Chapter 10
Virus-Like Particles-Based Mucosal Nanovaccines

Introduction

Vaccination is a key, highly cost-effective intervention to fight against infectious diseases that has led to eradication of smallpox. Other diseases are expected to be eradicated in the short term, including poliomyelitis. Despite these outstanding benefits derived from vaccination, many obstacles in the development and deployment of novel or improved vaccines remain (Sheerin et al. 2017). Most of the currently used vaccines are based on whole microorganisms or viruses in attenuated or killed forms. Although these vaccines possess high immunogenicity and efficacy, some aspects could be improved such as better reactogenicity and reduction of the risk of developing the disease in some population groups (e.g., immunocompromised individuals). The development of safer vaccines can be achieved by generating subunit vaccines, which are formulated with a few antigenic components from the pathogen. One of the challenges to address in this regard is the poor immunogenicity of the individual antigens, which is a frequent issue due to their low molecular complexity and absence of PAMPs in the formulation to stimulate innate immunity mechanisms that subsequently trigger the induction of adaptive immune responses. Therefore, the inclusion of adjuvants in the subunit vaccine formulations is needed to achieve induction of strong and protective immune responses (Bastola et al. 2017).

Molecular tools and knowledge on viral structure have allowed generating novel biomedical applications such as gene therapy vectors. However, the most highlighted application in this sense is the production of virus-like particles (VLPs), which are multimeric protein complexes that resemble a virus lacking genetic material. Thus, they are unable to replicate or cause the disease, but retain the immunogenic activity since they mimic the virus in shape, size, and surface antigenic determinants. The high immunogenicity of VLPs could avoid the need of accessory adjuvants (Charlton Hume et al. 2019). In fact, some VLPs have been used as immunostimulants able to mediate protection against some diseases without the use of specific antigens, which is attributed to the activation of innate immune system
mechanisms (Wiley et al. 2009). The interaction of VLPs with the innate immune system is of major importance for the induction of adaptive immune responses. The innate immune cells recognize and interact with VLPs on the basis of two major characteristics: size and surface geometry (Mohsen et al. 2018).

Therefore, VLPs are highly attractive targets for vaccine development in terms of safety and efficacy when compared to vaccines based on attenuated or inactivated viruses. VLPs structure comprises one or more structural proteins that can be arranged in one or several layers and in some cases the particle is enveloped by a lipid membrane (Zhao et al. 2013). In many cases, the target antigen conforms the VLPs structure, thus the vaccine can be easily produced and purified. In other cases, VLPs are adopted to carry unrelated antigens from the vaccine target pathogen.

For decades, the development of VLPs-based vaccines against several pathologies has led to great advances in biomedicine, resulting in the commercialization of vaccines to fight human diseases such as those against the hepatitis B and E viruses and the human papillomavirus (Donaldson et al. 2018; Park 2012). Moreover, several VLPs-based vaccines candidates are currently under clinical evaluation in schemes comprising parenteral administration; these include vaccines against Norovirus (Leroux-Roels et al. 2018), malaria (Chichester et al. 2018), and influenza (Pillet et al. 2018). The present chapter is rather focused on the vaccines evaluated in mucosal immunization schemes since these are simple and safe immunization modes that could also lead to substantial savings in the cost of vaccination campaigns since no trained personnel or sterile devices are required. In fact, the formulation of oral or nasal vaccines based on dried formulations may avoid the use of cold chain distribution, which is the most practical way to expand vaccination coverage in developing countries at low cost. Moreover, nasal and oral immunization allows inducing attractive immune responses. For instance, the nasal route has been shown to be superior to parenteral administration for VLPs-based vaccines at eliciting IgA at distal mucosal sites, which is critical to protect the port of entry of most of the pathogens. The oral route, under optimal schemes, induces IgA intestinal responses that protect against intestinal pathogens and also provides systemic humoral responses (Nardelli-Haeffiger et al. 1999; Buonaguro et al. 2005).

There are currently only few vaccines approved for human use based on mucosal immunization schemes. An intranasal influenza vaccine has been approved for clinical use by the FDA, which is based on live attenuated viruses (Treanor et al. 1999; Nichol et al. 1999; Belshe et al. 1998). This formulation deserves improvement, for instance, replacing attenuated virus by inactivated virus or VLPs could reduce the risk for immunocompromised individuals to develop the disease (Tamura et al. 2016). In the case of oral vaccines, the oral polio vaccine based on attenuated virus has been used for decades contributing to the decline of poliomyelitis cases; nonetheless the emergence of poliomyelitis cases caused by vaccine-derived strains has been reported (Bandyopadhyay et al. 2015). The oral vaccine against cholera is another example of the few mucosal vaccines approved for human use. It is based on the whole killed bacterium plus the recombinant cholera toxin B subunit (Bi et al. 2017).
Design and Production of VLPs

The generation of vaccines based on VLPs first comprises the expression of the structural protein(s) that serves as scaffold to assemble the VLP. This process can occur in vivo or in vitro. Expression of VLP-forming proteins is performed in a genetically engineered host that includes bacteria, yeast, insect cells, plant cells, and mammalian cells. The choice of the host basically depends on the cost and requirements in terms of post-translational modifications (Fuenmayor et al. 2017). For instance, glycosylation may impact the VLPs immunogenicity; it has been reported that mannosylation enhances the uptake of VLPs by antigen presenting cells (Al-Barwani et al. 2014). The case of VLPs resembling enveloped virus typically requires the use of mammalian cell lines as these possess the cellular machinery to properly produce such particles. VLPs resembling phages are naturally and efficiently expressed in recombinant *E. coli*, which is a low-cost system when compared to insect or mammalian cells. Plants have also been used as low-cost expression platforms to efficiently produce not only VLPs derived from plant viruses, but VLPs resembling mammalian viruses and even enveloped viruses (Pillet et al. 2018).

VLPs can be exploited for vaccination in several ways, which are represented in Fig. 10.1. VLPs can be assembled in vivo resembling the viral assembly, but can also be assembled in vitro (either spontaneously or induced by changes in pH and salinity) after purification of the VLP-forming protein. In many cases, the VLP-forming protein is the vaccine antigen itself; thus the VLPs-based vaccine is obtained in a straightforward manner. In other cases, the VLP from a specific virus is used as scaffold to display unrelated antigens from the target pathogen. In this case, there are basically two methodologies to address this objective: genetic fusion and chemical coupling. The former consists in designing chimeric VLPs comprising the VLP-forming protein and the unrelated antigen. The site for the insertion of the foreign protein sequence should be selected properly to avoid alterations in the assembly properties of the scaffold protein and allow displaying the foreign antigen. In some cases, limitations in the length of the foreign protein exist and this should be considered during VLP design. The fusion gene is generated and expressed in the proper host and the resulting VLPs generally display the unrelated antigen on the surface. In this way, the chimeric VLP serves as a carrier of unrelated epitopes, which offers the possibility of producing multivalent vaccines by including several epitopes in the chimeric protein (Wu et al. 2019; Kingston et al. 2019).

Another strategy consists in the chemical coupling of peptides to the surface of VLPs. This methodology has been widely applied in the case of phage-derived VLPs. Phages and their proteins can be efficiently produced in recombinant bacteria at low and rapid processes. For instance, VLPs derived from the bacteriophage Qβ have been used as carriers of unrelated antigens (synthetic peptides) that are displayed in a repetitive fashion on their surface. Coupling of peptides and VLPs is typically based on using heterobifunctional linkers using a two-step conjugation scheme. Another reported approach consisted in substituting methionine residues in VLPs with analogues containing terminal azide (azidohomoalanine: AHA) and alkyne (homoproparglyglycine: HPG) groups. Azide and alkyne functionalized small molecules and proteins are subsequently coupled to VLPs using click chemistry (Strable et al. 2008). The obtained Qβ VLPs are highly immunogenic in mice.
(Lechner et al. 2002) and humans (Maurer et al. 2005). Interestingly, since RNA from the host (*E. coli*) is captured inside VLPs during assembly, it serves as adjuvant acting as Toll-like receptor ligand, enhancing the vaccine efficacy especially for the induction of IgG2a/c-dominated antibody responses (Forsbach et al. 2007). Besides antigenic peptides, Qβ VLPs have been used also as carriers of carbohydrate epitopes and nicotine for the development of vaccines against Leishmania infection and tobacco dependence, respectively (Moura et al. 2017; Maurer and Bachmann 2007). Interestingly, Bessa et al. (2008) demonstrated no differences in

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**Fig. 10.1** Representation of the strategies to produce VLPs useful in vaccine development. The VLP-forming protein is obtained by heterologous expression in an appropriate host (e.g., bacteria, yeasts, plant cells, and insect cells). VLPs can be assembled in vivo or in vitro after purifying the VLP-forming protein in its monomeric form. Genetic modification allows producing chimeric VLP that display unrelated antigens, making possible the production of bi or multivalent vaccines (I). Conjugation approaches allow the covalent attachment of unrelated epitopes (synthetic peptides) onto the VLP surface (II).
the efficacy of Qβ-specific systemic IgG and IgA responses following subcutaneous versus intranasal immunization, which augurs an interesting potential for the development of mucosal nanovaccines.

Another example of a phage used as scaffold to generate VLPs is the *Salmonella typhimurium* bacteriophage P22, which is a short-tailed phage with a dsDNA genome. The viral capsid is composed of 415 copies of a 46 kDa coat protein (CP) and up to 300 copies of a 34 kDa scaffold protein (SP), which are encapsidated within the immature procapsid. Non-infectious VLPs can be assembled by co-expressing CP and SP resulting in a ~60 nm diameter cage. A heterologous protein can be included in the VLPs by fusing it to the scaffolding domain of the SP and co-expressing it with the CP (O’Neil et al. 2011). Thus, modularity avoids possible alterations of the CP and VLP assembly.

Plant viruses can also serve as a source of VLPs-forming proteins applied in vaccine development. This is the case of the following viruses: AlMV, CMV, CPMV, PapMV, PVX, and TMV (reviewed by Balke and Zeltins 2018). Importantly, two plant virus-derived vaccines are currently under evaluation in clinical trials: a malaria vaccine based on AlMV and the Pfs25 antigen under a Phase I clinical trial (https://clinicaltrials.gov/ct2/show/NCT02013687) and PapMV VLPs under evaluation as adjuvant for the seasonal flu trivalent vaccine (https://clinicaltrials.gov/ct2/show/record/NCT02188810).

Interestingly, VLPs can be functionalized or loaded with accessory molecules to improve vaccine efficacy. For instance, VLPs can be loaded with adjuvants such as CpGs, which are TLR9 ligands that favor antigen processing by the innate immune system. In fact, it has been proven that co-administration of VLPs loaded with CpGs plus VLPs displaying a target antigen results in the delivery of both the adjuvant and the antigen into the same APC, which simplify vaccine production (Mohsen et al. 2017). Another approach consists in genetically fusing immunostimulatory signals to VLPs. For instance, HIV VLPs containing CD40L (an inducer of costimulatory molecules in APCs) showed high immunogenicity against Gag (Franco et al. 2011), SIV VLPs containing glycosylphosphatidylinositol (GPI)-anchored GM-CSF (cytokine acting as adjuvant) induced higher levels of neutralizing antibodies than plain SIV VLPs (Skountzou et al. 2007), and chimeric rabies virus-like particles (cRVLPs) displaying either membrane-anchored flagellin or LTB induced enhanced immunogenicity and immunoprotective capacity in mice and dogs (Qi et al. 2015; Fig. 10.2).

Although VLPs-based vaccines have become a reality in the clinical realm, these are parenteral vaccines and the development of mucosal formulations has progressed at a lower degree. This chapter provides a general outlook on the ongoing research looking to develop mucosal vaccine formulations against relevant human and animal diseases (Table 10.1).
Influenza

The influenza virus is a serious threat to global health leading to a high economic burden. Since this pathogen mutates continuously, the new variants evade preexisting immunity and thus new vaccines are required that in addition must be produced in a short time. The 2009 H1N1 pandemic and human cases of H5N1 and H7N9 indicate the great need for platforms able to result in effective vaccines in a short time (Chen et al. 2014). Lee et al. (2018) explored the potential of using VLPs based on the M1 matrix protein and a tandem repeat of M2 ectodomains (M2e5x) derived from the human, swine, Avian 1, and Avian 2 influenza viruses as an approach to induce broad cross-protection against influenza virus strains variants. The insect cell-made VLPs were administered to mice i.n. (dose: 15 μg of M2e5x VLP total proteins, 0.9 μg M2e5x proteins) and boosting was performed at week 4. Animals were challenged with either the A/Phil (H3N2) or A/Viet (rgH5N1) virus. M2e5x VLPs reduced weight loss, attenuated inflammatory cytokines and cellular infiltrates, decreased viral loads, and induced germinal center phenotypic B and plasma cells. The vaccine induced M2-specific antibodies in sera and mucosal tissues, conferring effective cross-protection against heterosubtypic influenza viruses. A balanced Th2/Th1 response was evident according to measurements of IgG2a and IgG1. Both CD4+ and CD8+ T-cells had a role in the observed protective effects.
| Pathogen/disease       | VLPs description                                      | Production host              | Test species | Description of immunization trial                                                                 | Immunization results                                                                 | Reference                  |
|------------------------|--------------------------------------------------------|------------------------------|--------------|--------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------|---------------------------|
| Rotavirus diarrhea     | VLPs based on bovine rotavirus RF VP2 and VP6          | Baculovirus-insect cell system | Mice i.n. i.r. | Single dose: 10 μg 2/6-VLPs alone or with 10 μg LT-R192G (a mutant version of the heat labile enterotoxin from *E. coli*) | Only the i.n. administered vaccine alone induced antigen-specific IL-10 and IL-17 secreting T-cells. IL-10-, in contrast to IL-17-, secreting T-cells did not migrate to the mesenteric lymph nodes (MLN), whereas they were detected in cervical lymph nodes (CLN) and spleen. The inclusion of LT-R192G improved the immunogenicity in terms of activating the production of IL-2 and IL-4, increasing IL-17 secretion, and inducing antigen-specific CD4+CD25+Foxp3+ and Foxp3− T-cells in all studied organs (CLN, spleen, and MLN), but did not impact on IL-10 secreting T-cells. In the i.r. immunized animals, LT-R192G induced IL-2 and IL-17 secretion. Nonetheless, in contrast to the IN route, it did not allow IL-4 production. No neutralization studies were performed. | Alkadah et al. (2013)        |
### Table 10.1 (continued)

| Pathogen/disease | VLPs description | Production host | Test species | Description of immunization trial | Immunization results | Reference |
|------------------|------------------|-----------------|--------------|-----------------------------------|----------------------|-----------|
| Porcine reproductive and respiratory syndrome | VLPs based on viral surface proteins GP5-GP4-GP3-GP2a-M or GP5-M | Baculovirus-insect cell system | Pigs i.n. | Two doses at a 2-week interval Dosage: 650 μg of VLPs entrapped in PLGA nanoparticles, *Mycobacterium tuberculosis* whole cell lysate (M. tb WCL) was used as adjuvant Challenge: RRSV strain 1-4-4 | Immunooassays confirmed the presence of all the proteins in the VLPs, thus retaining antigenic determinants. Pigs i.n. immunized with VLPs entrapped in PLGA nanoparticles induced an anamnestic immune response; an elevation in IgG and IFN-γ production was observed after the challenge. The vaccinated group showed a two-log reduction in the lung viral load | Binjawadagi et al. (2016) |
| Nervous necrosis virus (NNV) | VLPs based on the orange-spotted grouper NNV (OSGNNV) capsid protein | *E. coli* | Orange-spotted grouper Immersion i.m. p.o. | Twice at a 2-week interval Dosage: immersion (250 μg/g fish body weight, 1 mg of VLPs per 10 L seawater, 30 min. immersion), i.m. injection (dose: 2 μg/g FBW, 0.1 μg VLPs per fish), or orally (dose: 20 μg/g FBW, feeding for 4 days with food containing 200 μg/g VLPs, consumption of 5% of body weight) | OSGNNV VLPs elicited strong humoral responses, being immersion and i.m. immunization the routes inducing higher humoral responses than the oral scheme. Upon challenge, the relative percent of survival values of immersion, injection, and oral immunization were 81.9, 61.4, and 52.3%, respectively | Chien et al. (2018) |
| Pathogen/disease | VLPs description | Production host | Test species | Description of immunization trial | Immunization results | Reference |
|------------------|------------------|-----------------|--------------|-----------------------------------|----------------------|-----------|
| Foot-and-mouth disease virus | VLPs based on VP60 and NT15 proteins from the rabbit hemorrhagic disease virus (RHDV) carrying a T-cell epitope of the 3A protein of foot-and-mouth disease virus (FMDV) into the VP60 sequence | Baculovirus-insect cell system | Mice i.n. i.m. | Twice at a 2-week interval Dosage: 20, 60, and 180 μg; the i.m. group received Montanide ISA 206 as adjuvant | The chimeric VLPs activated immature porcine bone marrow-derived dendritic cells (poBMDCs) in vitro. IgG and IgA antibodies against RHDV-VLPs were induced. The adjuvanted, i.m. immunized groups showed the highest response. Anti-RHDV-VLP IgA responses were higher in groups i.m. immunized. The adjuvant group exhibited the highest IFN-γ secreting cell numbers and lymphoproliferative specific T-cell responses against the 3A epitope and RHDV-VLP. No neutralization or challenge experiments were conducted | Crisci et al. (2012) |
| Macrobrachium rosenbergii nodavirus (MrNV) white tail disease (WTD) in freshwater giant prawn macrobrachium rosenbergii | VLPs based on the capsid protein from Macrobrachium rosenbergii nodavirus (MrNV) | Baculovirus-insect cell system | Giant prawn Oral | Feeding during 60 days Dosage: 10 μg of MrNV in 30 g of food. Daily intake of about 50–100 ng of VLPs during the test period Challenge: MrNV filtrate coated diets | Survival rates of 65 and 80% were achieved at 30 and 60 days post-vaccination, respectively; whereas the non-vaccinated group had 90% mortality. Pathogen load estimated by PCR decreased to 32 and 17% at 30 and 60 dpv, respectively. The vaccine induced the expression of Mramp | Citarasu et al. (2019) |
| Pathogen/disease | VLPs description | Production host | Test species | Description of immunization trial | Immunization results | Reference |
|------------------|------------------|-----------------|--------------|-----------------------------------|----------------------|-----------|
| Hepatitis E virus | Chimeric VLPs based on the truncated HEV ORF2 (aa 112–660) fused to a B-cell epitope from the glycoprotein D of herpes simplex virus | Baculovirus insect cell expression system | Mice p.o. | Four times at 2-week intervals Dosage: 50 μg of VLPs | The chimeric VLP showed morphology similar to that of the mature HEV virion. The target epitope was recognized by a specific antibody The chimeric VLPs induced specific IgG and IgA to both the inserted epitope and HEV-VLPs in intestinal secretions (responses were developed since 2 weeks post-first immunization). No immunoprotection assays were reported | Niikura et al. (2002) |
| Hepatitis E virus | VLPs based on the HEV capsid protein gene lacking 111 amino acids at the N-terminal | Baculovirus-insect cell system | Cynomolgus monkeys (*Macaca fascicularis*), p.o. | Dosage: 10 mg of the VLPs on days 0, 7, 21, 36, and 80 Challenge: intravenous injection of 2 mL of an HEV virus stock | rHEV VLPs induced serum IgM, IgG, and IgA responses without adjuvant requirement. Upon challenge, one monkey was fully protected and in another one the infection occurred, but the disease was not developed. Fecal IgA was not detected in any of the orally immunized monkeys | Li et al. (2004) |
| Pathogen/disease | VLPs description | Production host | Test species | Description of immunization trial | Immunization results | Reference |
|------------------|------------------|-----------------|--------------|-----------------------------------|----------------------|-----------|
| Hepatitis B      | VLPs based on the hepatitis B virus small surface protein (S-HBsAg) | Lettuce plants | Mice i.m. p.o. | Experiment I. Mice orally immunized with the lyophilize (22 μg SHBsAg/g) at days 0 and 28 (test doses: 10–10, 10–500, 500–10, 500–500, and 100–100) Experiment II. Mice i.m. primed (day 0) with 500 ng of S-HBsAg from Engerix_B (adjuvanted with 5% v/v alhydrogel) and subsequently boosted on days 42 and 84 with the lyophilize containing 5, 50, or 200 ng of the antigen Experiment III. Experiment II, but booster immunization was performed with the tissue extract adjuvanted with 10% v/v alhydrogel | I.m. priming with the commercial vaccine followed by double oral boosting with plant vaccine was comparably efficient as the standard i.m. vaccination, being boosting doses of 2 or 200 ng efficacious at enhancing systemic responses. None of the tested adjuvants improved the response to vaccination and the efficacy of plant lysate was lower than that registered for intact plant cells, suggesting that bioencapsulation of S-HBsAg favors vaccine activity not requiring accessory adjuvants | Pniewski et al. (2018) |

(continued)
| Pathogen/disease | VLPs description | Production host | Test species | Description of immunization trial | Immunization results | Reference |
|------------------|------------------|-----------------|--------------|-----------------------------------|----------------------|-----------|
| Hepatitis B      | VLPs based on the hepatitis B virus core antigen gene (HBc) in which aa 75–85 were replaced by the LAH gene fragment (coding for aa 76–130 of HA2) | *E. coli* | Mice i.n. i.p. | Thrice at 2-week intervals Dosage: 1, 5, or 25 μg of LAH-HBc VLPs alone or with 0.4% chitosan in a 1:1 ratio or 1 μg CTB i.p.: 1, 5, or 25 μg of LAH-HBc VLPs alone or with alum as adjuvant challenge: Sh2/H7N9, Gz54/H3N2, and PR8/H1N1 influenza viruses | The test VLPs induced, at the 25 μg dose, both humoral and cellular immune responses effectively and provide complete protection against a lethal challenge with the homologous H7N9 virus or heterologous H3N2 virus, whereas the i.m. administration provided weak protection. Either of the test adjuvants provided full protection, whereas unadjuvanted vaccines led to lower protection rates. The i.n. vaccine resulted in partial protection against a lethal challenge with the heterologous H1N1 virus | Zheng et al. (2016) |
| Pathogen/disease | VLPs description | Production host | Test species | Description of immunization trial | Immunization results | Reference |
|------------------|------------------|-----------------|--------------|-----------------------------------|----------------------|-----------|
| Cancer           | VLPs based on the L1 protein from HPV-16 or HPV-18 | Baculovirus-insect cell system | Mice i.m. p.o. | Thrice; on weeks 0, 2, and 6 Dosage i.m.: 0.3 μg of HPV-16 or HPV-18 VLPs Oral: 1, 3, or 9 μg of VLPs; alone or co-administered with LTR192G (10 μg) or CpG DNA (10 μg) | VLPs co-administered with LT R192G induced higher serum IgG titers than VLPs alone. CpG DNA did not boost the response as LT R192G. Immunization with HPV-16 VLPs plus LT R192G induced higher vaginal anti-VLP IgG and IgA antibodies than VLPs alone or plus CpG DNA. Similar results were obtained with HPV-18 VLPs; however, both adjuvants enhanced IgA responses. Only VLPs plus LT R192G induced positive mesenteric lymphoproliferative responses. Properties characteristically associated with virus-neutralizing antibody specificities appeared to be unaltered by the tested adjuvants | Gerber et al. (2001) |

(continued)
| Pathogen/disease                      | VLPs description                                               | Production host                              | Test species | Description of immunization trial                                                                 | Immunization results                                                                                                                                                                                                 | Reference               |
|--------------------------------------|-----------------------------------------------------------------|----------------------------------------------|--------------|----------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------|
| Simian immunodeficiency virus        | VLP based on the Gag and Env proteins from SIV coupled to the cholera toxin B subunit (CTB) | Baculovirus-insect cell system               | Mice i.n.    | Four times at 2-week intervals Dosage: 40 μg of VLPs, VLPs (40 μg) plus CTB (10 μg) or CT (10 μg), or VLPs (40 μg) conjugated with 10 μg of CTB | Co-administration of VLPs and CTB induced higher levels of anti-SIV gp160 IgG and IgA in mucosae (saliva, vaginal-wash samples, lung, and intestine) and higher neutralization activity when compared to VLPs alone. Conjugation of CTB to VLPs also enhanced the SIV VLP-specific antibodies in sera and in mucosae to similar levels. CTB-conjugated VLPs induced superior cellular responses (IFN-γ-producing splenocytes and cytotoxic-T-lymphocyte activities) respect to the VLPs/CTB mixture; and enhanced IgG1 and IgG2a serum levels, indicating enhanced Th1- and Th2-type cellular immune responses | Kang et al. (2003)       |
| Pathogen/disease | VLPs description | Production host | Test species | Description of immunization trial | Immunization results | Reference |
|------------------|------------------|----------------|-------------|-----------------------------------|---------------------|-----------|
| Norwalk virus    | VLPs based on the NV ORF2 (without ORF3) | Plants \((N. benthamiana)\) | Mice i.n. | Twice at a 3-week interval i.n. dosage: liquid formulations containing 25 μg VLPs alone, 25 μg VLPs + 25 μg of Murabutide (MB), 25 μg VLPs + 100 μg MB, 25 mg VLPs + 250 mg MB, 250 μg MB alone, 25 μg VLPs + 1 μg of cholera toxin (CT), or 25 μg VLPs + 25 μg of gardiquimod (GARD) s.c. dosage: 25 μg VLPs + alum | MB showed the optimal adjuvant effect when co-administered at 100 μg, based on the magnitude of VLPs-specific IgG, IgG1, IgG2a, and IgA production in serum and VLPs-specific IgA production at distal mucosal sites. I.n. vaccination using VLPs with MB induced humoral responses in a similar magnitude to those achieved by the i.n. co-administration of VLPs and CT or GARD and i.m. immunization with VLPs plus alum. Interestingly, in terms of the induction of mucosal immune responses, the MB groups were equivalent to CT and GARD and induced comparable systemic responses to those generated by the parenteral vaccine plus alum | Jackson and Herbst-Kralovetz (2012) |

(continued)
| Pathogen/disease | VLPs description | Production host | Test species | Description of immunization trial | Immunization results | Reference |
|------------------|------------------|-----------------|--------------|-----------------------------------|----------------------|-----------|
| Norwalk virus    | NV ORF2 (without ORF3) | Plants (N. benthamiana) | Pigs, Mice i.n. | Twice at a 2-week interval Pigs dosage: GelVac plus NV VLPs (10 μg) or GelVac NV VLPs (10 μg) + GARD (10 μg). Comparable liquid formulations comprising: NV VLPs (10 μg), NV VLPs (10 μg) + GARD (10 μg), or NV VLPs (25 μg) + GARD (10 μg) Mice dosage: 25 μg of VLPs alone or plus GARD (10 μg) in a GelSite liquid or PBS liquid formulation | Powder formulations, with or without the NV VLPs antigen, were similar in structure in dry form or when rehydrated in simulated nasal fluids. The GelVac powder induced higher antibody responses at the systemic and mucosal (aerodigestive and reproductive tracts) levels when compared to liquid formulations. Inclusion of GARD did not increase immunogenicity of the dry formulation, while a positive effect was recorded for liquid formulations. The authors hypothesized that the test formulation, upon in situ gelation, allows VLPs stabilization that ends up prolonging their presence on the mucosal surfaces overcoming mucociliary clearance and thus extending antigen uptake by APCs in NALT | Velasquez et al. (2011) |
| Pathogen/disease | VLPs description | Production host | Test species | Description of immunization trial | Immunization results | Reference |
|------------------|-----------------|----------------|-------------|-----------------------------------|---------------------|-----------|
| Norwalk virus    | VLPs based on the ORF2 (without ORF3) from GI and GII.4 NV | Plants (N. benthamiana) | Pigs i.n. | Twice at a 3-week interval Dosage: doses ranging 0.1–100 μg of VLPs | The humoral response magnitude followed a dose-dependent pattern in both serum and vaginal washes. The 15 μg dose allowed reaching the maximum antibody responses. The neutralizing potential was assessed in an assay based on estimating the capacity of VLPs to bind porcine gastric mucin after incubation with sera from immunized animals. The neutralizing activity for both GI and GII.4 –VLPs was proven | Springer et al. (2016) |

(continued)
| Pathogen/disease                     | VLPs description                                      | Production host               | Test species                                                                                     | Description of immunization trial                                                                                                                                                                                                                                                                  | Immunization results                                                                                                                                                          | Reference                      |
|-------------------------------------|-------------------------------------------------------|------------------------------|------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------|
| Norwalk virus, acute gastroenteritis | VLPs based on the VP1 protein from NV GI.1           | Baculovirus insect cell expression system | Healthy, 18–49 years old subjects were i.n. immunized twice at a 3-week interval Study 1 evaluated doses of 5, 15, and 50 μg of VLPs, while study 2 evaluated 50 and 100 μg dosages Monophosphoryl lipid A (MPL), a TLR4 agonist, was used as adjuvant and chitosan as mucoadhesive | The vaccine induced no serious adverse events, with nasal stuffiness, discharge, and sneezing as the most frequent side effects. Anti-VLPs IgG and IgA levels increased 4.8- and 9.1-fold, respectively, for the 100 μg dosage level. All subjects receiving either 50 or 100 μg doses presented IgA antibody secreting cells (ASCs) measured in peripheral blood mononuclear cells. ASCs expressed molecules associated with homing to mucosal and peripheral lymphoid tissues (CD19⁺CD27⁺CD62L⁺, integrin α4/β7⁺). A subset expressing exclusively mucosal homing molecules was also detected (CD19⁺CD27⁺CD62L⁻ integrin α4/β7⁻) | El-Kamary et al. (2010)                                                             |
| Pathogen/disease | VLPs description | Production host | Test species | Description of immunization trial | Immunization results | Reference |
|------------------|------------------|-----------------|--------------|-----------------------------------|----------------------|-----------|
| Human immunodeficiency virus (HIV) | VLPs based on the HIV-1 Gag (HIVIIIB strain) and env genes (HIVBaL strain) | HEK cells | Mice i.n./cheek injection | Intranasal prime was administered on day 0, after which two boosts were delivered Sub-cheek on days 14 and 28 (200 μg dose). CALV and TLR ligands were co-administered at 25 or 100 μg, respectively | VLPs coupled with the TLR3 ligand dsRNA complexed to CALV and in combination with VLPs (CALV(dsRNA) + VLPs) induced the strongest humoral response against the target HIV antigens. Importantly, antibodies against clade c 96ZM651 gp120 were induced. A Th1 response was evidenced by IgG subclass analysis. The most potent neutralizing antibodies against HIV strain MN.3 was also generated by CALV(dsRNA) + VLPs, as well as a significant increase in germinal center B-cells and T follicular cells | Poteet et al. (2016) |
| Group A streptococcus/several infections | VLPs, based on the murine polyomavirus (MuPyV) VP1 protein, displaying the J81 antigen peptide from Group A Streptococcus | E. coli | Mice i.n. | Thrice at 3-week intervals Dosage: 92 μg of wt-VP1, VP1-GCN4-J8i-GCN4, or VP1-GCN4-J8i-J8i-GCN4 VLPs Challenge: GAS reference strain M1 | Both versions of VLPs, when delivered i.n. to outbred mice without adjuvant, induced significant titers of J8i-specific IgG and IgA antibodies, indicating significant systemic and mucosal responses, respectively. GAS colonization in the throats of challenged animals was reduced in these immunized mice, a mild protection against a lethal challenge was observed | Rivera-Hernandez et al. (2013) |

(continued)
| Pathogen/disease                          | VLPs description                                                                 | Production host                  | Test species | Description of immunization trial                                                                 | Immunization results                                                                 | Reference                        |
|------------------------------------------|----------------------------------------------------------------------------------|----------------------------------|--------------|---------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|----------------------------------|
| Severe acute respiratory syndrome coronavirus | VLPs based on the SARS spike (S) protein and the influenza M1 protein             | Baculovirus insect cell expression system | Mice i.m.    | Twice at a 3-week interval Dosage: 0.8 μg or 4 μg of SARS S or the VLPs vaccine, with or without aluminum hydroxide i.n.: 0.8 μg or 4 μg of SARS S or the VLPs vaccine Challenge: mouse-adapted SARS-CoV strain v2163 | The SARS VLPs vaccine at the 0.8 μg dose completely protected mice upon i.m. or i.n. administration. The SARS VLPs vaccine (4 μg dose) without adjuvant reduced lung virus titer below detectable levels, protected mice from weight loss, and elicited high level of neutralizing antibodies against SARS-CoV SARS S protein was also protective, but only when i.m. administered along with aluminum hydroxide | Liu et al. (2011)                |
| Respiratory syncytial virus              | VLPs based in bacteriophage P22, containing the matrix (M) and matrix 2 (M2) proteins from RSV | E. coli                          | Mice i.n.    | Two doses at a 3-week interval Dose: 100 μg of P22-Ctrl or P22-M/M2 i.n. challenge: using 2 × 10⁶ pfu of the RSV A2 strain | The test VLPs stimulated CD8+ T-cell memory responses against both antigens and induced tissue-resident T-cell populations. Challenged animals showed reduced lung viral titers | Schwarz et al. (2016)            |
| Pathogen/disease                  | VLPs description                                                                 | Production host                        | Test species | Description of immunization trial | Immunization results                                                                 | Reference |
|----------------------------------|----------------------------------------------------------------------------------|----------------------------------------|--------------|-----------------------------------|-------------------------------------------------------------------------------------|-----------|
| Respiratory syncytial virus      | VLPs based on the matrix protein (M) and fusion glycoprotein (F) of RSV          | Vero cells-adenoviral vector           | Mice i.n.    | Single dose                        | Dosage: 10 or 30 μg of VLPs or 2 × 10⁶ pfu/mouse                                    | Jiao et al. (2017) |
|                                  |                                                                                  |                                        |              | Challenge: RSV-long virus          | VLPs presented a positive immunoreactivity and function when compared to the RSV virion in vitro. The i.n. administered vaccine induced a Th1 polarized response, an effective mucosal virus-neutralizing antibody, and CD8+ T-cell responses at both mucosal and systemic levels, whereas the i.m. immunization only induced such effective response at the systemic level. Upon RSV challenge, i.n. immunized mice showed increased viral clearance, but decreased signs of enhanced lung pathology and fewer eosinophils when compared to mice immunized with formalin-inactivated RSV (FI-RSV). I.n. RSV VLPs induced a response that lasted for up to 15 months |          |
| Pathogen/disease | VLPs description | Production host | Test species | Description of immunization trial | Immunization results | Reference |
|------------------|------------------|-----------------|--------------|-----------------------------------|----------------------|-----------|
| Influenza virus/flu | VLPs based on M1, HA/M1, and cVLPs containing HA and M1 proteins derived from the influenza Aichi virus co-incorporated with GPI-anchored CCL28 | Baculovirus insect cell expression system | Mice i.n. | Twice at a 2-week interval Dosage: 1 μg of Aichi HA and 0.5 μg of CCL28 (membrane-bound or soluble) | cVLPs induced significantly higher and sustainable levels of virus-specific antibody responses, especially IgA levels and hemagglutination inhibition (HAI) titers, more than 8-month post-vaccination when compared to influenza VLPs without CCL28 or influenza VLPs co-administered with soluble CCL28. Upon challenge with H3N2, the cVLPs-treated group showed strong recall responses, and reduced viral load and inflammatory responses. cVLPs resulted in 20% cross-protection against drifted (Philippines) and 60% protection against homologous (Aichi) H3N2 viruses | Mohsen et al. (2017) |
| Pathogen/disease and cancer model | VLPs description | Production host | Test species | Disease and immunization results | Reference |
|---------------------------------|-----------------|-----------------|-------------|---------------------------------|-----------|
| Influenza virus and cancer model | VLPs based on the murine leukemia virus (MLV) capsid protein Gag, variant specific surface proteins (VSPs) from Giardia lamblia, and surface antigens such as the influenza virus hemagglutinin (HA) and neuraminidase (NA) proteins fused to the transmembrane domain and cytoplasmic tail of the vesicular stomatitis virus (VSV-G) | HEK293 cells | Mice | VLPs surface displayed HA and VSP. The chimeric VLPs carrying VSPs were protected from degradation and activated antigen presenting cells in vitro. Orally administered VLPs–VSPs, but not plain VLPs, generated robust immune responses in terms of serum IgG1 and IgG2a and IgA in feces and bronchoalveolar lavage. When parenterally administered VLP–VSPs showed a superior immunogenicity with respect to plain VLPs, but did not induce mucosal humoral responses. VLP–VSPs induced full protection against an influenza virus challenge, whereas plain VLP induced no protection. VLP–VSPs induced robust IFN-γ T-cell responses and in vitro cytotoxicity against the tumor cells with almost complete control of tumor growth. | Serradell et al. (2019) |
### Table 10.1 (continued)

| Pathogen/disease | VLPs description | Production host | Test species | Description of immunization trial | Immunization results | Reference |
|------------------|------------------|-----------------|--------------|-----------------------------------|---------------------|-----------|
| Influenza virus  | VLPs based on the Qbeta phage, plain or functionalized with either the influenza virus derived ectodomain of the M2 protein (for humoral responses induction) or the p33 peptide derived from LCMV (for CTL responses induction). The latter conjugate contained CpG as accessory adjuvant | *E. coli* | Mice i.n. s.c. | Two doses at a 2-week interval Dosage: 50 μg of M2-Qb-VLP; 15 or 150 μg for Qb-p33 | Mice i.n. or s.c. immunized with either plain VLPs or VLPs-M2 showed strong and comparable IgG responses in serum and lung and IgA in serum. Only the i.n. administration induced IgA production in the lung. I.n. immunized animals showed large numbers of germinal centers (GC), as well as memory B-cells in the spleen and plasma cells in the bone marrow. I.n. immunization with VLPs-M2 also provided significant protection against an i.n. challenge with a lethal dose (4×LD50) of the influenza virus strain PR8. I.n. immunization with VLPs-p33 induced relatively inefficient cytotoxic T-cell responses, resulting in low numbers of specific T-cells and poor effector cell differentiation | Bessa et al. (2008) |
| Pathogen/disease | VLPs description | Production host | Test species | Immunization results | Test species | Reference |
|------------------|------------------|----------------|-------------|---------------------|-------------|----------|
| Influenza virus | VLPs based on the M1 matrix protein and a tandem repeat of M2 ectodomains (M2x5) derived from human, swine, Avian 1, and Avian 2 influenza viruses | Baculovirus-insect cell expression system | Mice i.n. | Twice at a 1-month interval | Dose: 15 μg of M2x5 VLPs (0.9 μg of M2e5x) | Lee et al. (2018) |
|                  |                  |                |             |                     | Challenge: A/Phil (H3N2) or A/Viet virus |          |
|                  |                  |                |             |                     | M2e5x VLPs induced fully, broad cross-protection associated with humoral and cellular immune responses. M2x5 VLPs reduced weight loss, attenuated inflammatory cytokines and cellular infiltrates, decreased viral loads, and induced germinal center phenotypic B and plasma cells. Both CD4 and CD8 T-cells had a role in the observed protective effects. |          |
|                  |                  |                |             |                     |              |          |
| Influenza virus | VLPs containing M1 only | Baculovirus-insect cell system | Mice i.n. | Twice at a 4-week interval | Dose: 10 μg for VLPs, 0.5 μg for flagellin | Wang et al. (2010) |
|                  |                  |                |             |                     |              |          |
|                  |                  |                |             |                     | HA/FliC/M1 cVLPs induced robust cellular responses and enhanced systemic and mucosal antibody responses when compared to plain influenza VLPs and provided full protection against a challenge with either the homologous or heterosubtypic virus. Plain VLPs did not induce protection upon co-administration with the heterosubtypic challenge. Upon co-administration with plain VLPs, soluble flagellin exhibited a moderate adjuvant effect evidenced by enhanced systemic and partial heterosubtypic protection. |          |
| Pathogen/disease | VLPs description | Production host | Test species | Description of immunization trial | Immunization results | Reference |
|------------------|------------------|-----------------|--------------|-----------------------------------|----------------------|-----------|
| Influenza virus  | H5N1 VLPs composed of the viral HA, NA, and M1 proteins and membrane-anchored forms of the *E. coli* heat-labile enterotoxin B subunit protein (LTB) or the Toll-like receptor 5 ligand flagellin (Flic) | Baculovirus insect cell expression system | Mice | i.m.: Twice at a 3-week interval p.o.: Four weekly immunizations Dosage i.m.: 10 μg p.o.: 100 μg (2^10 HA units) Challenge: A/meerkat/Shanghai/SH-1/2012 or A/duck/Jilin/JL-SIV/2013 | Mice intramuscularly or orally immunized with VLPs containing LTB or Flic generated greater humoral and cellular immune responses than those generated using H5N1 VLPs without LTB or Flic. Intramuscular immunization with VLPs protected mice from a lethal challenge with homologous or heterologous H5N1 viruses irrespective of whether the VLPs additionally included LTB or Flic. In contrast, oral immunization of mice with LTB- or Flic-VLPs conferred substantial protection against a lethal challenge with both homologous and heterologous H5N1 influenza viruses, whereas mice immunized orally with VLPs lacking LTB and Flic universally succumbed to infection. Mice immunized orally with LTB- or Flic-VLPs showed 10-fold higher virus-specific IgG titers than mice immunized with H5N1-VLPs lacking LTB or Flic | Ren et al. (2018) |
| Pathogen/disease | VLPs description | Production host | Test species | Description of immunization trial | Immunization results | Reference |
|------------------|------------------|-----------------|--------------|-----------------------------------|---------------------|-----------|
| Influenza virus  | VLPs based on the NP\textsubscript{163} or NP fused to the truncated scaffold protein (SP) and the P22 coat protein | E. coli         | Mice i.n.    | Five times, daily Dosage: 100 μg of P22 VLPs Challenge: A/PR8/8/34 strain (PR8, H1N1) | Both vaccine versions were able to induce strong protective immune responses in mice against a challenge with either H1N1 or H3N2 influenza viruses. The protective response was partially attributed to NP-specific CD8+ T lymphocyte responses. In terms of humoral responses, the full length NP-based vaccine induced anti-NP antibodies, whereas truncated NP did not. These data correlated with a lower protection rate in mice immunized with the truncated NP-based vaccine | Patterson et al. (2013) |
| Influenza virus  | VLPs based on the hemagglutinin (HA) of H7 influenza virus | Silkworm pupae, Recombinant baculovirus | Mice         | Doses: H7 VLPs containing 16,384 HA titers Group 1: two immunizations at a 1 month interval Group 2: two immunizations at a 1 week interval Group 3: three immunizations at 1 week intervals Group 4: mice i.p. immunized with two doses Chickens Immunized three times orally with 16,384 HA titers of H7 VLPs or H5-Fukushima VLPs | HA VLPs showed positive immunoreactivity with the authentic anti-H7 antibodies. Also H5 and H7 VLPs could produce HI antibody in chickens and mice upon oral immunization. The antibodies elicited upon oral immunization were confirmed in fluorescent antibody analysis and Western blotting in Korea H5-BmNPV and H7 HA-BmNPV recombinant infected BmN cells. No challenge experiments were reported | Nerome et al. (2017) |
Patterson et al. (2013) reported a vaccine based on P22 VLPs obtained by co-expressing in *E. coli* the P22 coat protein with either a truncated version of the NP antigen (NP<sub>163</sub>) or the full length NP, which was fused to the truncated scaffold (SP). VLPs carrying either NP versions were i.n. administered to mice daily for 5 days (dose: 100 μg of P22 VLPs). Both vaccine versions were able to induce strong protective immune responses in mice against a challenge with either the H1N1 or H3N2 influenza viruses. The protective response was in part attributed to an NP-specific CD8+ T lymphocyte response. In terms of humoral responses, the full length NP-based vaccine induced anti-NP antibodies, whereas the truncated NP did not. These data correlated with a lower protection rate in mice immunized with the truncated NP-based vaccine. According to the results, the reported vaccine is a potential candidate not requiring accessory adjuvants to achieve protection.

Another VLPs-based influenza vaccine candidate has been proposed by Nerome et al. (2017), which was produced by expressing the H7 influenza virus hemagglutinin (HA) in silkworm pupae. Mice were orally immunized with HA VLPs in different schemes comprising two (with 1 month or 1-week interval) or three immunizations (with a 1-week interval). A two-dose i.p. scheme was also employed. Chickens were immunized three times orally with 16,384 HA titers of H7 VLP or H5-Fukushima VLP. HA VLPs showed positive immunoreactivity with the authentic anti-H7 antibodies. H5 and H7 VLPs triggered humoral responses in chickens and mice upon oral immunization. The antibodies elicited upon oral immunization were confirmed by fluorescent antibody analysis and Western blotting in Korea H5-BmNPV and H7 HA-BmNPV recombinant infected BmN cells. No challenge experiments were reported. This platform is advantageous as it would allow for large-scale production of the vaccine at relatively low costs since the use of expensive culture media, required for insect cells in culture, is avoided.

Ren et al. (2018) explored the idea of producing influenza VLPs decorated with membrane-anchored forms of the *E. coli* heat-labile enterotoxin B subunit protein (LTB) or the Toll-like receptor 5 ligand flagellin (Flic) as accessory mucosal adjuvants. For this purpose, H5N1 VLPs (composed of the viral HA, NA, and M1 proteins) were produced in insect cells (Fig. 10.3). Mice were subjected to three weekly doses of VLPs (100 μg) administered by i.m. or oral routes. VLPs containing LTB or Flic generated higher humoral and cellular immune responses than plain influenza VLPs. Intramuscular immunization with plant VLPs, VLPs-CTB, or VLPs-Flic protected mice from a lethal challenge with homologous or heterologous H5N1 viruses. In contrast, in orally immunized mice only VLPs-LTB and VLPs-Flic conferred substantial protection against a lethal challenge with both homologous and heterologous H5N1 influenza viruses (40% of survival rate), whereas mice immunized with plain VLPs succumbed to infection (Fig. 10.4). Mice immunized orally with LTB- or Flic-VLPs showed tenfold higher virus-specific IgG titers than mice immunized with plain VLPs. Thus, these accessory adjuvants are promising tools that can be applied in the development of vaccines against other pathogens.

Wang et al. (2010) used insect cell-made VLPs consisting of the HA and M1 proteins plus Flic (HA/Flic/M1 cVLPs) or M1 plus flagellin (Flic/M1 cVLPs). The authors previously proved that Flic inclusion in the VLPs allowed protecting mice,
i.m. immunized, from a challenge with a heterosubtypic strain. In this study, mice were i.n. immunized twice with 10 μg of VLPs at 4-week interval. HA/Flic/M1 cVLPs induced robust cellular responses and enhanced systemic and mucosal antibody responses when compared to plain influenza VLPs; moreover, the vaccine provided full protection against a challenge with either homologous or heterosubtypic viruses. Plain VLPs did not induce protection upon heterosubtypic challenge. Upon co-administration with plain VLPs, soluble flagellin exhibited a moderate adjuvant effect evidenced by an enhancement of systemic and mucosal responses and partial heterosubtypic protection.

Another VLPs-based influenza vaccine was reported by Mohsen et al. (2017). Chimeric influenza VLPs were generated by expressing M1 or HA/M1 and glycosylphosphatidylinositol (GPI)-anchored CCL28M1. The latter is a mucosa-associated epithelial chemokine, which binds to CCR3 and CCR10 chemokine receptors and is involved in the migration of antibody secreting cells (ASCs) into mucosal tissues, thus exerting adjuvant effects. The vaccine was evaluated in mice, which were i.n. immunized twice at a 2-week interval with 1 μg of HA and 0.5 μg of CCL28 (membrane-bound or soluble). cVLPs induced enhanced humoral responses when compared to influenza VLPs without CCL28 or influenza VLPs co-
administered with soluble CCL28. Upon a challenge with H3N2, the cVLP-treated group showed strong recall responses and reduced viral load and inflammatory responses. cVLP resulted in 20% cross-protection against drifted (Philippines) and 60% protection against homologous (Aichi) H3N2 viruses (Fig. 10.5).

A vaccine based on chimeric VLPs derived from the hepatitis B virus core antigen gene (HBc) was reported by Zheng et al. (2016). aa 75–85 from HBc were replaced by the long alpha-helix (LAH) gene fragment (coding for aa 76–130 of HA2), which is conserved among different influenza A strains and thus offers the opportunity for developing broad-spectrum influenza vaccines. E. coli-made VLPs were obtained (Fig. 10.6) and tested in mice at 1, 5, or 25 μg doses. Mice were i.n. immunized thrice at 2-week intervals with VLPs alone or co-administered with chi-
tosan or 1 μg of CTB. Another group received VLPs i.p. plus alum. The test VLPs induced, at the 25 μg dose, effective humoral and cellular immune responses and provided complete protection against a lethal challenge using the homologous H7N9 virus or the heterologous H3N2 virus, while i.m. administration provided weak protection. Either of the test adjuvants provided full protection, whereas unadjuvanted vaccines led to lower protection rate. The i.n. vaccine resulted in partial protection against a lethal challenge with a heterologous H1N1 virus (Fig. 10.7).

An immunization approach against influenza based on VLPs was described by Bessa et al. (2008). The authors produced VLPs derived from the Qβ phage (E. coli-produced); plain or functionalized with the influenza virus derived ectodomain of the M2 protein (VLPs-M2, for humoral responses induction) or the p33 peptide (VLPs-p33), which is the major CTL epitope of the lymphocytic choriomeningitis virus (as a model CTL epitope). The latter conjugate contained CpG as an accessory adjuvant. Mice were i.n. or s.c. immunized with plain VLPs or VLPs-M2, with the latter inducing strong IgG responses in serum and lung and IgA in serum. Only mice
immunized i.n. induced IgA production in the lung and a large numbers of germinal centers (GC), as well as memory B-cells in the spleen and plasma cells in the bone marrow. i.n. immunization with VLPs-M2 also provided significant protection against an i.n. challenge with a lethal dose (4 × LD50) of the influenza virus strain PR8. In contrast, i.n. immunization with VLP-p33 induced relatively inefficient cytotoxic T-cell responses, resulting in low numbers of specific T-cells and poor effector cell differentiation. This report supports the use of Qβ phage for nasal vaccine production. Surprisingly since the publication of this report no additional efforts have been reported to apply Qβ phage in the development of vaccines against relevant pathogens, especially those for which humoral responses are critical for protection.

An outstanding approach for oral vaccination has been recently reported by Serradell et al. (2019), which consists in producing VLPs based on the murine leukemia virus (MLV) capsid protein Gag, variant specific surface proteins (VSPs) from *Giardia lamblia*, and surface antigens such as the influenza virus hemagglutinin (HA) and neuraminidase (NA) proteins fused to the transmembrane domain and the cytoplasmic tail of the G protein from the vesicular stomatitis virus (VSV-G). The VLPs produced in HEK293 cells were orally or s.c. administered to mice four

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**Fig. 10.6** Characterization of the VLPs-based influenza vaccine based on the HBc scaffold. (a) Schematic representation of the LAH-HBc structure. The LAH region (76–130 aa of HA2) from the H7N9 virus was inserted into the HBc fragment (1–149 aa of HBc) by replacement of aa 75–85. (b) Expression of the LAH-HBc protein by SDS-PAGE analysis. (c) Western blot analysis of the LAH-HBc protein using an anti-his tag monoclonal antibody. (d) Electron microscopy of VLPs composed of LAH-HBc with a diameter around 30 nm (Taken from Zheng et al. 2016. Permit number 4595381026220)
times at a 1-week interval (100 μg dose for oral and 10 μg for s.c. immunization). This strategy allowed displaying on the VLP surface both HA and VSP. The latter was shown to protect VLPs from degradation and activate APC using in vitro assays. Orally administered VLPs–VSPs, but not plain VLPs (expressing HA only), generated robust immune responses in terms of serum IgG1 and IgG2a and IgA in feces and bronchoalveolar lavage. When VLPs–VSPs were administered parenterally; they showed superior immunogenicity with respect to plain VLPs, but did not induce mucosal humoral responses. Interestingly, VLPs–VSPs induced full protection against an influenza virus challenge, whereas plain VLPs did not. The authors also challenged mice with murine AB1 malignant mesothelioma cells expressing HA, observing that VLPs–VSPs controlled tumor growth almost completely. Further analysis confirmed that VLPs–VSPs induced robust IFN-γ T-cell responses and in vitro cytotoxicity against the tumor cells (Fig. 10.8). This is an outstanding approach offering a promising path for the development of oral vaccines not requiring adjuvants.
Fig. 10.8  Protective efficacy of oral vaccines based on VLPs decorated with variant specific surface proteins (VSPs) from *Giardia lamblia*. The vaccine carries HA as antigen and immunoprotection was evaluated in mice challenged with influenza virus or tumor cells expressing HA. (a, b) Ten days after immunization with H5N1 VLPs, mice were i.n. challenged with a mouse-adapted influenza virus, n = 5. A Kaplan–Meier life survival curve analysis was performed using the log-rank Mantel-Cox method for curve comparison analysis (a). Body weight is presented as percentage of the initial average weight registered at day 0. Changes in body weight were evaluated for 2 weeks (b). (c, d) Ten days after the last immunization, mice immunized with H1N1 VLPs were injected with AB1-HA tumor cells. The tumor volume growth is reported from two independent experiments, n = 8 (c). After tumors harvesting and weighing (31 days after tumor inoculation), representative tumor photographs are shown from two independent experiments, n = 8 (d). (e) Ten days after immunization with H1N1 VLPs, the titer of neutralizing antibodies was measured in
Respiratory Syncytial Virus

The human respiratory syncytial virus (RSV) is the commonest causative agent of acute low respiratory illness in infants and immunocompromised adults (Collins and Crowe 2007). No licensed vaccine against RSV is available since the development of safe and effective candidates has been challenging (Hall 2001). A vaccine candidate against the respiratory syncytial virus (RSV) reported by Schwarz et al. (2016) consisted of *E. coli*-made VLPs assembled with the matrix (M) and matrix 2 (M2) proteins, which were coencapsidated within the P22 phage VLP by fusing the C-terminal 162 residues of SP to the C-terminus of the M/M2 protein chimera. The vaccine (after i.n. administration to mice using two doses of 100 μg at 3-week intervals) stimulated both CD4+T-cell and CD8+T-cell memory responses in the lung against M and M2. Importantly, challenged animals showed reduced lung viral titers. This is a promising approach since no accessory adjuvants were required.

A study by Jiao et al. (2017) consisted in the evaluation of VLPs carrying the matrix protein (M) and the fusion glycoprotein (F) as vaccine candidate against the respiratory syncytial virus (RSV), which was produced in Vero cells. Mice were i.n. or i.m. immunized with VLPs (single dose: 10 μg or 30 μg) or inactivated RSV (2 × 10⁶ pfu/mouse for i.n. and FI-RSV at a dose of 1.875 mg/mouse for i.m.). VLPs presented a positive immunoreactivity and function when compared to the RSV virion in vitro. The i.n. administered vaccine induced Th1 polarized response and effective mucosal virus-neutralizing antibody and CD8+ T-cell responses at both mucosal and systemic levels, whereas i.m. immunization only induced effective response at the systemic level. Upon a challenge with RSV, i.n. immunized mice showed increased viral clearance, but decreased signs of enhanced lung pathology and fewer eosinophils when compared to mice immunized with formalin-inactivated RSV (FI-RSV). i.n. administered VLPs induced a response that lasted up to 15 months. Therefore, this approach is promising for developing an effective and safe mucosal vaccine against RSV infection, although production in mammalian cells is costly when compared to other systems (e.g., bacteria, yeast, or plant cells).

Fig. 10.8 (continued) sera using a standard microneutralization assay (*n* = 8 from two independent experiments). (f, g) Ten days after immunization with H1N1 VLPs, mice were injected with AB1-HA cells; 31 days afterwards they were sacrificed. IFN-γ was measured in HA re-stimulated splenocyte supernatants, *n* = 8 from two independent experiments (f). An in vitro cytotoxicity assay using, as target cells, splenocytes and CFSE-labeled AB1-HA was performed. The quantification of dead cells using CFSE+ cells from two independent experiments, *n* = 6, is shown (g). (h) AB1-HA or 4T1-HA tumor cells were inoculated (at day 0; *n* = 10) and upon tumor detection, half of the mice were vaccinated (arrows). Data were analyzed by one-way ANOVA and Tukey’s multiple comparison test (d–g) or by two-way ANOVA and Bonferroni post-tests (c). Values represent mean±s.e.m. **p < 0.01, ***p < 0.001. (Taken from Serradell et al. 2019)
**Severe Acute Respiratory Syndrome Coronavirus**

The severe acute respiratory syndrome coronavirus (SARS-CoV) first emerged as an infectious agent in 2003, causing severe and sometimes fatal respiratory disease in humans, with a fatality rate of 10% and a remarkable negative economic and social impact (Peiris et al. 2004). Chimeric VLPs were obtained by using insect cells expressing the SARS spike (S) protein and the influenza M1 protein. Mice were immunized i.m. (dose: 0.8 μg or 4 μg of SARS S or VLP vaccine, with or without aluminum hydroxide) or i.n. (doses: 0.8 μg or 4 μg of SARS S or VLP vaccine). Boosting was performed at day 21. The SARS VLP vaccine at the 0.8 μg dose completely protected mice when i.m. or i.n. administered. The SARS VLP vaccine (4 μg dose) without adjuvant reduced lung virus titer below detectable levels, protected mice from weight loss, and elicited a high level of neutralizing antibodies against SARS-CoV. Soluble SARS S protein was also protective but only when i.m. administered along with aluminum hydroxide. Since this vaccine was produced under the same procedures followed for the production of an influenza VLP vaccine successfully evaluated in phase I and phase II clinical studies, the authors propose this as a platform of facile implementation allowing a rapid vaccine production in case of a SARS pandemic.

**Group A Streptococcus**

Group A Streptococcus (GAS) causes more than 517,000 annual deaths derived from rheumatic fever, rheumatic heart disease, poststreptococcal glomerulonephritis, and invasive infections (Carapetis et al. 2005). Rivera-Hernandez et al. (2013) designed chimeric VLPs based on the murine polyomavirus (MuPyV) VP1 protein, displaying the J8i antigen peptide from Group A Streptococcus (GAS). The J8i epitope was inserted at the 293 amino acid position of VP1 in a single (VP1-GCN4-J8i-GCN4) or repeated (VP1-GCN4-J8i-J8i-GCN4) element, including Gly-Ser linkers as flanks. These chimeric proteins were expressed in E. coli and assembled in vitro into VLPs (Fig. 10.9), which were administered to mice i.n. thrice on days 0, 21, and 42 (dose: 92 μg). The studied VLPs induced significant anti-J8i IgG and IgA responses, thus generating systemic and mucosal responses, respectively. Interestingly, upon a challenge with GAS, bacterial colonization in the throats was significantly lower in VLP-immunized mice that also showed mild protection against a lethal challenge (35% vs. 10% survival for the negative controls; Fig. 10.10). Improvements of this vaccine could comprise the use of accessory adjuvant and dose adjustment.

**Fig. 10.9** (continued) flow field flow fractionation (AF4) analysis of (a) VP1-GCN4-J8i-GCN4 VLPs, (b) VP1-GCN4-J8i-J8i-GCN4 VLPs, and (c) wt-VP1 VLPs. Transmission electron micrograph analysis of (I) VP1-GCN4-J8i-GCN4 VLPs, (II) VP1-GCN4-J8i-J8i-GCN4 VLPs, and (III) wt-VP1 VLPs (100 nm scale bars) (Taken from Rivera-Hernandez et al. 2013. Permit number 4595390043559)
Fig. 10.9 Characterization of chimeric VLPs based on the murine polyomavirus (MuPyV) VP1 protein, displaying the J81 antigen peptide from Group A Streptococcus (GAS). Asymmetrical
Norovirus (NoV) is an important etiologic agent of acute gastroenteritis that infects individuals of all ages, children especially (Paula et al. 2018). Plant-made VLPs for use in immunization against Norwalk virus are highly cost-attractive, but have shown low immunogenic potential. Looking to enhance the efficacy of this vaccine, some approaches have been explored. VLPs based on the NV ORF2 (without ORF3) expressed in plants (N. benthamiana) have been applied by Jackson and Herbst-Kralovetz (2012) using i.n. immunization schemes to determine the effect of various adjuvants (Murabutide, MB, an adjuvant that targets the nucleotide-binding oligomerization domain-containing protein 2 (NOD2) receptor; gardiquimod, GARD, which is a TLR7 agonist; and the cholera toxin, CT, a well-known mucosal adjuvant). Mice were i.n. immunized twice at 3-week intervals with VLPs (25 μg) alone or supplemented with one of the following: MB (levels: 25, 100, or 250 μg), GARD (25 μg), or CT (1 μg). MB showed the optimal adjuvant effect when co-administered at a 100 μg dose, based on the magnitude of VLP-specific IgG, IgG1, IgG2a, and IgA production in serum and VLP-specific IgA production at distal mucosal sites. i.n. vaccination using VLPs with MB induced humoral responses in similar magnitudes to those achieved by i.n. co-administration of VLPs and CT or GARD and i.m. immunization with VLP plus alum. Interestingly in terms of the induction of mucosal immune responses, the MB groups were equivalent to CT and GARD and induced comparable systemic responses to those achieved by the parenteral vaccine plus alum.

**Norwalk Virus**

Norovirus (NoV) is an important etiologic agent of acute gastroenteritis that infects individuals of all ages, children especially (Paula et al. 2018). Plant-made VLPs for use in immunization against Norwalk virus are highly cost-attractive, but have shown low immunogenic potential. Looking to enhance the efficacy of this vaccine, some approaches have been explored. VLPs based on the NV ORF2 (without ORF3) expressed in plants (N. benthamiana) have been applied by Jackson and Herbst-Kralovetz (2012) using i.n. immunization schemes to determine the effect of various adjuvants (Murabutide, MB, an adjuvant that targets the nucleotide-binding oligomerization domain-containing protein 2 (NOD2) receptor; gardiquimod, GARD, which is a TLR7 agonist; and the cholera toxin, CT, a well-known mucosal adjuvant). Mice were i.n. immunized twice at 3-week intervals with VLPs (25 μg) alone or supplemented with one of the following: MB (levels: 25, 100, or 250 μg), GARD (25 μg), or CT (1 μg). MB showed the optimal adjuvant effect when co-administered at a 100 μg dose, based on the magnitude of VLP-specific IgG, IgG1, IgG2a, and IgA production in serum and VLP-specific IgA production at distal mucosal sites. i.n. vaccination using VLPs with MB induced humoral responses in similar magnitudes to those achieved by i.n. co-administration of VLPs and CT or GARD and i.m. immunization with VLP plus alum. Interestingly in terms of the induction of mucosal immune responses, the MB groups were equivalent to CT and GARD and induced comparable systemic responses to those achieved by the parenteral vaccine plus alum.

![Fig. 10.10](image-url) Protective potential of the VLPs-based vaccine against Group A Streptococcus. Mice were challenged intranasally with GAS. (a) Survival percentage following an intranasal challenge with the M1 GAS reference strain. (b) Percentage of mice dead or alive with positive swabs following the challenge with GAS. Data for VP1-GCN4-J8i-GCN4 VLPs and VP1-GCN4-J8i-J8i-GCN4 VLPs were collected into one group, while data for the PBS and wt-VLPs groups were collected as a negative control group. Statistical comparison of the two immunized groups vs. the collected control group was performed using a standard log-rank test. (Taken from Rivera-Hernandez et al. 2013. Permit number 4595390043559)
A dry powder formulation (GelVac) based on an inert in situ gelling polysaccharide (GelSite), obtained from Aloe vera, has been assessed in a pig nasal immunization model by Velasquez et al. (2011). The authors compared immunogenicity of the dry powder VLP formulation vs. equivalent antigen/adjuvant liquid formulations. For this purpose test animals were immunized twice at 3-week intervals with one of the following: 10–12 mg/naris of GelVac alone, GelVac containing VLPs (10 μg), or GelVac VLPs (10 μg) + 10 μg of gardiquimod (GARD, a TLR7 agonist used as accessory adjuvant). In parallel, liquid formulations lacking GelVac and containing the same amount of NV VLPs were tested. Powder formulations, with or without VLPs, were similar in structure in dry form or when rehydrated in simulated nasal fluids. The GelVac powder induced higher antibody responses at systemic and mucosal (aerodigestive and reproductive tracts) levels when compared to liquid formulations. Inclusion of GARD did not increase immunogenicity of the dry formulation, while a positive effect was recorded for the liquid formulations. The authors hypothesized that the test formulation, upon in situ gelation, allows VLPs stabilization that ends up prolonging their presence on the mucosal surfaces overcoming mucociliary clearance and thus extending antigen uptake by APCs in the NALT. The same research group subsequently evaluated VLPs resembling GI or GII.4 norovirus to identify the optimal dose in guinea pigs. Pigs were i.n. immunized twice at 3-week intervals (Springer et al. 2016). Test doses ranged 0.1–100 μg of VLPs. The humoral response magnitude followed a dose-dependent pattern in both serum and vaginal washes. The 15 μg dose allowed reaching maximum antibody responses. The neutralizing potential was assessed in an assay based on estimating the capacity of VLPs to bind porcine gastric mucin after incubation with sera from immunized animals. The neutralizing activity for both GI and GII.4 VLPs was proven, nevertheless no challenge assays were reported. These are interesting outcomes as no accessory adjuvants were used and suggest that bivalent vaccines targeting both GI/GII.4 noroviruses could be produced. Dry powder vaccines offer high stability such that cold-chain free vaccines could become a reality. Bahamondez-Canas and Cui (2018) have reviewed this specific topic recently.

Interestingly, a clinical trial for the Norwalk VLP vaccine GI.1 genotype has been performed by El-Kamary et al. (2010). The vaccine was produced in insect cells and adjuvanted with monophosphoryl lipid A (MPL, which is a TLR-4 agonist) derived from the detoxified Salmonella minnesota lipopolysaccharide and the mucoadherent agent chitosan. Healthy subjects, 18–49 years old, were i.n. immunized twice at 3-week intervals. Study 1 evaluated 5, 15, and 50 μg of VLPs and study 2 evaluated 50 and 100 μg dosages. The vaccine induced no serious adverse events with nasal stuffiness, discharge, and sneezing as the most frequent side effects. Anti-VLPs IgG and IgA levels increased 4.8- and 9.1-fold, respectively, for the 100-μg dosage level (Fig. 10.11). All subjects that received either 50 or 100 μg doses presented IgA antibody secreting cells (ASCs) measured in peripheral blood mononuclear cells. ASCs expressed molecules associated with homing to mucosal and peripheral lymphoid tissues (CD19+CD27+CD62L+, integrin α4/β7+). A subset expressing exclusively mucosal homing molecules was also detected (CD19+CD27+CD62L-integrin α4/β7+). Therefore, this vaccine is considered safe and highly immunogenic that could progress into phase II clinical trials.
Fig. 10.11  Immunogenicity of a VLPs-based vaccine against Norwalk virus evaluated in a Phase I clinical trial. VLPs-specific immunoglobulin G (IgG) and immunoglobulin A (IgA) geometric mean antibody titers are presented, by group in studies 1 and 2. In study 1, 28 adult subjects were randomized sequentially by group to receive two doses of (1) 5 mg of Norwalk VLP vaccine (five subjects, squares) or adjuvant control (two subjects, diamonds), (2) 15 mg of Norwalk VLP vaccine (five subjects, triangles) or adjuvant control (two subjects, diamonds), or (3) 50 mg of Norwalk VLP vaccine (ten subjects, circles) or adjuvant control (four subjects, diamonds). (a) Serum IgG geometric mean titers from study 1. (b) Serum IgA geometric mean titers from study 1. In study 2, 61 healthy adult subjects were enrolled at four sites and randomized 2:2:1:1, respectively, to receive either two doses of (1) 50 mg of Norwalk VLP vaccine (20 subjects, circles), (2) 100 mg of Norwalk VLP vaccine (20 subjects, triangles), (3) adjuvant control (10 subjects, filled diamonds), or (4) true placebo (11 subjects, open diamonds) consisting of a puff of air (no dry powder). (c) Serum IgG geometric mean titers from study 2. (d) Serum IgA geometric mean titers from study 2. All doses were delivered intranasally, and the 2-dose regimen was separated by 21 days. (Taken from El-Kamary et al. 2010. Permit number 4595390605207)
**Polio Virus**

The polio virus (PV) multiplies in the intestine and can invade the central nervous system causing irreversible paralysis in 1/200 infected people. At present, there is a need for subunit vaccines able to confer mucosal protection without the use of attenuated strains given the risk of reversion to pathogenic forms (Bandyopadhyay et al. 2015). Daniell et al. (2018) have developed a plant-made vaccine against PV by expressing, in lettuce, VLPs based on a chimeric protein comprising the VP1 protein from PV and the cholera toxin B subunit (CTB-VP1). The assembly of VP1-VLPs of 22.3 nm in size was evidenced. Mice were primed s.c. with IPV and boosted three times with 20 mg of lyophilized tissue and squalene and/or saponin plus Protegrin-1 (PG-1) and/or human antimicrobial peptide (LL37) as adjuvants and additives, respectively. Enhanced anti-PV IgG1 and IgA, as well as neutralizing activity (80–100% seropositivity of Sabin 1, 2, and 3), were induced upon boosting with the plant-made VLPs when compared to the treatment based on an IPV single dose or boosting with CTB-VP1 VLPs without IPV priming. These are considered very promising results since population worldwide is receiving IPV at a single dose, thus a booster cold-chain free vaccine might be used to aid in polio eradication.

**Hepatitis**

The hepatitis B virus infection is a major public health problem worldwide. It is estimated that 30% of the population show serological evidence of current or past infection. Although vaccines are available and have led to an important decrease in infections, new vaccines are still required to fight this pathogen. The group headed by Pniewski et al. (2018) has been working on the development of a plant-made vaccine against hepatitis B, which is formulated with lettuce tissues expressing the hepatitis B virus small surface protein (S-HBsAg) assembled into VLPs within the plant cell. A recent study was focused on assessing several schemes to determine the potential of this low-cost vaccine to serve as boosting agent when combined with the i.m. administration of the conventional vaccine produced in yeast (Engerix_B). i.m. priming with the commercial vaccine followed by double oral boosting with plant-made VLPs is comparably efficient as standard i.m. vaccination, with boosting doses of 2 or 200 ng being efficacious to enhance systemic responses (Fig. 10.12). None of the tested adjuvants improved the response to vaccination and the efficacy of plant lysate was lower than intact plant cells, which suggests that bioencapsulation of S-HBsAg into the plant cell favors vaccine activity not requiring accessory adjuvants (Fig. 10.13). This is a relevant study since plant-based platforms offer low production cost and the freeze-dried plant material is stable at room temperature, thus the formulation of this booster vaccine can be easily performed by preparing capsules or tablets containing plant powder.
Fig. 10.12 Immune response of serum anti-HBs antibodies. Mice were i.m. immunized at day 0 with 500 ng of S-HBsAg (Engerix_B) followed by two oral boosts on days 42 and 84 using tissue extract containing 0, 5, 50, or 200 ng of S-HBsAg. Significant results are marked by asterisks for pre-immune (day 3), while hashes are used for pre-boost (day 39). (1) Anti-HBs serum antibodies in control groups (i.m. primed with PBS and 2 p.o. boosts with tissue extract having no S-HBsAg) and (2) anti-HBs S-IgA in any group. No results are shown for antibodies <2 mIU/mL (Taken from Pniewski et al. 2018. Permit number 4595391037784)

Hepatitis E is generally a self-limiting, acute, and rarely fatal disease that is treated with anti-virals; these are often insufficient and unsafe, thus the development of vaccines for HEV is an important goal (Nishiyama et al. 2019). Niikura et al. (2002) reported the production in insect cells of chimeric VLPs comprised of the truncated hepatitis E virus ORF2 (aa 112-660), fused to a B-cell epitope of glycoprotein D from the herpes simplex virus. The chimeric VLPs showed morphology similar to that of the mature HEV virion and displayed the target epitope according to an immunoassay. Mice were orally immunized four times with 50 μg of VLPs at 2-week intervals. The chimeric VLPs induced specific intestinal IgG and IgA to both the inserted epitope and HEV-VLP in intestinal secretions. The authors proposed optimizing the dose of VLPs to enhance significant humoral responses. No immunoprotection assays were reported.

Li et al. (2004) produced VLPs resembling the hepatitis E virus (HEV) by expressing, in insect cells, the HEV capsid protein gene lacking 111 amino acids at the N-terminal. The vaccine was administered orally to cynomolgus monkeys (Macaca fascicularis) in a scheme based on 10 mg VLPs doses administered on days 0, 7, 21, 36, and 80, with a subsequent i.v. HEV challenge. The test vaccine induced specific serum IgM, IgG, and IgA responses without adjuvants. Upon chal-
challenge one monkey was fully protected and in another one infection occurred, but it did not develop the disease. Fecal IgA was not detected in any of the orally immunized monkeys.

**Human Papillomavirus**

The human papillomavirus (HPV) is a sexually transmitted virus responsible for the development of cervical cancer, anal cancer, head and throat cancers, and genital area warts (Aksoy et al. 2017). An insect cell-made vaccine against HPV was evaluated by Gerber et al. (2001), the vaccine was based on VLPs assembled with the L1 protein from HPV-16 or HPV-18. Mice were immunized i.m. (dose: 0.3 μg of VLPs) or orally (doses: 1, 3, or 9 μg of VLPs alone or co-administered with 10 μg of LTR192G or 10 μg of CpG DNA). Boosting doses at weeks 2 and 6 were administered. The VLPs co-administered with LT R192G induced higher serum IgG titers than VLPs alone. Immunization with HPV-16 VLPs plus LT R192G induced higher vaginal anti-VLP IgG and IgA antibodies than VLPs alone or plus CpG DNA. Similar
results were obtained with HPV-18 VLPs, with both adjuvants enhancing IgA responses. Only VLPs plus LT R192G induced positive mesenteric lymphoproliferative responses. Properties characteristically associated with virus-neutralizing antibody specificities appeared to be unaltered by the tested adjuvants.

**Immunodeficiency Viruses**

The human immunodeficiency virus (HIV) is a pathogen to which no licensed vaccines are available, being its genetic variability and mechanisms for immune evasion the factors hampered vaccine development (Rahman and Robert-Guroff 2019). Poteet et al. (2016) have reported HIV VLPs composed of HIVIIIB Gag and HIVBaL gp120/gp41 envelope, which were produced in HEK cells. VLPs were coupled to VesiVax conjugatable adjuvant lipid vesicles (CALV) containing one of the following Toll-like-receptor (TLR) ligands: PAM3CAG for TLR2, dsRNA for TLR3, MPLA for TLR4, and resiquimod for TLR7/8. Mice were immunized thrice at 2-week intervals with 200 μg VLPs dose. Priming was i.n. and boostings were performed by sub-cheek boosts. VLPs coupled with the TLR3 ligand, dsRNA, complexed to CALV and in combination with VLPs (CALV(dsRNA) + VLPs) induced the strongest humoral response against the target HIV antigens. Importantly, antibodies against clade c 96ZM651 gp120 were induced. A Th1 response was evidenced by IgG subclass analysis. The most potent neutralizing antibodies against HIV strain MN.3 were also generated by CALV(dsRNA) + VLPs, as well as a significant increase in germinal center B-cells and T follicular cells. This type of immunization schemes exemplifies how mucosal vaccination can be combined with boosts administered by injection in order to optimize the induced immune profile.

Kang et al. (2003) generated VLPs from the Simian immunodeficiency virus (SIV) comprising Gag and Env proteins, expressed in insect cells. These VLPs were conjugated to the cholera toxin B subunit to determine if immunogenicity is enhanced by a subtransmucosal carrier. Mice were immunized i.n. on weeks 0, 2, 4, and 6 with 40 μg of VLPs, VLPs (40 μg) plus CTB (10 μg) or CT (10 μg), or VLPs (40 μg) conjugated with 10 μg of CTB. The major results were: co-administration of VLPs and CTB induced higher levels of anti-SIV gp160 IgG and IgA in mucosae (saliva, vaginal-wash, lung, and intestine) and higher neutralization activity when compared to VLPs alone. Conjugation of CTB to VLPs also enhanced SIV VLP-specific antibodies in sera and mucosae to similar levels. CTB-conjugated VLPs induced superior cellular responses (IFN-γ-producing splenocytes and cytotoxic-T-lymphocyte activities) with respect to the VLPs/CTB mixture, along with enhanced IgG1 and IgG2a serum levels, indicating enhanced Th1- and Th2-type cellular immune responses.
Current Status of Animal Mucosal VLP-Based Vaccines

Alkadah et al. (2013) reported a rotavirus vaccine, produced in insect cells, based on VLPs composed of VP2 and VP6 from bovine rotavirus. The authors tested the effect of including LT-R192G, which is a mutant version of the heat labile enterotoxin from *E. coli* in immunization schemes based on either i.n. or i.r. routes. A single dose of the i.n. administered vaccine alone induced antigen-specific IL-10 and IL-17 secreting T-cells. IL-10-, in contrast to IL-17-, secreting T-cells did not migrate to the mesenteric lymph nodes (MLN), while they were detected in cervical lymph nodes (CLN) and spleen. The inclusion of LT-R192G improved immunogenicity in terms of activating the production of IL-2 and IL-4, increasing IL-17 secretion and inducing antigen-specific CD4+CD25+Foxp3+ and Foxp3−T-cells in CLN, spleen, and MLN, while IL-10 secreting T-cells were unmodified. When i.r. administered, the vaccine plus LT-R192G induced IL-2 and IL-17 production; nonetheless, unlike the i.n. scheme, it did not trigger IL-4 production. No neutralization studies were performed to determine and compare the protective potential of these vaccination approaches.

Binjawadagi et al. (2016) evaluated a vaccine against the porcine reproductive and respiratory syndrome (PRRS) using insect cell-made VLPs containing the GP5-GP4-GP3-GP2a-M or GP5-M proteins. A whole cell lysate from *Mycobacterium tuberculosis* (M. tbWCL) was co-administered as adjuvant. VLPs were administered alone or entrapped in PLGA nanoparticles (average size of 300 nm). Immunoassays confirmed the presence of all proteins in the VLPs, suggesting the retention of antigenic determinants. Pigs i.n. immunized with VLPs (two doses of 650 μg at 2-week intervals) entrapped in PLGA nanoparticles induced an anamnestic immune response since elevation of IgG and IFN-γ production was observed after a challenge. The vaccinated group showed a two-log reduction in lung viral load. The authors suggested that efficacy should be improved, e.g., by including other viral structural proteins or producing VLPs carrying heterodimer (GP5-M) and heterotetramer (GP2a-E-GP3-GP4) interacting complexes, which are expected to resemble the native virion.

A VLPs-based vaccine candidate against infection by the nervous necrosis virus (NNV) was produced by expressing recombinant orange-spotted grouper NNV (OSGNNV) capsid protein in *E. coli*. The test vaccine was evaluated in orange-spotted grouper, which were immunized twice at 2-week intervals by immersion (250 μg/g fish body weight, 1 mg of VLPs in 10 L-seawater, 30 min of immersion), intramuscular injection (dose: 2 μg/g FBW, 0.1 μg VLPs per fish), or orally (dose: 20 μg/g FBW, feeding for 4 days with food containing 200 μg/g VLPs, consumption of 5% of body weight). OSGNNV VLPs elicited strong humoral responses, with the immersion and i.m. routes inducing higher humoral responses than the oral scheme. Upon challenge, the relative percent of survival values for immersion, injection, and oral immunizations were: 81.9, 61.4, and 52.3, respectively. This type of vaccine is highly promising as no accessory adjuvants are required and immunization by immersion or feeding is practical for implementation in aquaculture. In addition,
E. coli-expressed proteins are cheaper to produce when compared to insect and mammalian cell-based production.

Crisci et al. (2012) reported a VLPs-based vaccine candidate against the foot-and-mouth disease virus (FMDV). The vaccine consisted of chimeric insect cell-made VLPs based on the rabbit hemorrhagic disease virus (RHDV) carrying a T-cell epitope of the 3A protein from FMDV. The vaccine was evaluated in pigs immunized either i.n. or i.m. (doses: 20, 60, and 180 μg/pig; administered twice at 2-week intervals), having MontanideTM ISA 206 as adjuvant for i.m. immunization. Chimeric VLPs activated immature porcine bone marrow-derived dendritic cells (poBMDCs) in vitro. IgG and IgA antibodies against RHDV-VLPs were induced. The adjuvanted i.m. immunized groups showed the highest humoral response. The adjuvanted group exhibited the highest IFN-γ secreting cell numbers and lymphoproliferative specific T-cell responses against the 3A epitope and RHDV-VLP. No neutralization or challenge experiments were conducted.

Citarasu et al. (2019) produced a vaccine against the Macrobrachium rosenbergii nodavirus (MrNV), which is the causative agent of the white tail disease (WTD) in freshwater giant prawn (Macrobrachium rosenbergii). The vaccine comprised insect cell-made VLPs assembled with the MrNV capsid protein. The obtained VLPs were used to orally immunize larvae. For this purpose, animals were fed with VLP-supplemented feed during 60 days (10 μg of MrNV was added to 30 g of feed, leading to a daily intake of about 50–100 ng of VLPs during the test period). After either 30 or 60 days of vaccination period, prawns were orally challenged with virulent MrNV. Survival rates of 65 and 80% were achieved at 30 and 60 days post-vaccination, respectively, whereas the non-vaccinated group had a 90% mortality. Pathogen load estimated by PCR decreased to 32 and 17% at 30 and 60 days post-vaccination, respectively. The vaccine induced the expression of Mramp, which is believed to be involved in innate immune response against MrNV infection. No data on the induction of adaptive immune responses was presented.

Conclusive Remarks

VLPs stand as the most promising agents in the development of subunit vaccines, thanks to their high immunogenicity, stability, versatility, and safety. In terms of toxicity, it is clear that VLPs constitute one of the safest nanoparticulate systems as these are conformed by proteins, which contrast with metallic and polymeric nanoparticles that depending on the size, shape, and doses could lead to toxic effects. Overcoming the challenge of developing mucosal vaccines is with no doubt being driven by VLPs-based approaches, with substantial advances over the last years. Despite that the majority of the VLPs-based vaccines developed thus far have been evaluated in parenteral immunization schemes, the reports summarized in the present chapter indicate that there is a substantial potential to achieve the development of mucosal VLPs with a real possibility of becoming agents for the fight against relevant diseases in both humans and animals. The case of the influenza
vaccines indicates that there is a remarkable niche for mucosal VLPs vaccines as some candidates are effective and in some cases they possess higher efficacy than the parenteral vaccination scheme. It is clear that the use of chimeric VLPs carrying accessory adjuvants to target TLRs is an effective approach to enhance vaccine efficacy.

The key perspectives identified for this topic comprise: (1) developing VLPs-based vaccines targeting specific receptors at the mucosal tissues to increase vaccine efficacy. As transport through M-cell is a critical step in the induction of immunity by mucosal routes, targeting these cells by specific ligands can increase the uptake of the antigen and increase the access for uptake by antigen presenting cells, which is the first step for the induction of robust adaptive immune responses. For this purpose, an M-cell-targeting peptide ligand Co1 has been described (Kim et al. 2010). This concept has been proven with a vaccine against the porcine epidemic diarrhea virus in which the COE antigen fused to Co1 exhibited higher immunogenicity in terms of inducing systemic and mucosal immune responses (Huy et al. 2012). To the best of our knowledge, this approach has not been applied for VLPs-based vaccines. Another goal could expand the use of the nontoxic subunit of the cholera toxin (CTB) or the B subunit of the *E. coli* heat labile enterotoxin (LTB), both subunits bind to the GM1 ganglioside present in the membrane of the epithelial cells and allow the antigen to be transported into the submucosa. (2) Although several promising VLPs-based vaccine candidates are found in the literature, most of the vaccines are produced in insect or mammalian cells, which are expensive production platforms. Expanding the use of low-cost platforms such as plants is a relevant perspective. This technology has led to influenza vaccine candidates that are currently under clinical trials, but administered by a parenteral route (Pillet et al. 2018). (3) Another attractive approach is the one recently reported by Serradell et al. (2019), which is expected to lead to oral vaccine candidates against several infectious diseases. In addition, the fact that this vaccination approach resulted effective in a cancer model makes this vaccine highly attractive for the field of cancer immunotherapies given that most of the promising vaccines against cancer are administered by parenteral routes. Therefore, innovative oral cancer vaccines using this approach are envisaged. (4) A detailed exploration of the innovative immunization routes is a point that deserves consideration for VLPs-based vaccines. Sublingual immunization has been recently highlighted as very attractive as this allows inducing robust immune responses (Bahceciler et al. 2014). Therefore, expanding the evaluation of VPLs-based vaccines by the s.l. route will open new possibilities to address vaccinology challenges. For instance, the group headed by Kweon is working on the evaluation of a s.l. vaccine against the Group A Streptococcus based on VLPs assembled with the VP1 structural protein from the murine polyomavirus (MuPyV) produced in *E. coli* (Seth et al. 2016).

VLPs are advantageous agents for vaccine development that will lead, with no doubt, to more advances in the clinic for the fight against infectious and non-communicable diseases. It is of interest that VLPs-based vaccine candidates against emerging pathogens such as the Zika virus (Yang et al. 2017), Nipah virus (Walpita et al. 2018), Chikungunya virus (Salazar-González et al. 2015), and Ebola virus
(Rosales-Mendoza et al. 2017) are under development. However, the vaccine candidates have been mainly evaluated in parenteral immunization schemes; their evaluation in schemes based on mucosal immunization remains a relevant pending goal knowing that mucosal vaccination could lead to higher efficacy than the schemes based on parenteral administration. The use of prime-boosting schemes should be also studied in a more systematic way to achieve better immune profiles, i.e., optimal immune responses at both the systemic and mucosal levels. Another innovation expected in the following years is related to applying computer-based modeling methods to ensure proper antigen spatial conformation, especially in chimeric VLPs where conformational epitopes are critical for vaccine efficacy (Liljeroos et al. 2015). The generation of more sophisticated VLPs containing antigens arranged in multimeric arrays on carrier surfaces, as well as different immunostimulating components, is envisaged.

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