models of autoimmune diseases. Journal of leukocyte biology **70:**849-860.

219. **Heeger PS, Forsthuber T, Shive C, Biekert E, Genain C, Hofstetter HH, Karulin A, Lehmann PV.** 2000. Revisiting tolerance induced by autoantigen in incomplete Freund's adjuvant. J Immunol **164:**5771-5781.

220. **Stills HF, Jr.** 2005. Adjuvants and antibody production: dispelling the myths associated with Freund's complete and other adjuvants. ILAR journal / National Research Council, Institute of Laboratory Animal Resources **46:**280-293.

221. **Kindler V, Sappino AP, Grau GE, Piguet PF, Vassalli P.** 1989. The inducing role of tumor necrosis factor in the development of bactericidal granulomas during BCG infection. Cell **56:**731-740.

222. **Wanstrup J, Christensen HE.** 1965. Granulomatous Lesions in Mice Produced by Freund's Adjuvant; Morphogenesis and Phasic Development. Acta pathologica et microbiologica Scandinavica **63:**340-354.

223. **Kensil CR, Wu JY, Soltysik S.** 1995. Structural and immunological characterization of the vaccine adjuvant QS-21. Pharmaceutical biotechnology **6:**525-541.

224. **Campbell JB, Peerbaye YA.** 1992. Saponin. Research in immunology **143:**526-530;discussion 577-528.

225. **Rimmelzwaan GF, Osterhaus AD.** 1995. A novel generation of viral vaccines based on the ISCOM matrix. Pharmaceutical biotechnology **6:**543-558.

226. **Ennis FA, Cruz J, Jameson J, Klein M, Burt D, Thipphawong J.** 1999. Augmentation of human influenza A virus-specific cytotoxic T lymphocyte memory by influenza vaccine and adjuvanted carriers (ISCOMS). Virology **259:**256-261.

227. **Bauer S, Wagner H.** 2002. Bacterial CpG-DNA licenses TLR9. Current topics in microbiology and immunology **270:**145-154.

228. **Verthelyi D, Kenney RT, Seder RA, Gam AA, Friedag B, Klinman DM.** 2002. CpG oligodeoxynucleotides as vaccine adjuvants in primates. J Immunol **168:**1659-1663.

229. **Akira S, Takeda K.** 2004. Toll-like receptor signalling. Nat Rev Immunol **4:**499-511.

230. **Takeda K, Akira S.** 2004. TLR signaling pathways. Seminars in immunology **16:**3-9.

231. **Uwiera RR, Gerdts V, Pontarollo RA, Babiuk LA, Middleton DM, Griebel PJ.** 2001. Plasmid DNA induces increased lymphocyte trafficking: a specific role for CpG motifs. Cellular immunology **214:**155-164.

232. **Hartmann G, Weiner GJ, Krieg AM.** 1999. CpG DNA: a potent signal for growth, activation, and maturation of human dendritic cells. Proc Natl Acad Sci U S A **96:**9305-9310.

233. **Jakob T, Walker PS, Krieg AM, Udey MC, Vogel JC.** 1998. Activation of cutaneous dendritic cells by CpG-containing oligodeoxynucleotides: a role for dendritic cells in the augmentation of Th1 responses by immunostimulatory DNA. J Immunol **161:**3042-3049.

234. **Chu RS, Targoni OS, Krieg AM, Lehmann PV, Harding CV.** 1997. CpG oligodeoxynucleotides act as adjuvants that switch on T helper 1 (Th1) immunity. The Journal of experimental medicine **186:**1623-1631.

235. **Duthie MS, Windish HP, Fox CB, Reed SG.** 2011. Use of defined TLR ligands as adjuvants within human vaccines. Immunological reviews **239:**178-196.

236. **Lahiri A, Das P, Chakravortty D.** 2008. Engagement of TLR signaling as adjuvant: towards smarter vaccine and beyond. Vaccine **26:**6777-6783.

237. **Griffin DE.** 2001. Alphaviruses, p. 917-962. *In* Knipe DM, Fields BN, Howley PM (ed.), Fields Virology, 4th ed. Lippincott Williams & Wilkins, Philadelphia, PA.

238. **Weaver SC, Ferro C, Barrera R, Boshell J, Navarro JC.** 2004. Venezuelan equine encephalitis. Annual review of entomology **49:**141-174.

239. **Wang E, Bowen RA, Medina G, Powers AM, Kang W, Chandler LM, Shope RE, Weaver SC, Cysticercosis Working Group in P.** 2001. Virulence and viremia characteristics of 1992 epizootic subtype IC Venezuelan equine encephalitis viruses and closely related enzootic subtype ID strains. Am J Trop Med Hyg **65:**64-69.

240. **Berge TO, Banks IS, Tigertt WD.** 1961. Attenuation of venezuelan equine encephalomyelitis virus by in vitro cultivation in guinea-pig heart cells Am. J. Epidemiol. **73:**209-218.

241. **Kinney RM, Johnson BJ, Welch JB, Tsuchiya KR, Trent DW.** 1989. The full-length nucleotide sequences of the virulent Trinidad donkey strain of Venezuelan equine encephalitis virus and its attenuated vaccine derivative, strain TC-83. Virology **170:**19-30.

242. **Strauss JH, Strauss EG.** 1994. The alphaviruses: gene expression, replication, and evolution. Microbiological reviews **58:**491-562.

243. **Laakkonen P, Ahola T, Kaariainen L.** 1996. The effects of palmitoylation on membrane association of Semliki forest virus RNA capping enzyme. The Journal of biological chemistry **271:**28567-28571.

244. **Klimstra WB, Nangle EM, Smith MS, Yurochko AD, Ryman KD.** 2003. DC-SIGN and L-SIGN can act as attachment receptors for alphaviruses and distinguish between mosquito cell- and mammalian cell-derived viruses. J Virol **77:**12022-12032.

245. **Ludwig GV, Kondig JP, Smith JF.** 1996. A putative receptor for Venezuelan equine encephalitis virus from mosquito cells. J Virol **70:**5592-5599.

246. **Bernard KA, Klimstra WB, Johnston RE.** 2000. Mutations in the E2 glycoprotein of Venezuelan equine encephalitis virus confer heparan sulfate interaction, low morbidity, and rapid clearance from blood of mice. Virology **276:**93-103.

247. **Rose PP, Hanna SL, Spiridigliozzi A, Wannissorn N, Beiting DP, Ross SR, Hardy RW, Bambina SA, Heise MT, Cherry S.** 2011. Natural resistance-associated macrophage protein is a cellular receptor for sindbis virus in both insect and mammalian hosts. Cell host & microbe **10:**97-104.

248. **Paredes AM, Ferreira D, Horton M, Saad A, Tsuruta H, Johnston R, Klimstra W, Ryman K, Hernandez R, Chiu W, Brown DT.** 2004. Conformational changes in Sindbis virions resulting from exposure to low pH and interactions with cells suggest that cell penetration may occur at the cell surface in the absence of membrane fusion. Virology **324:**373-386.

249. **Strauss EG, Rice CM, Strauss JH.** 1984. Complete nucleotide sequence of the genomic RNA of Sindbis virus. Virology **133:**92-110.

250. **Wang YF, Sawicki SG, Sawicki DL.** 1991. Sindbis virus nsP1 functions in negative-strand RNA synthesis. J Virol **65:**985-988.

251. **Shirako Y, Strauss JH.** 1994. Regulation of Sindbis virus RNA replication: uncleaved P123 and nsP4 function in minus-strand RNA synthesis, whereas cleaved products from P123 are required for efficient plus-strand RNA synthesis. J Virol **68:**1874-1885.

252. **Lemm JA, Rumenapf T, Strauss EG, Strauss JH, Rice CM.** 1994. Polypeptide requirements for assembly of functional Sindbis virus replication complexes: a model for the temporal regulation of minus- and plus-strand RNA synthesis. The EMBO journal **13:**2925-2934.

253. **Linger BR, Kunovska L, Kuhn RJ, Golden BL.** 2004. Sindbis virus nucleocapsid assembly: RNA folding promotes capsid protein dimerization. Rna **10:**128-138.

254. **Garoff H, Huylebroeck D, Robinson A, Tillman U, Liljestrom P.** 1990. The signal sequence of the p62 protein of Semliki Forest virus is involved in initiation but not in completing chain translocation. The Journal of cell biology **111:**867-876.

255. **Kinney RM, Johnson BJ, Brown VL, Trent DW.** 1986. Nucleotide sequence of the 26 S mRNA of the virulent Trinidad donkey strain of Venezuelan equine encephalitis virus and deduced sequence of the encoded structural proteins. Virology **152:**400-413.

256. **de Curtis I, Simons K.** 1988. Dissection of Semliki Forest virus glycoprotein delivery from the trans-Golgi network to the cell surface in permeabilized BHK cells. Proc Natl Acad Sci U S A **85:**8052-8056.

257. **von Bonsdorff CH, Harrison SC.** 1978. Hexagonal glycoprotein arrays from Sindbis virus membranes. J Virol **28:**578-583.

258. **Gorchakov R, Frolova E, Frolov I.** 2005. Inhibition of transcription and translation in Sindbis virus-infected cells. J Virol **79:**9397-9409.

259. **Ehrenkranz NJ, Ventura AK.** 1974. Venezuelan equine encephalitis virus infection in man. Annual review of medicine **25:**9-14.

260. **de la Monte S, Castro F, Bonilla NJ, Gaskin de Urdaneta A, Hutchins GM.** 1985. The systemic pathology of Venezuelan equine encephalitis virus infection in humans. Am J Trop Med Hyg **34:**194-202.

261. **Gleiser CA, Gochenour WS, Jr., Berge TO, Tigertt WD.** 1962. The comparative pathology of experimental Venezuelan equine encephalomyelitis infection in different animal hosts. The Journal of infectious diseases **110:**80-97.

262. **Jackson AC, SenGupta SK, Smith JF.** 1991. Pathogenesis of Venezuelan equine encephalitis virus infection in mice and hamsters. Veterinary pathology **28:**410-418.

263. **Aronson JF, Grieder FB, Davis NL, Charles PC, Knott T, Brown K, Johnston RE.** 2000. A single-site mutant and revertants arising in vivo define early steps in the pathogenesis of Venezuelan equine encephalitis virus. Virology **270:**111-123.

264. **Charles PC, Walters E, Margolis F, Johnston RE.** 1995. Mechanism of neuroinvasion of Venezuelan equine encephalitis virus in the mouse. Virology **208:**662-671.

265. **Davis NL, Brown KW, Greenwald GF, Zajac AJ, Zacny VL, Smith JF, Johnston RE.** 1995. Attenuated mutants of Venezuelan equine encephalitis virus containing lethal mutations in the PE2 cleavage signal combined with a second-site suppressor mutation in E1. Virology **212:**102-110.

266. **Davis NL, Grieder FB, Smith JF, Greenwald GF, Valenski ML, Sellon DC, Charles PC, Johnston RE.** 1994. A molecular genetic approach to the study of Venezuelan equine encephalitis virus pathogenesis. Archives of virology. Supplementum **9:**99-109.

267. **Grieder FB, Davis BK, Zhou XD, Chen SJ, Finkelman FD, Gause WC.** 1997. Kinetics of cytokine expression and regulation of host protection following infection with molecularly cloned Venezuelan equine encephalitis virus. Virology **233:**302-312.

268. **Tonkin DR, Whitmore A, Johnston RE, Barro M.** 2012. Infected dendritic cells are sufficient to mediate the adjuvant activity generated by Venezuelan equine encephalitis virus replicon particles. Vaccine **30:**4532-4542.

269. **Charles PC, Trgovcich J, Davis NL, Johnston RE.** 2001. Immunopathogenesis and immune modulation of Venezuelan equine encephalitis virus-induced disease in the mouse. Virology **284:**190-202.

270. **Ryman KD, Klimstra WB.** 2008. Host responses to alphavirus infection. Immunological reviews **225:**27-45.

271. **White LJ, Wang JG, Davis NL, Johnston RE.** 2001. Role of alpha/beta interferon in Venezuelan equine encephalitis virus pathogenesis: effect of an attenuating mutation in the 5' untranslated region. J Virol **75:**3706-3718.

272. **Brooke CB, Schafer A, Matsushima GK, White LJ, Johnston RE.** 2012. Early activation of the host complement system is required to restrict central nervous system invasion and limit neuropathology during Venezuelan equine encephalitis virus infection. The Journal of general virology **93:**797-806.

273. **Brooke CB, Deming DJ, Whitmore AC, White LJ, Johnston RE.** 2010. T cells facilitate recovery from Venezuelan equine encephalitis virus-induced encephalomyelitis in the absence of antibody. J Virol **84:**4556-4568.

274. **Davis NL, Powell N, Greenwald GF, Willis LV, Johnson BJ, Smith JF, Johnston RE.** 1991. Attenuating mutations in the E2 glycoprotein gene of Venezuelan equine encephalitis virus: construction of single and multiple mutants in a full-length cDNA clone. Virology **183:**20-31.

275. **Davis NL, Willis LV, Smith JF, Johnston RE.** 1989. In vitro synthesis of infectious venezuelan equine encephalitis virus RNA from a cDNA clone: analysis of a viable deletion mutant. Virology **171:**189-204.

276. **Lee JS, Hadjipanayis AG, Welkos SL.** 2003. Venezuelan equine encephalitis virus-vectored vaccines protect mice against anthrax spore challenge. Infection and immunity **71:**1491-1496.

277. **Lee JS, Pushko P, Parker MD, Dertzbaugh MT, Smith LA, Smith JF.** 2001. Candidate vaccine against botulinum neurotoxin serotype A derived from a Venezuelan equine encephalitis virus vector system. Infection and immunity **69:**5709-5715.

278. **Marth T, Kleen N, Stallmach A, Ring S, Aziz S, Schmidt C, Strober W, Zeitz M, Schneider T.** 2002. Dysregulated peripheral and mucosal Th1/Th2 response in Whipple's disease. Gastroenterology **123:**1468-1477.

279. **Pushko P, Geisbert J, Parker M, Jahrling P, Smith J.** 2001. Individual and bivalent vaccines based on alphavirus replicons protect guinea pigs against infection with Lassa and Ebola viruses. J Virol **75:**11677-11685.

280. **Velders MP, McElhiney S, Cassetti MC, Eiben GL, Higgins T, Kovacs GR, Elmishad AG, Kast WM, Smith LR.** 2001. Eradication of established tumors by vaccination with Venezuelan equine encephalitis virus replicon particles delivering human papillomavirus 16 E7 RNA. Cancer research **61:**7861-7867.

281. **Wilson JA, Bray M, Bakken R, Hart MK.** 2001. Vaccine potential of Ebola virus VP24, VP30, VP35, and VP40 proteins. Virology **286:**384-390.

282. **Herbert AS, Kuehne AI, Barth JF, Ortiz RA, Nichols DK, Zak SE, Stonier SW, Muhammad MA, Bakken RR, Prugar LI, Olinger GG, Groebner JL, Lee JS, Pratt WD, Custer M, Kamrud KI, Smith JF, Hart MK, Dye JM.** 2013. Venezuelan equine encephalitis virus replicon particle vaccine protects nonhuman primates from intramuscular and aerosol challenge with ebolavirus. J Virol **87:**4952-4964.

283. **Hevey M, Negley D, Pushko P, Smith J, Schmaljohn A.** 1998. Marburg virus vaccines based upon alphavirus replicons protect guinea pigs and nonhuman primates. Virology **251:**28-37.

284. **Hooper JW, Ferro AM, Golden JW, Silvera P, Dudek J, Alterson K, Custer M, Rivers B, Morris J, Owens G, Smith JF, Kamrud KI.** 2009. Molecular smallpox vaccine delivered by alphavirus replicons elicits protective immunity in mice and non-human primates. Vaccine **28:**494-511.

285. **Johnston RE, Johnson PR, Connell MJ, Montefiori DC, West A, Collier ML, Cecil C, Swanstrom R, Frelinger JA, Davis NL.** 2005. Vaccination of macaques with SIV immunogens delivered by Venezuelan equine encephalitis virus replicon particle vectors followed by a mucosal challenge with SIVsmE660. Vaccine **23:**4969-4979.

286. **Thompson JM, Whitmore AC, Konopka JL, Collier ML, Richmond EM, Davis NL, Staats HF, Johnston RE.** 2006. Mucosal and systemic adjuvant activity of alphavirus replicon particles. Proc Natl Acad Sci U S A **103:**3722-3727.

287. **Tonkin DR, Jorquera P, Todd T, Beard CW, Johnston RE, Barro M.** 2010. Alphavirus replicon-based enhancement of mucosal and systemic immunity is linked to the innate response generated by primary immunization. Vaccine **28:**3238-3246.

288. **LoBue AD, Lindesmith L, Yount B, Harrington PR, Thompson JM, Johnston RE, Moe CL, Baric RS.** 2006. Multivalent norovirus vaccines induce strong mucosal and systemic blocking antibodies against multiple strains. Vaccine **24:**5220-5234.

289. **LoBue AD, Thompson JM, Lindesmith L, Johnston RE, Baric RS.** 2009. Alphavirus-adjuvanted norovirus-like particle vaccines: heterologous, humoral, and mucosal immune responses protect against murine norovirus challenge. J Virol **83:**3212-3227.

290. **Steil BP, Jorquera P, Westdijk J, Bakker WA, Johnston RE, Barro M.** 2014. A mucosal adjuvant for the inactivated poliovirus vaccine. Vaccine **32:**558-563.

291. **Kinney RM, Chang GJ, Tsuchiya KR, Sneider JM, Roehrig JT, Woodward TM, Trent DW.** 1993. Attenuation of Venezuelan equine encephalitis virus strain TC-83 is encoded by the 5'-noncoding region and the E2 envelope glycoprotein. J Virol **67:**1269-1277.

292. **Halstead SB, Lan NT, Myint TT, Shwe TN, Nisalak A, Kalyanarooj S, Nimmannitya S, Soegijanto S, Vaughn DW, Endy TP.** 2002. Dengue hemorrhagic fever in infants: research opportunities ignored. Emerging infectious diseases **8:**1474-1479.

293. **Kliks SC, Nimmanitya S, Nisalak A, Burke DS.** 1988. Evidence that maternal dengue antibodies are important in the development of dengue hemorrhagic fever in infants. Am J Trop Med Hyg **38:**411-419.

294. **Guy B, Barban V, Mantel N, Aguirre M, Gulia S, Pontvianne J, Jourdier TM, Ramirez L, Gregoire V, Charnay C, Burdin N, Dumas R, Lang J.** 2009. Evaluation of interferences between dengue vaccine serotypes in a monkey model. Am J Trop Med Hyg **80:**302-311.

295. **White LJ, Parsons MM, Whitmore AC, Williams BM, de Silva A, Johnston RE.** 2007. An immunogenic and protective alphavirus replicon particle-based dengue vaccine overcomes maternal antibody interference in weanling mice. J Virol **81:**10329-10339.

296. **Adkins B, Bu Y, Guevara P.** 2002. Murine neonatal CD4+ lymph node cells are highly deficient in the development of antigen-specific Th1 function in adoptive adult hosts. J Immunol **169:**4998-5004.

297. **Tiru M, Hallander HO, Gustafsson L, Storsaeter J, Olin P.** 2000. Diphtheria antitoxin response to DTP vaccines used in Swedish pertussis vaccine trials, persistence and projection for timing of booster. Vaccine **18:**2295-2306.

298. **Pihlgren M, Friedli M, Tougne C, Rochat AF, Lambert PH, Siegrist CA.** 2006. Reduced ability of neonatal and early-life bone marrow stromal cells to support plasmablast survival. J Immunol **176:**165-172.

299. **Pihlgren M, Schallert N, Tougne C, Bozzotti P, Kovarik J, Fulurija A, Kosco-Vilbois M, Lambert PH, Siegrist CA.** 2001. Delayed and deficient establishment of the long-term bone marrow plasma cell pool during early life. Eur J Immunol **31:**939-946.

300. **Whitehead SS, Blaney JE, Durbin AP, Murphy BR.** 2007. Prospects for a dengue virus vaccine. Nature reviews. Microbiology **5:**518-528.

301. **Sabchareon A, Wallace D, Sirivichayakul C, Limkittikul K, Chanthavanich P, Suvannadabba S, Jiwariyavej V, Dulyachai W, Pengsaa K, Wartel TA, Moureau A, Saville M, Bouckenooghe A, Viviani S, Tornieporth NG, Lang J.** 2012. Protective efficacy of the recombinant, live-attenuated, CYD tetravalent dengue vaccine in Thai schoolchildren: a randomised, controlled phase 2b trial. Lancet **380:**1559-1567.

302. **Weiskopf D, Angelo MA, de Azeredo EL, Sidney J, Greenbaum JA, Fernando AN, Broadwater A, Kolla RV, De Silva AD, de Silva AM, Mattia KA, Doranz BJ, Grey HM, Shresta S, Peters B, Sette A.** 2013. Comprehensive analysis of dengue virus-specific responses supports an HLA-linked protective role for CD8+ T cells. Proc Natl Acad Sci U S A.

303. **Moran TP, Collier M, McKinnon KP, Davis NL, Johnston RE, Serody JS.** 2005. A novel viral system for generating antigen-specific T cells. J Immunol **175:**3431-3438.

304. **Osorio JE, Brewoo JN, Silengo SJ, Arguello J, Moldovan IR, Tary-Lehmann M, Powell TD, Livengood JA, Kinney RM, Huang CY, Stinchcomb DT.** 2011. Efficacy

of a tetravalent chimeric dengue vaccine (DENVax) in Cynomolgus macaques. Am J Trop Med Hyg **84:**978-987.

305. **Murphy BR, Whitehead SS.** 2011. Immune response to dengue virus and prospects for a vaccine. Annual review of immunology **29:**587-619.

306. **Rothman AL.** 2011. Immunity to dengue virus: a tale of original antigenic sin and tropical cytokine storms. Nat Rev Immunol **11:**532-543.

307. **Zaghouani H, Hoeman CM, Adkins B.** 2009. Neonatal immunity: faulty T-helpers and the shortcomings of dendritic cells. Trends Immunol **30:**585-591.

308. **Langrish CL, Buddle JC, Thrasher AJ, Goldblatt D.** 2002. Neonatal dendritic cells are intrinsically biased against Th-1 immune responses. Clinical and experimental immunology **128:**118-123.

309. **de Brito CA, Goldoni AL, Sato MN.** 2009. Immune adjuvants in early life: targeting the innate immune system to overcome impaired adaptive response. Immunotherapy **1:**883-895.

310. **Futata EA, Fusaro AE, de Brito CA, Sato MN.** 2012. The neonatal immune system: immunomodulation of infections in early life. Expert review of anti-infective therapy **10:**289-298.

311. **Prendergast AJ, Klenerman P, Goulder PJ.** 2012. The impact of differential antiviral immunity in children and adults. Nat Rev Immunol **12:**636-648.

312. **Barnard DL.** 2009. Animal models for the study of influenza pathogenesis and therapy. Antiviral Res **82:**A110-122.

313. **Stamboulian D, Bonvehi PE, Nacinovich FM, Cox N.** 2000. Influenza. Infectious disease clinics of North America **14:**141-166.

314. **Kohlmeier JE, Woodland DL.** 2009. Immunity to respiratory viruses. Annual review of immunology **27:**61-82.

315. **Marchant A, Goetghebuer T, Ota MO, Wolfe I, Ceesay SJ, De Groote D, Corrah T, Bennett S, Wheeler J, Huygen K, Aaby P, McAdam KP, Newport MJ.** 1999.

Newborns develop a Th1-type immune response to Mycobacterium bovis bacillus Calmette-Guerin vaccination. J Immunol **163:**2249-2255.

316. **Wu CY, Demeure C, Kiniwa M, Gately M, Delespesse G.** 1993. IL-12 induces the production of IFN-gamma by neonatal human CD4 T cells. J Immunol **151:**1938-1949.

317. **Dowling DJ, Tan Z, Prokopowicz ZM, Palmer CD, Matthews MA, Dietsch GN, Hershberg RM, Levy O.** 2013. The ultra-potent and selective TLR8 agonist VTX-294 activates human newborn and adult leukocytes. PLoS One **8:**e58164.

318. **Levy O, Suter EE, Miller RL, Wessels MR.** 2006. Unique efficacy of Toll-like receptor 8 agonists in activating human neonatal antigen-presenting cells. Blood **108:**1284-1290.

319. **Olafsdottir TA, Lingnau K, Nagy E, Jonsdottir I.** 2009. IC31, a two-component novel adjuvant mixed with a conjugate vaccine enhances protective immunity against pneumococcal disease in neonatal mice. Scandinavian journal of immunology **69:**194-202.

320. **Kenney RT, Edelman R.** 2003. Survey of human-use adjuvants. Expert review of vaccines **2:**167-188.

321. **Craig CP, Reynolds SL, Airhart JW, Staab EV.** 1969. Alterations in immune responses by attenuated Venezuelan equine encephalitis vaccine. I. Adjuvant effect of VEE virus infection in guinea pigs. J Immunol **102:**1220-1227.

322. **Howard RJ, Craig CP, Trevino GS, Dougherty SF, Mergenhagen SE.** 1969. Enhanced humoral immunity in mice infected with attenuated Venezuelan equine encephalitis virus. J Immunol **103:**699-707.

323. **Hidmark AS, Nordstrom EK, Dosenovic P, Forsell MN, Liljestrom P, Karlsson Hedestam GB.** 2006. Humoral responses against coimmunized protein antigen but not against alphavirus-encoded antigens require alpha/beta interferon signaling. J Virol **80:**7100-7110.

324. **Carroll TD, Matzinger SR, Barro M, Fritts L, McChesney MB, Miller CJ, Johnston RE.** 2011. Alphavirus replicon-based adjuvants enhance the immunogenicity and effectiveness of Fluzone (R) in rhesus macaques. Vaccine **29:**931-940.

325. **Moran TP, Burgents JE, Long B, Ferrer I, Jaffee EM, Tisch RM, Johnston RE, Serody JS.** 2007. Alphaviral vector-transduced dendritic cells are successful therapeutic vaccines against neu-overexpressing tumors in wild-type mice. Vaccine **25:**6604-6612.

326. **Nishimoto KP, Laust AK, Wang K, Kamrud KI, Hubby B, Smith JF, Nelson EL.** 2007. Restricted and selective tropism of a Venezuelan equine encephalitis virus-derived replicon vector for human dendritic cells. Viral immunology **20:**88-104.

327. **Thompson JM, Nicholson MG, Whitmore AC, Zamora M, West A, Iwasaki A, Staats HF, Johnston RE.** 2008. Nonmucosal alphavirus vaccination stimulates a mucosal inductive environment in the peripheral draining lymph node. J Immunol **181:**574-585.

328. **Schafer A, Brooke CB, Whitmore AC, Johnston RE.** 2011. The role of the blood-brain barrier during Venezuelan equine encephalitis virus infection. J Virol **85:**10682-10690.

329. **Cruz CC, Suthar MS, Montgomery SA, Shabman R, Simmons J, Johnston RE, Morrison TE, Heise MT.** 2010. Modulation of type I IFN induction by a virulence determinant within the alphavirus nsP1 protein. Virology **399:**1-10.

330. **Thompson JM, Whitmore AC, Staats HF, Johnston R.** 2008. The contribution of type I interferon signaling to immunity induced by alphavirus replicon vaccines. Vaccine **26:**4998-5003.

331. **Thompson JM, Whitmore AC, Staats HF, Johnston RE.** 2008. Alphavirus replicon particles acting as adjuvants promote CD8+ T cell responses to co-delivered antigen. Vaccine **26:**4267-4275.

332. **Holmgren J, Czerkinsky C.** 2005. Mucosal immunity and vaccines. Nature medicine **11:**S45-53.

333. **Mestecky J.** 1987. The common mucosal immune system and current strategies for induction of immune responses in external secretions. Journal of clinical immunology **7:**265-276.

334. **Elson CO, Ealding W, Lefkowitz J.** 1984. A lavage technique allowing repeated measurement of IgA antibody in mouse intestinal secretions. Journal of immunological methods **67:**101-108.

335. **Stetson DB, Medzhitov R.** 2006. Type I interferons in host defense. Immunity **25:**373-381.

336. **Grewal HM, Karlsen TH, Vetvik H, Ahren C, Gjessing HK, Sommerfelt H, Haneberg B.** 2000. Measurement of specific IgA in faecal extracts and intestinal lavage fluid for monitoring of mucosal immune responses. Journal of immunological methods **239:**53-62.

337. **Chen J, Fang F, Li X, Chang H, Chen Z.** 2005. Protection against influenza virus infection in BALB/c mice immunized with a single dose of neuraminidase-expressing DNAs by electroporation. Vaccine **23:**4322-4328.

338. **Chen Z, Matsuo K, Asanuma H, Takahashi H, Iwasaki T, Suzuki Y, Aizawa C, Kurata T, Tamura S.** 1999. Enhanced protection against a lethal influenza virus challenge by immunization with both hemagglutinin- and neuraminidase-expressing DNAs. Vaccine **17:**653-659.

339. **Galarza JM, Latham T, Cupo A.** 2005. Virus-like particle vaccine conferred complete protection against a lethal influenza virus challenge. Viral immunology **18:**365-372.

340. **Dadaglio G, Sun CM, Lo-Man R, Siegrist CA, Leclerc C.** 2002. Efficient in vivo priming of specific cytotoxic T cell responses by neonatal dendritic cells. J Immunol **168:**2219-2224.

341. **Muthukkumar S, Goldstein J, Stein KE.** 2000. The ability of B cells and dendritic cells to present antigen increases during ontogeny. J Immunol **165:**4803-4813.

342. **Sun CM, Fiette L, Tanguy M, Leclerc C, Lo-Man R.** 2003. Ontogeny and innate properties of neonatal dendritic cells. Blood **102:**585-591.

343. **Aksoy E, Albarani V, Nguyen M, Laes JF, Ruelle JL, De Wit D, Willems F, Goldman M, Goriely S.** 2007. Interferon regulatory factor 3-dependent responses to lipopolysaccharide are selectively blunted in cord blood cells. Blood **109:**2887-2893.

344. **De Wit D, Olislagers V, Goriely S, Vermeulen F, Wagner H, Goldman M, Willems F.** 2004. Blood plasmacytoid dendritic cell responses to CpG oligodeoxynucleotides are impaired in human newborns. Blood **103:**1030-1032.

345. **Cusumano V, Mancuso G, Genovese F, Cuzzola M, Carbone M, Cook JA, Cochran JB, Teti G.** 1997. Neonatal hypersusceptibility to endotoxin correlates with increased tumor necrosis factor production in mice. The Journal of infectious diseases **176:**168-176.

346. **Dufour JH, Dziejman M, Liu MT, Leung JH, Lane TE, Luster AD.** 2002. IFN-gamma-inducible protein 10 (IP-10; CXCL10)-deficient mice reveal a role for IP-10 in effector T cell generation and trafficking. J Immunol **168:**3195-3204.

347. **Spellberg B.** 2008. Dr. William H. Stewart: mistaken or maligned? Clinical infectious diseases : an official publication of the Infectious Diseases Society of America **47:**294.

348. **Adkins B, Bu Y, Cepero E, Perez R.** 2000. Exclusive Th2 primary effector function in spleens but mixed Th1/Th2 function in lymph nodes of murine neonates. J Immunol **164:**2347-2353.

349. **Adkins B, Du RQ.** 1998. Newborn mice develop balanced Th1/Th2 primary effector responses in vivo but are biased to Th2 secondary responses. J Immunol **160:**4217-4224.

350. **Barrios C, Brawand P, Berney M, Brandt C, Lambert PH, Siegrist CA.** 1996. Neonatal and early life immune responses to various forms of vaccine antigens qualitatively differ from adult responses: predominance of a Th2-biased pattern which persists after adult boosting. Eur J Immunol **26:**1489-1496.

351. **Wong OH, Huang FP, Chiang AK.** 2005. Differential responses of cord and adult blood-derived dendritic cells to dying cells. Immunology **116:**13-20.

352. **Ito T, Allen RM, Carson WFt, Schaller M, Cavassani KA, Hogaboam CM, Lukacs NW, Matsukawa A, Kunkel SL.** 2011. The critical role of Notch ligand Delta-like 1 in the pathogenesis of influenza A virus (H1N1) infection. PLoS Pathog **7:**e1002341.

353. **Dewar AL, Doherty KV, Woods GM, Lyons AB, Muller HK.** 2001. Acquisition of immune function during the development of the Langerhans cell network in neonatal mice. Immunology **103:**61-69.

354. **Simpson CC, Woods GM, Muller HK.** 2003. Impaired CD40-signalling in Langerhans' cells from murine neonatal draining lymph nodes: implications for neonatally induced cutaneous tolerance. Clinical and experimental immunology **132:**201-208.

355. **Suri-Payer E, Amar AZ, Thornton AM, Shevach EM.** 1998. CD4+CD25+ T cells inhibit both the induction and effector function of autoreactive T cells and represent a unique lineage of immunoregulatory cells. J Immunol **160:**1212-1218.

356. **Wang G, Miyahara Y, Guo Z, Khattar M, Stepkowski SM, Chen W.** 2010. "Default" generation of neonatal regulatory T cells. J Immunol **185:**71-78.

357. **Strebel PM, Papania MJ, Dayan GH, Halsey NA.** 2008. Measles vaccine, Vaccines, 4th ed. W. B. Saunders, Philadelphia.

358. **Zellweger RM, Miller R, Eddy WE, White LJ, Johnston RE, Shresta S.** 2013. Role of humoral versus cellular responses induced by a protective dengue vaccine candidate. PLoS Pathog **9:**e1003723.