Live Attenuated Vaccines for Respiratory Syncytial Virus

Michael N. Teng

Abstract In the five decades since the identification of respiratory syncytial virus (RSV) as an important pediatric pathogen, no effective vaccine has been developed. Previous attempts to develop inactivated RSV vaccines resulted in vaccine-enhanced disease, resulting in a greater focus on the generation of live attenuated RSV vaccines. However, identifying a live attenuated vaccine candidate that is appropriately attenuated and sufficiently immunogenic has proven to be difficult. Recently, reverse genetics systems have been developed for RSV, allowing researchers to introduce specific mutations into the genomes of recombinant vaccine candidates. These systems provide a means of determining the effects of known attenuating mutations and identifying novel methods of attenuating the virus without decreasing immunogenicity. In addition, different mutations can be combined in a single genome to fine-tune the level of attenuation and immunogenicity to achieve the proper balance in a viable vaccine candidate. Current research into RSV attenuation includes investigation of point mutations responsible for temperature sensitivity, nontemperature-sensitive attenuating mutations, and deletion of nonessential viral genes that play roles in viral RNA synthesis and/or inhibition of innate immune responses. Development of an effective RSV vaccine will likely rely on using reverse genetics systems to optimize the attenuation and immunogenicity of a live vaccine candidate, while preserving viral replication in vitro.

Keywords Live attenuated vaccines · Paramyxoviruses · Pediatric vaccines · Recombinant vaccines · Respiratory infections · Vaccine development

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M.N. Teng
Department of Biochemistry and Molecular Biology, Pennsylvania State University, University Park, PA 16802, USA
and
Nanomedicine Research Center, Divisions of Translational Medicine and Allergy & Immunology, Department of Internal Medicine, University of South Florida College of Medicine, 12901 Bruce B. Downs Blvd., Tampa, FL 33612, USA
e-mail: mteng@health.usf.edu

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1 Respiratory Syncytial Virus

Respiratory syncytial virus (RSV) is the most important etiologic agent of pediatric viral respiratory infection and remains a major cause of morbidity and mortality among infants. Infection rates for RSV in infants have been found to be 68.8 per 100 children for the first year of life, reaching 82.6 per 100 children for the second year [1]. Lower respiratory tract illness (LRTI) is more common during year 1, though LRTI occurs frequently during year 2. Approximately half of all children are reinfected by age 2, but most children experience only 1 LRTI [1]. RSV infection accounts for between 70,000 and 120,000 hospitalizations in the United States of infants under 6 months of age and ~70% of hospitalizations due to bronchiolitis [2–5]. Severe RSV infection has been associated with long-term effects such as asthma and wheezing and can cause significant mortality in high-risk groups, such as premature infants or children with immunodeficiency, chronic pulmonary disease, or cardiovascular disease [6–9]. In addition, RSV infection is a serious complication in immunocompromised subjects, particularly bone marrow transplant patients, and the elderly [10].

Previously, RSV bronchiolitis was thought to be caused by an overactive antiviral immune response, similar to allergic asthma [11–13]. However, recent evidence indicates that severe RSV disease is likely due to virus-induced cell death and sloughing of apoptotic cells into the lumen of the bronchioles [14]. Examination of autopsy specimens from fatal cases of RSV bronchiolitis showed the presence of overwhelming RSV antigen and massive apoptotic sloughing of epithelial cells, but a relative dearth of infiltrating T cells. In addition, infants who suffered nonfatal cases of RSV showed decreased expression levels of cytokines, particularly IFN-γ, IL-17, IL-4, and IL-6, compared to infants infected by influenza [14, 15]. Cytokine expression levels in RSV-infected infants did not appear to correlate with the severity of RSV infection. However, viral replication levels directly correlated with the severity of RSV disease [14, 16]. Thus, severe RSV LRTI is likely due to high levels of RSV replication in ciliated and nonciliated airway cells, resulting in cell death and a large influx of neutrophils and macrophages. This hypothesis also fits with the time course of RSV infection and the observation that corticosteroids are ineffective in treating RSV bronchiolitis [17]. These results suggest that reducing viral replication levels by the induction of protective immune responses via vaccination is likely to reduce the morbidity and mortality due to RSV infection.

Infection by RSV causes severe disease in the very young (infants under 6 months of age) and the elderly [18]. One distinctive characteristic of RSV infection is that it does not induce long-lived immunity upon exposure, resulting in recurrent infection throughout life. Reinfections occur frequently throughout life, though the symptoms of subsequent infection are generally milder [18]. Thus, the target populations for RSV vaccines would be individuals at the extremes of age. In both populations, lung function is suboptimal due to relatively inelastic lungs, either due to developmental immaturity or loss of elasticity. Premature infants are particularly susceptible to severe RSV disease due to interrupted lung development, leading to

decreased lung function with reduced airway diameter and increased smooth muscle. In addition, both populations present challenges to vaccination because of deficiencies in their immune responses. For infants, there are two major hurdles to effective immunization: (1) developmental immaturity of the immune system and (2) presence of maternal antibodies. Neonatal immune responses are both quantitatively and qualitatively different from those in adults, and these differences persist throughout the first year of life. The neonatal immune system appears to be biased toward Th2-like responses, although Th1 responses can be induced in neonates with certain stimuli including certain microbes [19–21]. This effect is likely due in part to immaturity of dendritic and other accessory cell populations. Serum antibodies derived from the mother pose a challenge for vaccine take, as seen in the experience with the measles virus vaccine. In contrast, premature infants born before 28 weeks of gestation, when maternal antibody transfer occurs, have increased susceptibility to RSV. Premature infants born closer to full term are likely better protected, as maternal antibody levels are proportional to gestational age.

At the other end of the age spectrum, immunosenescence is a hurdle for RSV vaccination in the elderly population. Not only are adaptive immune responses blunted in the elderly, but innate immune function appears to be decreased as well [22–24]. Protection from RSV by vaccination will likely require the induction of both B- and T-cell responses in the elderly, similar to influenza vaccination [19, 25, 26]. Thus, a more complete understanding of the mechanisms responsible for immunosenescence is required to improve the efficacy of RSV vaccines in the elderly.

Immunologic protection from RSV infection requires induction of high-affinity neutralizing antibody responses. Both infants and the elderly show decreased B-cell responses compared with healthy adults [27–29]. Moreover, these two populations display a limited ability to generate diversity in their antibody responses to antigenic stimulation [27, 30]. The exact mechanisms for these defects are not well understood. However, increasing the diversity and affinity of the immunoglobulin response in vaccinees is essential for efficient protection.

2 Agent

RSV is an enveloped virus classified in the family Paramyxoviridae in the order Mononegavirales, and is the prototype member of the *Pneumovirus* genus. The nonsegmented, negative-sense RNA genome of RSV is 15,222 nucleotides long and contains 10 genes from which 11 proteins are translated (Fig. 1). The genome is encapsidated by the viral nucleocapsid (N) protein, and this ribonucleocapsid complex serves as the template for viral transcription and RNA replication. RSV enters cells by direct fusion of its envelope with the plasma membrane and replicates solely in the cytoplasm. RSV packages its own viral RNA-dependent RNA polymerase (RdRP), which is essential for the initial transcription of its

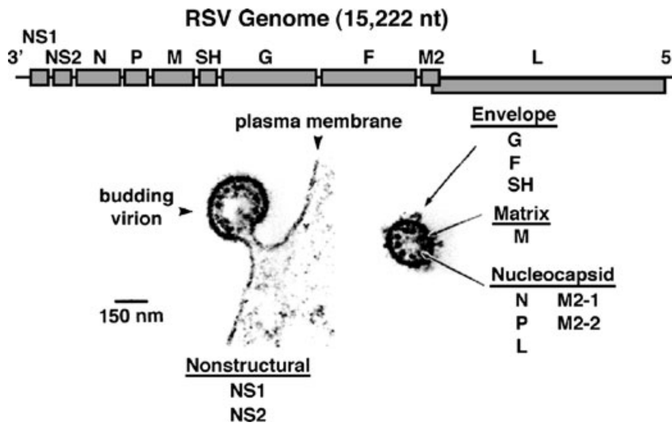

Fig. 1 RSV genome and virion structure. The M2 gene overlaps with the L gene. Photograph by Anthony Kalica (courtesy of Peter Collins, NIAID)

genome upon infection. The RdRP for RSV transcription is minimally composed of P, M2-1, and L. L encodes the large enzymatic subunit of the polymerase, and P is an essential cofactor for RNA synthesis. M2-1 is specific for the viral transcriptase and is an antitermination/processivity factor. The polymerase complex accesses the genome at a single promoter at the 3′ end of the genome and initiates transcription at the first gene (NS1). Each gene is bounded by conserved transcription initiation and termination signals and is separated from the adjacent genes by a variable length of intergenic sequence. The linear array of viral genes is transcribed sequentially in a start/stop fashion, resulting in a polar gradient of mRNA production, whereby genes proximal to the 3′ promoter are transcribed more efficiently than those that are promoter-distal. At a low frequency, the RdRP will fail to terminate, resulting in an oligocistronic or "readthrough" mRNA that is terminated at a subsequent transcription termination signal, or will fail to reinitiate, resulting in transcription attenuation and a gradient of expression inversely proportional to the distance from the 3′ end of the genome. After primary transcription has occurred, the polymerase complex begins replicating the viral genome, synthesizing a full-length copy of the vRNA called the antigenome (cRNA). The regulation of the switch from transcription to replication by RdRP is not clear; however, the M2-2 protein is thought to be involved in this process. The antigenome is also encapsidated by N protein and serves as a template for synthesis of more vRNA. In infected cells, there is more vRNA than cRNA [10]. Encapsidated vRNA interacts with the matrix (M) protein and traffics to the plasma membrane where the viral N interacts with the cytoplasmic tails of the attachment (G) and fusion (F) proteins. Virion morphogenesis occurs at lipid raft domains in the membrane where F is localized. In addition to G and F, the RSV viral envelope contains a small hydrophobic (SH) protein of unknown function. Importantly, G and F are the major neutralizing antigens for RSV. The two remaining RSV proteins, NS1 and NS2, are nonstructural proteins that have been

shown to inhibit IFN-β induction and signaling but are otherwise dispensable for viral replication in vitro [31, 32].

3 Treatment

Currently, there are no effective antiviral drugs to treat RSV infection. Ribavirin has been previously used to treat severe RSV disease, but the efficacy of this treatment is questionable and the cost is high [33–35]. Supportive care with supplemental oxygen is the most common treatment option, although treatment with corticosteroids and/or β-agonists has been tried with limited success [35]. Nebulized hypertonic saline with or without epinephrine has been found to decrease length of stay in infants hospitalized with viral bronchiolitis [36, 37]. Immunoprophylaxis has been the mainstay for the prevention of RSV infection in high-risk infants. Synagis (palivizumab), a recombinant humanized monoclonal antibody to the RSV F protein, has been shown to be effective in preventing infection in premature infants and children with underlying risk factors for severe RSV disease [38–40]. The recent development of a higher affinity monoclonal antibody to F has improved the efficacy profile of RSV immunoprophylaxis [41, 42]. However, Synagis treatment is not cost-effective in normal populations due to the need to administer the drug monthly during RSV season and the lower incidence of hospitalization for severe RSV bronchiolitis.

4 RSV Vaccines

Although RSV is the most important cause of viral lower respiratory tract disease in infants, initial attempts to develop an RSV vaccine by using inactivated virus met with failure. In the early 1960s, vaccination of infants with a formalin-inactivated (FI)-RSV vaccine not only failed to protect against RSV disease during the following RSV season but some vaccinees developed enhanced disease upon infection with RSV, resulting in increased rates of severe pneumonia and two deaths [43–45]. Studies on autopsy samples as well as in the mouse model suggested that the enhanced disease due to FI-RSV vaccination was due to an imbalanced T helper cell response, predisposing the vaccinees to a response resembling allergic asthma upon subsequent infection by RSV (reviewed in [46]). More recently, it has been determined that the FI-RSV vaccine has reduced the capacity for inducing high avidity antibodies, due to reduced TLR stimulation, likely resulting in the deposition of complement in the lungs [47, 48].

In the intervening years, a number of different approaches have been evaluated including subunit vaccines, vectored vaccines, and live attenuated vaccines; however, as of the writing of this chapter there remains no licensed RSV vaccine. Currently, the most promising vaccine candidates for RSV are live attenuated viruses. These

viruses have several benefits: (1) enhanced RSV disease has not been observed either after natural infection or vaccination with live attenuated viruses [49–53]; (2) administration of live attenuated RSV vaccines induces balanced immune responses that more closely match natural immunity compared with parenterally administered subunit (or inactivated) vaccines [54, 55]. Also, vaccination with live attenuated viruses intranasally would likely induce better local immunity compared with intramuscular injection of subunit or killed vaccines [56]. Live attenuated RSV vaccines have been in development for several decades, using a combination of cold passage (*cp*) and chemical mutagenesis to induce temperature sensitivity (*ts*) (reviewed in [57, 58]). The initial RSV vaccine candidates were either under- or over-attenuated, with reversion of one of the *ts* mutants in vaccinated children [50, 59–61]. However, children vaccinated with these live attenuated viruses did not show enhanced disease upon subsequent infection with RSV [62]. Therefore, further development of live attenuated vaccine candidates was performed, combining cold passage and chemical mutagenesis to generate temperature-sensitive RSV. A spectrum of *cpts*RSV vaccine candidates were produced by this method, with a range of temperature sensitivity in culture and attenuation in animal models (Fig. 2a) [53, 63–66]. Candidate vaccines from this method were immunogenic and protected against RSV challenge in both rodent and nonhuman primate models. Two candidate vaccines (*cpts*248/955 and *cpts*530/1009) were chosen for testing in the clinic [53]. These candidates induced protective immune responses in seronegative children; however, both candidates were underattenuated in this age group, precluding further analysis in infants (Table 1). One additional candidate, *cpts*248/404, was found to be sufficiently attenuated and immunogenic in seronegative children and was tested in 1- to 3-month-old infants [49]. However, *cpts*248/404 caused nasal congestion in these infants, an unacceptable adverse effect in this population [49].

Production of live attenuated RSV vaccine candidates by mutagenesis and screening for temperature sensitivity is a laborious and inefficient process. Therefore, it is essential to develop a method of systematically deriving *ts*RSV and identifying additional attenuating mutations that can be incorporated into RSV vaccine candidates. The recent advent of reverse genetics systems for RSV has allowed the development of live attenuated RSV vaccine candidates encoding specific attenuating mutations, rather than relying on random mutagenesis. The ability to generate recombinant RSV (rRSV) from cDNAs also allows the identification of novel viral targets for attenuation through the investigation of the virus–host interactions important for viral pathogenesis. Reverse genetics systems for RSV rely on the coexpression of the viral polymerase components (N, P, M2-1, and L) with a complete copy of the viral genome [67, 68]. Coexpression is achieved by transfection of plasmids encoding each of the viral polymerase genes and a plasmid encoding the full-length cDNA of the viral genome into cultured cells. Expression from the plasmids is driven by the bacteriophage T7 RNA polymerase, which is supplied exogenously. For the purposes of vaccine development, T7 RNA polymerase is expressed by cotransfection of an expression plasmid with the other plasmids into qualified Vero cells [69]. Upon expression of viral components,

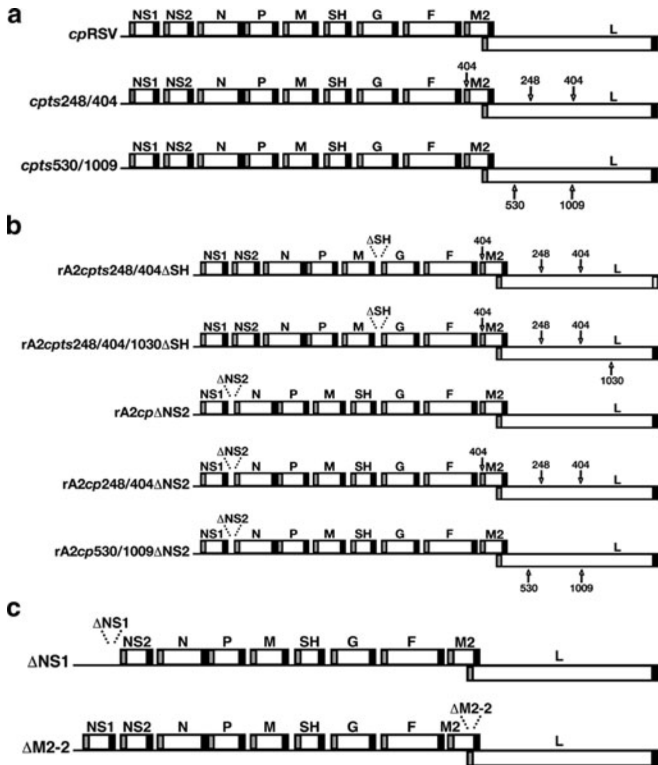

Fig. 2 RSV vaccine candidates. (**a**). Genomic organization of biologically derived, temperature-sensitive RSV vaccine candidates. *Arrows* indicate relative position of the attenuating mutations corresponding to the mutant, indicated on the *left*. (**b**). Recombinant RSV vaccine candidates. ts point mutations are identified as in (**a**). Deletions are indicated with *dashed lines*. (**c**). Potential recombinant RSV vaccine candidates. ts point mutations are identified as in (**a**). Deletions are indicated with *dashed lines*

transcription and replication of the viral genome initiates the RSV infectious cycle, resulting in the production of infectious rRSV. The cDNA copy of the viral genome can be mutated by standard molecular biology techniques in order to attenuate the resultant rRSV.

Initial studies using rRSV focused on two different means of attenuating RSV. The first method involved combining the known mutations from the *cpts*RSV isolates in rRSV strain A2 (rA2) to increase attenuation of the vaccine candidates. This resulted in the generation of rA2*cpts*248/404/1009 and rA2*cpts*248/404/1030, combining the *cpts*248/404 mutations with those of 530/1009 and 530/1030 [70]. These new mutants were more attenuated than the *cpts*248/404 parental virus, indicating that some mutations have additive effects in attenuation. However, these studies also showed that certain mutations are incompatible with others, as the rA2*cpts*248/404/530 could not be recovered, due to incompatibility of the 530

Table 1 Clinical trials on live attenuated RSV vaccine candidates

Vaccine candidate	Attenuation phenotype	Immunogenicity	References
Biologically derived			
*cp*RSV	Underattenuated in seropositive children	Mild (adults)	[53]
*cpts*248/955	Underattenuated in seronegative children	Good (seronegative children)	[53]
*cpts*530/1009	Underattenuated in seronegative children	Good (seronegative children)	[53]
*cpts*248/404	Underattenuated in infants (partial reversion)	Good (seronegative children) Mild (infants)	[49]
Recombinant			
rA2cpts248/404ΔSH	Underattenuated in seronegative children	Good (seronegative children)	[52]
rA2cpts248/404/1030ΔSH Ongoing trials	Sufficiently attenuated in infants (partial reversion)	Good (seronegative children) Poor (infants)	[52]
rA2cpΔNS2	Underattenuated in seropositive children	Mild (seropositive children)	[79]
rA2cp248/404ΔNS2	Underattenuated in seronegative children	Moderate (seronegative children)	[79]
rA2cp530/1009ΔNS2	Sufficiently attenuated in seronegative children	Poor (seronegative children)	[79]
Vectored			
MEDI-534 (rB/HPIV3-RSV-F) Ongoing trials	Attenuated in seropositive children	Poor (seropositive children)	[117]

mutation with, particularly, the 248 mutation [70]. Therefore, it would be desirable to have a panel of attenuating mutations from which to select to incorporate into rRSV vaccine candidates, so that the level of attenuation can be properly tuned. In order to increase the number of attenuating mutations that could potentially be combined in a vaccine candidate, specific viral proteins have been mutagenized to replace charged amino acids with a noncharged amino acid (e.g., alanine). This procedure has been employed to identify a number of mutations in both P and L that result in attenuation of RSV, both in culture and in rodents [71–73]. These mutations thus add to the panel of mutations available for inclusion in future vaccine candidates, either alone or in combination with the previously identified *cpts* L mutations.

Another avenue of attenuation for RSV has been the deletion of nonessential genes. Gene deletion should be more stable than the point mutations responsible for temperature sensitivity, reducing the risk of reversion to virulence of the vaccine candidate. rRSVs (rA2) lacking one or a combination of NS1, NS2, M2-2, and SH were generated and shown to be attenuated in preclinical trials [31, 74–76]. RSV lacking SH (rA2ΔSH) replicated similarly to wild-type (wt) RSV (rA2) in culture but showed a low level of attenuation in the respiratory tracts of rodents and nonhuman primates [77]. Because clinical trials indicated that rA2cpts248/404

was only slightly underattenuated, the SH gene deletion was incorporated into this vaccine candidate to increase the level of attenuation (Fig. 2b). However, this vaccine candidate (rA2cpts248/404ΔSH) was not further attenuated in adults, seropositive or seronegative children (Table 1) [52]. It was not possible to determine from these observations whether the SH deletion mutation confers attenuation to RSV in humans, even though rA2ΔSH was attenuated in mice and chimpanzees. These results indicate that attenuation of RSV by combining different mutations is not necessarily additive. However, subsequent addition of the 1030 mutation to rA2cpts248/404ΔSH resulted in a virus that was more *ts* and more attenuated in seronegative children [52]. Further trials in seronegative infants showed that rA2cpts248/404/1030ΔSH was well tolerated and appropriately attenuated (Table 1) [52]. Only a minority of vaccinees produced increased neutralizing antibody responses, even after a second dose of the vaccine virus. However, replication of the second dose of vaccine was significantly reduced, indicating that some protective immunity had been induced by the initial dose [52].

Preclinical testing of RSV lacking NS1 or NS2 (rA2ΔNS1 and rA2ΔNS2, respectively) showed that these viruses were deficient in replication in culture and also attenuated in rodents and nonhuman primates [31, 32, 76, 78]. In chimpanzees, rA2ΔNS2 displayed an attenuation phenotype similar to rA2cpts248/404, and rA2ΔNS1 was significantly more attenuated in both the upper and lower respiratory tracts [74, 75]. However, both deletion mutants induced levels of serum-neutralizing antibodies against RSV to levels comparable or slightly lower than wt RSV. In addition, chimpanzees immunized with rA2ΔNS2 were protected against subsequent challenge with RSV. Therefore, an NS2-deletion rA2 derivative was then tested in clinical trials as a vaccine for the elderly because it was less attenuated in chimpanzees than the *cpts*248/404 vaccine candidate (Fig. 2b) [79]. rA2cpΔNS2 was shown to be overattenuated in adults; however, it was also underattenuated in children, a contraindication for testing in infants (Table 1). The NS2 deletion virus was further attenuated by inclusion of the *ts* mutations 248/404 or 530/1009. These vaccine candidates were more attenuated than their parental strains and modestly immunogenic when tested in seronegative children [79].

5 Live Vectored RSV Vaccines

An alternative means of delivering RSV antigens in attenuated virus vaccines has been the use of heterologous viral vectors expressing RSV F and/or G. Early efforts focused on vaccinia viruses (VV) expressing RSV proteins. VV-F and VV-G together were immunogenic and protective in the mouse model of RSV; however, these VV recombinants did not induce protective immunity in chimpanzees [80–83]. In addition, VV is likely too virulent to use as a vector for current vaccine development. More recently, use of the attenuated modified vaccinia Ankara as a vector for RSV antigens has shown some efficacy, though a prime-boost strategy may be required to elicit sufficiently protective immunity [84, 85].

Adenovirus vectors were initially used to immunize against RSV F and G over 15 years ago and, with the advent of replication-deficient adenovirus vectors, have been further investigated more recently [86–90]. Adenovirus-vectored F and/or G have been shown to provide protection to RSV in mice and ferrets; however, this vaccine modality does not immunize chimpanzees against RSV, indicating that this strategy will likely not be clinically useful [88, 89]. Alphavirus replicons have also been tested for their ability to vaccinate against RSV [91–94]. Immunization via either the intranasal or intramuscular route with Venezuelan equine encephalitis virus replicons expressing RSV F induces balanced Th1/Th2 immunity, protects mice and cotton rats against RSV challenge, and induces serum antibodies in macaques [91, 92].

The recent proliferation of reverse genetics systems for the paramyxovirus family has provided the possibility that RSV antigens can be expressed in the context of a number of different paramyxoviruses, including Sendai virus, Newcastle disease virus (NDV), and human parainfluenza viruses (HPIV) 1, 2, and 3 (reviewed in [95–97]). Sendai virus and NDV are murine and avian viruses, respectively, and thus are naturally attenuated in humans due to host range restriction. NDV is a strong inducer of IFN-β and may therefore provide better stimulation of dendritic cell (DC) maturation and T-cell responses than RSV infection [98]. Both of these vector systems have been shown to be immunogenic and protective against RSV challenge in animal model systems [98–102].

An additional consideration is the possibility of combining vaccines against multiple pediatric viral pathogens into a single recombinant virus. Infection of children by HPIV1 and HPIV2 generally occurs later in life (approximately 6 months of age), so immunization would occur in older infants. Thus, an HPIV1- or HPIV2-vectored RSV vaccine may be useful as a booster to prevent secondary disease or as a vaccine in the elderly. In addition, attenuated HPIV1 and HPIV2 are being developed for use as vaccine candidates [103–109].

Because HPIV3 is also an important cause of pediatric respiratory tract disease, significant effort has been put into developing a live attenuated HPIV3 vaccine that could also be used as a vector for an RSV vaccine (Table 1). One candidate vaccine utilizes the bovine PIV3 (BPIV3) backbone, which has been shown to be safe and immunogenic in infants [110, 111]. In order to generate a bivalent HPIV3/RSV vaccine, the BPIV3 F and HN genes were replaced by their HPIV3 counterparts and RSV F was inserted into the B/HPIV3 chimera; thus, the resulting virus expresses both RSV and HPIV3 surface antigens. Recombinant B/HPIV3-RSV-F was slightly more attenuated than the parent virus, but remained immunogenic and was protective against both RSV and HPIV3 in animal model systems [112–115]. This vaccine candidate (MEDI-534) has recently been tested in clinical trials. Although the vaccine was attenuated and safe, it was minimally immunogenic in both adults and children, indicating that further modification may be required [116, 117]. However, the major advantage of this approach is that the viral vector is also a vaccine, thus providing protection against multiple pathogens. Because the RSV F protein is likely not incorporated into its viral envelope, RSV-specific antibodies were ineffective at neutralizing the chimeric virus [112], suggesting it could be also used as to boost anti-RSV immune responses.

6 Future Directions

There remain a number of challenges to the development of an efficacious RSV vaccine. First, it will be important to develop additional animal models for RSV challenge that more faithfully represent the target populations of infants and the elderly. Although nonhuman primate models have yielded important information on both vaccine safety and immunogenicity, these models also have not recapitulated some aspects of the replication of vaccine candidates in humans. For example, ΔNS2 was immunogenic in chimpanzees but not in seropositive children [75, 79]. In addition, the partial reversion of the *ts* phenotype seen with the 248/404/1030 mutations in infants was not detected in animal experiments [49, 52, 70, 118]. Defining the correlates of protection and attenuation in animal models will aid in the selection of vaccine candidates for clinical trials. In addition, a model that recapitulates stimulation of the immature immune system in the presence of maternal antibodies will be important for the development of a pediatric RSV vaccine.

Perhaps the most important challenge in the development of an effective RSV vaccine has been achieving the proper balance between immunogenicity and attenuation. The rA2cpts248/404/1030ΔSH vaccine candidate, which was appropriately attenuated in infants, was only mildly immunogenic [52]. It is possible to enhance immunogenicity of vaccines by increasing the dose or boosting with multiple inoculations. However, the target population of a pediatric RSV vaccine would be infants who are entering their first RSV season, thus shortening the window in which immunization would be effective. Therefore, a better understanding of the induction of immune responses in the target populations for RSV vaccines will be essential. Identifying signals (e.g., TLR agonists, cytokines) that can induce DC maturation and/or activate other antigen-presenting cell populations stimulate Th1 responses that can augment the immunogenicity of an RSV vaccine. For example, studies in mice suggest that deletion of NS1 results in a virus that has enhanced capacity to induce DC maturation, likely due to increased production of IFN-β [119]. In addition, NS1 appears to play a role in viral replication beyond IFN antagonism, indicating that deletion of this gene might be both attenuating and immunomodulatory [31].

An alternative method to enhancing immune responses that has been explored is the expression of cytokine genes as an additional transcription unit in rRSV [120–122]. Stable expression of additional gene products in the rRSV genome has been shown for a variety of genes [123]. rRSV encoding GM-CSF as an additional gene shows reduced replication in the respiratory tracts of mice with a concomitant increase in the number of pulmonary DCs and in the expression of IFN-γ and IL-12 [121]. By contrast, insertion of genes for the cytokines IL-4 and IFN-γ into rRSV results in viruses that caused increased pathogenesis after immunization and/or challenge [120]. Skewing of the T helper response can have adverse effects on secondary exposure and even to unrelated viruses [124]. Thus, significant care must be taken in identifying specific immunomodulators that will

increase the immunogenicity of an RSV vaccine candidate without causing enhanced disease.

One potential mechanism of improving B-cell responses to RSV is increasing the expression of the RSV F and G proteins, which serve as the major protection antigens [18]. Because of the linear nature of the RSV genome, the promoter-proximal genes are expressed to a greater extent than the promoter-distal genes [18]. Rearrangement of the gene order in the related vesicular stomatitis virus (VSV) has been shown to result in genome site-specific levels of expression for the viral genes [125]. These rearranged viruses displayed an attenuated phenotype both in vitro and in vivo and were able to vaccinate pigs against subsequent VSV infection [126, 127]. For RSV, rearrangement of the gene order in a recombinant virus, such that the F and/or G genes are the promoter-proximal, resulted in an approximately twofold increase in protein expression [128]. Unlike VSV, these viruses replicated slightly better than wt virus in culture and similarly to wt in the respiratory tracts of mice [128]. Thus, gene rearrangement alone in the context of RSV is not attenuating. In addition, shifting F to a promoter-proximal position resulted in an increase in anti-F serum antibody responses in mice, suggesting that increased F expression may be desirable in a vaccine candidate [128]. Expression of F and G might be further increased by optimizing the codon usage of these genes for translation [129]. Combining these relatively small increases in antigen expression might allow for an additive effect for vaccination. Studies with anti-RSV F antibody prophylaxis show significant increases in efficacy with even minor increases in antibody titer [130, 131]. Thus, increasing the amount of antigen available for presentation to the immune system may allow for a more robust anti-RSV response.

RSV G is unique among paramyxovirus attachment proteins in that it is produced in both a membrane-bound and a secreted form. Secreted G (sG) is produced from the G mRNA by alternative initiation from a downstream AUG [132, 133]. Ablation of this translation initiation codon in rRSV results in RSV that produces only membrane-bound G [134]. Studies have shown that the sG can act both as an antigenic decoy in vitro and as an immunomodulatory factor in mice [135]. Importantly, sG appeared to affect restriction of RSV replication in vivo by both anti-G and anti-F antibodies through a mechanism involving FcγR-bearing immune cells [135]. Thus, a vaccine candidate that does not express sG may have increased immunogenicity and may be more efficiently controlled by the immunity induced. In addition, sG showed proinflammatory functions in the lungs of mice, likely via its CX3C (fractalkine) motif [135]. Because pulmonary inflammation is associated with increased pathogenicity of RSV, removal of this factor may result in decreased reactogenicity. However, sG may be necessary for vaccine take in infants in the presence of maternal antibody. Further studies will clarify these disparate effects of sG on RSV pathogenesis and immunity. An alternative to ablating the expression of sG might be removal of the CX3C motif from G; studies have shown that mutagenesis or deletion of this sequence does not affect viral replication in vitro or in mice [136].

One important characteristic of vaccine candidates is genotypic and phenotypic stability. Genomic stability is important during the scaling up of production for the vaccine viruses, which undergo multiple rounds of replication and thus have a greater chance for mutation. In addition, phenotypic stability is essential during vaccination, during which reversion to virulence can cause increased pathogenicity and shedding. In this case, the attenuated phenotype is more important than specific genotype provided that immunity to the major protective antigens is achieved. Deletion of nonessential viral genes should provide the most stable attenuating mutations because genetic recombination of RSV is extremely rare and has only been observed in the laboratory under optimal conditions [137]. In addition to the NS2 and SH deletion viruses, RSVs lacking NS1 or M2-2 (Fig. 2c) are significantly attenuated and protective in animal models and are potentially good vaccine candidates [31, 74, 76, 78].

All of the *ts* mutations identified in the RSV vaccine candidates that have undergone clinical trials are point mutants. *ts*248, *ts*530, *ts*1009, and *ts*1030 are all missense mutations within the viral polymerase (or L protein), and *ts*404 is a point mutation in the M2 gene start sequence [65, 66, 70]. Characterization of virus shed from vaccinees has shown that these point mutations can readily revert, resulting in less *ts* RSV, in some cases despite the "stabilization" of these mutations in rRSV by changing two residues of the specific codon encoding the *ts* mutant. For example, analysis of nasal wash specimens from seronegative infants vaccinated with rA2cpts248/404/1030ΔSH showed that approximately one-third of the samples had lost a measure of their *ts* phenotype, displaying a 1–3°C increase in permissive temperature [52]. Sequencing of these clinical specimens identified reversion mutations at either the *ts*248 or the *ts*1030 mutation [52]. Although these partial revertants retained four of the five attenuating mutations and a measure of attenuation, these results demonstrate the difficulty of using point mutations to attenuate RNA viruses, which encode an error-prone viral polymerase. To counteract this problem, there are a number of possibilities to generate genotypically and phenotypically stable *ts* RSV vaccine candidates.

It is possible to generate phenotypically stable attenuated RSV viruses by introducing several *ts* point mutations in a variety of places in the RSV genome. The difficulty with this approach is that some combinations of mutations might increase the attenuation of the vaccine virus beyond the level required for inducing protective immunity. In addition, some *ts* mutations are not compatible with each other, resulting in a nonviable virus [70]. Thus, the spectrum of mutations that can be combined would have to be empirically defined. The benefit to this strategy is that reversion at any one site should be compensated by the presence of the additional attenuating mutations. However, as seen with rA2cpts248/404/1030ΔSH, particular mutations have a more prominent effect on attenuation of the vaccine virus and reversion at these sites may result in a significant loss in attenuation.

One method of preventing reversion is to "stabilize" existing *ts* mutations by altering the codon usage to require two mutation events in order for the mutant to revert to the wt phenotype. Theoretically, the viral polymerase would not be likely

to introduce two mutations at the same site. Recently, Luongo et al. have constructed rRSV that have mutations at position 831 of L (*ts*248) encoding every possible amino acid residue. Although most mutants could be recovered, only two mutants were found to confer temperature sensitivity (831I and 831F) to the rRSV in addition to the 831L mutation [138]. Furthermore, neither 831I nor 831F was as attenuated as 831L in the respiratory tracts of mice, suggesting that 831L has an attenuating function beyond temperature sensitivity. Interestingly, using the different codons for Leu resulted in different frequencies of reversion (to wt genotype) or pseudoreversion (to wt phenotype) [138]. These data suggest that careful selection of mutant codons may offer a strategy for increasing genotypic stability of attenuating point mutations. However, the genetic code precludes certain mutations from being "stabilized" by this method, as not all mutations can be made with two nucleotide differences from the wt assignment.

A novel potential mechanism of providing genotypic stability for point mutations is increasing the fidelity of the viral polymerase. Recent studies with poliovirus (PV) have shown that mutations that alter replication fidelity and/or replication speed of the PV RdRp produce attenuated viruses that protect mice transgenic for the PV receptor from a lethal challenge with wt PV [139–141]. Furthermore, mutation of a single amino acid residue that is conserved in all viral RdRps appears to control both replication speed and replication fidelity. This amino acid residue is a lysine that is present in conserved structural motif D of the RdRp [142, 143]. In the PV model, changes to this residue produce slow, high-fidelity RdRps [143]. Biochemical analysis shows that mutation of the homologous lysine in HIV RT and T7 RNA polymerase results in similar effects on polymerase speed and fidelity [143]. Thus, application of this technology to RSV could allow the identification of an additional attenuating mutation and could prevent or delay the emergence of more virulent variants of the vaccine candidates. Combinations of L mutations that increase polymerase fidelity and known attenuating mutations could allow for even finer tuning of vaccine efficacy and prevent outgrowth of more virulent viruses, which could then be spread to naive individuals.

7 Summary

Much progress has been made recently toward the development of an effective, live attenuated RSV vaccine; however, a number of hurdles remain. Most importantly, achieving the proper balance of attenuation and immunogenicity has been difficult because of the lack of animal models and immune correlates to investigate induction of immune responses in infants, a target population for RSV vaccines. Future studies into the molecular biology of the virus may lead to novel ways to address current difficulties in RSV vaccine development.

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