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Review

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Bioengineering of Virus-like Particles for the Prevention or Treatment of Allergic Diseases

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ABSTRACT

Recent findings on the mechanism of allergen-specific immunotherapy (AIT) have revisited the role of immunoglobulin G (IgG) as the development of specific blocking IgG antibodies appeared critical for the successful suppression of T-helper 2 (Th2)-biased allergic responses. Consequently, any form of molecular AIT-promoting potent allergen-specific neutralizing antibodies would be preferred to conventional administration of allergen extracts. The potent immunogenicity of virus-like particles (VLPs) could be harnessed for that purpose. The particle size (20–200 nm) optimizes uptake by antigen-presenting cells as well as lymphatic trafficking. Moreover, the display of antigens in repetitive arrays promotes potent B cell activation for the development of sustained antibody responses. The presentation of self-antigens on the particle surface was even capable to break B cell tolerance. In this review, we describe the immunomodulatory properties of the 3 VLP-based strategies designed so far for the treatment of allergic disease: VLP packaged with CpG motifs as well as chimeric particles displaying pro-Th2/Th2 cytokines or allergens (full-length or B cell epitopes).

Keywords: Nanoparticles; allergen; blocking antibodies; CpG motif; autoantibodies

INTRODUCTION

Atopic subjects sensitized to allergens from different sources develop exacerbated inflammatory responses initiated by epithelial-derived pro-T-helper (Th2) mediators (interleukin [IL]-33, IL-25, thymic stromal lymphopoietin [TSLP] and granulocyte macrophage colony-stimulating factor [GM-CSF]) and the activation of innate lymphoid type 2 cells producing Th2 cytokines IL-5 and IL-13.[1,2] Allergen presentation by dendritic cells (DCs; conventional DC2s) in this Th2-prone milieu leads to the differentiation of allergen-specific Th2 cells and the production of specific immunoglobulin E (IgE) antibodies by allergen-specific B cells.[1] Allergen-specific IgE mediates immediate phase response, marked by basophil/mast cell degranulation as well as IgE-facilitated antigen presentation by B cells.[2] IL-4, IL-5 and IL-13 released by specific Th2 cells control the late and the chronic phases of allergic response characterized by eosinophilia, airway hyperresponsiveness or mucus hypersecretion by goblet cells (**Fig. 1**).
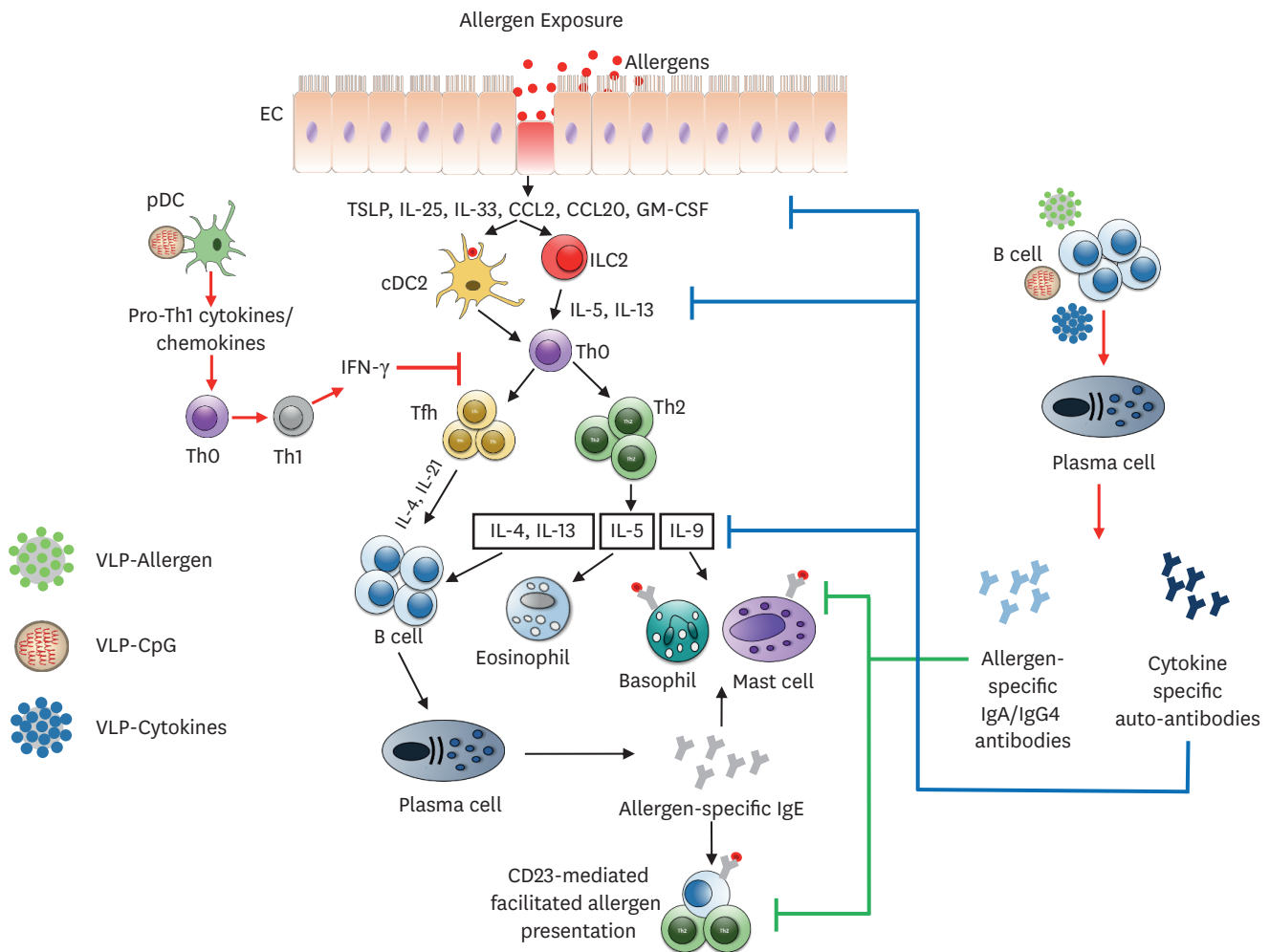
Fig. 1. Mechanisms of allergic responses and immunomodulatory effects mediated by different VLP-based therapeutic approaches. Whereas VLPs encapsulating CpG mediate robust Th1 humoral and cell-mediated immune responses, particles displaying pro-Th2/Th2 cytokines or allergens stimulate strong polyclonal blocking IgG responses for the down-regulation of allergic inflammation.

VLP, virus-like particle; Th, T-helper; Ig, immunoglobulin; TSLP, thymic stromal lymphopoietin; IL, interleukin; CCL, C-C motif chemokine ligand; GM-CSF, granulocyte macrophage colony-stimulating factor; pDC, plasmacytoid dendritic cell; cDC, conventional dendritic cell; ILC2, innate lymphoid type 2 cell; EC, epithelial cell; Tfh, T follicular helper.

Allergen-specific immunotherapy (AIT) is the unique specific treatment capable to desensitize allergic patients through installation of long-term immune tolerance by frequent subcutaneous (SCIT) or sublingual administration of natural allergen extracts.[3] The efficacy and safety of AIT have been demonstrated in large clinical trials for venom allergy, allergic rhinitis, mild allergic asthma and more recently for food allergy.[4,5] The cellular and molecular mechanisms of AIT are not, up to now, completely depicted, but include early desensitization of basophils/mast cells, development of peripheral allergen-specific regulatory T (Treg) and regulatory B cells producing immunosuppressive IL-10, and stimulation of non-inflammatory allergen-specific blocking IgG4 antibodies.[2]

Unfortunately, several issues are commonly associated with conventional AIT which negatively impact on the clinical efficacy[3]: 1) the quality and the standardization of allergen extracts containing contaminated non-allergenic proteins and microbial components susceptible to trigger adverse innate immune events, 2) the potential induction of early IgE-

and late T cell-mediated side effects through frequent administrations of folded allergens displaying IgE binding and T cell epitopes and 3) the duration of AIT (3 to 5 years) for the reinstatement of long-term tolerance, affecting patient adherence.

During the last 2 decades, molecular immunotherapeutic approaches were continuously tailored to improve the safety and efficacy of AIT.[6] However, the optimization of these molecular strategies remains cumbersome and empirical due to the lack of validated candidate biomarkers predictive for successful AIT. However, AIT promoting the development of allergen-specific IgG4 antibodies seem to be the most promising treatment option as the blocking activities of IgG have been correlated with the clinical efficacy of AIT. Remarkably, efficient Phase 1b studies using single passive immunization with anti-Fel d 1 monoclonal IgG4 antibodies have not only confirmed the key role of blocking IgG4 antibodies, but strengthened the concept of antibody-based passive AIT developed more than 100 years ago by Dunbar.[7]

Non-infectious virus-like particles (VLPs), made by spontaneous self-assembly of recombinant viral coat/envelop proteins in the absence of viral genetic material, are highly immunogenic and promote potent neutralizing antibody responses.[8] Moreover, these nanostructures can display arrays of heterologous antigens in order to optimize vaccine formulation.

The goal of the present review is to evidence the tremendous potential of VLP-based immunotherapies in the control of allergic diseases. Three main approaches will be described: VLP displaying allergens or pro-Th2/Th2 cytokines as well as VLP encapsulating CpG motifs.

VLPS: HIGHLY IMMUNOGENIC SELF-ASSEMBLING PROTEIN STRUCTURES

VLPs represent stable nanostructures resulting from the spontaneous self-assembly of recombinant viral structural protein(s).[8] Importantly, VLPs are non-infectious due to the lack of viral genomic material into the particle.[9] As VLPs adopt the conformation of authentic native viruses, these particles could constitute efficient human vaccines as evidenced by HBV and HPV subunit vaccines made by the self-assembly of HBV surface antigen and HPV L1 major capsid protein, respectively.[8]

Whereas subunit vaccines consisting of poorly immunogenic monomeric antigens require formulation with potent adjuvants, accumulating evidence showed that viral nanoparticle vaccines exhibit much superior immunogenicity through several intrinsic properties summarized in **Fig. 2**.

Optimal size for efficient drainage to lymph nodes (LNs) and uptake by antigen-presenting cell (APC)

LNs are key organs to stimulate adaptive B and T cell responses and the drainage of antigens to LNs depends largely on their size. Injected materials < 10 nm are commonly drained from the capillaries into systemic circulation. Remarkably, VLPs of 10- to 100-nm in diameter are freely drained to LNs by direct diffusion through 200-nm pores of the lymphatic vessel wall.[10] The active transport of VLPs to LNs can also be mediated by APCs at the injection sites (mainly DCs and macrophages) following particle uptake and processing. In the absence of specific functions targeting at APCs (through mannosylation notably), clathrin-dependent
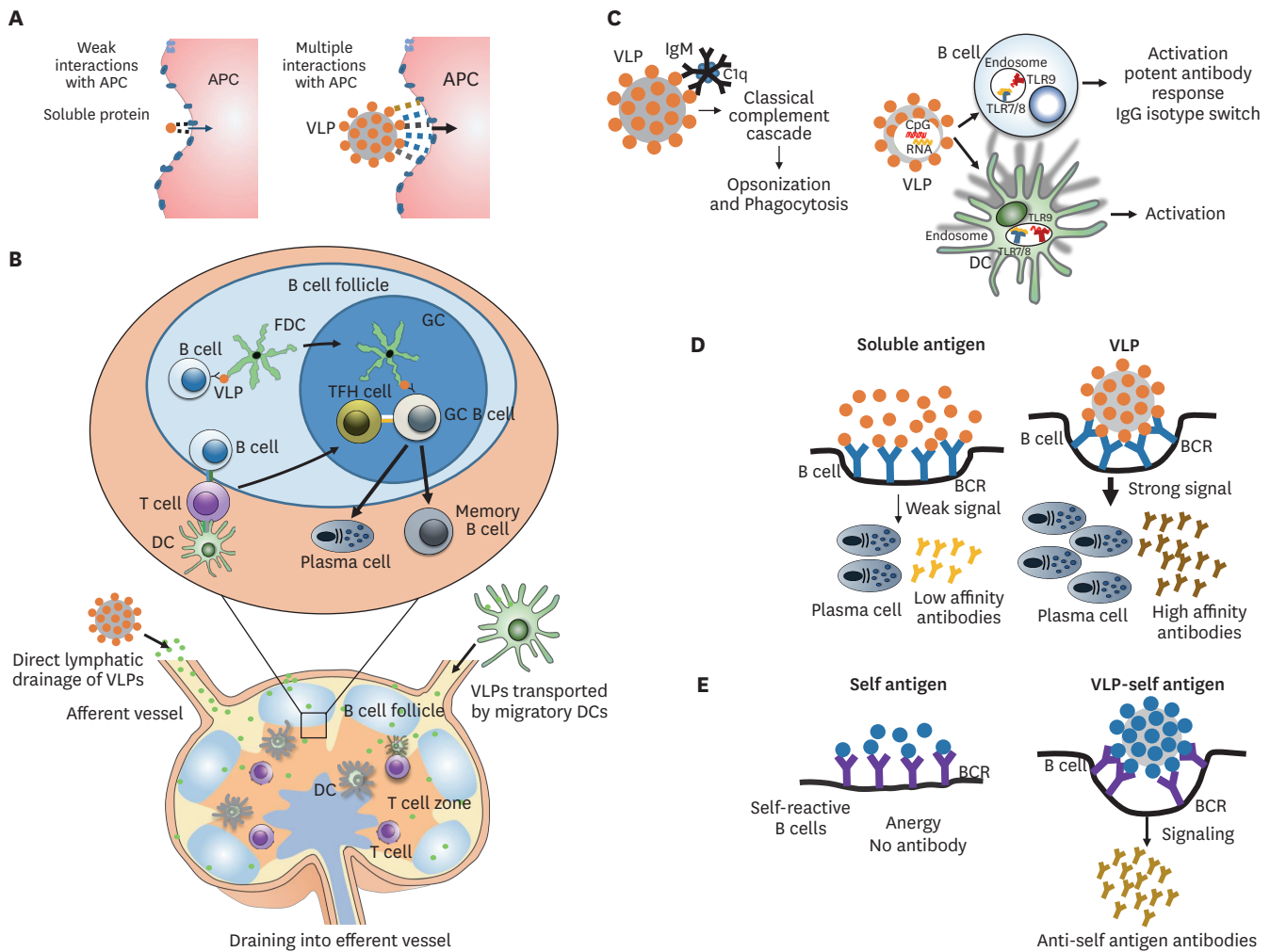
Fig. 2. Key properties of VLPs favoring their potent immunogenicity. Several immunological and physicochemical parameters can explain the superior immunogenicity of VLPs in comparison to soluble antigens. (A) Efficient uptake by antigen-presenting cells. (B) Direct trafficking to the lymph nodes. (C) Stimulation of innate immune signaling. (D) Optimal interactions with BCR for potent antibody response. (E) Induction of autoantibodies by the high density display of self-antigens on the VLP surface.

VLP, virus-like particle; BCR, B cell receptor; APC, antigen presenting cell; GC, germinal center; DC, dendritic cell; FDC, follicular dendritic cell; Tfh, T follicular helper; C1q, complement component 1q; CpG, unmethylated CpG motif rich DNA; TLR, Toll-like receptor; Ig, immunoglobulin.

macropinocytosis and phagocytosis represent the most prominent uptake mechanisms for VLPs.[10] The size and probably the shape of VLP greatly optimize a wide variety of interactions (ionic, hydrophobic and hydrophilic) between repetitive antigens and the APC surface in favor of the uptake.

Optimal interaction with B cells

When intact VLPs directly access LNs, the native state of the antigen and consequently the integrity of conformational epitopes for follicular B cell activation are conserved. The repetitive highly ordered and quite rigid para-crystalline antigenic surface found in viruses/VLPs is absent in the repertoire of human antigens and could represent a structure-dependent pattern capable to trigger rapid and strong antibody responses.

Contrary to monomeric antigens which interact poorly with a single B cell receptor (BCR), the array of epitopes displayed by VLPs can engage multiple specific BCRs simultaneously

on the B cell surface. Such potent cross-linking provides stimulatory BCR signaling for B cell activation and leading to T cell-independent antibody response.[11,] The strength of such BCR clustering depends on several parameters such as the BCR affinity for the antigen, antigen valency and antigen spacing (density and steric arrangement). Reports showed that 15–20 haptens or 60 peptide epitopes per particle, both spaced by 5–10 nm, represent ideal epitope spacing for optimal B cell activation.[11,12] Comparative immunogenicity studies on different forms of vesicular stomatitis virus G protein confirmed the positive correlation between antigen density and intensity of antibody responses.[13] Finally, low-density display of viral antigens can be actually used by the virus to evade host neutralizing antibody responses as shown for the naturally sparse human immunodeficiency virus (HIV)-1 envelope glycoprotein trimers (up to 23 nm).[14] The contribution of the rigidity of VLP to the production of antibodies is a matter of debate as comparative studies using capsid-based particles and enveloped ones produced conflicting data.[15]

Innate immune activation

Although natural IgM antibodies display low affinity for VLP surface, its pentameric structure allows higher avidity interactions with repetitive antigen organization.[16] Such binding triggers C1q complement protein activation for the initiation of the classic complement cascade, resulting in phagocytosis and antigen processing by APCs.[17]

The association of IgM/IgG and complement proteins to VLPs can facilitate the binding of VLP to the complement receptor CD21 on the B cell surface.[18] Such immune complexes are subsequently shuttled from B cells to follicular DCs within B cell follicles, a key event for the germinal center reaction.[18,19]

VLPs can retain the viral capacity to package nucleic acids.[9] As the viral genome is not present for the heterologous expression of VLPs, the particles can package host-cell genetic materials during the assembly process. Typical examples are VLPs derived from ssRNA bacteriophages in bacteria which encapsulate host-cell RNA and potent Toll-like receptor (TLR)7/8 activators.[20] Moreover, it is possible to substitute in these particles packaged RNA by unmethylated CpG motif rich DNA sequences for TLR9 activation.[9]

Nucleic acids packaged within VLPs drastically amplify B cell response as B cell-intrinsic TLR signaling is shown to be critical for B cell differentiation and up-regulation of germinal-center formation leading to the generation of long-lived antibody-secreting plasma cells and memory B cells.[21] Of note, it was recently evidenced that few ssRNA-derived VLP-specific B cells represent primary APCs to promote differentiation of antigen-specific T follicular helper cells.[22] Consequently, the germinal center response was fully independent of DCs.

Finally, TLR7 or TLR9 signaling on B cells mediates the isotype switching of the antibody response elicited by VLPs packaging nucleic acids, bacterial RNA-directing specific IgG2a/c in mice and IgG1 in humans.[23]

Splintering of B cell tolerance by VLPs

Originally reported by Chackerian et al.,[24] the VLP display of self-antigens is capable to break B cell tolerance in order to induce potent autoantibody response, whereas the immune system is not responsive to soluble self-antigens even formulated with strong adjuvants or at high doses. The density of self-antigens on the particle surface was shown to be critical

for the efficient production of autoreactive IgG.[25] Studies in mouse models evidenced that the presence of foreign Th cell epitopes are indispensable to successfully overcome B cell tolerance to soluble self-antigens.[26] Consequently, VLPs displaying self-antigens bring double safety features: a repetitive structural self-antigen presentation together with assistance of VLP-specific cognate Th cells. VLP-self antigen safety was confirmed by preclinical studies evaluating active vaccination against IL-17 in mouse models of chronic inflammatory diseases.[27] Sustained anti-IL-17 antibody responses generated by VLP-murine IL (mIL)-17 returned to background level following completion of the immunization protocol. Finally, the induction of IL-17 levels by imiquimod (TLR7/8-dependent IL-17 activator) in vaccinated mice did not promote endogenous boosting of anti-IL-17 response, confirming the importance of VLP T cell epitopes to break B cell tolerance.

Harnessing the geometry of viruses for antigen scaffolds

Several strategies have been explored for the presentation of foreign antigens at high density on the surface of these protein scaffolds to generate chimeric VLPs. Classic methods for VLP multimeric decoration consist in either genetic fusion or chemical conjugation using cross-linkers.

Incorporation of foreign antigen into self-assembling protein by genetic fusion induces display of foreign proteins in a viral-like configuration.[15,28] Such fusion can take place at the termini or into a loop of the scaffold protein. Commonly observed folding issues at the level of foreign antigen or core protein can preclude chimeric VLP assembly or negatively impact antibody production. Consequently, this method is best suited for the display of small peptides but restrict antigen-specific antibody production to responses against single exposed epitopes.

Chemical conjugation of proteins to preassembled VLPs is an alternative strategy for the modular display of larger antigens.[28] VLPs and antigens are produced and purified separately and subsequently coupled by cross-linkers which usually react with lysine residues on VLP surface and single cysteine residues present or incorporated into the antigen. Of note, the introduction of cysteine residues can be challenging and induce antigen misfolding. The presence of multiple lysine residues onto the VLP surface results commonly in heterogeneous coupling reaction and antigen orientation or even destabilization of the protein scaffold.

Tag/Catcher plug-and-display decoration is a new elegant approach for chimeric VLP production.[15] The conjugation system is based on the splitting of fibronectin-binding protein from *Streptococcus pyogenes* into the peptide (SpyTag, 13 aa) and the protein fragment (SpyCatcher, 116 aa). Spontaneous isopeptide bond is formed when the 2 split units are mixed in solution. Self-assembly of Acinetobacter phage AP205 coat protein fused with SpyCatcher or SpyTag molecule generates VLPs capable to plug and display SpyTag- or SpyCatcher-antigen respectively.[29,30] Such versatile and modular VLP platform successfully displayed structurally different antigens ranging from 2.2 to higher than 300 kDa.[29,31] The unidirectional antigen multimerization offered by such approaches was shown to induce higher biologically active antibodies than when antigen orientation is uncontrolled (as for chemical conjugation).[15]

VLP-BASED IMMUNOTHERAPIES FOR THE TREATMENT OF ALLERGIC DISEASE

Three different immunotherapeutic approaches exploited the immunogenicity of VLPs for the control of allergic disease: VLPs encapsulating CpG motifs, VLPs decorated with allergens or cytokines.

Bacteriophage-based VLP packaged with CpG motifs

As explained above, ssRNA bacteriophage-based VLPs spontaneously encapsulate bacterial RNA during the self-assembly of the coat protein. However, these particles can be disassembled and reconstituted in the presence of synthetic CpG-rich oligodeoxynucleotides (ODNs) for the generation of VLPs packaged with these TLR9 ligands.[9] In humans, TLR9 is mainly expressed by plasmacytoid DCs and B cells. Whereas pDCs produce TLR9-dependent type I interferons (notably IFN-α), it is well known that the main effects of TLR9 signaling are the release of Th1-promoting cytokines and chemokines such as monocyte inflammatory protein-1, IFN-γ-inducible 10-kDa protein and IgG class switching in B cells.[32] According to the pro-Th1 activity of unmethylated CpG ODNs, VLPs packaged with TLR9 ligands were evaluated in clinical trials for reprogramming Th2-biased allergic response.

The potential anti-allergy effects of QbG10, a Qβ bacteriophage-based VLP loaded with type A CpG ODN, were first investigated in a phase I/II trial, enrolling 20 house dust mite (HDM)-allergic patients treated with subcutaneous injections of HDM allergen extracts.[33] QbG10 was well tolerated and the typically observed mild adverse effects were only attributed to HDM extracts. Administrations of QbG10 boosted specific IgG1 and IgG4 responses in all patients. Specific IgE levels transiently increased at the initiation of SCIT, but dropped at the end of the protocol. Conjunctival allergen provocation tests evidenced that QbG10 induced a 100-fold increase in allergen tolerance. Symptoms of rhinitis and allergic asthma were drastically and significantly reduced, this sharp decline in the symptoms was prolonged for at least 38 weeks after treatment.

A second trial assessed the clinical efficacy of QbG10 in allergen-free immunotherapy. In this phase IIb study (n = 299), 2 different doses (0.5 or 1 mg) of QbG10 were subcutaneously administered 6 times in HDM-sensitized patients with allergic rhinoconjunctivitis symptoms.[34]

The treatment was not only safe, but reduced average combined symptom and medication scores significantly at a higher dose than placebo. Quality of life after treatment of patients at 1 mg QbG10 dose improved markedly as well. Finally, patients in the high-dose group tolerated a 10-fold increase in conjunctival provocation dose.

Although promising clinical data were similarly obtained with QbG10 for the control of mild-to-moderate persistent allergic asthma in patients upon steroid withdrawal,[35] VLPs carrying CpG ODN did not trigger any benefits in a phase 2b study for the treatment of moderate to severe allergic asthma.[36] Due to these disappointing results, no further investigations on QbG10 were pursued to our knowledge.

VLP displaying full-length allergens or allergen peptides

Several VLPs decorated with allergens or allergen peptides were evaluated for the immunomodulation of the allergic response (**Table 1**). The first vaccine candidate used p1 protein, a protein scaffold from yeast retrotransposon Ty to present an immunodominant

Table 1. Design and testing of VLP displaying allergen or allergen-derived B/T cell epitope

VLP platform	Antigen	Size (nm)	Mode of display	Injection route	Test	Ref.
Ty	Der p 1 peptide	60	Genetic fusion	Intraperitoneal	Therapeutic vaccinations in Der p 1 sensitization mice model	37
	Asp f 2 peptides			Subcutaneous	Therapeutic vaccinations in Asp f 2 sensitization mice model	38
AAV-2	OVA peptide	N.A.	Genetic fusion	Subcutaneous	Immunogenicity study and safety evaluation in mice model of ovalbumin-induced active anaphylaxis	39
MoMLV	Art v 1	90 (shielded Art v 1) 110 (surface exposed Art v 1)	Genetic fusion	Intranasal	Prophylactic vaccinations in mugwort pollen extract-induced mice asthma model	42
Qβ	Fel d 1	N.A. but 30 nm before coupling	Chemical conjugation	Subcutaneous	Treatment of Fel d 1-induced active anaphylaxis in mice	43
					In vitro allergenicity and binding studies	44
CuMVTT	Fel d 1	N.A. but 39 nm before coupling	Chemical conjugation	Subcutaneous	Treatment of Fel d 1-induced active anaphylaxis in mice	27
				Intramuscular	Safety and Immunogenicity studies in cats	46
				Subcutaneous	Impact of cat vaccinations with VLP-Fel d 1 on cat allergic patients owning cats	47
	Ara h 1			Intraperitoneal	Treatment of peanut allergen extract-induced active anaphylaxis in mice	48
	Ara h 2				Therapeutic vaccinations in chronic model of peanut allergy	
AP205	Der p 2	50	SpyCatcher/SpyTag conjugation	Intramuscular	Prophylactic vaccinations in HDM-induced mice asthma model	49

VLP, virus-like particle; Ty, Yeast retrotransposon Ty; N.A., not available; AAV-2, adeno-associated virus-2; OVA, ovalbumin; MoMLV, moloney murine leukemia virus; Qβ, bacteriophage Qβ; CuMVTT, cucumber mosaic virus-based virus-like particle containing a tetanus toxoid universal T cell epitope; AP205, acinetobacter phage AP205; HDM, house dust mite.

T cell epitope of Der p 1 (aa 111-139) in order to optimize cell-mediated immune response.[37] In Der p 1-sensitized mice, Ty-Der p 1 was shown to down-regulate IL-5 secretion from Der p 1-specific Th2-biased response and to abrogate Der p 1-specific T-cell proliferation as well. These positive effects were not accompanied by IFN-γ release, suggesting that Ty-Der p 1 mediates anergy of Der p 1-specific Th2 cells instead of a shift from Th2 to Th1 polarization. Ty-based VLP displaying major T cell epitopes of *Aspergillus* allergen Asp f 2 similarly triggered T cell tolerance as well as modulated B-cell response, reducing specific IgE, IgG2a, but not IgG1 or IgG2b levels.[38] However, the abrogation of T cell response was not long lasting and could be restored following subsequent allergen challenge.

VP3 capsid protein from adeno-associated virus 2 (AAV-2) was genetically fused with B cell epitope of ovalbumin (OVA), resulting in chimeric VLP containing 60 copies of VP3-OVA.[39] Although AAV-OVA uptake by mouse immune cells was impaired by the OVA peptide insertion, the chimeric particle adjuvanted with alum or Montanide stimulated higher than or equal to OVA-specific IgG1 antibody response compared to monomeric OVA formulated with alum, respectively. Direct ELISA IgE, rat basophil leukemia (RBL-2H3) assays and *in vivo* anaphylaxis experiments confirmed that AAV-OVA administration hampered the typical development of specific IgE observed by immunization with OVA-alum, leading to the prevention of anaphylactic reaction upon intravenous OVA challenge.

Moloney murine leukemia virus (MoMLV)-based VLPs, produced by co-expression of Gag/Pol core proteins in eukaryotic cells, were engineered to present immunodominant T cell epitopes in the context of HLA Class II molecules and to express costimulatory elements (CD80, CD86 or CD58).[40] MoMLV-VLPs decorated with major mugwort pollen allergen Art v 1 epitopes (aa 25-36) could strongly activate Art v 1-specific T cells in the presence of CD80 or CD86, and without the intervention of any APCs. Omission of costimulatory molecule

expression trigged Art v 1-specific T cell anergy. Remarkably, the same particles expressing CD58 stimulated the differentiation of allergen-specific Tr1 cells secreting IL-10/IFN-γ. Unfortunately, the potential of this approach to modulate allergic responses was not further explored by animal studies.

Two other MoMLV-VLPs were recently designed: a particle co-exposing OVA-specific T cell epitopes as well as GM-CSF- and VLP- delivering full-length Art v 1.[41,42]

The co-display of OVA-specific CD4+ and CD8+ T cell epitopes together with GM-CSF induced expansion of murine CD11b+ splenocytes leading to the optimal activation and proliferation of OVA-specific T cells.[41] The weaker T cell proliferation measured with an equimolar mixture of VLPs displaying either OVA peptide or GM-CSF suggested that VLPs co-displaying arrays of adjuvant and antigens constitute efficient APC-targeting vaccines. Two MoMLV-VLPs delivering Art v 1 were created in order to compare the immunomodulatory properties of shielded or surface-exposed allergen.[42] For that purpose, the allergen was respectively fused to the MoMLV matrix protein p15 or to the glycophosphatidylinositol acceptor sequence of CD16b. Both VLPs were shown to equally stimulate Art v 1-specific T cells. Strikingky, shielded Art v 1 was clearly hypoallergenic, whereas the surface-exposed form of the allergen could activate similar levels of basophil degranulation in comparison to recombinant monomeric Art v 1. The differences in allergenicity between the 2 forms of Ar t v 1 was confirmed by intranasal sensitization experiments in Art v 1-specific mice. Whereas VLPs decorated with Art v 1 induced modest specific IgG or IgE, the particle encapsulating allergen was non-immunogenic. Intranasal prophylactic vaccinations with shielded Art v 1 prevented the development of mugwort pollen-associated allergic sensitization as well as airway inflammation. These beneficial effects were associated with the development IFN-γ/IL-10 cytokine responses and up-regulation of lung-resident Foxp3+ Treg cells. To our knowledge, this VLP platform shielding multi-copies of full-length allergens could constitute a unique nanoparticle strategy to modulate the allergic response through induction of T cell tolerance, but not through development of blocking antibodies.

Key detailed studies characterized the immunological properties of Qβ-derived VLPs displaying the recombinant form of major cat allergen Fel d 1 (rFel d 1).[43,44] Around 60-70 rFel d 1 molecules could be successfully chemically conjugated to each VLP, consisting of 180 copies of the Qβ coat protein. However, sodium dodecyl sulphate-polyacrylamide gel electrophoresis analysis evidenced heterogeneous Qβ-Fel d 1 population. The chimeric particle induced much more IgG responses than unconjugated rFel d 1 mixed with Qβ. Interestingly, rFel d 1 displayed on the particle surface was hypoallergenic, poorly activated degranulation of basophils from cat-allergic patients and non-reactogenic in rFel d 1-sensitized mice. Vaccination with a single dose of Qβ-Fel d 1 could efficiently desensitize rFel d 1-allergic mice. Fel d 1-specific IgG was shown to be the key effector molecule for the shutdown of allergic response. The protective role of Fel d 1-specific IgG in systemic anaphylaxis was mediated by FcγRIIb signaling, but independent of Treg cells and IL-10.

The hypoallergenicity of conjugated rFel d 1 could not be attributed to B cell epitope modifications once the allergen was displayed on VLPs because chimeric particles were able to generate potent IgG responses and to be recognized by human monoclonal IgE anti-Fel d 1.[44] Qβ-Fel d 1 failed to trigger the activation of human stem cell-derived mast cells, whereas equimolar amounts of rFel d 1 caused dose-dependent cell degranulation. Surface plasmon resonance studies and signaling pathway analysis revealed that, once displayed on VLP, rFel

d 1 was shown to weakly bind to immobilized IgE, and consequently, initiated poorly FcεRI signaling cascade for effective mast-cell degranulation

The low diffusion capacity of VLP-rFel d 1 together with the high allergen concentration present on each particle could explain the failure of this chimeric VLP to activate human mast cells. This concentration effect could be related to the well-known bell-shaped mast cell activation for which supraoptimal allergen concentration engages inhibitory mechanisms.[45]

As Th cells up-regulate the development of B cell response,[11] incorporation of potent T cell epitopes into VLPs could boost antibody development. According to the widespread pre-existing T cell memory against tetanus toxin (TT), universal TT-derived Th-cell epitope (aa 830-843) was fused to the cucumber mosaic virus (CuMV) coat protein.[27] Similar to Qβ, self-assembly of this fusion protein in bacteria generated CuMVTT-VLPs packaging host cell RNA. As previously observed with the Qβ-VLP platform, chemical conjugation of rFel d 1 to CuMVTT-VLP drastically increased the immunogenicity of the allergen, but prevented anaphylactic reactions in Fel d 1-sensitized mice.[27] Of note, an innovative approach to cat allergy treatment involved cat vaccinations with CuMVTT-rFel d 1.[46] In that context, Fel d 1 represents a self-antigen. CuMVTT-rFel d 1 was well tolerated and triggered potent anti-Fel d 1 high-affinity neutralizing antibodies in cats. The development of these anti-Fel d 1 IgG responses considerably reduced the allergen levels in cat tears, and consequently the allergenicity of this cat secretion. Interestingly, a recent but small (10 cat-allergic participants) clinical trial evidenced that cat immunizations with CuMVTT-rFel d 1 could relieve clinical symptoms of cat owners.[47]

The same CuMVTT-VLP platform was used to display the peanut allergen Ara h 1 or Ara h 2.[48] Despite weak allergen-coupling efficiency (20% to 30%), CuMVTT-rAra h 1/rAra h 2 was shown to be hypoallergenic and able to develop in peanut-sensitized mice protective IgG response against anaphylaxis. Moreover, in a chronic animal model of peanut allergy, CuMVTT-rAra h 1 restrained eosinophilic and mast cell infiltration of gastrointestinal tissues after oral challenge to peanut. The protective effects mediated by CuMVTT-rAra h 1 was dependent on inhibitory FcγRIIb receptor engagement highlighting the key role of IgG complexed with allergen in regulating anaphylaxis. Such protection was transferrable as passive immunization with IgG antibodies generated with CuMVTT-rAra h 1 could similarly control anaphylactic reactions.

The first VLP-allergen designed by SpyCatcher/SpyTag Plug and Display technology consisted in the coupling of the major HDM allergen Der p 2 to the coat protein of Acinetobacter phage AP205.[49] AP205 VLP-rDer p 2 preparations were remarkably homogenous. Complete covalent bond formation producing chimeric VLP subunits was achieved by the simple mixing of SpyCatcher-AP205 coat protein with rDer p 2-SpyTag. As AP205-VLP is formed from 180 copies of the coat protein, the same copy number of rDer p 2 was displayed on each particle. Whereas coated monomeric rDer p 2 and VLP-rDer p 2 shared similar reactivity to Der p 2-specific human IgE, competitive inhibition assays showed that VLP-Der p 2 in solution displays a stronger IgE binding capacity than rDer p 2. Strikingky and contrary to monomeric rDer p 2, VLP-rDer p 2 was unable to trigger degranulation of RBL-SX38 cells primed with human Der p 2-specific IgE. Unadjuvanted VLP-rDer p 2 elicited much higher anti-Der p 2 blocking IgG responses than monomeric rDer p 2 mixed with untagged VLP. Finally, in a mouse model of HDM allergy (intraperitoneal sensitization using rDer p 2 formulated with alum and intranasal challenges with HDM allergen extracts), prophylactic vaccination could

prevent development of HDM-induced allergic inflammation as evidenced by the absence of specific IgE and by a marked reduction in airway eosinophilia. Protection was associated with IFN-γ secretion as well as potent induction of specific IgG-blocking antibodies.

Despite the promising preclinical data obtained from several VLP-allergen vaccine candidates, their clinical evaluations were curiously limited to a single phase I safety and immunogenicity trial in healthy subjects (n = 24) on Qβ-VLP carrying a Der p 1 B cell epitope.[50] For that purpose, 2 unadjuvanted VLP doses (10 or 50 μg) as well as 2 routes of injections (subcutaneous or intramuscular) were compared. Qβ-Der p 1 vaccinations were well tolerated and induced dose-dependent IgG1 and IgG3 responses to Qβ and its corresponding Der p 1 peptide. Comparable specific antibody levels were obtained with both routes of injection. Surprisingly, 2 booster doses did not influence the IgG development, and high antibody levels were detected for 4 months after the first immunization, followed by a 75% drop in antibody titers within 1 year. Of note, the blocking capacity of Der p 1-specific IgG was not tested in that study.

VLP displaying Th2 or Th2-cytokines

A range of monoclonal therapeutic antibodies targeting Th2-specific or epithelial-derived innate cytokines, including IL-4, IL-5, IL-13, TSLP, IL-33, IL-25, as well as their corresponding receptors were developed for the treatment of respiratory allergic or allergic skin diseases.[51] Although administration of these biologics are not curative, a large collection of clinical trials evidenced their capacity to improve the quality of life of patients with poorly controlled allergic conditions. The selection of a biologic-based personalized treatment should depend on disease endotypes and predictive biomarkers. However, the cost of passive antibody therapy is high because of frequent high-dose administrations of these biologics. Undesired side-effects, including the generation of anti-antibody responses, are usually observed. Moreover, the design, production and purification of biologics are still a long-lasting and expensive process.

Therefore, different VLP platforms decorated with Pro-Th2 or Th2cytokines were evaluated for their capacity to generate high polyclonal autoantibody titers. Their efficacy to control allergic inflammation was exclusively tested in preclinical studies up to now (**Table 2**).

The first VLP displaying endogenous Th2 cytokine consisted in truncated hepatitis B core antigen (HBcAg) fused to a B cell epitope of mIL-4.[52] In an acute OVA-induced asthma model, vaccinations with HBcAg-mIL4 elicited high levels of anti-mIL4 IgG which prevented the induction of OVA-specific IgE as well as eosinophil infiltrates in the lung and goblet cell hyperplasia. Similar protective results were reproduced with HBcAg-mIL-13 peptide which suppressed IL-13 response in the lungs.[53] Interestingly, HBcAg-IL-13 treatment was more efficient in the control of OVA-specific allergic response when administered intranasally, eliciting notably IL-13-specific IgA in mucosal tissues.[54] This vaccine was also capable to prevent chronic airway inflammation and remodeling,[55] but failed to revert established chronic airway inflammation and hyperresponsiveness.

As IL-23/Th17 pathways contributed to allergic disorders such as atopic dermatitis (AD) and severe and steroid-resistant asthma, HBcAg was fused with a peptide of murine IL-23p40.[56] Active immunization against mIL-23p40-induced sustained and long-lasting autoanti-p40 IgG response which reduced significantly OVA-specific skin and airway inflammation.

Table 2. Design and testing of VLP displaying cytokine or cytokine-derived B cell epitope

VLP platform	Antigen	Size (nm)	Mode of display	Injection route	Test	Ref.
HBcAg	mIL-4 peptide	25	Genetic fusion	Subcutaneous	Prophylactic vaccinations in OVA-induced mice asthma model	52
	mIL-13 peptide				Prophylactic or therapeutic vaccinations in OVA-induced mice asthma model	55
	mIL-23 p40 peptide				Prophylactic vaccinations in OVA-induced mice atopic dermatitis or asthma model	56
	mIL-33			Subcutaneous/ intranasal	Prophylactic vaccinations in OVA-induced mice asthma model	57
Qβ	mIL-5 mEotaxin	N.A. but 30 nm before coupling	Chemical conjugation	Subcutaneous	Prophylactic vaccinations in OVA-induced mice asthma model (single or combination)	58
CuMVTT	cIL-31	N.A. but 39 nm before coupling	Chemical conjugation	N.A.	Prophylactic vaccinations in HDM-induced canine atopic dermatitis model	59
	eIL-5			Subcutaneous	Prophylactic vaccinations in seasonal IBH affected horses (1 year)	60
					Prophylactic vaccinations in seasonal IBH affected horses (2 years)	61
	eIL-31				Prophylactic vaccinations in seasonal IBH affected horses	62
	eIL-5 mIL-5				Safety studies in healthy mice or IBH-affected horses	65

VLP, virus-like particle; N.A., not available; HBcAg, hepatitis B virus core antigen; OVA, ovalbumin; mIL, murine interleukin; Qβ: bacteriophage Qβ, CuMVTT: cucumber mosaic virus-based virus-like particle containing a tetanus toxoid universal T cell epitope; HDM, house dust mite; IBH, insect bite hypersensitivity.

The entire sequence of mature murine alarmin IL-33 (aa 1-149) was successfully inserted into the main loop of truncated HBcAg.[57] Expressed chimeric HBcAg-mIL-33 retained its ability to assemble into VLP. The vaccine was highly immunogenic in mice, even in the absence of adjuvants as shown by the induction of elevated anti-mIL-33 IgG and IgA titers. Moreover, a low specific IgG1/IgG2a ratio indicated that HBcAg-mIL-33 is particularly prone to mediate Th1-biased response. In an OVA challenge model of asthma, prophylactic vaccinations with these chimeric VLPs prevented airway inflammation and notably the release of mIL-33 in the bronchoalveolar lavage fluid. The Qβ-based VLP platform was used to display murine IL-5 or eotaxin by chemical conjugation, both cytokines playing key roles in eosinophil recruitment and activation.[58] As previously observed with Qβ-allergen,[43,48] heterogeneous chimeric Qβ particles were obtained by the chemical conjugation of IL-5 or eotaxin on the particle surface. Nevertheless, both VLPs could break self-tolerance and triggered potent anti-cytokine autoantibodies. The 2 vaccines similarly reduced OVA-mediated eosinophilic airway inflammation.

Veterinary vaccines were designed against AD in dogs and insect bite hypersensitivity (IBH) in horses[59-62] and consisted in CMVTT-VLPs decorated with canine or equine IL-5 and IL-31.

AD is frequently diagnosed in dogs and causes extensive IL-31-dependent itching, affecting the quality of life of animals and the owners.[63] IBH is the most common skin allergic disease of horses characterized by seasonal pruritic allergic dermatitis caused by *Culicoides* insect bites.[64] Th2-derived IL-31 was shown to play a crucial role in pruritus development. Moreover, predominant inflammatory cells accumulating in IBH skin lesions are eosinophils, and the severity of IBH was associated with the eosinophil levels in the blood.[64]

Dogs with HDM-induced AD and treated with CMVTT-cIL-31 mounted robust anti-IL-31 autoantibody responses.[59] A net decrease in symptoms of itching was observed in immunized dogs. The level of reduced scratching was correlated with the anti-cIL-31 antibody titers. Unadjuvanted CMVTT-eIL-5 was shown to be well tolerated in horses and to elicit strong

anti-eIL-5 autoantibodies.[60] The anti-eIL-5 response persisted for at least 6 months after the last boost but declined later, suggesting that the anti-eIL-5 response was reversible. In the treatment year and as compared to pre-season, an average reduction of 50% in IBH lesion scores was measured in about 50% of horses by active immunization against eIL-5. Comparable blood eosinophil counts between vaccinated and placebo groups indicated that anti-IL-5 responses affect mainly eosinophilic infiltrations into skin lesions. The semi-crossover follow-up study along a second treatment year in vaccinated horses revealed that a single booster dose of the vaccine mediated better and durable protection against IBH, due to a more stable antibody response.[61]

In the same horse model of IBH, CMVTT-VLP displaying equine IL-31 similarly reproduced the anti-inflammatory effects previously observed with the display of eIL-5: potent induction of anti-eIL-31 IgG levels and a significant reduction in lesion scores.[62]

The safety of vaccines targeting self-antigens needs to be particularly addressed before any future clinical trials, to preclude autoimmune adverse effects. Imperatively, the optimal control of autoreactive B cell response requires that induction of antibodies must be reversible and cannot be mediated by endogenous antigens in soluble form. Moreover, induction of T cell response against self-antigens must be strictly prevented. The recently characterized safety profile of CMVTT-eIL-5 could provide some guarantees.[65] Long-term vaccinations of horses with the chimeric VLP did not activate eIL-5 reactive T cells. In mice, the challenge of VLP-mIL-5-vaccinated mice with soluble mIL-5 did not boost the pre-established autoantibody response, whereas a booster dose of the vaccine significantly increased anti-mIL-5 IgG levels. The development of eIL-5-reactive antibody response was reversible as the autoreactive antibody concentrations from horses vaccinated with CMVTT-eIL-5 returned to pre-immune levels after each IBH season, but increased again following each boost of the vaccine.

DISCUSSION AND PERSPECTIVES

The pivotal role of allergen-specific blocking IgG responses for the successful treatment of allergic disease has recently been revisited. The IgG4 isotype is considered to be the main antibody subclass involved in IgE-blocking activity, and the European Academy of Allergy and Clinical Immunology guidelines recommended selecting biologically active allergen-specific IgG4 as a marker of therapy compliance.[66] The similar clinical efficacies observed with a single passive immunization with monoclonal IgG4 antibodies against Fel d 1 or 2 years of cat allergen extracts-based SCIT strengthened the importance of this blocking antibody response.[7] Of note, a single monoclonal antibody against the Ara h 2 peanut allergen has recently been shown to confer protection in a mouse model of peanut allergy.[67]

Consequently, VLP platforms displaying allergens would fulfil conditions for the development of potent antibody response. Particularly, the display of allergens in repetitive array optimizes BCR cross-linking stimulation to produce IgG with high affinity, a prerequisite for successful allergen neutralization.[68]

Data from different preclinical studies confirmed the potential of this molecular AIT. Remarkably, VLPs decorated with a single major folded allergen (Fel d 1, Ara h 1/2 or Der p 2) were hypoallergenic and could confer protection towards inflammatory responses mediated

by allergen cocktails (**Fig. 1**). Engagement of the inhibitory Fc receptor (FcγRIIb) by a single allergen/IgG complex would be sufficient to inhibit basophil/mast cell activation mediated by other allergens.[43,48] Therefore, these new vaccines could be effective for the most common allergies (*i.e.*, mite and pollen) showing patterns of multiple sensitizations. Translation of VLP-allergen-based AIT into humans will inevitably need large clinical controlled trials to compare their efficacy as well as safety with those measured for conventional allergen extract administrations. Attention will be paid to the longevity of the allergic desensitization. Although some VLP-allergen vaccine candidates display auto-adjuvanticity by the presence of host cell RNA into the particles, identification of judicious formulations with adjuvants will be crucial for warranting the development of prolonged blocking antibody response in humans. This optimization will be particularly decisive for vaccines based on VLP platform which do not encapsulate nucleic acids. Together with aluminium salts, conventionally used for allergen-based SCIT, other adjuvants such as L-tyrosine and MPL, Imiquimod could be promising for VLP formulations.

In comparison with monomeric soluble protein expression and purification, the scaling-up of VLP carrier production is more challenging in terms of stability, yield or reproducibility. More importantly, consistent allergen display on the particle surface is a key issue to warrant reproducible results. To our knowledge, the dependence of protective antibody response on allergen density optimization remains to be explored as well. Nevertheless, we speculate that this parameter is likely flexible as judged by successful suppression of allergic response already obtained with particles displaying only 30 to 50 copies of allergens.[43,48] Despite these challenges, it must be pointed out that 2 biotech companies, Anganys (Val-de-Reuil, France; www.angany.com) and Allergy Therapeutics (Worthing, UK; http://www.allergytherapeutics.com), were committed to this path and currently evaluated different VLP platforms for the development of molecular AIT against HDM, cat and peanut allergies.

Among different methods for modular decoration of VLPs, SpyCatcher/SpyTag Plug and Display technology can not only offer consistent antigen conjugation, but overcome issues associated with genetic fusion such as antigen size and steric hindrance. This modular display concept, associated with a single particulate scaffold, could rapidly generate any VLP-based vaccine, the race for creating a vaccine being particularly critical in the context of pandemics as coronavirus disease 2019.

The 2 other VLP-based approaches presented in this review are VLP-encapsulating CpG and particles decorated with endogenous pro-Th2/Th2 cytokines. As these molecules trigger allergen-independent immunomodulation, these particles could be suitable for the treatment of any allergic patients and particularly for subjects developing polysensitizations.

Phase I and II trials evidenced the potent immunomodulatory capacities of VLP carrying CpG for the treatment of allergic disorders. Remarkably, the absence of allergen administration prevented any risk of anaphylactic reactions. However, larger clinical studies are necessary to confirm the efficacy and safety of this allergen-free immunotherapy and particularly to elucidate the longevity and tolerability of the induced Th1 response (**Fig. 1**).

Several clinical studies evidenced the beneficial effects of therapeutic monoclonal antibodies targeting Th2 cytokines or their corresponding receptors for the treatment of uncontrolled asthma.[51] Notably 4 biologicals (2 anti-IL-5, 1 anti-IL5Rα and 1 anti-IL4Rα) were approved for eosinophilic asthma.[69] As mAb-based passive immunizations typically face several

downsides, strategies based on active vaccinations with VLP displaying endogenous pro-Th2/Th2 cytokines could substitute the use of these biologicals. Data collected from different preclinical studies confirmed that VLP decorated with cytokines can break B cell tolerance to promote anti-cytokine polyclonal autoantibody responses at titers sufficient to mediate the suppression of allergic responses (**Fig. 1**). A recent report on VLPs displaying murine or equine IL-5 highlighted the reversibility of the B cell response together with the maintenance of T cell tolerance.[65] Such long-term B and T cell safety could strengthen the clinical potential of this strategy to treat allergic patients. Of note, long-term face to face comparative clinical studies between VLP-cytokine and its corresponding anti-cytokine mAbs remain to be initiated. Several unanswered key questions need to be addressed before drawing a conclusion on the applicability of VLP-cytokine in allergy immunotherapy: 1) Can the inhibition of pro-Th2/Th2 cytokines compromise innate defense? 2) Does the retained biological activity of the cytokines displayed on VLP produce local or systemic side effects? 3) Is the blockade of a single pro-Th2/Th2 cytokine sufficient for the overall suppression of the allergic response? 4) Does the inhibition of one pro-Th2/Th2 cytokine globally affect the tightly regulated cytokine network?

Careful attention will need to be paid as well to the putative existence of pre-existing immunity directed to VLP carrier. Such VLP-specific antibodies could have a negative impact on the functionality of the 3 VLP-based vaccines described in this review. Moreover, these carrier-specific antibodies reduced the development of antibodies against antigens conjugated to the same carrier through a process called carrier-induced epitopic suppression.[70] In that context, the selection of VLP platforms derived from bacteriophages or plant viruses, both unable to infect humans, would minimize the pre-existing immunity issue.

In conclusion, the present review highlighted the applicability of VLP technology for the treatment or prevention of allergic disease. Although the future of these new vaccine candidates will depend on successful efficacy and safety trials, we believe that the revolution of molecular AIT takes a decisive step for the abandon of allergen extract use.

ACKNOWLEDGEMENTS

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