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REVIEW

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Progress in the development of virus-like particle vaccines against respiratory viruses

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ABSTRACT

Introduction: Influenza virus, human respiratory syncytial virus (RSV), and human metapneumovirus (HMPV) are important human respiratory pathogens. Recombinant virus-like particle (VLP) vaccines are suggested to be potential promising platforms to protect against these respiratory viruses. This review updates important progress in the development of VLP vaccines against respiratory viruses.

Areas Covered: This review summarizes progress in developing VLP and nanoparticle-based vaccines against influenza virus, RSV, and HMPV. The PubMed was mainly used to search for important research articles published since 2010 although earlier key articles were also referenced. The research area covered includes VLP and nanoparticle platform vaccines against seasonal, pandemic, and avian influenza viruses as well as RSV and HMPV respiratory viruses. The production methods, immunogenic properties, and vaccine efficacy of respiratory VLP vaccines in preclinical animal models and clinical studies were reviewed in this article.

Expert opinion: Previous and current preclinical and clinical studies suggest that recombinant VLP and nanoparticle vaccines are expected to be developed as promising alternative platforms against respiratory viruses in future. Therefore, continued research efforts are warranted.

1. Introduction

Respiratory viruses cause infections in the upper or lower respiratory tracts. Viral infections in the lower respiratory tracts can cause several respiratory syndromes such as bronchitis, pneumonia, and bronchiolitis [1]. In particular, children and the elderly are greatly affected. There are several important viruses that infect epithelial cells in the human respiratory tracts: respiratory syncytial virus (RSV); influenza A and B viruses; parainfluenza 1, 2, and 3; and adenovirus [2]. Other viruses infecting human respiratory tracts include rhinovirus, human metapneumovirus (HMPV), severe acute respiratory syndrome coronavirus, human coronavirus NL63 and HKU1, parainfluenza 4, and bocavirus [1,3].

Among the respiratory viruses, influenza is the leading cause of respiratory illness. Globally 5–15% of human populations are affected by influenza virus annually. According to the report of World Health Organization, every year 250,000 to 500,000 deaths occur due to the infection of influenza virus [4]. Different platforms for influenza vaccines have been available on the market, such as inactivated split vaccine, live attenuated influenza vaccines (LAIV) and recombinant purified subunit protein vaccines [5,6].

Human respiratory syncytial virus (RSV) causes respiratory tract infections in infants and young children worldwide, leading acute bronchiolitis and viral pneumonia [7]. An extensive epidemic study indicates that approximately 45% of hospital admissions and in-hospital deaths are due to RSV-induced acute lower respiratory infection in children younger than 6 months [8]. Thirty-three million cases worldwide in children under at age 5 are estimated to be infected with RSV-associated acute lower respiratory infections and about 10% of patients were being hospitalized, resulted in about 1-3% of in-hospital deaths [8]. Palivizumab, the licensed monoclonal antibody (Ab), has been used as a prophylactic drug to prevent severe RSV disease in high-risk children [9]. Since palivizumab has safety and efficacy concerns such as anaphylaxis and hypersensitivity reactions, palivizumab is not recommended for the therapeutic-treatment post-RSV infection [10]. There are neither effective therapies nor any licensed vaccines commercially available in the market against RSV so far.

Another important respiratory virus is human metapneumovirus (HMPV) which is also a leading cause of acute lower respiratory tract infection globally. Pediatric population and immunocompromised patients are greatly susceptible to HMPV, causing substantial morbidity and mortality worldwide [11,12]. However, there is currently no HMPV-specific vaccine available.

Virus-like particles (VLPs) are developed and reported, suggesting effective vaccine platforms against respiratory viruses such as influenza virus, RSV, and HMPV infections (Figure 1)

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[13–16]. VLPs are non-replicating as well as noninfectious because they do not contain viral genetic materials. Viral structural proteins such as envelope viral glycoproteins and capsid core proteins can be expressed and self-assembled into a particulate morphology of VLPs in cell cultures *in vitro*. VLPs can be produced in bacteria, mammalian cell lines, insect cell lines, yeast, and plant cells [17]. VLPs produced by recombinant baculovirus – derived constructs exhibited higher and broader immune responses compared to those by mammalian – expressed VLP vaccines [18]. It is possible that residual baculoviruses in VLP preparations activates innate immune response at the site of inoculation. VLPs provide an immunogenic platform effectively eliciting T cell and B cell immune responses due to repetitive and high density of viral proteins displayed on the surfaces of VLPs [19,20].

In this review, we attempted to cover the production, immunogenic properties, and vaccine efficacy of VLP vaccines, from preclinical and clinical studies reporting on respiratory viruses of influenza viruses, RSV and HMPV.

2. Influenza VLP vaccines

2.1. Production and immunogenicity of influenza VLP vaccines targeting seasonal influenza viruses

Different approaches have been reported to generate influenza VLP vaccines. Influenza VLPs can be composed of various structural influenza proteins, such as hemagglutinin (HA), neuraminidase (NA), matrix (M1) and M2 proteins Figure 1(a). VLPs containing four structural proteins derived from influenza virus A/Udorn/72 (H3N2) were produced in insect cells via the recombinant baculovirus (rBV) expression system [21]. Three HA, NA, and M1 protein-encoding genes cloned into a single baculovirus construct were expressed for the generation of influenza virus VLPs [22–24] (Table 1). Also, individual rBVs expressing HA and M1 were co-infected together into insect cells to produce influenza VLPs [16,25,26]. There are several advantages of using the baculovirus expression vector system (BEVS). Proteins expressed in BEVS are soluble and functionally active. Other advantages of BEVS include post-translational modifications and higher yields for secreted proteins.

Mammalian cell-expressed VLPs were reported to be produced in human embryonic kidney 293T cells with transfection of DNA plasmids expressing influenza surface proteins and core matrix proteins [30] (Table 2). In brief, mammalian cells were co-transfected with three different cloned recombinant plasmids such as M1, HA, and NA, respectively, M1 from A/Puerto Rico/8/1934 strain, NA from A/Thailand/1(KAN-1)/2004 and codon-optimized HA protein from clade 2 H5N1.
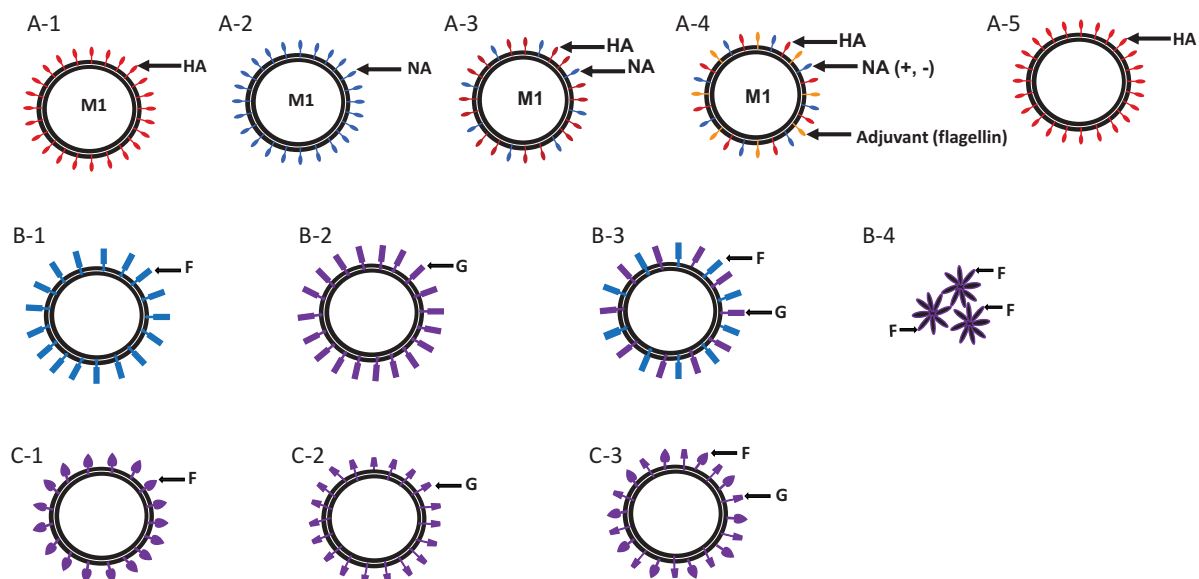

Figure 1. Diagrams for influenza VLPs, RSV VLPs, and HMPV VLPs.

(a) Influenza VLPs expressing hemagglutinin (HA) (A-1), neuraminidase (NA)(A-2), both HA and NA (A-3), HA, NA together with adjuvant flagellin (A-4), or plant-made VLPs containing HA (A-5). Influenza matrix protein (M1) was used as a core protein. Influenza VLPs are generated by insect/rBV system, mammalian cells or plant – derived. Plant-derived VLPs are produced in *Nicotiana benthamiana* infiltrated in batches with an Agrobacterium inoculum containing influenza antigen expression cassette. (b) RSV VLPs and nanoparticle by insect/rBV system. VLPs expressing RSV fusion protein (F) (B-1), RSV glycoprotein (G) (B-2) or both F and G (B-3) on influenza M1 core protein. RSV F nanoparticle (B-4) extracted and purified from insect cell membranes composed of multiple RSV F oligomers arranged in the form of rosettes. (c) HMPV VLPs: HMPV VLPs derived from retroviral core particles are generated in human embryonic kidney epithelial (293-f) cells by expressing fusion protein (F) (C-1), glycoprotein (G) (C-2) or both F and G (C-3) proteins.

Table 1. Seasonal influenza VLP vaccines in animal models.

Source	Component	Protection determined by mouse survival (%)	Host	Reference
H1N1	HA+NA+M1	Homologous virus (NC/99, H1N1) A/Mexico/4482/09	Mice, Ferrets	[53,55]
	NA+B gag	Not available	NA	[62]
	HA	Not available	Mice	[45]
	HA+M1	100%, A/California/04/2009 80%, A/PR/8/1934	Mice	[54]
	NA+M1	100%, homologous virus (A/PR/8/34); 100%, heterosubtypic virus (A/Phil/82)	Mice	[52]
	HA+M1+ flagelin	(1)100%, homologous virus (A/PR/8/34); 67%, heterosubtypic virus (A/Phil/82) (2)100%, homologous (A/PR/8/34) 100%, heterosubtypic virus (A/Phil/82)	Mice	[51,56]
	M1+ M2e+ Flagellin	A/Philippine/2/82 A/PR/8/34	Mice	[77,78]
	HA+M1+ CT, alum, CpG, MPL	100%, homologous virus (A/PR/8/34)	Mice	[89]
	HA+NA+M1+ M2/CpG	Homologous virus (1918 influenza A)	Mice	[27]
	M2 (A/WSN/33)/inactivated PR8	2009 H1N1, H3N2, H5N1	Mice	[58]
H3N2	HA+M1 HA+M1/IL-12	(1) Not available (2) A/HK (H3N2) (3) NY/04 (H3N2) (4) A/Hong Kong/68 (H3N2)	(1) Dog (2) Mice, Ferrets	[25,28,29,53]
	HA (LAH)+ HBc	Not available	Mice	[85]
	HA+NA+M1	Not available	Mice, Ferrets	[22]
	HA+M1/GPI-CCL28	20%, A/Philippines/2/1982 60%, A/Aichi/2/1968	Mice	[84]
H1, H3	HA+M1	100%, homologous (A/PR/8/34, A/Aichi/2/68), 100%, heterologous (A/WSN/33)	Mice	[16]
H1N1, H3N2, Influenza B	HA+NA+M1	A/New Caledonia/20/1999 A/New York/55/2004	Mice, Ferrets	[53]
H1N1	HA (plant-made)	Stable, interact and activate antigen-presenting cells	Mice	[44,45]
H1N1, H3N2,	HA (plant-made)	Proper antigenicity (E+, HI+, VN+, MN+)	Mice, chicken, ferrets	[43]

E: ELISA; VN: virus neutralization; MN: virus microneutralization; HI: hemagglutination inhibition.

Plant cells are being used for expression and production of recombinant HA influenza VLP vaccines, which are currently under clinical trials to evaluate the immunogenicity, safety, and efficacy [43] (Tables 1 and 2). Various forms of recombinant HA, including monomers, trimers, virus-like particles or chimeric proteins were obtained by plant-based technology. Nicotiana benthamiana plants are vacuum infiltrated in batches with an Agrobacterium inoculum containing an HA expression cassette. VLPs obtained by harvesting plant cells, homogenizing, and purification. Plant-made VLPs showed morphological stable structures over time, eliciting both humoral and cellular responses in mice, ferrets, rabbits, or chickens [43–45] (Tables 1, 2, 4). A plant-derived H1-VLP vaccine was reported to induce significantly higher levels of IgG antibodies, CD4+, and CD8+ T cell responses compared to inactivated virus vaccine in aged mice [45].

Taken together, influenza VLPs can be composed of HA, NA, or M1 either by containing all of these three proteins or two proteins HA or NA with M1. These VLP vaccines containing structural proteins from seasonal influenza virus H1N1 or H3N2 subtypes were immunogenic inducing both humoral antibodies and cellular immune responses [46].

Studies have reported that H1N1 or H3N2 influenza VLPs could provide protection against homologous and heterologous protection with a different degree of efficacy depending on antigenic closeness [16,17,22,47–52] (Table 1). The rBV expression system produced seasonal trivalent VLP vaccines derived from influenza A virus such as A/New Caledonia/20/1999 H1N1, A/New York/55/2004 H3N2, and influenza B virus B/Shanghai/367/2002 were able to elicit substantial levels of hemagglutination inhibition (HAI) titers against homologous and heterologous virus challenge in mouse and ferret models [53] (Table 1). In this study, mice were immunized with 3 μg, 6 μg, or 12 μg of VLP and ferrets immunized with 15 μg, 3 μg, or 0.6 μg [53]. Trivalent VLP vaccines were found to elicit higher levels of influenza-specific CD8+ T cell responses in mice than those with commercial egg-derived trivalent-inactivated vaccine (TIV). H1N1 or H3N2 influenza VLPs vaccinations provided higher vaccine efficacies against homologous or heterologous virus challenge infections compared to that against heterosubtypic influenza virus, showing less body weight loss (0–12%) in animal models after challenge with homologous or heterologous virus [25,50–52,54–56]. These results indicate that the efficacies of influenza VLP vaccines are encouraging in preclinical studies as an alternative vaccine platform. However, protective efficacies vary with diverse VLP vaccines containing different antigenic components and contents. Most efficacy studies were reported in in-bred mouse models, which might not predict what will happen in humans after vaccination. Influenza VLP vaccines need to be better characterized, tested in out-bred, more relevant animal models, and developed for improved cross-protective immunity for consideration of future vaccines.

2.2. Influenza VLP vaccine against potential pandemic influenza virus

An influenza pandemic is an outbreak of a novel influenza virus that can spread worldwide. During the 2009 influenza pandemic, we observed significant shortages and delays of egg-based vaccines in the global supply [5]. Technology of non-replicating VLPs represents an alternative option since it is immunogenic, safe, and

Table 2. Avian influenza VLP vaccines in animal models.

H5N1	HA+NA+M1	100%, homologous and heterologous virus (A/Viet Nam/1203/2004, A/Indonesia/05/2005) 100%, homologous virus (A/chicken/Korea/ES/2003, H5N1)	Mice Ferrets Chicken	[31–33]
	HA + NA+ Bgag/M8 HA + NA+ Bgag/Alum HA + NA+ Bgag/Addavax HA + NA+ Bgag/Poly(I:C)	100%, rgH5N1 (A/Vietnam/1203/2004, A/Thailand/1(KAN-1)/2004, A/Puerto Rico/8/1934)	Mice	[87]
	HA+M1 HA	100%, homologous virus (A/Viet Nam/1203/2004) H5N1 HPAI H5N1 HPAI	Mice Chicken	[34,59]
	HA+NA+M1+ M2	100%, homologous virus (A/Hanoi/30,408/2005, H5N1)	Mice	[35]
	COBRA HA+M1 COBRA HA+M1/Alum	A/Whooper Swan/Mongolia/244/2005	Mice Ferrets	[30]
	HA+NA+Gag	homologous virus (A/Viet Nam/1203/04) and heterologous virus (A/Indonesia/5/05)	Mice	[36]
	HA+NA+M1/CFA	100%, A/meerkat/Shanghai/SH-1/2012 (clade2.3.2.1) (H5N1)	Mice	[37]
	HA+NA+M1/NP	With NP: 100%, heterologous virus A/goose/GD/1996, H5N1, clade 2.3.4 Without NP: 50%, heterologous virus clade 2.3.4	Chicken	[38]
H7N9	HA+NA+M1+ Iscomatrix	NA, A/Shanghai/1/2013	Human Ferrets	[24,39,40,60,80]
	HA+NA+M1+ Iscomatrix Plant derived-HA-VLP Plant derived-HA+ alum HA+NA+M1/Novasome rH9/Novasome	Homologous A/Anhui/1/2013 100%, A/Anhui/1/2013 A/Hong Kong/1073/99 (H9N2)	Mice Ferrets Rat Ferrets Mice	
	TM of H3N2 HA+H7-HA+M1	A/Chicken/Guangdong/53/2014 A/Chicken/Guangdong/MCX/2014 A/Chicken/Guangdong/ZSM/2017	Mice	
H7N3, H7N9, H5N1	HA+ NA+M1+ Iscomatrix	100%, A/Anhui/1/2013	Mice	[81]
H9N2	HA+M1/ISA70 HA+NA/Freund's HA+NA+M1/Novasome HA+NA+M1	NA, homologous H9N2 (A/Chicken/Korea/01310/2001) NA NA, homologous H9N2 (A/Hong Kong/1073/99)	Chicken chickens Mice Ferrets	[23,41,88]
H1, H5	M2e5x+M1 Split vaccine (prime) + M2e5x VLP (Boost)	100%, homologous (A/Viet Nam/1203/2004) Not protected 100%, A/Philippine/2/81 (H3N2), rgH5N1 A/Brisbane/59/2007 (BR/59)	Mice Chicken Mice Ferrets	[42,64,66,68,69]
	M2e5x+HA+M1 M2e5x+ Flagellin M2e5x+M1	100%, homologous virus (A/Viet Nam/1203/2004) 100%, A/Philippines/2/82 A/Philippines/2/82 A/California/4/2009 rgH5N1	Mice Mice Mice	[42,78] [63,64,67]
	LAIV+M2e5xVLP	rgH5N1 A/PR/8/34 A/Philippines/2/1982	Mice	[70]
	HA+NA+M1+ M2	Not available	Mice	[76]
	HA+M1/GPI-ISM	100%, heterologous A/Vietnam/1203/2004	Mice	[83]
H1N1, H3N8, H5N1, H7N3	HA+M1	100%, H1N1, H2N1, H7N9, H7N1, H10N1, H11N1 90%, H5N1, 88.3%, H10N1	Mice	[50]
H1N1, H3N2	HA+M1	100%, H1N1 A/PR/8/34, H3N2 A/Aichi/2/68, H1N1 A/WSN/33	Mice	[47]
H5N8	HA+NA	Not available	Mice	[26]
H5/H7/H9/H10	HA+NA+ gag (immunodeficiency virus gag protein)	Not available	NA	[62]
H5, H7	HA+ DNA plasmid	A/WSN/1933, A/California/07/2009 (CA09), A/Cambodia/P0322095/2005 (CAM05), A/swine/Jiangxi/1/2004, A/chicken/Jiangsu/7/2002, A/Hong Kong/1/1968, A/Guizhou/54/1989, A/Netherlands/219/2003	Mice	[61]
H5	HA (plant-made)	Interact and activate antigen-presenting cells	Mice	[44]

independent of egg supplies. Previous studies reported influenza VLPs containing the HA, NA, and M2 surface proteins from highly pathogenic avian influenza viruses: A/Chicken/FPV/Rostock/1934 (H7N1) and A/Thailand/KAN-1/04 (H5N1), inducing high titers of neutralizing antibodies in mice [57]. Influenza 2009 H1N1 VLPs and H5N1 VLPs generated by using the rBV expression system-induced encouraging protective immunity in mice and ferrets [54,58,59] (Tables 1 and 2). H7 HA VLPs assembled with HA of wild-type (HA-WT) or HA transmembrane (TM) domain replaced by that from H3N2 subtype (HA-TM) were generated [60]. H7 VLPs-TM induced better protection against homologous and heterologous H7N9 viruses challenge, as observed with less weight loss and higher

survival rates compared to H7 VLPs-WT [60]. A bivalent heterologous DNA and VLP prime-boost vaccine was reported to provide protection against H1, H5, H9, and H3 and H7 viruses [61]. Mice were primed with DNA plasmid twice at weeks 0 and 3 and boosted with VLP. The immune sera from vaccinated mice were able to neutralize homologous, heterologous (H5, H7) and heterosubtypic H1 virus after passive transfer [61]. Irina Tretyakova and her colleague reported a quadrivalent pseudotype influenza VLP vaccine containing four different types of HA (H5N1, H7N9, H9N2, and H10N8) proteins, NA and bovine immunodeficiency virus gag protein (B gag) as an alternative to M1 matrix protein in insect cells [62] (Table 2). Electron microscopic observation results

demonstrated that average sizes of the B gag core protein assembled VLPs are in a range of 150 nm to 200 nm, which was larger than the influenza virus M1 core protein assembled VLPs. These results indicate that vaccine efficacies from avian influenza VLPs are encouraging. B gag core protein assembled VLPs might incorporate more spike antigens than the M1 assembled VLPs.

2.3. Universal influenza VLP vaccines

Influenza virus surface antigenic proteins HA and NA change over time and result in generating novel viruses that are antigenically different to become new HA and/or new HA and NA proteins in influenza viruses. New research efforts to develop universal influenza VLPs containing HA, NA, and M2 proteins are going on for broader immunity.M2e as an extracellular domain of M2 is conserved among influenza A viruses and has been used for cross-protective antigenic target [63]. The cross-protective efficacy was observed after vaccination of mice with M2e5x VLP vaccines containing heterologous tandem M2e repeats (M2e5x) derived from human, swine, and avian influenza viruses [63]. This heterologous tandem M2e5x repeat construct contains multiple M2e conserved epitopes to increase the density of M2e epitopes. A comparative study of vaccine efficacy has been conducted in mice between M2e5x VLP and wild-type M2 expressed VLP. This study demonstrated that M2e5x VLP immunization induced 100% protection against A/California/4/2009 (H1N1), A/PR/8/34 (H1N1), A/Philippines/2/82 (H3N2), and A/Vietnam/1203/2004 (H5N1). However, wild-type M2 VLP-vaccinated mice showed a significant loss in body weight with a substantial delay in recovering body weight [64] (Table 2). M2e5x VLP vaccination could induce long-lasting cross-protection against A/PR/8/34 (H1N1) and reassortant A/Vietnam/1203/2004 (H5N1) virus by passively transferring immune sera from vaccinated mice to the naïve mice [64]. Another experimental data demonstrated that M2e5x VLP vaccine has the ability to show more cross-protective efficacy in BALB/c mice compared to C57BL/6 mice [63,65], suggesting that immunogenicity and efficacy are dependent on host strains. The efficacy of cross-protection by M2e VLP immunity is mediated by weak non-neutralizing immune responses [66–68]. Supplemented HA-based influenza vaccination with M2e5x VLP vaccines could significantly enhance cross-protection in mice, chicken, and ferret [66,69,70]. These results indicate that higher vaccine efficacy or cross-protection induced by M2e VLPs can be induced by supplemented influenza vaccination.

2.4. Chimeric influenza or adjuvanted VLPs vaccines

Different adjuvants were tested in the influenza vaccination with M2e epitopes, which include Freund's adjuvant, monophosphoryl lipid A, cholera toxin subunits, heat-labile endotoxin, flagellin, and profilin, providing certain levels of broader and enhanced cross-protection [71–78]. ISCOMATRIX is composed of purified fractions of Quillaia saponaria extract, cholesterol, and phospholipid [79] and was tested with influenza VLP vaccine [80]. A/Anhui/1/2013 (H7N9) based HA-NA-M1 VLP vaccine with ISCOMATRIX adjuvant was shown to induce 3-4-fold higher levels of serum HAI and NA antibody titers and to provide better protection in mice against heterosubtypic A/Shanghai/1/2013 (H7H9), A/Chicken/Jalisco/CPA/2012 (H7N3)

virus and heterologous A/Indonesia/05/2005 (H5N1) virus, compared to HA-NA-M1 VLP only vaccination [81].

Influenza VLPs were engineered to express molecular adjuvants or immunostimulatory molecules on the surfaces of VLPs. A flagellin molecular adjuvant was engineered to be incorporated into influenza VLP vaccines via a membrane-anchoring domain [51,56] (Figure 1(a) A-4). Flagellin-containing influenza VLPs were produced in insect Sf9 cells by co-infecting with rBVs expressing PR8 HA, M1, and membrane-anchored flagellin. This chimeric flagellin plus HA VLP vaccination-induced heterosubtypic protection against challenge with A/Philippines (H3N2) virus [51]. Also, flagellin VLP was found to play an effective adjuvant role in promoting IgG isotype-switched long-lasting antibody induction and protection of influenza vaccines in a CD4-deficient condition in mice [82].

Using a protein transfer technology, glycosylphosphatidy-linositol – anchored immunostimulatory molecules (GPI-ISMs) are incorporated onto influenza VLPs [83]. Mice immunized with VLPs modified by protein transfer with GPI-GM-CSF induced higher levels of IgG antibody responses and protection against a heterologous influenza virus challenge than unmodified VLPs in mice [83]. Intranasal immunization with chimeric VLPs containing influenza HA antigen and GPI-CCL28 was shown to induce long-lasting mucosal immunity against H3N2 virus infection [84]. GPI-anchored CCL-28 chemokine co-expressed on the surface of influenza HA VLPs was shown to be effective in reducing lung viral titers after virus challenge in vaccinated mice. Vaccination of mice with influenza HA VLPs containing GPI-anchored CCL-28 enhanced lung IgA and serum IgG antibody responses, compared to the standard influenza HA VLP vaccine.

Yeast expression system rather than using the bacterial expression system has been used to avoid bacterial endotoxins [85]. In this study, a long alpha helix domain (55 amino acid residues) from the conserved HA stalk region was genetically fused to the hepatitis B virus (HBV) core protein and expressed via the yeast expression system [85]. The hydrophobic stalk domain fragment was presented in immunogenic spikes on the surfaces of self-assembled HBV core protein VLP [85]. This chimeric HA stalk – HBV core VLPs produced in yeast cells were immunogenic, inducing IgG antibody responses to both group 1 and group 2 HA proteins in mice after vaccination in combination with adjuvant CpG (ODN 2395) [85].

M8 as a sequence-optimized 5′-triphosphate-containing RNA (5′pppRNA) RIG-I agonist has been used as adjuvant to enhance influenza VLP efficacy [86]. Immunization with a novel M8 adjuvanted VLPs (M8-VLP) containing H5N1 influenza virus HA and NA induced humoral and cellular mediated protective responses and protection against influenza virus challenge [87]. Intramuscular immunization with M8-VLP induced long-lasting protective antibody responses against influenza virus in mice. Intramuscular immunization with Freund's adjuvanted H9N2 influenza VLP containing HA and NA induced high levels of HAI titers with significant reduction in virus shedding and substantial homologous protection in chickens [88]. Adjuvants Alum, CpG DNA, monophosphoryl lipid A (MPL), poly IC, gardiquimod, and cholera toxin (CT) have been used to assess immunity in combination with

influenza VLPs in mice [89]. Alum, CpG, MPL, or CT adjuvanted VLPs showed higher levels of antibodies in both sera and mucosa. Alum, MPL, or CT adjuvanted VLPs showed higher HAI and virus-neutralizing activities compared to a non – adjuvanted control.

These studies support the concept that chimeric VLPs or adjuvanted influenza VLP vaccines can be more effective in inducing antibody responses and protection than standard VLP controls. However, most of these chimeric VLPs or adjuvanted influenza VLP vaccines were tested in mouse models and remain unclear for efficacy and safety in translational application to humans.

3. RSV VLP vaccines

3.1. RSV VLP vaccine components and platforms

Human respiratory syncytial virus (RSV) belongs to the pneumoviridae family, containing fusion protein (F) and attachment (G) glycoprotein on the surface of the virion, which are directed toward outside from the virion membrane. The F glycoproteins are relatively conserved among the different RSV A and B strains. RSV F and G glycoproteins contain B cell and T cell epitopes to generate a protective-immune response in hosts after natural infection [90–92]. Although RSV contains another surface glycoprotein, SH glycoprotein, but naturally it is weakly immunogenic [93]. RSV VLP vaccines have been generated by expressing RSV G or F glycoproteins. As seen in Figure 1(b) and Table 3, Newcastle diseases virus (NDV) VLPs containing the ectodomain of RSV G and F proteins have been generated. Recombinant chimeric RSV-NDV constructs of RSV G and F protein fused to the cytoplasmic tail (CT) and the transmembrane (TM) domain of NDV hemagglutinin-neuraminidase (HN) and F protein were generated to produce RSV VLP vaccines based on NDV structural proteins [94,95]. Interaction between the TM-CT of chimeric RSV-NDV glycoproteins and NDV – nucleoprotein (NP) and matrix M protein was necessary for efficient production of chimeric RSV-NDV VLP vaccines in avian cells by DNA transfection [95,96]. Both chimeric F(F/F) and G (H/G) proteins were together co-presented on the surface of NDV-VLP with the help of NDV structural proteins for VLP assembly and budding [94]. Chimeric RSV – NDV VLP vaccines containing RSV G or RSV G + F proteins were immunogenic and prevented the replication of RSV in mice without causing vaccine-enhanced RSV lung histopathology [94,95].

RSV F proteins have two forms of conformation, one is a metastable pre-fusion F protein conformation and another is a stable post-fusion F protein form. A pre-fusion form of RSV-F protein contains potent neutralizing epitopes of sites Ø and V, which are not exposed in the post-fusion form [90,98]. Pre-fusion F conformation-stabilized protein was reported by introducing pre-fusion stabilizing mutations (DS-Cav1) of 2 cystine disulfide bond formation and 2 point mutations of cavity filling as well as a foldon oligomer stabilizing domain [90]. For presentation of pre-fusion F protein on the surfaces of VLPs, the pre-fusion stabilizing DS-Cav1 pre-fusion F with or without foldon domain was fused to the TM of NDV F protein [99]. A recent study reported that NDV VLPs containing pre-

fusion F stabilized single chain F with deletions of the peptide 27 sequence and cleavage sites and point mutations could induce higher RSV neutralizing antibodies in cotton rats, compared to NDV VLP with DS-Cav1 F [100].

RSV VLPs were also generated by using influenza matrix protein M1 as a core protein [101]. RSV G and F full-length glycoproteins were expressed in insect cells via rBVs containing genes encoding G or F proteins [101]. Both RSV-F and RSV-G VLPs were observed in spherical shapes with spikes on their surfaces in morphology. RSV F VLPs displayed a range of size distribution at approximate 80–100 nm in diameter, whereas G-VLPs were similar but somewhat more heterogeneous in size [101]. RSV VLPs were also reported to be generated by transfection of mammalian HEK293 cells with three types of expression plasmids, containing codon optimized RSV-G, F and M proteins [102]. RSV VLPs appear to mimic native RSV in morphology and structure, as examined by transmission electron microscopy.

A region within the central RSV G protein located between amino acids 131 to 230 was reported to contain protective epitopes, inducing T helper cell (Th cell) responses and eosinophilia [103,104]. A tandem repeat gene encoding this RSV G protein region containing T cell and B cell epitopes and CX3C motif was utilized to generate RSV G fragment VLPs [105]. G VLPs containing tandem repeat G fragment proteins could provide protection against RSV after vaccination of mice and challenge with RSV [105]. A different platform of RSV F protein nanoparticle vaccines was generated for advanced clinical trials. A near full-length RSV F protein with transmembrane domains, containing mutations to enhance the expression on insect cell surfaces using the rBV expression system, was purified under detergent conditions and formulated into nanoparticles, and found to be highly immunogenic in inducing neutralizing antibodies in cotton rats [106]. In a follow up study, prefusogenic F nanoparticle vaccine was found to induce neutralizing antibodies competitive with monoclonal antibodies targeting to multiple antigenic sites present on pre-F and post-F conformation [107]. Taken together, RSV G or F proteins could be presented on VLPs or formulated into nanoparticles utilizing different strategies, which were immunogenic and could induce protective immune responses in animal models.

3.2. Vaccine efficacy of RSV VLP vaccines

Alum-adjuvanted formalin-inactivated RSV (FI-RSV) vaccines failed to provide protection in children in clinical trials because vaccine-enhanced respiratory disease upon RSV infection was observed during winter epidemic season [108]. There are safety concerns in applying inactivated or subunit RSV vaccines to naïve young children. As implied by the absence of licensed RSV vaccines until now, it is challenging difficult to develop effective and safe RSV vaccines inducing protective RSV neutralizing immunity but avoiding RSV vaccination-induced respiratory disease after RSV infection. In a comparison study of RSV F VLP and alum-adjuvanted FI-RSV, F VLP intranasal immune mice induced T helper type 1 (Th1)-biased IgG2a antibodies, neutralizing immunity against RSV, CD8α⁺ CD103⁺ dendritic cells, F-specific IFN-γ⁺ and TNF-α⁺ CD8⁺ T cells, displaying least weight loss and no sign of histopathology and

Table 3. Pneumovirus VLP vaccines in animal models.

Source	Component	Protective immunity	Host	Reference
RSV A2	RSV G/NDV (NP, M)	Lung virus replication inhibited, neutralizing antibody induced, no immunopathology	Mice	[94,95]
	RSV F DNA + F VLP + G VLP (FFG-VLP)	Lung viral replication below the detection limit, neutralizing activity, ASC response, no weight loss, pulmonary inflammation prevented	Cotton rats	[118]
	RSV F + G + DNA-F Influenza M1 (FFG-VLP)	Clearance of lung virus loads, the absence of eosinophil infiltrates, no inflammatory cytokine-secreting cells	Mice	[112,113]
	RSV-G + F + M/alum/MPLA	Neutralizing antibody response, virus clearance	Cotton rats	[102]
	hMPV-M + RSV Pre-F/F + RSV Post F/F + Alum	Prevented detectable viral replication, Th1-mediated immune response, no severe bronchiolitis, neutralizing antibody elicited	Mice	[110]
	RSV F/influenza M1	Least weight loss, no sign of histopathology and eosinophilia IgG2a antibody response, Th1 immune response	Mice	[109,114,115]
	RSV G (1-780bp, 450-780bp)/influenza M1	Lung virus replication inhibited, no enhanced inflammatory response	Mice	[105]
	RSV G, RSV F/M1	IgG2a dominant antibody response, lung virus loads decreased	Mice	[101]
	RSV G + RSV F/M1	Mixed VLP F+ VLP G provided a high level of protection, no vaccine-induced immunopathology	Mice	[111]
	RSV F/M1	FG VLP and F VLP: no pulmonary inflammation,	Cotton rat	[117]
	RSV G/M1	G VLP: moderate lung inflammation,		
	RSVFG/M1	F VLP and FG VLP: antibody secreting cell responses		
	NDV-RSV H/G+ Pre- F/F, H/G+ post-F/F	Lung virus replication inhibited, neutralizing antibody induced	Mice	[97]
	H/G + RSV pre-F + NDV-NP + NDV-M, H/G + post-F + NDV-NP + NDV-M	Neutralizing antibody induced, pulmonary inflammation reduced	Cotton rat	[99,120]
	RSV F nanoparticle	Neutralizing antibodies induced, virus replication inhibited, no sign of disease enhancement	Cotton rat	[106]
HMPV	HMPV F, G	Homologous and heterologous virus, neutralizing antibody induced, lung virus titer reduced	Mice	[13]
	HMPV F, G, M	NA	NA	[123]
	HMPV F, M	Neutralizing antibody induced, lung virus titer reduced	Mice	[14]
	HMPV F, M	Lung virus replication inhibited	Mice	[124]

eosinophilia [109]. In contrast, alum-adjuvanted FI-RSV immune mice showed severe lung histopathology and eosinophilia together with high levels of IL-4$^+$ and TNF α$^+$ CD4$^+$ T cell responses despite lung viral control after RSV challenge [109].

Chimeric RSV-NDV VLPs containing ectodomains of RSV F and G or G protein alone confer protection against RSV challenge infection in mice without displaying enhanced respiratory lung inflammation [94,95]. Immunization of mice with RSV VLP vaccines containing either F or G proteins induced RSV A2 -specific IgG, IgG1, and IgG2a antibodies in sera and in lung extracts [101]. RSV VLP vaccination of mice induced neutralizing antibodies, correlating with reducing lung viral loads and protection in mice after RSV challenge infection [101]. RSV VLPs composed of RSV G, F, M proteins elicited high titers of IgG antibody responses to homologous RSV A2 and heterologous RSV B strain challenge [102]. Mammalian cell-derived RSV VLP vaccines adjuvanted with combination of alum and monophosphoryl lipid A induced higher levels of IgG titers than non-adjuvanted VLPs [102].

RSV VLP vaccines with combined post-fusion and pre-fusion F glycoproteins afforded higher neutralizing titers, T helper type 1 (Th1) immune responses, and more effective prevention of lung viral replication, compared to either post-fusion F or pre-fusion F VLP vaccination after challenge in mice [110]. Vaccination of mice with combined RSV F VLP F + G VLP induced Th1 CD8 T cell responses and provided additive protection and prevented lung immunopathology after challenge but RSV G alone VLP vaccination resulted in substantial lung histopathology after RSV challenge [111]. VLP vaccine containing tandem repetitive RSV G fragment was able to provide recall B cell responses and protection against lung viral replication after RSV challenge [105]. Also, immunization with combined RSV VLP vaccines (F + G) with F DNA (FFG VLP) induced Th1-biased IgG2a isotype antibody responses to RSV F and more effective in preventing lung histopathogloy after RSV challenge than immunity induced by live RSV prior infection [112].

In addition, combined FFG VLP vaccination induced higher ratios of CD11c$^+$ versus CD11b$^+$ and IFN-γ$^+$ CD8 versus CD4 T cells in addition to effective clearing lung viral loads [113]. Interestingly, prime immunization of mice with RSV F VLP vaccine induced Th1-biased immune response, preventing the induction of lung histopathology due to subsequent FI-RSV vaccination [114]. Unique immunogenic properties of RSV F VLP platforms were evident by a study reporting that soluble F protein vaccination exacerbated pulmonary histopathology upon RSV challenge but not when presented on VLPs in a mouse model [115]. Most individuals are considered to be infected with RSV by ages two to three years old but live RSV infection did not provide long-term protective immunity. A recent study demonstrated that avian cell-derived VLP vaccines containing ectodomains of RSV G and pre-F/F proteins were highly effective in inducing higher levels of RSV neutralizing antibodies even in the presence of prior RSV infection in a mouse model compared to the RSV re-infected mice [116]. Cotton rats are known to be more permissive to RSV infection and thus considered to be a preferred animal model than mice for RSV pathogenesis and vaccine efficacy studies. VLP vaccines containing two chimeric RSV ectodomain glycoproteins (chimeric pre-fusion F/F and H/G) were shown to confer protection against RSV replication upon RSV challenge in cotton rats [99]. NDV-RSV VLPs containing stabilized pre-fusion F/F chimera protein were more effective in inducing higher titers of serum neutralizing activity, compared to low dose live RSV prior infection [99]. Pre-F/F VLPs immunization protected cotton rats and did not result in enhanced lung inflammation after RSV challenge. Combined F + G VLP with F DNA vaccination of cotton rats was more effective in inducing RSV neutralizing antibody titers than RSV F but both combined FFG VLP and F VLP vaccination prevented the induction of vaccine-induced inflammation in the lungs, in contrast to FI-RSV [117,118].

Maternal vaccination with RSV VLP or F nanoparticle vaccines provided insight into protecting infants from RSV infection. RSV F

nanoparticle protein or chimeric NDV-RSV VLP-induced immunity could be transferred from mothers to the infants [119]. Pregnant cotton rats were intramuscularly immunized with RSV pre-fusion F/G VLP or RSV F nanoparticle protein vaccines to evaluate the protective role of maternally transferred immunity in infants through the placental barrier [119,120]. The offspring pups born to vaccinated cotton rats were protected against RSV infection and pulmonary inflammation [120]. RSV F nanoparticle protein vaccine induced polyclonal palivizumab-competitive high neutralizing activities against RSV A and B viruses after active and passive immunization of cotton rats [119]. These results indicate that studies on RSV vaccines based on VLP and nanoparticle platforms are expected to provide important insight into developing effective and safe RSV vaccines.

4. HMPV VLP vaccines

4.1. Production of HMPV VLP vaccines

Human metapneumovirus (HMPV) is also an enveloped virus belonging to the pneumoviridae family and contains M protein as core protein which is encircled by lipid membrane expressing three HMPV surface antigens (F, G, SH). The F protein is required for virus assembly, fusion, and entry and the primary target of neutralizing antibodies [121,122]. HMPV-VLP was produced by co-expression of F and G proteins along with the core matrix protein M through the mammalian cell-based DNA transfection [123]. This study has also revealed some new findings about HMPV VLP assembly, that HMPV-G protein itself can independently facilitate the formation of VLP and additionally, that expression of HMPV-F protein on the surface of VLP is largely dependent on its association with the HMPV-G [123]. Another group of researchers has produced HMPV VLP by using retrovirus-derived Gag core protein [13]. Murine leukemia virus Gag protein was used as a core particle to generate HMPV VLPs containing HMPV- G and F glycoproteins from either lineage A or B [118]. HMPV VLPs have been also generated by expressing viral proteins matrix (M) and F proteins in suspension-adapted human embryonic kidney epithelial (293-F) cells through mammalian cell-based expression system [14]. In this study, full-length M and F viral protein sequences were derived from pathogenic clinical HMPV (genotype A2) isolates TN/94-49 and TN/92-4, respectively. Recently, another group of researchers reported the generation of HMPV VLPs by the mammalian cell-based expression system which expressed RSV prefusion F protein (deletion of p27 and introduction of disulfide bonds to stabilize pre-fusion F) [99] or post fusion F protein [110]. Metapneumovirus

M protein was used as core protein and both codon optimized RSV Pre-fusion F and post-fusion F sequences were fused with the TM of hMPV-F protein to generate RSV-HMPV VLPs [110].

4.2. Vaccine efficacy of HMPV VLP vaccines

HMPV VLP vaccines displaying F and G surface glycoproteins of lineage A or B provided protection against HMPV challenge in mice [13] (Table 3). HMPV VLP immunized mice showed serum neutralizing antibodies and significant reduction of lung viral titers after challenge. Adjuvanted HMPV VLP expressing HMPV F and matrix (M) could provide complete protection against HMPV replication in the lungs of mice [14]. HMPV VLP immunization induced F-specific IgG antibody and CD8+ T cell response recognizing an F protein epitope. HMPV VLP vaccines adjuvanted with either TiterMax Gold or α-galactosylceramide enhanced neutralizing-antibody responses in mice [107]. Other studies demonstrated that serum neutralizing antibodies alone might not be sufficient for long-lasting protection against reinfection with HMPV [114] and that T cell immunity together would be important for protection against reinfection or long-term protection [124]. Importantly, HMPV VLP vaccination did not cause enhanced respiratory disease upon viral challenge [124]. These previous studies indicate that HMPV vaccines based on VLPs produced in mammalian cells could be effective in inducing protective immunity against HMPV.

5. Clinical studies of VLP vaccines against respiratory viruses

5.1. Clinical studies of influenza VLP vaccines

Seasonal influenza VLP vaccines might provide comparable or more effective protection over inactivated split vaccines produced in egg substrates. A phase-I/II, double-blind, dose dependent vaccine efficacy study was conducted in Mexico against 2009 pandemic H1N1 virus to evaluate the vaccine safety and immunogenicity of influenza HA-VLPs produced in insect cells via the rBV expression system [125] (Table 4). H1N1 VLP vaccine was safe and well tolerated in 4500 healthy adults at 18–64 ages after vaccination with VLP vaccines (5-, 15-, or 45-µg HA dose) [125]. The low dose (5 µg of HA) HA VLP vaccine was found to be immunogenic and safe after vaccination. The local adverse events were observed in the moderate (15-µg HA) or high dose (45-µg HA) groups. The low dose (5 µg of HA) subjects or placebo group did not display local and serious adverse events after vaccination.

Table 4. Clinical trial studies.

Source	Component	Protective immunity	Host	Reference
H1N1, H5N1	HA+NA+M1	Neutralizing antibody induced and 79% sero-protection rate achieved.	18–64 years old	[125]
H5N1	HA+NA+M1	61% seroconversion rate based on HAI and 76% seroconversion rate based on MN.	18 to 40 years old	[126]
H7N9	HA VLP+ ISCOMATRIX	Neutralizing antibody induced against homologous strain and the heterologous H7-A/Netherlands/219/03 strain		[80]
H1N1	gH1-Qbeta/alhydrogel	65.4% seroconversion in adjuvanted group and 65.4% seroconversion in non-adjuvanted group.	21 and 64 years old	[130]
H5N1	HA	HI+, MN+	18-60 years old	[43,45]
RSV-A2	RSV F protein nanoparticle	Neutralizing antibody induced	Older adult	[137]
SV-A2	RSV F protein nanoparticle	Neutralizing antibody induced	Healthy women	[133]

MN: virus microneutralization; HI: hemagglutination inhibition.

Notably, in the follow up clinical studies, 82-92% of all influenza VLP vaccinated subjects were observed to have sero-protective antibody levels (≥40 HAI titer) after boost [125]. Another phase I/II dose dependent clinical trial approved by Food and Drug administration was conducted in healthy adults to determine the efficacy of rBV/insect cell-derived A/Indonesia/05/2005 (H5N1) VLP vaccines [126]. In this clinical study, different doses of H5 HA (15, 45, or 90 µg of HA) were tested and found to induce functional antibodies, preferentially recognizing the oligomeric form of hemagglutinin [126]. Low HAI titers were observed in the 15 µg dose-immunized subjects whereas high HAI titers and cross-reactivity against other clade 2 viruses were induced in individuals with 45- and 90-µg H5 HA VLP dose immunization. There were no serious local or systemic adverse events, and only a single subject was discontinued due to an adverse event. An additional clinical study on avian influenza VLP vaccines, unadjuvanted H7N9 VLPs vaccine exhibited poor antibody responses [80]. In contrast, ISCOMATRIX™ adjuvanted VLP vaccines elicited HAI responses and raised high-quality antibody immune responses against avian influenza in naïve humans [80].

The safety and immunogenicity of the *N. benthamiana* plant cell-derived influenza H5 HA (A/Indonesia/5/05) VLP vaccines were assessed in a phase I human clinical trial [127]. To license an influenza vaccine, the immunogenic properties of vaccines should exhibit remarkable serologic responses as measured by HAI titers, single radial hemolysis (SRH) and microneutralization titers. A randomized, double-blind, placebo-controlled phase I clinical trial was performed among 18–60 years old healthy adults at the McGill University Health Center to assess the efficacy of plant cells derived, alum-adjuvanted A/Indonesia/5/05 H5N1 VLP by intramuscular immunization with 5, 10, or 20 µg vaccine dose [128]. The immunogenicity of vaccine (H5 HA VLP) was dose dependent, and 96% of vaccinated subjects with a high dose showed detectable microneutralization titers. In the clinical trials above, it has been reported that in the high dose 20 µg HA group after getting the first dose and the 10-µg HA group after receiving the second dose, 8.3% of the vaccinated subjects experienced transient headaches. However, the adverse events were mild-to-moderate and self-limited. Another phase I clinical trial has been completed with plant cell derived HA seasonal influenza VLP vaccine to evaluate the immunogenicity, safety, and efficacy [43]. In this clinical study, 18–49 years old subjects were intramuscularly administered H1-VLPs at 5-, 13-, and 28-µg H1 HA-VLPs. The clinical results were found to be safe and well tolerated at all dose levels and even 5-µg dose of VLPs induced greater than 1:40 HAI titers in 83% of the subjects in this low-vaccine dose group [43] (Table 4). Two randomized Phase II clinical trials in 18 to 49 and over 50 years old adults have shown that the 30-µg dose of quadrivalent plant-derived influenza VLP vaccine produced consistent humoral and cellular responses [129]. Recently, phase III clinical trials are ongoing to evaluate the efficacy, safety, and immunogenicity of plant-made influenza quadrivalent VLP vaccines during the 2018–2019 influenza season in elderly adults 65 years of age and older (ClinicalTrials.gov Identifier: NCT03739112). In this phase III trial, quadrivalent VLP influenza Vaccine (30-µg HA/strain) will be compared with standard seasonal vaccine (15-µg HA/strain).

A phase I double-blinded, randomized clinical trial has been conducted in healthy volunteer with bacteriophage gH1-Qbeta VLP vaccine with and without alhydrogel adjuvant to determine the efficacy and immunogenicity of bacteriophage-derived influenza VLP vaccine. The healthy subjects were intramuscularly immunized with 100-µg gH1-Qbeta VLP vaccine containing 42 µg HA antigen showed similar antibody-mediated immunogenicity and a comparable safety profile to commercially available vaccines [130].

These clinical studies support that plant cell-derived influenza VLP vaccines would be safe and immunogenic. Further clinical studies on quadrivalent VLP vaccine safety and dose-escalating studies in comparison with conventional egg-derived influenza vaccines are being planned.

5.2. Clinical studies of RSV nanoparticle vaccines

RSV F protein nanoparticle vaccines produced from insect cells via the rBV expression system are in phase III clinical trials [131,132]. A study of a RSV recombinant fusion (F) nanoparticle vaccine in healthy women of childbearing age demonstrated that the vaccine was safe, immunogenic, and appeared to be effective in reducing RSV infections (Clinical Trials Registration: NCT01704365) [133]. RSV F protein nanoparticle vaccination in older adults induces functional immunity to RSV (ClinicalTrials.gov number NCT01709019) [134].

In a phase III study with the elderly (≥60 years), the insect cell-derived recombinant RSV F nanoparticle vaccine failed to demonstrate significant efficacy of protection against RSV moderate to severe lower respiratory disease in older adults, partially due to a low-attack rate.

There are no clinical trials yet to evaluate the efficacy of RSV and HMPV vaccines based on VLP platforms, suggesting technological challenges in manufacturing clinical-grade RSV and HMPV VLP vaccines stabilizing pre-fusion F conformation and the scale-up processes of VLP vaccine production.

6. Expert opinion

6.1. Influenza VLP vaccines

VLPs provide an alternative platform to generate an effective vaccine probably due to its multifarious desirable properties. VLPs can be self-assembled, expressing one or multi proteins with dense epitopes or chimeric proteins being presented on the VLP surface. VLP vaccines can be produced in cell cultures with different expression systems and are compatible for formulating with various adjuvant systems. Influenza VLP vaccines present individual HA or NA or in combinations on the VLP surface as native-like functional glycoproteins. Both HA and NA proteins on VLPs can elicit HA and NA specific antibodies. However, due to the immune-dominant properties of HA protein, it could induce more HA-specific antibody responses than NA. To avoid immunodominant effects of HA, a separate individual NA VLP or multivalent NA (N1, N2) VLP vaccine could be an alternative approach.

Influenza VLP vaccine production can be scalable through the insect or plant cell-based expression system. Compared to conventional influenza split vaccine or attenuated vaccines produced in embryonated egg substrates, influenza VLP vaccines would be stable in antigenic properties due to the lack of growth adaptation. An additional advantage is that

manufacture of influenza VLP vaccine does not require handling live viruses and growth adaptation. In the case of pandemic outbreaks, it will be possible to produce and distribute insect cell-derived pandemic VLP vaccines within a short time period than egg-growth vaccines. Egg cell-based live attenuated influenza vaccines and inactivated split influenza vaccines would have a limitation in manufacturing capacity to produce sufficient vaccine doses to meet the surge in high vaccine demand during a pandemic outbreak.

In contrast to the plant cell expressed VLPs, rBV/insect cell-based expression system has flexibility to co-express various influenza viral structural proteins on the surface of the VLPs. Preclinical as well as clinical studies reported that immunization with insect cell-derived HA-NA-M1 VLPs can induce effective serum neutralizing antibodies with high HAI titers and NA inhibiting antibodies. Although HA immunity plays a major role in providing homologous protection, NA immunity would contribute to providing heterologous cross protection. In the clinical trials with influenza VLP vaccines produced in plant cells or in the insect cells, there were no serious adverse events reported so far. The combined VLP technology and innovative approaches of plant and insect cell expression systems will lead to developing effective influenza VLP vaccines in near future.

Production of recombinant purified protein subunit vaccines would cost high prices due to multiple purification steps but provide high safety features. To overcome low immunogenicity of soluble proteins, a technology of formulating protein nanoparticle vaccines would be highly desirable. Nanoparticle preparations of recombinant proteins (influenza virus HA conserved stalk domains, tandem repeat M2e proteins) expressed in insect cells, and purified to high purity, were immunogenic and effective in inducing cross-protective immunity in mice [135]. Combining the technology of VLP vaccine and protein nanoparticle vaccine technology would provide a promising vaccination strategy. Utilizing desirable properties of non-replication and high immunogenicity, VLP-based vaccine technologies are worthwhile to be further developed toward more effective and safe vaccines against viral pathogens difficult to control. Cross-protective influenza conserved antigens (M2e, conserved stalks, NA) are poorly immunogenic and thus developing effective VLP vaccines to present these cross protective antigens should be a future direction. VLP vaccines containing conserved cross-protective epitopes but inducing non-neutralizing immunity such as M2e would not induce high protective immunity, a strategy of supplementing HA-based vaccines with VLPs containing conserved epitopes might provide new vaccination enhancing cross-protective efficacy of current influenza vaccines. With encouraging outcomes from preclinical mouse studies and clinical trials of influenza VLP vaccine platforms, further development of VLP vaccines presenting influenza-conserved antigens in an immunogenic form would provide new approaches to develop cross-protective and universal influenza vaccines against seasonal and pandemic viruses.

6.2. RSV and HMPV VLP vaccines

RSV VLP vaccines were demonstrated to be unique in conferring protection against RSV while preventing vaccine-enhanced respiratory disease upon RSV infection [93,131]. These preclinical outcomes of RSV VLP vaccines in mice and cotton rat animal studies provide evidence warrantying further development of effective and safe RSV vaccines. It would be a future direction to develop RSV VLP platforms more stably presenting pre-fusion F proteins, compared to the current pre-F version with DS-Cav1. RSV pre-fusion stabilizing mutations (DS-Cav1) with a foldon stabilizer retained pre-F site Ø epitopes and site II neutralizing epitopes in soluble proteins [136]. It is likely that additional mutations together with DS-Cav1 or alternative mutations in RSV F will be required for further stabilization of pre-fusion F protein antigens on VLP platforms [99,100]. RSV F nanoparticle formulations of recombinant F proteins produced in the insect cell expression system are under advanced clinical trials of maternal vaccination to protect infants although the vaccine phase III clinical trial in the elderly was not successful [132,133,137]. An alternative vaccination strategy of heterologous prime with RSV pre-fusion F VLP and boost with pre-F protein nanoparticles might be a future study to develop effective and safe RSV vaccination. HMPV VLP vaccines were reported to provide protection against subgroups A and B in a mouse model but the prevention of vaccine-enhanced disease by HMPV VLPs remain to be further tested in more relevant animal models.

6.3. General consideration in VLP vaccine technology

A consideration in VLP vaccine technology is that host cell- and vector-derived components are randomly packed together into the assembled VLPs and sometimes abortive packaging can happen which would have impact on generating immune responses to vaccine antigens. There is a probability that protective immune responses would be dependent on the manufacturing process and resulting different batches of VLP vaccines. It is also expected that host immune responses to non-vaccine components in VLP vaccines would be induced after VLP vaccination although immune responses to non-vaccine antigen vector components would not have adverse effects since most enveloped viruses (influenza, RSV, HMPV) would contain host cell-derived non-viral components. Since VLP platform vaccines are effective in priming immune responses in naïve hosts, a strategy of heterologous VLP prime and protein boost vaccination would enhance immune responses to vaccine antigens, minimizing the immune responses to non-vaccine vector components. Advancing VLP vaccine technology should be continued to maximize the protective vaccine antigen contents in immunogenic conformation as for future research efforts.

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