



Virus-Like Particles as Carrier Systems to Enhance Immunomodulation in Allergen Immunotherapy

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Abstract

Purpose of Review Utilization of virus-like particles (VLPs) is considered to improve allergen-specific immunotherapy (AIT). AIT aims at the efficient uptake of the target allergen by antigen-presenting cells (APCs) subsequently inducing adaptive allergen-specific immune responses to induce tolerance. The purpose of this review is to describe the immune-modulating properties of VLPs per se and to summarize the application of VLPs as antigen carriers, preferably for Th2 cytokines or allergens, with and without simultaneous administration of adjuvants in order to modulate allergic immune responses.

Recent Findings Currently, a broad variety of approaches considering the origin of the VLPs, the choice of the adjuvant and antigen, and the coupling of the antigen are under preclinical investigation.

Summary The data provide evidence that VLPs used as carrier for antigens/allergens strongly increase antigen immunogenicity, and might be suitable to prevent allergies. However, systematic studies in mice showing the immunological mechanism and data from clinical studies are scarce.

Keywords Allergen carrier · Delivery system · Protein transfer vector · Virus-like particles · Modular vaccines

Abbreviations

AA-VLP	Adeno-associated VLPs
ACT-VEC	Activating vector
AHR	Airway hyperreactivity
AIT	Allergen-specific immunotherapy
APC	Antigen-presenting cell
BCR	B cell receptor
CMV	Cucumber mosaic virus
CTL	Cytotoxic T lymphocytes
DC	Dendritic cell
FDA	Food and Drug Administration
FEV1	Forced expiratory volume 1 s
GM-CSF	Granulocyte macrophage-colony stimulating factor

gp33	Glycoprotein 33
GPI	Glycosylphosphatidylinositol
HBcAg	Hepatitis B core antigen
H E K 2 9 3	Human embryonic kidney (293) cells
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HPV	Human papilloma virus
i.m.	Intramuscular
LV-VLP	Lentiviral virus-derived VLPs
MHC	Major histocompatibility complex
MLV-VLPs	Murine-leukemia-virus-derived VLPs
MVA	Modified vaccinia virus Ankara
ODN	Oligodesoxynucleotide
Ova	Ovalbumin
pDCs	Plasmacytoid DC
PRR	Pattern recognition receptor
PTV	Protein transfer vectors
s.c.	Subcutaneous
SIV	Simian immunodeficiency virus
SLAM	Signaling lymphocyte activation molecule
TGF- β 1	Transforming growth factor beta 1
TLM	Translocation motif
T _{reg} cells	Regulatory T cells

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This article is part of the Topical Collection on *Allergens*

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Ty	Yeast transposon
VLP	Virus-like particle
VNP	Viral nanoparticles
Wt	Wild-type

Introduction

Immunologically, type I allergies are caused by exaggerated Th2-mediated immune responses, typically directed against otherwise harmless environmental antigens. In allergic patients, contact with the respective allergen triggers IgE-mediated degranulation of mast cells and eosinophils resulting in allergic inflammation.

To date, allergen-specific immunotherapy (AIT) is the only available curative treatment option, while emergency medication only suppresses acute allergic symptoms. AIT is accomplished by subcutaneous (s.c.) or sublingual administration of increasing amounts of crude allergen extracts derived from allergen sources such as pollen or house dust mites that contain a mixture of different allergenic proteins [1]. Although different allergen extracts can be successfully used to treat allergies, AIT sometimes has disadvantages like the risk of inducing anaphylactic reactions, low patient compliance due to the long duration of the treatment, and low extract quality and underrepresentation of certain allergens in these extracts, as well as poor treatment efficacy in polysensitized patients that prevent many patients from undergoing AIT [2].

Therefore, novel strategies have been proposed to enhance efficacy, safety, and convenience of allergy treatment. Among others, these novel strategies include the use of (1) new delivery routes (intranodal, transcutaneous, epicutaneous, or sublingual); (2) recombinantly produced allergens, hypoallergens, allergoids, and peptide-based vaccines; and novel adjuvants derived from bacteria and viruses such as (3) bacterial extracts and (4) TLR ligands, as well as (5) new formulations and delivery systems (e.g., virus-like particles (VLPs), DNA vaccination, modified vaccinia virus Ankara (MVA)-based vaccines, aggregates, nanoparticles, or liposomes) (reviewed in [3, 4]).

The focus of this review is to summarize and discuss the available studies describing the usage of VLPs for the treatment of allergic diseases.

Immunological Properties of Virus-Like Particles

VLPs are multimeric structures that resemble the morphology of the native virus but lack its genomic material. Thus, they are unable to replicate, precluding any possibility of reversion mutations or pathogenic infection. VLPs are composed of proteins that form the viral capsid [5–8]. These proteins can

be arranged in single, double, or triple layers [7]. As ordered nanoscale architectures, VLPs display a densely repetitive array of epitopes facilitating their interaction with components of the immune system [5, 9, 10]. Analyses by Zeltins et al. revealed that at least 110 different VLPs have been constructed from viruses belonging to 35 different virus families [11]. VLPs range from 20 to 200 nm in size and can be either non-enveloped or enveloped, spherical, or filamentous [8, 12]. Furthermore, a novel type of VLPs, named chimeric VLPs, has been developed. Here, the VLPs serve as carrier systems in order to present foreign epitopes [7, 8]. For example, in the case of the human papilloma virus (HPV), the VLPs are formed by a single structural protein [7] while a chimeric HIV-VLP, named activating vector (ACT-VEC), consists of SIV-derived VLPs displaying a variety of HIV envelope proteins and is currently evaluated in non-human primates for its efficacy in the prevention of AIDS [6].

VLPs can be produced either by removing the genetic material and recovering only the structural capsid proteins or by heterologous overexpressing of the structural proteins in suitable cell lines. Following expression, VLPs self-assemble into their final shape, usually imitating the symmetry of the parental virus [10]. Currently, five main platforms are used to produce VLPs: bacteria, yeast, baculovirus/insect systems, mammalian cells, and plants [6]. Although more than 170 different expression systems are currently used to produce VLPs, almost 30% of all described VLPs are generated in bacterial expression systems (mainly *Escherichia coli*). The usage of bacteria and yeast for VLP production represents the most scalable and cost-effective approach. Here, yields can vary from 0.75 to 700 µg of VLP protein per milliliter of culture. Nevertheless, bacteria and yeast lack the ability to perform post-translational modifications which can sometimes be necessary to generate an optimal immune response toward certain antigens [6, 7]. The baculovirus/insect system can produce protein quantities comparable with those obtained from bacteria or yeast but VLPs must be purified from baculovirus contaminants [6]. For the production of VLPs in mammalian cells, two approaches are currently used: (1) stable cell lines encoding the protein of interest, or (2) transient infection with the antigen of choice. In contrast to the systems mentioned before, mammalian cells can perform complex post-translational modifications most accurately, but achieve lower production yields of only 0.018–10 µg VLP protein per milliliter [6, 7]. The most common plants used for VLP production are *Nicotianum tabacum* and *Arabidopsis thaliana*; others include potato or tomato plants [7].

VLP-based vaccines offer a safe and effective alternative to live-attenuated or inactivated vaccines, can be developed and produced within 3–12 weeks, and offer a strong immunogenicity due to their highly repetitive and ordered structure [6, 8, 12]. Therefore, they provide a promising platform for facilitating recognition, uptake, and processing of antigens by the immune system [8, 10].

Several VLPs have already been licensed and commercialized, while others have entered clinical trials or are still in research. The hepatitis B vaccine was the first VLP-based vaccine approved by the Food and Drug Administration (FDA) in 1986 [13]. Since their discovery, advances in production, purification, and adjuvant optimization led to the licensing of seven VLP-based vaccines approved for human usage [9, 12]. These include Cervarix®, Gardasil®, and Gardasil9® for HPV, Hecolin® for hepatitis E, Recombivax HB® and Sci-B-Vac™ for hepatitis B, and recently a VLP-based vaccine for malaria, called Mosquirix™ [12]. Clinicaltrials.gov (2018) currently lists 95 studies for VLP-based vaccines. Among these studies, numerous clinical trials for VLP-based influenza, malaria, or cancer vaccines are investigated concerning their applicability, but only three studies using VLPs loaded with allergens are reported (reviewed in [14]).

In clinical trials, it has been shown that the efficacy of VLPs is highly dependent on the route of administration [10]. Most approved vaccines are administered by intramuscular (i.m.) or s.c. application. However, Cubas et al. demonstrated that intradermal administration of VLPs induced significantly higher antibody production and antigen-specific T cell responses compared to other immunization routes [15]. Particulate structures are able to diffuse through the 200 nm pores of the lymphatic vessels. Therefore, VLPs with an average size of 30 nm are able to drain into the lymphatic system allowing them to efficiently traffic from the site of injection to the draining lymph nodes [5]. Studies in mice revealed that VLPs accumulate in the draining lymph node soon after s.c. footpad injection [9]. In addition to free lymphatic drainage, VLPs can also be actively transported via specialized immune cells. Several studies showed that VLPs are easily taken up by antigen-presenting cells (APC) such as skin-derived macrophages and dendritic cells (DC) [6–8]. Recently, the predominant subsets of APC were classified and include CD11b⁺F480⁺ macrophages, CD8[−]CD11c⁺, CD8[−]CD11b⁺, and CD8⁺CD11c⁺ DC, as well as CD45R⁺B220⁺ B cells [9]. Intradermal injection of mice with hepatitis B core antigen (HBcAg)-VLPs induced the expression of costimulatory surface molecules such as CD40, CD80, and CD86 on DC [16]. Exogenous antigens, such as VLPs, are usually presented on MHC class II in order to activate CD4⁺ T helper cells that in turn produce cytokines and promote the antibody production of antigen-specific B cells. Thus, VLP-derived peptides presented on MHC class II result in the generation of protective IgG antibody titers via activation of T helper cells. Interestingly, several studies showed that VLP can also induce efficient cytotoxic T cell (CTL) responses via cross-presentation [10, 16]. Ruedl et al. used the model antigen glycoprotein 33 (gp33) to demonstrate that gp33-VLPs were presented on MHC class I via cross-presentation and induced activation of CTL in vivo [17]. Furthermore, HPV-derived

VLPs induce maturation of DCs and were shown to be presented on MHC class I [16]. In contrast to soluble antigens, the highly organized and repetitive structures of VLPs promote cross-linking of the B cell receptors (BCRs). BCRs can efficiently bind to VLPs via low affinity/high avidity interactions and trigger endocytosis [9]. Due to their ability to present antigens on both MHC class II and class I, VLPs represent potent stimulators of adaptive immune responses.

Adjuvants are immune stimulatory substances that are added to vaccines to enhance their immunogenicity, potency, and efficacy. VLPs by themselves induce both BCR cross-linking and strong T helper and CTL responses without any adjuvant. Nevertheless, VLP formulations with additional adjuvant may enhance the quality, magnitude, and duration of immune responses as well as the stability of VLP-based vaccines. Therefore, the licensed VLP-based vaccines are mainly adjuvanted with classical aluminum compositions such as aluminum hydroxyphosphate in Gardasil® [5, 8]. Novel adjuvant classes such as liposomes, emulsions, cytokines, and bacterial toxins, as well as pattern recognition receptor (PRR) agonists like CpG are used to improve the immune stimulatory capacities of VLPs [5, 18]. Storni et al. demonstrated that packaging CpG into VLPs increased their immune-stimulating effects while reducing the side effects observed upon separate administration of CpG and VLP [19]. In addition, it was shown that HBcAg-VLPs resulted in efficient CTL responses when CpG was packaged into VLPs [12]. Thus, although VLPs by themselves are potent stimulators of humoral immune responses alone, additional adjuvants can be used to modify the desired type of immune response.

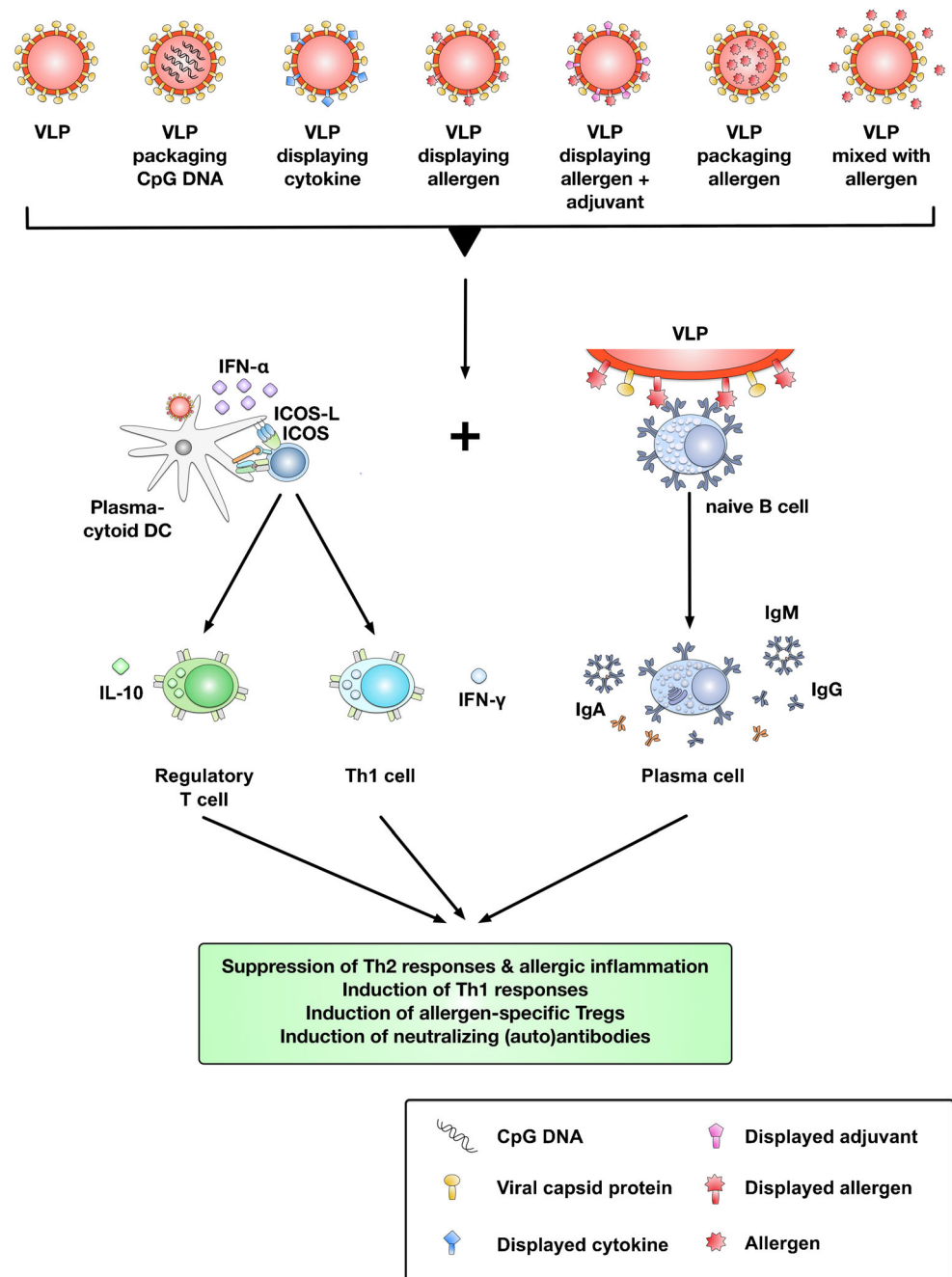
Application of VLPs in the Treatment of Allergic Diseases

When analyzing the usage of VLPs for the treatment of allergies, two general types of strategies are published. In order to achieve the desired immune modulation, the used VLPs can be either applied with or without allergen (extract). If allergen is contained within the used VLP preparations, the allergen can either be packaged into the VLPs, displayed on the surface of the VLP, or the allergen (extract) is simply mixed with the VLPs (Fig. 1).

Usage of VLP Preparations That Does Not Include Allergen Cargo Proteins

The available literature describes two general strategies for the usage of VLPs to treat allergic diseases that do not include packaging, display, or mixing of allergens with VLPs: (1) the modulation of Th2-biased immune responses by packaging immune activating TLR9-ligands into the VLPs, and (2) the

Fig. 1 Experimental approaches using VLPs for the treatment of allergic diseases and their immunological effects



induction of autoantibodies directed against different Th2-promoting cytokines by vaccination with VLPs displaying the respective cytokines (or cytokine-derived peptides) on their surface (for summary see Table 1).

All published strategies using isolated VLPs without additional protein sequences used to treat allergies are based on the VLP CYT003-QβG10 [20•, 21•]. CYT003-QβG10 consists of a protein shell derived from the bacteriophage Qβ (consisting of approx. 180 Qβ molecules, ~30 nm in size) that encapsulates approximately 60 copies of the negatively charged bacterial DNA oligomer G10 during its self-assembly process. G10 itself is a bacterial-derived A-type CpG

oligodesoxynucleotide (ODN) rich in non-methylated CpG motifs that acts as a ligand for the innate immune receptor TLR9 [20•, 21•]. A-type CpG ODNs consist of a phosphodiester backbone that is rapidly digested by DNase type I in vivo, normally limiting its effectiveness in clinical settings [22•]. However, by packaging G10 into the Qβ-VLP, G10 is protected from degradation, likely does not trigger antinuclear antibodies, and can efficiently be targeted to INF-α producing plasmacytoid DCs (pDC) in vivo [20•, 21•, 22•]. By combining the highly ordered and immunogenic Qβ-VLP structure with the potent TLR9-ligand G10, CYT003-QβG10 is able to efficiently activate the immune system and

Table 1 VLP-based strategies to treat allergic diseases

Paper	VLP used	Vaccination target	Investigated species	Immunological effects
Klimek et al., 2011	Bacteriophage Q β -derived VLPs packaging CpG motif G10 (CYT003-Q β G10)	No antigen included	Human	• Significant improvement in rhinoconjunctivitis symptoms and quality of life scores in house dust mite allergic patients treated with CYT003-QβG10 compared to placebo within 2 months • Median 10-fold increase in allergen tolerance determined by conjunctival provocation test in the high-dose group • No drug-related serious adverse event, mild-to-moderate injection site reactions most frequently observed adverse events
Beeh et al., 2013	Bacteriophage Q β -derived VLPs packaging CpG motif G10 (CYT003-Q β G10)	No antigen included	Human	• Higher occurrence of injection site reactions (injection site erythema, pruritus, and swelling) with CYT003-QβG10 than placebo • Significantly reduced symptom scores and medication usage in patients with mild-to-moderate persistent allergic asthma after treatment with CYT003-QβG10 for 12 weeks • Reduction in peripheral blood eosinophils and no deterioration in lung function in CYT003-QβG10-treated patients compared to placebo
Ma et al., 2007	Truncated hepatitis B core antigen (HBcAg)	IL-4	Mouse	• Induction of neutralizing anti-IL-4 IgG autoantibodies • Suppression of ovalbumin-specific IgE production, eosinophil accumulation in BALF, goblet cell hyperplasia, tissue inflammation, and methacholine-induced respiratory inflammation
Fattelschoss et al., 2018	Cucumber mosaic virus containing tetanus toxoid universal T cell epitope	IL-5	Horse	• Induction of neutralizing anti-IL-5 IgG autoantibodies • Significant reduction in clinical lesion and disease scores • Reduction in eosinophil inflammation in lesions but little change in blood eosinophil counts
Landi et al., 2010	Bacteriophage Q β	IL-5 plus cotaxin	Mouse	• Induction of neutralizing anti-IL-5 and cotaxin IgG autoantibodies • Reduction of eosinophilic lung inflammation in an ovalbumin-based mouse model of allergic airway inflammation
Ma et al., 2007a; Ma et al., 2007b; Ma et al., 2013	HBcAg	IL-13	Mouse	• Induction of neutralizing anti-IL-13 autoantibodies (2007a) • Suppression of ovalbumin-induced lung inflammation (goblet cell hyperplasia, lung tissue inflammatory cell infiltration, IL-13 and IL-5 in BALF, and pulmonary hyperresponsiveness to inhaled methacholine) (2007a) • Inhibition of total and ovalbumin-specific IgE (2007a) • Induction of IL-13-specific IgA responses in multiple mucosal tissues and higher titers of IgG in serum upon intranasal vaccination compared to subcutaneous vaccination (2007b) • More effective suppression of ovalbumin-induced Th2 responses (IL-13 production and eosinophil accumulation) upon intranasal vaccination (2007b) • Suppression of IL-13, TGF-β1, and IL-12p40 production in the lung, accumulation of inflammatory cells in BALF, lung mucus production, and collagen deposition (2013) • Suppression of ovalbumin challenge-induced increases in airway resistance, tissue resistance, and tissue elastance, both acute and after a 4-week recovery phase (2013)
Guan et al., 2012	HBcAg	IL-23p40	Mouse	• Induction of neutralizing IgG autoantibodies to IL-23, p40, and IL-12 that inhibited IL-23-dependent IL-17 production in vitro • Suppression of ovalbumin-induced lung inflammation (neutrophil and eosinophil infiltration, reduction of IL-5, IL-13, IL-23, and IL-17 in BALF, decrease in ovalbumin-specific IgE, IgG1, and total IgE)
Long et al., 2014	HBcAg	IL-33	Mouse	• Induction of neutralizing IL-33 IgG and IgA autoantibodies • Shift from Th2- to Th1-like responses (lowered ratio of allergen-specific IgG1/IgG2a in vaccinated mice)

Table 1 (continued)

Paper	VLP used	Vaccination target	Investigated species	Immunological effects
Ma et al., 2016	HBcAg	Transforming growth factor beta 1 (TGF-beta 1)	Mouse	• Suppression of ovalbumin-induced goblet cell hyperplasia, eosinophilia, lung inflammation, and IL-33 production in BALF • Increased IL-4, IL-13, and IL-33 levels in BALF with no significant effects on lung tissue inflammation and airway goblet cell hyperplasia (prophylactic treatment) • Reduced airway subepithelial collagen deposition, but no change in lung inflammation, airway resistance, tissue resistance, and tissue elastance as well as Th2-cytokine production (therapeutic treatment)
Senti et al., 2009	Bacteriophage Q β -derived VLPs packaging CpG motif G10 (CYT003-QbG10)	HDM extract mixed to VLPs	Human	• Significantly reduced symptoms of rhinitis and allergic asthma in Qβ-treated patients within 10 weeks of treatment that lasted at least 38 weeks post-treatment • Increase in allergen-specific IgG, transient increase in allergen-specific IgE, and reduced skin reactivity
Harris et al., 1997 Hirschberg et al., 1999	Ty (yeast transposon)-VLP	Der p 1 ₁₁₁₋₁₃₉ (house dust mite allergen)	Mouse	• Induction of allergen-specific CTL response • Secretion of IFN-γ • Abrogated specific T cell and IL-5 responses (in vivo)
Svirshchevskaya et al., 2002	Ty (yeast transposon)-VLP	Asp f 2 T cell epitope (<i>A. fumigatus</i>)	Mouse	• Abrogated specific T cell responses (in vivo)
Svirshchevskaya et al., 2006	Ty (yeast transposon)-VLP	Asp f 2, Der p 1, Fel d 1	Mouse	• Induction of allergen-specific IgG1
Kündig et al., 2006	Bacteriophage Q β -derived VLPs	Der p 1 _{16mer} (house dust mite allergen)	Human	• Phase I safety and immunogenicity (induction of IgG1 and IgG3) • No difference between i.m. and s.c. application
Schmitz et al., 2009	Bacteriophage Q β -derived VLPs	Fel d 1	Mouse	• Qβ-Feld1 prevents anaphylaxis in Fel d 1-sensitized mice, mediated by IgG rather than IL-10 or T_{reg}
Engeroff et al., 2018	Bacteriophage Q β -derived VLPs	Fel d 1	Human	• Qβ-Feld1 displays reduced allergenic potency using human mast cells
Manzano-Szalai et al., 2014	Adenovirus associated (AA)	Ova ₃₂₃₋₃₃₉	Mouse	• Induction of Ova-specific IgG1 (but not IgE) • Impaired uptake into APCs
Uhlig et al., 2015	Lentivirus (LV), MV-GP pseudotyped	Ova	Mouse	• VLPs targeting SLAM as entry receptor • Induction of IFN-γ and IL-2 induction from Ova-specific CD8⁺ T cells
Akhras et al., 2017	Hepatitis B virus (HBV)	Ova	Mouse	• Induction of Ova-specific CD8⁺ T cell responses in vitro
Gogesch et al., 2018	Monolev-murine-leukemia virus (MLV), displaying GM-CSF	Ova (peptides)	Mouse	• Expansion of CD11b⁺ APCs • Proliferation of Ova-specific CD4⁺ and CD8⁺ T cells
Kratzer et al., 2018	Monolev-murine-leukemia virus (MLV),	Art v 1 (<i>Artemisia vulgaris</i> allergen)	Mouse	• Shielding of Art v 1 (but not surface-exposed Art v 1) protects from mediator release and did not induce humoral immune response • Induction of Th1 and T_{reg} and reduction of AHR

to mimic an infectious agent without the ability to replicate and cause disease.

Klimek et al. reported a phase II clinical trial in which CYT003-Q β G10 (either 0.5 or 1 mg) was administered 6 times weekly without additional allergen to 299 patients with house dust mite allergy [20••]. The treatment with CYT003-Q β G10 was generally safe and well-tolerated, with no drug-related serious adverse events and only mild to moderate injection site reactions [20••]. Here, Klimek and co-workers reported a significant improvement in both rhinoconjunctivitis symptom and quality of life scores as well as a median tenfold increase in allergen tolerance determined by conjunctival provocation test in patients treated with a high dose of CYT003-Q β G10 compared to placebo [20••]. Therefore, the authors suggested CYT003-Q β G10 to be a suitable candidate for allergen-independent treatment combining a favorable safety profile with a therapeutic benefit within 2 months [20••].

In a follow-up study, Beeh et al. examined the clinical efficacy, safety, and tolerability of CYT003-Q β G10 (dose 0.9 mg) in a proof-of-concept, double-blind, randomized trial including 63 patients with mild to moderate persistent allergic asthma (allergens not specified) [21••]. In line with the results by Klimek et al., CYT003-Q β G10 had a good safety profile with no reported drug-related serious adverse events [21••]. Moreover, despite steroid withdrawal, treatment with CYT003-Q β G10 for 12 weeks resulted in significantly reduced symptom scores and medication usage, as well as reduced peripheral blood eosinophilia compared to placebo control [21••]. Here, lung function determined by forced expiratory volume 1 s (FEV1) remained unchanged over the course of the study in CYT003-Q β G10-treated patients, while FEV1 values were significantly reduced in placebo controls [21••]. Taken together, these results suggest an anti-inflammatory effect of CYT003-Q β G10 even when applied without allergen [21••].

Remarkably, in both studies, CYT003-Q β G10 was able to improve allergic symptoms without co-applying the respective allergen. Here, the promising immunological effects of CYT003-Q β G10 were suggested to be caused by the ubiquitous presence of house dust mite allergens in the environment of the study participants combined with the following immunological effects of the administered VLPs: (1) efficient uptake of CYT003-Q β G10 by pDCs via anti-CYT003 antibodies and Fc γ Rs as well as BCR on B cells in vivo due to its repetitive and highly ordered structure [23]; (2) a TLR9-mediated production of IFN- α from pDCs that suppresses Th2-responses via a degradation of GATA-3 [24]; (3) the induction of Th1-biased immune responses [20••, 21••]; and (4) an induction of regulatory T cell subsets by the stimulated pDCs [25, 26].

In line with these results, Senti and co-authors reported the application of CYT003-Q β G10 admixed with house dust mite extract in a single-center, open-label phase I/IIa study

including 20 patients suffering from HDM allergy [22••]. Here, application of CYT003-Q β G10 plus house dust mite extract significantly reduced symptoms of rhinitis and allergic asthma in Q β -treated patients within 10 weeks of treatment [22••]. These clinical improvements were paralleled by an increase in allergen-specific IgG, a transient increase in allergen-specific IgE, and reduced skin reactivity, and lasted at least 38 weeks post-treatment [22••].

The second strategy using VLPs without allergen molecules leverages the strong immune activating potential of the highly ordered and repetitive VLPs in order to break self-tolerance, resulting in the induction of neutralizing autoantibodies directed against the key cytokines necessary to induce and maintain allergic inflammation. Here, different VLPs have been reported that target the in vivo production of IL-4 [27], IL-5 plus eotaxin [28], IL-13 [27, 29, 30], IL23p40 [31], and IL-33 [32], as well as transforming growth factor beta 1 (TGF- β 1) [33••] in preclinical settings (see also Table 1). In these studies, the VLP types used were either derived from truncated HBcAg [27, 29–32, 33••, 34], cucumber mosaic virus (CMV) containing a tetanus toxoid universal T cell epitope [35], or the bacteriophage Q β [28].

In summary, all tested constructs were reported to efficiently induce the production of cytokine-specific IgG autoantibodies. Moreover, studies by Long et al. and Ma et al. reported the induction of cytokine-specific IgA antibodies upon intranasal [29] or s.c. [32] VLP application.

In line with the original hypothesis, VLPs inducing autoantibodies directed against IL-4 [27], IL-5 and eotaxin [28], IL-13 [29, 30, 34], IL-33 [32], and IL23p40 [31] were consistently shown to ameliorate allergic inflammation in different mouse lung asthma models. Here, the different VLPs suppressed allergen-induced goblet cell hyperplasia and eosinophilia and Th2-cytokine production in bronchoalveolar lavage fluid (BALF), as well as systemic allergen-specific IgE production. Moreover, VLPs inducing autoantibodies against IL-5 were shown to suppress eosinophil-mediated skin inflammation in horses [35].

Among these promising studies, VLPs inducing autoantibodies against TGF- β 1 were reported to be a noteworthy exception [33••]. While therapeutic in vivo application of these VLPs reduced airway subepithelial collagen deposition but had no effect on the established lung inflammation, functional lung parameters, or Th2-cytokine production, prophylactic vaccination even resulted in increased levels of the Th2 cytokines IL-4, IL-13, and IL-33 in BALF and had no significant effects on lung tissue inflammation and airway goblet cell hyperplasia [33••].

These results suggest that targeting TGF- β 1 has complex immunological effects in vivo, while the neutralization of IL-4, IL-5, IL-13, and IL-33 was able to prevent allergic inflammation. Therefore, not every cytokine seems to be equally suited as a target for such vaccination approaches.

Usage of VLP Preparations as Antigen (Allergen) Carrier

When using VLPs for antigen delivery, VLPs can be designed either to encapsulate or to display any antigen of interest (see Fig. 1). The respective antigens can be attached to the viral capsid proteins either by chemical coupling or genetic fusion to form chimeras with the respective capsid proteins (reviewed in [36]). However, the development of chimeric VLPs is frequently limited by technical reasons: (1) The self-assembly of the capsid proteins can be affected by the nature and the molecular size of the fusion protein and (2) conformational epitopes of the antigen may be destroyed by insertion into the viral capsid protein. So far, several antigens comprising Th2-cytokines (IL-13, IL-4, IL-5), eotaxin, or HLA and allergens have been co-administrated with VLPs for the intervention of Th2-biased immune response (see above).

Early studies combining VLPs with allergens aimed to activate allergen-specific CD8⁺ T cells [37] in order to downregulate IgE responses. A Ty-VLP, which refers to a yeast transposon (Ty) and a yeast expression vector encoding the structural core protein of TYA gene p1, was utilized to generate a fusion protein with peptides from the major house dust mite (*Dermatophagoides pteronyssinus*) allergen Der p 1, TYA:Derp1-peptide. Ty-VLPs are particles formed by about 300 copies of the p1 protein. Here, immunization of mice with Ty-VLP displaying Der p 1_{111–139}, but without adjuvant, induced the following: (1) An allergen-specific CTL response demonstrated by the lysis of Der p 1_{111–139}-pulsed target cells ex vivo and (2) secretion of INF- γ in vivo [38]. Notably, in the presence of alum, these VLPs primed a CD4⁺ Th1 cell response [38]. The treatment of Der p 1-sensitized mice with Der p 1_{111–139} hybrid Ty-VLPs (single i.p. application of 100 μ g corresponding to 5 μ g of peptide) abrogated allergen-specific T cell proliferation and IL-5 production while INF- γ production was not affected, suggesting the induction of anergic Th2 cells [38]. However, the study did not address any potential effect on clinical symptoms and allergen-specific antibody responses. Further studies followed the same concept by using prominent T cell epitopes from Asp f 2 (the major allergen from *Aspergillus fumigatus*) expressed on VLPs [39]. Asp f 2-sensitized mice receiving a mixture of Asp f 2 hybrid Ty-VLPs (single s.c. dose of 500 μ g corresponding to 10 μ g of peptide) showed abrogated T cell responses. However, the treatment was not sufficient to induce long-term tolerance and the study did not monitor the allergen-specific IgE response. The same authors [40] showed that Ty-VLPs (s.c. application of 100 μ g on 3 consecutive days) can be used as carrier for Asp f 2-, Der p 1-, and Fel d 1- (major allergen from cats *Felis domesticus*) derived B cell epitopes to induce IgG1 antibodies reactive with the intact allergen, respectively. The study demonstrated the

immunogenicity in mice but did not evaluate a potential preventive effect [40].

Other studies utilized VLPs derived from bacteriophage Q β produced in *E. coli* which naturally encapsulate bacterial RNA (TLR7/8-ligands) during the self-assembly process (reviewed in [41]). Initially, Q β -VLPs have been applied for chemical conjugation of a synthetic 16-mer peptide from Der p 1 (Q β -Derp1) displaying B cell epitopes [42]. Using Q β -Derp1, for the first time, a VLP:allergen carrier was evaluated for safety and immunogenicity in a phase I study with 24 healthy human subjects. Injection (3 times s.c. and i.m. application, respectively) of 10 μ g and 50 μ g Q β -Derp1 without further adjuvant was well tolerated, and both Q β - and Der p 1-specific IgG (mainly IgG1 and IgG3) antibodies were induced after 4 weeks in response to a single dose [42]. Here, no difference was observed between s.c. and i.m. application. Notably, in particular, Der p 1-specific antibody levels started decreasing after 4 months [42]. Of note, neither Q β -VLP alone nor admixed with Der p 1 was tested as controls.

The development of modular VLP vaccines should overcome the restrictions of chimeric VLPs generated by genetic fusion of individual antigens to the capsid proteins by employing more robust and flexible means for displaying allergens on the surface of VLPs (reviewed in [36]). This approach involves the separate synthesis of VLPs and the target antigen/allergen. Both components are subsequently assembled in vitro. Here, the use of the hetero-bifunctional conjugation reagent SMPH (succinimidyl-6 [(β -maleimidopropionamido)hexanoate]) was suggested for covalent linkage of antigens (e.g., allergenic phospholipase A2) to Q β -derived VLPs using amines exposed on the surface of VLPs and SH groups of the antigen [36]. One important study [43••] describes the increased immunogenicity of major cat allergen Fel d 1 coupled Q β -VLP (Q β -Feld1). Here, coupling efficiency was calculated to be approximately 40%, corresponding to 70 molecules of Fel d 1 per VLP consisting of 180 Q β subunits. Single application of Q β -Feld1 (s.c. 50 μ g corresponding to 20 μ g Fel d 1), but not Q β alone, was sufficient to prevent allergen-specific anaphylaxis and Th2 immune responses in Fel d 1-sensitized mice [43••]. This effect was mediated by allergen-specific IgG rather than by the induction IL-10 or T_{reg} cells [43••]. Notably, this study for the first time demonstrated the underlying molecular mechanism for the therapeutic effect of a VLP as allergen carrier in an experimental allergy model. Moreover, degranulation assays using human whole blood [43••] and differentiated human mast cells [44] showed almost negligible allergenic potency of recombinant (r) rFel d 1 conjugated to Q β -VLP compared to non-conjugated rFel d 1. Generation of VLPs as allergen carriers by chemical coupling, rather than by genetic conjugation, is suggested not to be restricted by the size and structure of the antigen. Although chemical conjugation of the antigen should not affect the conformation of epitopes displayed on

the surface of VLPs, the available data suggest either some structural changes, masking of allergen epitopes upon coupling, and/or insufficient steric display of Fel d 1 on the surface of the VLP not able to cross-link Fc RI on mast cells. Importantly, in this study, chemical coupling did not impair capsid integrity [44]. A further advantage of this strategy is that a single and broadly used VLP carrier can be generated with consistent quality that is subsequently conjugated to different antigens. It remains to be investigated whether VLP-allergen conjugates have advantages in comparison to VLPs just admixed with allergens (reviewed in [41]). Although both Q β -VLPs encapsulating CpG-motifs and Q β -VLPs displaying allergen on their surface have been tested (see above), Q β -VLPs displaying allergens and simultaneously encapsulating CpG have not been evaluated so far.

Manzano-Szalai et al. described adeno-associated VLPs (AA-VLP) for display of the model allergen Ovalbumin (Ova) using the Ova_{323–339} B cell epitope [45]. The Ova peptide extended by a Gly-Linker was expressed as fusion protein with a modified AAV-2 capsid protein VP3 in HEK293 cells [45]. The AA-VLP (~25 nm diameter) was shown to be composed of 60 copies of the VP3 capsid subunit [45]. This approach allows the integration of peptides of up to 34 amino acids (aa) after aa₅₈₇ of the capsid protein [45]. Here, immunization (3 times s.c. in a 2-week interval) of mice with AA-VLP-Ova (11.2 μ g) plus Montanide or alum as adjuvants induced strong Ova-specific IgG1 but not IgE response in comparison to Ova alone [45]. Moreover, immunization induced also wtAA-VLP-specific IgG production [45]. Notably, AA-VLPs without adjuvant were not tested [45]. Although the insertion of the Ova-peptide into VP3 impaired the uptake into APCs in vitro, a substantial antibody response to Ova was achieved. However, the authors suggested uptake via preformed anti-Ova, anti-AA-VLP antibodies, or BCRs of the respective specificity to mediate the immunogenic effects in vivo [45]. Therefore, anticarrier antibodies do not necessarily interfere with vaccine efficacy as neutralizing antibodies. However, an antibody (receptor)-independent uptake was not reported.

One strategy employed targeted protein transfer vectors (PTV) combining lentiviral-derived VLPs (LV-VLP, > 100 nm diameter) that display the genetically fused Ova and which were pseudotyped with measles virus glycoproteins which use human SLAM (signaling lymphocyte activation molecule) as main entry receptor on Ova-specific lymphocytes and APCs [46]. Ex vivo and in mice studies Ova-PTV triggered IFN- γ and IL-2 production from Ova-specific CD8⁺ T cells. Another approach followed the strategy of modular VLP vaccines, but applying non-covalent attachment of the antigen (using Ova) to the VLPs [47]. Here, the HBcAg was genetically fused to the cell permeable translocation motif (TLM) mediating cell entry [47]. The HBV-VLPs (~32 and

38 nm diameter) are formed by either 90 or 120 dimers of the HBcAg which was further modified by insertion of a strep-tag to allow coupling of tetrameric streptavidin-antigen fusion proteins (using Ova) to the surface of the particles and its co-delivery into the cytoplasm of APCs to induce antigen-specific CD8⁺ T cell responses [47]. These universal carrier VLPs represent a promising platform to present any allergen of interest to the immune system. However, both VLP-protein carrier systems (PTVs and HBV-VLPs) have not been evaluated in preclinical studies in respect to their potential preventive effect on Th2-biased allergies.

Two reports refer to the utilization of Monoley-murine-leukemia-virus-derived VLPs (MLV-VLPs) as allergen carrier. Gogesch et al. [48••] showed MLV-VLPs (~120–150 nm diameter) co-displaying GM-CSF (granulocyte macrophage-colony stimulating factor) as non-classical adjuvant and Ova-derived immune dominant peptides, both displayed on the MLV-VLP protein, to expand murine CD11b⁺ APCs which in turn induced proliferation and activation of Ova-specific CD4⁺ and CD8⁺ T cells [48••]. Notably, the strongest immune modulating effect was achieved when both, antigen and adjuvant, were presented on the same particle [48••], suggesting the immune activating effects of these MLV-VLPs to be caused by the simultaneous targeting of APCs with antigen and adjuvant arranged in a highly repetitive fashion. So far, this approach was neither tested for humoral immunogenicity nor in a mouse allergy model. A second study reports the preventive effect of MLV-like nanoparticles (VNP of ~100 nm diameter) encapsulating the major allergen Art v 1 in a humanized mouse model of mugwort (*Artemisia vulgaris*) allergy [49••]. Art v 1 was expressed as a fusion protein with the MLV matrix protein MA p15 which directs the allergen into viral core particles coated with a HEK293-derived envelope exosomes after budding. In a separate approach, Art v 1 (18% of the mass of VNPs) was exposed on the surface of VNP by fusion to the glycosylphosphatidylinositol (GPI) acceptor sequence of CD16b [49••]. Notably, in contrast to VNPs with surface-exposed Art v 1, VNPs shielding the allergen (1) did not trigger degranulation of RBL cells sensitized with Art v 1-specific IgE, and (2) upon i.n. application did not induce allergen-specific antibody production (neither IgG nor IgE). However, in a prophylactic vaccination setting, VNPs encapsulating Art v 1 (2 times i.n. bi-weekly application) induced a T_H1/T_{reg}-biased immune response and improved airway hyperreactivity (AHR) in comparison to empty control VNPs [49••]. Currently, this is the only study which addresses the encapsulation of an allergen into VLPs. However, the overall amount of Art v 1 packaged was not determined. Moreover, VLPs carrying a lipid envelope possess the capacity of including additional immune-modulating proteins.

Finally, the concept of chemical coupling was transferred to peanut allergens [50].

Summary and Conclusion

Among several types of nanoparticles (reviewed in [51]) which are suggested for allergen-specific immunotherapy, VLPs display specific features and properties. VLPs display a size of 20 to 200 nm, which matches perfectly with an efficient entry into lymphatic vessels and uptake by DC (reviewed by [52]). Like liposomes, VLPs are biodegradable, but have to be resistant to gastrointestinal digestion [51], a feature which is required for treatment of food allergy in the gastrointestinal tract. Moreover, disassembling and assembling of viral capsid proteins during the generation and purification of VLPs can be easily utilized for packaging of adjuvants, likewise CpG and ssRNA, which otherwise are unstable (e.g., due to the *in vivo* exposure to DNases and RNases). Although also antigens/allergens theoretically can be encapsulated into VLPs to increase their concentration, while preventing degradation and avoiding IgE recognition [49•], in the majority of approaches, the antigen/allergen is displayed on the surface of the particles. Due to technical reasons, packaging of high amounts of proteins into VLPs might be less feasible for VLPs compared to other nanoparticles. Moreover, encapsulation of antigens does not promote antigen-specific T cell-independent B cell activation by cross-linking the allergen-specific BCR.

Coupling of antigens/allergens can be achieved either by genetical fusion or chemical conjugation. Chemical conjugation of antigens is accompanied with the advantage that VLPs which are produced as one batch under defined conditions can be utilized as flexible platform for the preparation of modular vaccines for different antigens. Moreover, for VLPs which are generated without genetic modification of capsid proteins, likewise by surface display of the antigen, the VLP assembly is not affected. Consequently, non-modified VLPs are likely more robust, resulting in higher immunogenicity. Notably, using VLPs as carrier system, the antigens/allergens are displayed in a highly ordered and repetitive structure which is a requisite to increase immunogenicity. Optimizing the density (loading capacity) of antigens on VLPs, which likely affects the ratio of the antigen- to VLP-specific immune responses [42], is the objective of future studies. In line with this, it has been suggested that IgG/IgM antibodies which are raised against VLPs (e.g., Q β) [42] interfere with the immune modulating effect, e.g., by neutralizing and opsonizing the VLPs thereby affecting uptake into APCs. Notably, in case of the bacteriophage Q β , no preexisting immunity in humans has been described [53]. Moreover, utilization of VLPs which lack envelope proteins, e.g., MLV-VLP [48•], does not have self-adjuncting effects.

Recently, evidence has been provided that clinical efficacy in house dust mite allergic patients can be achieved by Q β -VLPs loaded with CpG motifs, but without co-applied allergens (reviewed in [41]). Therefore, VLPs loaded with adjuvant but without allergens have been discussed as suitable stand-alone AIT products (reviewed in [4]). However,

possible explanations for the observed apparent allergen-independent effects of the VLPs might be (1) the direct effect of CpG on TLR9 expressing mast cells; (2) CpG-induced INF- α production which induces indoleamine 2,3 dioxygenase (IDO) from DC, which suppresses T cells and favors T_{reg} induction; and (3) a potential concomitant environmental exposure to HDM (reviewed in [4, 41]). Here, it still remains interesting to investigate whether VLPs other than Q β -VLPs (without bacterial CpG ODN) and the application of Q β -VLPs with strictly controlled lack of simultaneous allergen exposure have similar effects.

Up to now, almost all studies applying VLPs (with the exception of TGF- β 1 displaying VLPs [33•]) for allergy treatment have demonstrated promising immune modulating effects in (mouse) *in vivo* models (e.g., reduced capacity to induce mediator release from mast cells, suppression of Th2 responses, induction of Th1-biased immune responses, and induction of neutralizing antibodies). However, with the notable exception of CYT003-Q β G10, the suggested approaches so far have not progressed to human clinical trials. Therefore, neither optimal treatment dosages, application routes, nor the magnitude and duration of potential therapeutic effects, as well as potential side effects, have been determined.

Another open question is how the application of VLPs can be further improved. Here, one obvious strategy is to leverage the advantage that VLPs can co-deliver allergen, immune-stimulating VLP proteins, and additional adjuvants to a single antigen-presenting cell to efficiently boost allergen-specific immune responses without the risk of causing potentially detrimental allergen-specific Th2 immune responses by bystander activation. Of note, while both VLPs plus adjuvant and VLPs displaying/packaging allergens have been repeatedly described in the available literature, studies that combine VLPs with both allergen and adjuvant are still rather scarce (the only two described studies are Schmitz et al. combining Q β -VLP-Feld1 with bacterial RNA [43•] and Gogesch et al. [48•] combining MLV-VLPs with Ova and GM-CSF).

Furthermore, there is currently only one study describing the packaging of allergens into VLPs [49•]. The packaging of the allergen protected humanized mice from allergen-induced mediator release and prevented the induction of humoral immune response while still inducing Th1 and T_{reg} responses and reducing AHR [49•]. Here, more studies packaging allergens into the VLPs are needed to evaluate if this strategy can replicate the beneficial immune-modulating effects repeatedly demonstrated for VLPs displaying the allergen on their surface.

In summary, the application of VLPs for the treatment of allergic diseases has repeatedly shown promising immune-modulating effects and therefore clearly has potential to improve allergy treatment in the future. However, further studies are needed to investigate both the underlying mechanisms of the protective immune responses and their safety, applicability, and efficacy in human clinical trials.

Compliance with Ethical Standards

Conflict of Interest The authors declare no conflicts of interest relevant to this manuscript.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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