Review

Future VIROLOGY

Nanoparticle vaccines against respiratory syncytial virus

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Respiratory syncytial virus (RSV) is a leading cause of respiratory disease in infants, the elderly and immunocompromised individuals. Despite the global burden, there is no licensed vaccine for RSV. Recent advances in the use of nanoparticle technology have provided new opportunities to address some of the limitations of conventional vaccines. Precise control over particle size and surface properties enhance antigen stability and prolong antigen release. Particle size can also be modified to target specific antigen-presenting cells in order to induce specific types of effector T-cell responses. Numerous nanoparticle-based vaccines are currently being evaluated for RSV including inorganic, polymeric and virus-like particle-based formulations. Here, we review the potential advantages of using different nanoparticle formulations in a vaccine for RSV, and discuss many examples of safe, and effective vaccines currently in both preclinical and clinical stages of testing.

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Respiratory syncytial virus (RSV) is a major respiratory pathogen, accounting for approximately 7% of deaths in children less than 1 year of age [1]. It is the leading cause of lower respiratory tract infections (LRTIs) in infants, resulting in more than $300 million in annual medical costs due to RSV-related hospitalizations in the USA [2,3]. RSV also represents a major health burden for immunocompromised patients and the elderly in the USA, causing an estimated 14,000 deaths annually in adults over the age of 65 [4]. Repeated infections occur because natural exposure to the virus affords short-lived and incomplete protective immunity [5,6]. While primary RSV infection results in symptomatic LRTI, high-risk groups are susceptible to more serious outcomes including pneumonia, bronchiolitis and increased mortality [7,8]. Despite the global health burden caused by RSV infections, there is currently no licensed RSV vaccine.

RSV is a negative-sense, single-stranded RNA virus of the *Pneumoviridae* family. The genome encodes for 11 proteins including three surface proteins, the attachment (G) protein, the small hydrophobic (SH) protein and the fusion (F) protein [9]. The G protein facilitates attachment between the virus and a host cell via potential binding with CX3CR1 [10]. Following attachment, the RSV F protein facilitates fusion between the virus and the targeted host cell, leading to the release of the viral genome [11]. Fusion is thought to occur via interaction between RSV F and its recently identified putative receptor, IGF1R as well as nucleolin [12,13]. The F and G glycoproteins are the major targets of neutralizing antibody responses, with the human antibody repertoire targeting these viral proteins composed primarily of IgG and IgA subtypes [14,15]. In contrast, T-cell responses to RSV are to a broader array of the virus-encoded proteins, with T-cell epitopes identified in numerous viral proteins including F, G, matrix (M), nucleocapsid (N) and the polymerase (L) [16–19]. RSV F exists in various conformations, residing in a metastable prefusion structure prior to fusion and undergoing a conformational change to a postfusion state after fusion occurs [20]. The prefusion conformation contains unique antigenic sites that present promising new targets for vaccine design. Additionally, the majority of the neutralizing activity in human serum is attributed to antibodies that target epitopes only exposed when the protein is in the prefusion conformation [21,22]. Most vaccines currently in testing utilize the F or G protein as they can induce both T-cell and antibody responses.

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Vaccination is a cost-effective, widely implemented strategy for fighting infectious diseases. It has aided in the successful control of detrimental infections including polio, measles, small pox and rubella [23]. RSV vaccines are currently being developed to target numerous populations including the elderly, pregnant women and children. A wide range of RSV vaccine candidates that span all vaccine modalities are currently in development and testing including live-attenuated, nanoparticle-based, subunit-based, vector-based, as well as prophylactic agents and passive monoclonal antibodies. While many of these strategies represent promising candidates, nanoparticle-based formulations in particular are a safe and easily modifiable delivery method for inducing immunity in all populations. Here, we review the current literature on the use of nanoparticles in a vaccine for RSV, and discuss many examples of effective vaccines currently in development.

Improving the design of an RSV vaccine

Current challenges impeding RSV vaccine development

The path toward an RSV vaccine began in the 1960s when a formalin-inactivated RSV (FI-RSV) vaccine was tested in seronegative children. In this trial, vaccinated children exhibited enhanced respiratory disease (ERD) following a natural infection, resulting in the death of two children [24–27]. Later, studies utilizing a mouse model of vaccine-enhanced disease demonstrated that the immune response mounted by FI-RSV was characterized by a failure to elicit a memory CD8 T-cell response, the induction of non-neutralizing antibodies and a mixed Th1 and Th2 pathogenic CD4 T-cell response [28–33]. This resulted in uninhibited viral replication in the lungs and extensive cellular infiltration [24]. The failure of the FI-RSV vaccine has led to prolonged concerns about vaccine safety. Thus, one challenge in RSV vaccine development is that any vaccine candidate needs to demonstrate outstanding safety in both preclinical and early clinical trials.

Due to the inaccessibility of lung tissue, most human RSV studies primarily rely on blood, serum and limited fluid samples from the upper airways. The difficulty in directly sampling cells from the lower respiratory tract has made it challenging to precisely define the correlates of immunity in humans. Neutralizing antibodies can be protective, as infants and children with mild disease exhibit increased levels of neutralizing antibodies compared with those with severe disease [34]. The protection afforded by neutralizing antibodies is further demonstrated by the success of palivizumab, a monoclonal antibody against the RSV F protein. Prophylactic administration of palivizumab to high-risk individuals reduced the rate of severe RSV-associated hospitalization by approximately 50% [35,36]. However, palivizumab is expensive to administer and ineffective when given after RSV infection, therefore, additional mediators of protection are needed [37]. Mucosal IgA antibodies are also believed to contribute to protection against RSV reinfection. In mice, passive administration of IgA reduced viral titers in the lungs [38]. In humans, the ability to successfully establish an RSV infection inversely correlated with pre-existing virus-specific nasal IgA titers in adults experimentally infected with the Memphis 37 RSV strain [39,40]. Individuals with lower antibody titers were also more likely to be hospitalized following a natural RSV infection [41,42]. However, RSV-specific antibody titers have been shown to decrease over time [43]. In mice, IgG titers in the lungs are nearly undetectable 30 days after RSV infection, and nasal IgA titers wane 180 days post-RSV challenge in adults [39,44–46]. Thus, while RSV-specific antibodies are positive indicators of protection, the observed decline in antibody titers over time may indicate that the induction of antibodies alone may not be sufficient to induce long-term protection from RSV infection.

Studies in both human and animal models of RSV have identified a protective role for T cells. Depletion of either CD4 or CD8 T cells in wildtype (WT) mice prior to an acute RSV infection resulted in increased weight loss and enhanced viral replication [47,48]. Similarly, the transfer of RSV-specific T cells into naive mice is sufficient to protect against RSV-induced weight loss compared with nonspecific T cells [48]. In humans experimentally challenged with RSV, preexisting RSV-specific CD8 T cells isolated from the bronchial alveolar lavage fluid correlate with reduced overall symptom score [49]. Despite the protection mediated by T cells, natural exposure to RSV fails to boost T-cell responses [50–52]. Infants that succumbed to a fatal RSV infection exhibited low numbers of CD8 T cells in their lung tissue [53]. The difficulties associated with studying resident T-cell dynamics in the human lung make it difficult to corroborate findings from the animal models. Overall, another challenge in vaccine development is determining the ideal balance between virus-specific T cells and antibodies to generate long-lasting protection against RSV.

Infants, adults and elderly populations each have varying levels of pre-existing memory to RSV. The effect of previous virus exposure and the overall responsiveness of the immune system suggests that the characteristics of a vaccine will likely need to vary for each target population. The first exposure to RSV infection typically

occurs before 2 years of age and often presents with more severe symptoms, resulting in a large portion of RSV-associated morbidity and mortality occurring in children during their first year of life [54–57]. Therefore, it would be advantageous to provide infants with enhanced RSV-specific immunity to make it through their first RSV season. Newborn infants possess RSV-specific neutralizing serum antibodies at levels similar to those of their mother by way of maternal antibody transfer. However, maternally derived antibodies gradually decline over time and are nearly undetectable by 6–7 months of age [45,58]. Using mathematical modeling, a maternal vaccine that is able to extend the duration of fetal-transferred maternal antibodies by 4 months has been estimated to reduce the RSV infant infection rate by 31.5% [54]. Thus, an effective maternal vaccine would have the potential to protect newborn children through their first RSV season and substantially reduce RSV-associated morbidity and mortality in this highly vulnerable population.

Infants are at a greater risk for severe disease following RSV infection, and are therefore an important target population for early vaccination efforts. However, infant vaccination poses a number of obstacles. The immunological immaturity of infants may make it challenging to induce a robust and long-lasting RSV-specific immune response through vaccination. Compared with adults, infants have a reduced frequency of plasmablasts and exhibit decreased somatic hypermutation of immunoglobulin genes [59,60]. As a result, infants may develop reduced antibody responses following infection or vaccination. This is supported by a study showing that infants less than 9 months of age develop diminished levels of RSV-specific antibodies compared with children over 1-year old following natural infection [61]. The age of vaccination will also play a role, as pre-existing levels of circulating maternal antibodies can suppress the magnitude of the immune response generated by either a subsequent natural RSV infection or a live-attenuated RSV vaccine [61–63]. Finally, vaccination of RSV-naive children presents the risk of priming for ERD [27,64]. Therefore, infant vaccination strategies must demonstrate outstanding safety and may require more than one dose of vaccine to induce adequate levels of long-term protection.

Vaccines are currently being developed for elderly adults, as they represent a substantial portion of RSV burden [4]. With aging, the adaptive immune system exhibits a reduced repertoire of naive T cells and increases in predominantly dysfunctional memory cells with diminished proliferative capacity [65,66]. This lowered immune responsiveness known as immune senescence makes it more difficult to induce an effective immune response through vaccination. RSV-specific responses including neutralizing antibody titers have been shown to diminish with age [67–69]. Peripheral blood mononuclear cells (PBMCs) from adults greater than 60 years old exhibited an immunosuppressive phenotype, characterized by an increased frequency of regulatory T cells and increased RSV-specific IL-10 production [70]. *In vitro* stimulated PBMCs from older adults were deficient in RSV-specific IFN-γ and TNF production compared with adults age 20–30 [70,71]. Similarly, aged mice exhibited a diminished CD8 T-cell response, as measured by a reduced frequency of RSV-specific cells in the lung and a decreased capacity to produce IFN-γ compared with young mice [72]. Thus, a vaccine tailored to target elderly populations will need to maximize the immune response generated in order to provide protection. Overall, there remains no clear consensus on the best population to vaccinate, and further studies are needed to determine the best vaccine platform to maximize efficacy.

Use of nanoparticle delivery methods

Nanoscale vaccines incorporate a given target antigen, drug or protein into a nanosized vehicle for delivery. Their chemistry and small size, typically 10–500 nm, make nanoparticle-based vaccine platforms an appealing alternative to conventional vaccine modalities. The versatility of nanoparticle-based designs derives from the physiochemical properties of the nanoparticles. Charge, solubility and hydrophobicity can all be adjusted by changing the manufacturing process and/or composition [73,74]. These properties subsequently influence the load capacity and the release kinetics of the nanoparticles [75,76]. Thus, a nanoparticle-based delivery method can be tailored to be compatible with the target pathogen and the delivery method of any vaccine.

Incorporation of the antigen of interest can be achieved by either encapsulation within the nanoparticles, or conjugation to display the antigen on the particle surface [77]. Encapsulation enhances the stability of the antigen, maintaining its native structure [78,79]. Poly(lactic-*co*-glycolic acid) nanoparticles prevent the degradation of hepatitis B surface antigen for 60 days in guinea pigs [80]. Similarly, the fusion protein of *Yersinia pestis* encapsulated in nanoparticles made from 1,8-bis(*p*-carboxyphenoxy)-3,6-dioxaoctane and 1,6-bis-(*p*-carboxyphenoxy)hexane remains stable for over 60 days in mice [81]. This sustained release profile may improve the presentation and uptake by various antigen-presenting cells (APCs) [76,82,83]. This occurs by formation of an antigen depot effect, allowing for prolonged exposure of the antigen to immune cells [84,85]. Additionally, human and murine macrophages

phagocytose a higher concentration of antigen when it is encapsulated into polymeric nanoparticles compared with free antigen alone [86–89]. Thus, nanoparticle-based vaccine designs prevent rapid degradation of the encapsulated antigen, and maintain antigen stability to prolong antigen availability.

Nanoparticles themselves have been shown to have immunomodulatory properties. Numerous polymer-based and inorganic nanoparticles can activate the inflammasome, leading to downstream immune activation and cytokine production. Polystyrene nanoparticles induce Nod-like receptor protein 3 (NLRP3)-dependent production of IL-1β in human macrophages [90]. Similarly, titanium dioxide nanoparticles stimulate IL-1β, IL-6 and TNF production through the induction of caspase-1 and reactive oxygen species production in human lung monocytes [91,92]. Additionally, uptake of nanoparticles by murine bone-marrow-derived dendritic cells (DCs) enhances the expression of major histocompatibility complex class II (MHC-II) and costimulatory molecules CD80 and CD86 [87,89]. Thus, by boosting early proinflammatory cytokine responses and enhancing the activation of APCs, the nanoparticles themselves can serve as an adjuvant.

These adjuvant properties lead to enhanced antigen-specific adaptive immune responses. Nanoparticle encapsulation of ovalbumin or hepatitis B core antigen resulted in enhanced CD4 and CD8 T-cell proliferation, and increased IFN-γ production in mice compared with free antigen [93–95]. Intranasal delivery of chitosan nanoparticles adsorbed with cholera toxin induced higher titers of IgA compared with free peptide [96]. Size can also modulate both the magnitude and the phenotype of the effector T-cell subset induced. Recombinant RSV F protein administered with synthetic 80-nm nanoparticles induced higher serum IgG titers, neutralizing antibodies and IFN-γ-producing CD4 and CD8 T cells compared with particles greater than 150 nm in size [97]. Similarly, mice immunized with ovalbumin-containing nanoparticles exhibited elevated IL-4 production when the particles were greater than 100 nm, but enhanced IFN-γ when the particles were 40–60 nm [98]. Important for viral infections, many nanoparticle formulations also enhance cross-presentation of antigen to CD8 T cells [99,100]. The effector T-cell phenotype of the resulting immune response is likely due to the influence that particle size plays on which APC population mediates transport of the particles [101]. Large particles (>500 nm) administered to mice are phagocytosed by APCs at the site of administration [102]. However, smaller nanoparticles between 20 and 100 nm travel through the lymphatics to the draining lymph node within 12–24 h postadministration, independently of DCs. Additionally, using extensive phenotyping of murine lungs, larger particles (**400–1000** nm) were taken up predominantly by $CD11b^+$ macrophages and plasmacytoid DCs, while smaller nanoparticles (<200 nm) were found in $F4/80^+$ macrophages [103]. Therefore, by altering the participating APC population, the size of the nanoparticles can impact the balance between a Th1- and Th2-driven immune response.

Due to their size, nanoparticle-based vaccines are an ideal method for inducing protective immunity within the lung mucosa. Particle size can be controlled to determine where the nanoparticles primarily deposit in the respiratory tract, as well as the amount of the inoculum that settles into the tissue [104,105]. Compared with micro-sized particles, nanoscale delivery mediates enhanced uptake into mucosal surfaces such as the nasal-associated lymphoid tissue [106]. In human studies, nanoparticles 50–200 nm in size deposit predominantly in the distal lung, while the smallest-sized particles (5–10 nm) settle in the upper respiratory tract [107]. Moreover, the maximum percentage of deposition is achieved with moderate (<200 nm)-sized nanoparticles, with approximately 75% of the dose retained in the mucosal tissue [103,108]. Intranasal vaccination against respiratory viruses has been shown to be more effective compared with systemic delivery of the same vaccine [109]. Intranasal delivery allows the vaccine to mirror a similar entrance mechanism to the virus itself. This allows for efficient delivery of antigen to the mucosal sites and the induction of a localized immune response.

Overall, the properties of nanoparticles make them an excellent vehicle for an RSV vaccine. There are a number of nanoparticle-based vaccine modalities that can be made from biological and/or synthetic materials, including polymeric and inorganic particles, virus-like particles (VLPs) and self-assembling protein particles. They all offer advantages and disadvantages to consider when developing a vaccine. Currently, there are many RSV vaccine candidates being tested in both preclinical testing and clinical trials using a wide variety of nanoparticle-based formulations (Table 1).

Nanoparticle-based vaccine candidates

Polymer-based nanoparticle vaccines

Polymer-based nanoparticles are complexes constructed from monomeric units via emulsion and evaporation techniques [110,111]. Many polymers, including poly(lactic-*co*-glycolic acid) and 1,6-bis-(*p*-carboxyphenoxy)hexane, use biocompatible materials that safely degrade into naturally removed by-products [112,113]. Due to this property,

Table 1. Preclinical and clinical nanoparticle-based vaccines for respiratory syncytial virus.

Nanoparticle-based vaccine candidate		Formulation	Antigen	Immune response measured	Ref.
Polymer	Advantages: biocompatible materials (nontoxic), easily tunable properties Disadvantages: low synthesis yield, reduced antigen loading	Poly-l-glutamic acid and poly-l-lysine	RSV G CX3C motif	IFN-γ production	[117–119]
				IgG2a	
				Reduced viral clearance	
		CPH:CPTEG	RSV F and G	IFN-γ, IL-6, TNF production	[122]
				Reduced viral clearance	
				IgA	
				Proliferation of RSV-specific CD4 T cells	
		Thermoresponsive polymer	Prefusion RSV F	IgG2a	[123,124]
				Serum-neutralizing antibodies	
				Reduced viral clearance	
		Polysorbate 80	RSV F trimers	Serum-neutralizing antibodies	[125–132]
				IgG	
				Reduced viral clearance	
				No evidence of histopathology	
Virus-like particles	Advantages: uniform surface protein expression, high production capacity Disadvantages: ensuring proper post-translational modifications	Influenza M1 core	RSV F and/or G	IgG2a, IgG, IgA	[140–144]
				Serum-neutralizing antibodies	
				IFN-γ production	
				RSV-specific CD4 T cells in the lung and BAL	
				RSV-specific CD8 T cells in the lung and BAL	
		RSV matrix (M) protein	RSV G and G	IgG	[146]
				Serum-neutralizing antibodies	
		NDV	Chimeric RSV F and G	IgG	[147–149,151,152]
				Serum-neutralizing antibodies	
				Reduced viral clearance	
		PIV5	RSV F or M2-1	IgG2a, IgG, IgG1	[153]
				Serum-neutralizing antibodies	
				IFN-γ production	
				No evidence of histopathology	
		Hepatitis B virus core proteins	RSV G fragment + $M2_{82-90}$ epitope	IgG2a, IgG, IgG1	[154]
				Serum-neutralizing antibodies	
				IFN-γ, IL-2, TNF production	
		Woodchuck hepadnavirus	RSV F	IgG	[155]
				Serum-neutralizing antibodies	
		Icosahedral nanoparticle	Prefusion RSV F	T follicular helper cells, Germinal center B cells	[156]
				Serum-neutralizing antibodies	
		Virosome	RSV F and G	IgG2a, IgG, IgG1, IgA	[157,158]
				Serum neutralizing antibodies	
				IFN-γ and IL-5 production	
				Reduced viral clearance	
Inorganic	Advantages: low production cost, high-quality reproducibility, antiviral properties Disadvantages: potential toxicity	Gold nanorods	RSV F	Proliferation of T cells	[169]
		Ferritin nanoparticle	Prefusion RSV F	Serum-neutralizing antibodies	[170]
				RSV F-binding serum antibodies	

BAL: Bronchial alveolar lavage; CPH: 1,6-bis-(*p*-carboxyphenoxy)hexane; CPTEG: 1,8-Bis(*p*-carboxyphenoxy)-3,6-dioxaoctane; NDV: Newcastle disease virus; PIV5: Parainfluenza virus 5; RSV: Respiratory syncytial virus.

polymer-based nanoparticles are nontoxic and many are already approved by the US FDA [112,114]. Additionally, the properties of the nanoparticles can be rapidly altered by changing the type or ratio of monomers, regulating the release kinetics to fit the desired application [81,115]. The downsides to polymer-based nanoparticles stem from the manufacturing process. Reduced antigen loading and low synthesis yield are common with emulsion methods, and would need to be optimized in order to scale up manufacturing of such vaccines [116]. Nevertheless, several RSV vaccine candidates based on polymer nanoparticles are currently in preclinical trials.

Researchers at the University of Georgia and Artificial Cell Technologies have developed a polymeric nanoparticle by layering poly-l-glutamic acid and poly-l-lysine polymers around a core of $CaCO_3$ and coating them with the RSV G protein CX3C motif [117,118]. Following two subcutaneous (s.c.) doses of the vaccine, mice exhibited a Th1-biased immune response characterized by IFN-γ production and high RSV-specific IgG2a antibody titers [117,119]. The vaccine also mediated enhanced viral clearance and protection from RSV-induced disease. Another vaccine candidate utilized polyanhydride polymers, a novel class of polymers that undergo surface erosion and maintain sustained antigen release [120,121]. Intranasal vaccination with polyanhydride nanoparticles encapsulating the postfusion RSV F and G proteins provided protection from RSV-induced pathology and reduced viral replication in a model of bovine RSV [122]. Thus, polymer-based nanoparticle vaccines are immunogenic and protective in different animal models of RSV.

Another novel polymer nanoparticle vaccine utilizes synthetic thermoresponsive polymer (TRP) chains that self-assemble at a predetermined transition temperature [123]. This allows the solution to be purified prior to particle formation, preventing any damage to the nanoparticles during the filtering process. The TRP nanoparticles express both prefusion RSV F trimers and a synthetic Toll-like receptor (TLR)-7/8a adjuvant, and can be customized to display multiple proteins [123,124]. Mice that received two doses of the vaccine s.c. exhibited high RSV-specific serum antibody titers and achieved complete clearance of an RSV A2 strain challenge by 4 days postinfection. Thus, a prefusion RSV F TRP nanoparticle vaccine induces a strong humoral immune response, however, further studies are needed to determine the magnitude of the T-cell response generated by the vaccine.

The furthest progressed vaccine candidate is a polymer formulation, ResVax, developed by Novavax. It consists of recombinant RSV F trimers stabilized around a core of polysorbate 80 polymer that is delivered with an aluminum phosphate adjuvant. ResVax was initially designed to be administered intramuscularly (i.m.) in older adult populations. Preclinical and Phase I clinical trials of the postfusion RSV F formulation demonstrated safety and a robust induction of RSV-neutralizing antibodies [125–127]. However, in Phase III clinical trials, the vaccine failed its primary objective to prevent RSV-associated moderate–severe LRTIs (NCT02608502) [128]. The vaccine was subsequently reevaluated as a maternal immunization strategy. Additionally, the RSV F protein was stabilized in a prefusion conformation that induces enhanced neutralizing antibodies in humans [20,22]. Phase I and II clinical trials of the prefusion RSV F vaccine in healthy pregnant and nonpregnant women demonstrated safety and robust antibody responses in both the mother and infant [129–131]. Nevertheless, results from the Phase III clinical trial show that the vaccine did not meet its primary objective to reduce the incidence of medically significant RSV-related LRTI in newborn infants during their first 90 days (NCT02624947) [132]. It did, however, achieve its secondary objective to reduce the incidence of RSV LRTI with hospitalization through 90 days of life by 44%. Overall, the biodegradability and modifiable properties of polymers make them an excellent format for a vaccine candidate. Despite the past failure of Phase III clinical trials, preclinical studies utilizing newly emerging technology demonstrate the potential of numerous other polymer-based nanoparticle vaccines for RSV.

Self-assembling proteins & VLP-based vaccines

VLPs are self-assembling structures composed of viral proteins, giving them a uniform morphology and size similar to native viral particles. They lack the viral genome necessary for infection and replication, making them a safe vehicle for a vaccine [133]. VLPs express multiple copies of protein antigens on their surface, which promotes epitope recognition and subsequent phagocytosis by APCs, boosting humoral and cellular immunity [134,135]. There are many systems for generating VLPs, including insect cells, mammalian cells or bacterial cells, each with its own strengths and drawbacks [134,136]. VLPs can be easily amplified for commercial use, as VLP-based vaccines using yeast expression systems are already licensed for human papillomavirus and hepatitis B virus [137,138]. A number of VLP-based RSV vaccine candidates are presently being tested in preclinical animal models.

VLP-based vaccine approaches often utilize *Spodoptera frugiperda* Sf9 insect cell expression systems due to the high production output of VLPs [139]. However, insect cells are unable to introduce the authentic post-translational modifications to proteins [136]. One group generated particles with an influenza M1 core expressing the RSV F

and/or G proteins on the surface [140]. Immunization of mice with two doses of the RSV F or RSV G VLP i.m. increased RSV-specific lung antibody titers and mediated protection from both weight loss and viral replication without inducing ERD [140–143]. Importantly, an additive effect on protective immunity was observed when RSV F and RSV G VLPs were administered together [141]. This suggests that the inclusion of multiple viral proteins may enhance the protective efficacy of an RSV vaccine. The RSV F VLP induced increased protection and a Th1-biased CD4 T-cell response in the lungs when administered to mice intranasally (i.n.), demonstrating a benefit of mucosal immunization [144]. This vaccine demonstrated robust protection from an RSV challenge infection.

There are also several studies reporting VLP-based vaccines generated by other eukaryotic expression systems. Production using mammalian cells allows for natural inclusion of post-translational glycosylation modifications to the proteins, however, VLP production using mammalian cells can be less efficient compared with bacterial expression systems [145]. Researchers at Emory University used human embryonic kidney 293 (HEK293) cells to generate VLPs assembled from the RSV matrix (M) protein that express the RSV F and G glycoproteins [146]. Cotton rats that received the VLP adjuvanted with alum i.m. exhibited increased serum IgG and neutralizing antibodies against both RSV A and RSV B strains. Additionally, animals were moderately protected from viral replication in both the upper and lower airways.

A second group used an avian cell expression system to generate particles composed of the Newcastle disease virus (NDV) structural proteins that express a chimeric protein consisting of the RSV G and RSV F ectodomains fused to the NDV hemagglutinin–neuraminidase (HN) cytoplasmic tail [147–149]. One potential drawback of this approach is that the use of a chimeric protein in a VLP can lead to insertions that are incompatible with final assembly of the VLP [150]. When administered i.m., cotton rats exhibited long-lasting RSV-specific antibody titers and partial viral clearance that waned 14 months postvaccination [46,148]. The cellular immune response generated by the vaccine was not evaluated in these studies. When assessed in a maternal immunization model, both pregnant dams and newborn pups exhibited durable RSV-specific antibody titers, resulting in moderate protection against viral replication in RSV challenged pups compared with pups from unvaccinated mothers [151,152].

Finally, there are many novel viral expression systems and self-assembling mechanisms being used to generate VLP-based RSV vaccines. A parainfluenza virus 5 (PIV5)-based amplifying VLP expressing the RSV F or M2-1 protein induced RSV-specific humoral and cellular immune responses when administered i.n. to mice [153]. The presence of only the PIV5 transcription machinery allows for gene amplification without the spread of infectious progeny, making it possible to achieve protection with a lower dose of VLP. Viral core proteins from hepatitis B virus and woodchuck hepadnavirus displaying either an RSV G fragment or an RSV F epitope stimulated RSV-specific neutralizing antibody titers and conferred protection against an acute RSV A2 strain challenge in mice [154,155]. In both murine and nonhuman primate studies, a recently developed self-assembling icosahedral nanoparticle complex of prefusion-stabilized F trimers induced an RSV-specific humoral immune response characterized by neutralizing antibodies and a robust frequency of germinal center B cells [156]. Lastly, an RSV virosome generated by mixing solubilized RSV F and G proteins, egg-derived lipids and the TLR4 agonist monophosphoryl lipid A induced elevated mucosal antibodies and protection from RSV replication without eliciting ERD when administered either i.n. or i.m. to cotton rats [157,158]. Altogether, the use of a self-assembling or VLP-based platform for an RSV vaccine has demonstrated success in numerous preclinical trials, demonstrating *in vivo* safety and efficacy.

Inorganic nanoparticle vaccines

Inorganic nanoparticles, such as metals and silica, are a promising vehicle for vaccine design. They have a low production cost and high reproducibility that would make large-scale manufacturing easy. Several metal formulations have also demonstrated antiviral properties against respiratory viruses including influenza virus and adenovirus [159–161]. Silver and gold nanoparticles have been proposed to cross the cell membrane and interact directly with virus-encoded proteins, blocking the function of the viral polymerase [162]. Additionally, inorganic nanoparticles can be delivered i.n. to induce robust antibody responses against respiratory viruses [163,164]. However, many inorganic nanoparticles are less suited for biological applications due to their tendency to aggregate. Therefore, they are commonly coated in an organic material such as a polymer [165]. Inorganic nanoparticles are also often nonbiodegradable, and can build up in the organs at high doses, driving potential toxicity [166]. Several studies assessing the toxicity of silver nanoparticles in the lungs of mice and rats demonstrated minor airway mucus thickening and neutrophil infiltration following intratracheal administration [167,168]. Nevertheless, several groups have developed promising RSV vaccine candidates that are being tested in preclinical studies.

Researchers at Vanderbilt University have developed gold nanorods coated in water-soluble cetyltrimethylammonium bromide that express native RSV F protein trimers on the surface [169]. Human DCs stimulated with the vaccine induced proliferation of naive T cells *in vitro*, demonstrating processing and presentation of the nanoparticles. Further studies are needed to extensively evaluate the efficacy of this vaccine candidate *in vivo*. Another inorganic formulation utilized a ferritin nanoparticle core expressing eight prefusion RSV F moieties on the surface. Vaccination of mice i.m. or *in vitro* stimulation of human PBMCs induced robust prefusion F-specific and neutralizing antibodies compared with the prefusion RSV F antigen alone [170]. Additional studies are needed to assess the cellular immune response generated by the ferritin-based nanoparticle vaccine candidate, as well as the protective capacity against an RSV challenge. Overall, preclinical studies on inorganic particles in both vaccine and therapeutic approaches demonstrate efficacy in reducing RSV viral replication and inducing an RSV-specific humoral immune response. Due to their nonbiodegradable properties, extensive analyses will need to be completed to ensure the safety of these particles in biological systems.

Conclusion

The successful development of an efficacious RSV vaccine has remained elusive despite decades of constant research. There are a number of challenges that continue to impede vaccine progress. The failure of the FI-RSV clinical trial in the 1960s has led to prolonged concerns about vaccine safety and the induction of ERD. There is also currently no clear consensus regarding the correlates of immunity for RSV. While mucosal antibodies and lung-resident T cells mediate protection in animal models, the inaccessibility of human lung samples makes it challenging to verify these findings in humans. Finally, as RSV reinfection commonly occurs in all age groups, further studies are needed to better understand the host immune response to RSV, and the best vaccination approach for each population. Despite these challenges, considerable advances have been made in the effort to develop a successful vaccine for RSV. Of particular interest is the application of nanoparticle delivery approaches.

There are a variety of particle formulations including VLPs, polymer-based or inorganic materials, each with their own strengths and weaknesses. While preclinical studies demonstrate protection and robust induction of RSV-specific immune responses, there are still challenges to overcome before nanoparticle-based vaccines can be widely implemented. FDA approval has only been achieved for a select few nanosized materials. Approval of additional formulations would require numerous safety studies, which may prove difficult for those materials such as silver that have previously demonstrated toxicity. Furthermore, to generate a widely implemented vaccine, the manufacturing of nanoparticles would need to be adapted for large-scale quality-controlled production. Achieving a uniform size may be challenging for materials such as polymers where synthesized particles fall into a range of sizes.

Several nanoparticle-based vaccine candidates have recently failed in human clinical trials. The inability to demonstrate sufficient protective efficacy during the Phase III clinical trial of ResVax by Novavax represents a recent failure of a nanoparticle-based RSV vaccine candidate. The company has speculated that the failure may have stemmed from a low overall frequency of individuals with RSV-related LRTIs, resulting in the study being underpowered. More importantly, we currently lack concrete serological markers of protection for RSV. Most vaccine strategies focus on inducing high titers of neutralizing antibodies or mucosal IgA, and ResVax generated both palivizumab-competing antibodies as well as serum-neutralizing antibodies. However, antibodies titers wane over time, leading to increased susceptibility to reinfection. This is demonstrated by an approximately 50% reduction in the efficacy of ResVax to reduce medically significant RSV-related LRTIs by 180 days. One potential reason for the incomplete immune response is the use of only one RSV-derived target protein. While the capacity of RSV F to elicit neutralizing antibody responses is well established, the protective capacity of RSV F-specific T cells remains unclear. The inclusion of additional internal RSV antigens such as N or M that may elicit more robust CD8 T-cell responses may provide the broader cellular immune responses necessary to induce long-lasting protection. As humans and animals recognize different epitopes on the RSV proteins, determining which RSV-derived antigens to include in a vaccine needs further research.

Another potential contributing factor to the failure of past clinical trials is the adjuvant selection. Adjuvants recognize various molecules and pattern recognition receptors, resulting in the activation of different APC populations and the production of polarizing cytokines. Thus, the predominant CD4 T-cell subset induced during the response can be influenced by the adjuvant. It is important to test multiple adjuvants to determine the ideal formulation, as many can induce different responses in animal models compared with humans. Nanoparticles offer the benefit of displaying any combination of viral antigens and adjuvants in one delivery vehicle, similar to a native virus.

Codelivering of the immunogen and adjuvant to the same cell allows a vaccine to direct the most beneficial T-cell response for protection without inducing pathology. Additionally, many of the nanoparticles have adjuvant-like properties, eliminating the need for added synthetic adjuvants.

An additional important consideration for RSV vaccination using nanoparticles is the immunization delivery method. To date, the most of the RSV vaccines employing nanoparticles have been delivered intramuscularly due to the easier route of administration and the lower regulatory hurdles involved in approval. However, intramuscular delivery of an RSV vaccine has the disadvantage of inducing far less mucosal immunity than intranasal delivery. The importance of tissue-resident memory T cells in providing protective immunity has become increasingly understood in the last few years. Thus, it may be necessary to further explore intranasal administration as a delivery route in order to elicit tissue-resident memory T cells in the lung that are capable of providing more robust protective immunity than peripheral T cell induced by intramuscular delivery. Overall, while further testing is needed to optimize their properties for human use, nanoparticle-based vaccines are a promising vaccine delivery method for RSV.

Future perspective

Decades of research into a vaccine for RSV has yielded numerous candidates that generally reduce RSV-induced disease and generate RSV-specific antibody responses. However, because serum antibody titers wane over time, it may be advantageous to produce a more complete immune response consisting of both humoral and cellular immunity. Memory T cells residing in the lung tissue correlate with protection against respiratory viruses including RSV, however, the contribution of T cells to vaccine-mediated protection has not been assessed in many studies.

As traditional vaccine modalities including subunit and inactivated formulations have not yet been able to translate into the clinic, new technologies have emerged. Despite the recent failures of nanoparticle-based RSV vaccine formulations, a Phase III trial of a nanoparticle-based influenza virus vaccine has recently proved successful, indicating the promise of this vaccine approach. The vaccine utilized a nano-sized VLP administered with a novel saponin-based adjuvant. Thus, the success of an RSV vaccine will likely rely on finding the right adjuvant/protein combination.

Executive summary

Improving the design of an respiratory syncytial virus vaccine

- The failed formalin-inactivated respiratory syncytial virus (FI-RSV) vaccine trials in the 1960s has led to prolonged concerned about vaccine safety. This has set a high expectation for the level of safety that must be exhibited by any future RSV vaccine.
- Both neutralizing antibodies and RSV-specific T cells are critical mediators of protection for RSV. However, the balance that is needed between these populations to generate a long-lasting and safe vaccine has not been determined.
- Infants, adults and elderly individuals all pose challenges for vaccine targets, and will likely require different vaccine platforms to maximize efficacy in each population.
- Nanoparticles enhance the stability of the encapsulated antigen, allowing for longer release time and enhanced uptake by antigen-presenting cells (APCs).
- Nanoparticle size and properties can be modified to modulate the activation of APCs, alter the cytokine production of T cells and vary the localization of vaccine deposition in the lungs.

Nanoparticle-based vaccine candidates

- Polymer-based nanoparticles are biodegradable, nontoxic and several are already approved by the US FDA for use in humans.
- Numerous polymer-based nanoparticle vaccines have demonstrated safety and a high level of immunogenicity in preclinical mouse and cotton rat models.
- A polymer-based prefusion RSV F nanoparticle vaccine demonstrated protection and efficacy in early-stage clinical trials. However, the vaccine failed to reduce RSV-related lower respiratory tract infections in Phase III clinical trials in both elderly adults and in a maternal immunization model.
- Virus-like particle (VLP) vaccines utilize self-assembling viral proteins to generate a nanometer-sized carrier. VLPs are generally safe and have a high-throughput production process.
- Preclinical models of VLP-based nanoparticle vaccines demonstrate robust humoral responses and protection from RSV viral replication. No RSV VLP candidates are currently in clinical trials.
- Inorganic nanoparticles are less studied and pose a risk of toxicity due to their composition. The particles have antiviral properties against respiratory viruses, and a few studies have demonstrated the induction of RSV-specific responses in animal models.

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