ACTA ABP BIOCHIMICA POLONICA

Vol. 61, No 3/2014
531–539
on-line at: www.actabp.pl

Review

Virus-like particles as vaccine

Jadwiga Chroboczek[1,2]✉, Inga Szurgot[2] and Ewa Szolajska[2]

[1]Therex, TIMC-IMAG, CNRS UMR 5525, UJF, La Tronche, France; [2]Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland

This review presents data on commercial and experimental virus-like particle (VLP) vaccines, including description of VLP vaccines against influenza. Virus-like particles are multimeric, sometimes multiprotein nanostructures assembled from viral structural proteins and are devoid of any genetic material. VLPs present repetitive high-density displays of viral surface proteins. Importantly, they contain functional viral proteins responsible for cell penetration by the virus, ensuring efficient cell entry and thus tissue-specific targeting, determined by the origin of the virus. The foremost application of VLPs is in vaccinology, where they provide delivery systems that combine good safety profiles with strong immunogenicity and constitute a safe alternative to inactivated infectious viruses. These stable and versatile nanoparticles display excellent adjuvant properties capable of inducing innate and cognate immune responses. They present both, high-density B-cell epitopes, for antibody production and intracellular T- cell epitopes, thus inducing, respectively, potent humoral and cellular immune responses. Uptake of VLPs by antigen-presenting cells leads to efficient immune responses resulting in control of pathogenic microorganisms.

Key words: virus-like particle, VLP vaccine, adenovirus dodecahedron, antigen-presenting cells, cell-mediated immunity

Received: 17 July, 2014; revised: 20 August, 2014; accepted: 04 September, 2014; available on-line: 18 September, 2014

VIRUS-LIKE PARTICLES

Virus-like particles (VLP) are multimeric, sometimes multiprotein nanostructures that are assembled from viral structural proteins and are devoid of any genetic material. These naturally occurring bionanomaterials often emulate the conformation of authentic viruses. VLPs contain repetitive high-density displays of viral surface proteins and as such are a highly adaptable platform for various applications. Importantly, they contain functional viral proteins responsible for cell penetration by the virus, which ensures efficient cell entry.

The foremost application of VLPs is in vaccinology, whereby they provide delivery systems that combine good safety profiles with strong immunogenicity. Traditionally, vaccines against viral diseases have been prepared from attenuated or inactivated infectious viral strains. VLPs, devoid of the viral genome but able to penetrate cells and tissues, are a much safer alternative. Moreover, they provide a polyvalent structure that can accommodate multiple copies of antigens and, in addition, are able to stimulate immune cells. Finally, they ensure tissue-specific targeting, determined by the origin of the virus.

These stable and versatile nanoparticles display excellent adjuvant properties capable of inducing innate and cognate immune responses. They present both, high-density B-cell epitopes for antibody production and intracellular T-cell epitopes, thus inducing respectively, potent humoral and cellular immune responses (Beyer *et al.*, 2001; Wang & Roden, 2013). Indeed, VLPs show potent adjuvant activity enhancing the immunogenicity of weakly immunogenic peptides and proteins. They direct antigenic peptides/proteins to immature dendritic cells (DC), activating DC maturation. Mature DCs are the key antigen presenting or antigen-presentig step (APC) that efficiently mediate antigen transport to lymphoid tissues for the initiation of T cell responses and induction of cell-mediated immunity (Zinkernagel, 2014). Uptake of the VLPs by APC leads to efficient immune responses and results in the control of pathogenic microorganisms.

Like parental viruses, VLPs can be either non-enveloped or enveloped, and spherical or filamentous (Fig. 1). Analysis of the published data performed by Zeltins (2013) revealed that at least 110 VLPs have been constructed from viruses belonging to 35 different families. They form spontaneously during the viral cycle or in heterologous systems upon expression of one or several viral structural proteins. Depending on the complexity of the VLPs, they can be produced from appropriate recombinant vectors in either prokaryotic or eukaryotic expression systems, or assembled under cell-free conditions. Furthermore, they can be formed from proteins derived from a single virus, in order to obtain immunity to this virus, or alternatively, on a platform derived from a single virus to present proteins/peptides derived from other microorganism or any cell/tissue (chimeric VLPs).

The yield of VLPs production is rather high and even in eukaryotic systems can approach the expression efficiency comparable to that observed for bacterial expression systems. Due to their high molecular weight, VLPs are purified from extracts of expressing cells by sucrose density centrifugation, usually followed by an additional step to remove cellular components loosely attached to them.

✉e-mail: jadwiga.chroboczek@imag.fr; wisia@ibb.waw.pl

Abbreviations: VLP, virus-like particle; DC, dendritic cells; APC, antigen-presenting cells; HBV, Hepatitis B virus; HPV, human papilloma virus; HBsAg, HBV surface antigen; HA, hemagglutinin; GLA-AF, glucopyranosyl Lipid A; CTL, cytotoxic T lymphocyte; SV40, Simian virus 40; Ad, adenovirus; Ad3, adenovirus serotype 3; DF, dodecahedra-fibre; DB, dodecahedra- base; Ang II, angiotensin II; MDC, myeloid dendritic cells; PBMC, peripheral blood mononucleated cell.

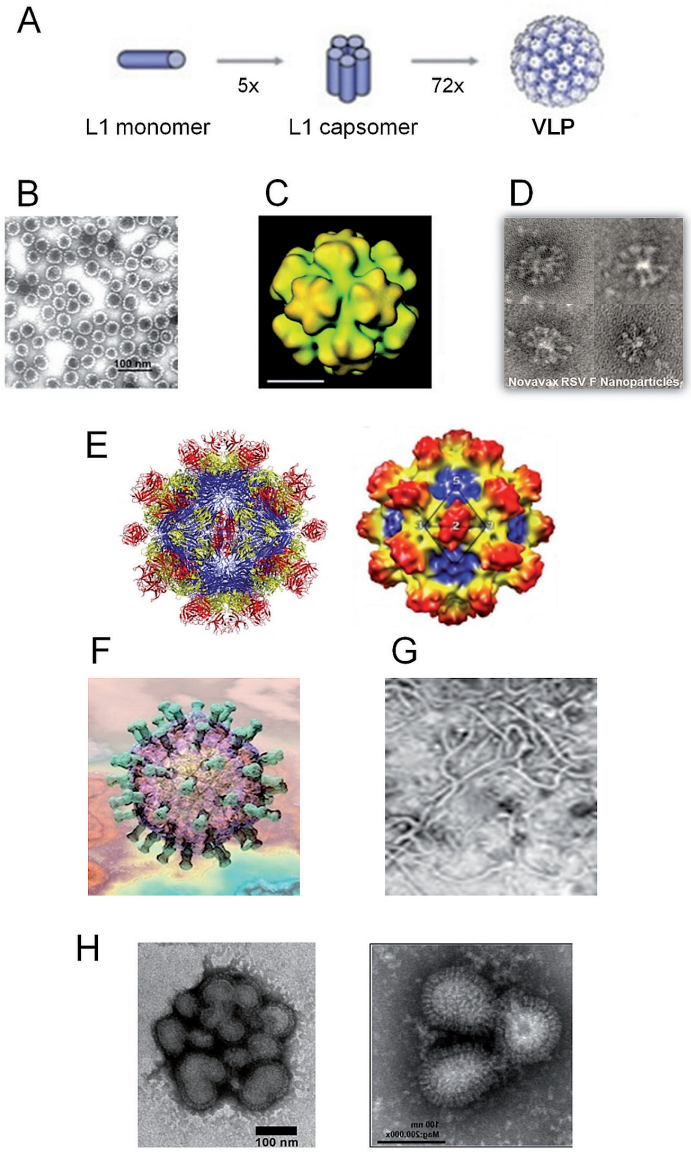

Figure 1. Different virus-like particles.
(**A**) Diagram of assembly of polyoma VLP from the L1 capsid protein. Five L1 monomers form spontaneously one pentameric L1 capsomer, 72 of such capsomers self-assemble into a VLP. From German Cancer Research site http://www.dkfz.de. (**B**) The hamster polyomavirus (HaPyV) VLPs formed from major capsid protein VP1. Electron microscopy (EM) reproduced from the site of the Vilnius University http://www.ibt.lt/en/laboratories/egil_en. (**C**) Cryo-electron microscopy reconstruction of the small BKV (polyoma family) VLP formed in a calcium-dependent manner from the P1 protein of a human BK virus. Bar, 10nm. After Nilsson *et al*. (2005). (**D**) Eight of respiratory syncytial virus (RSV) trimeric fusion proteins expressed in a baculovirus system form VLPs, visualized by EM. After Novavax. (**E**) Structure of the hepatitis E virus-like particle (HEV VLP). Left panel — atomic structure of HEV VLP. The 3 domains, S, P1, and P2 are colored blue, yellow, and red, respectively. Right panel — cryo-EM reconstruction at 14Å resolution. After Guu *et al*. (2009). (**F**) Bluetongue virus-like VLPs, requiring the simultaneous expression of four distinct proteins in varying amounts. VLPs are produced in *Nicotiana benthamiana* using the cowpea mosaic virus-based HyperTrans (CPMV-HT) and associated pEAQ plant transient expression vector system. After Thuenemann *et al*. (2013). (**G**) Ebola VLP visualized by EM. Reproduced from somapps.med.upenn.edu site. (H) Influenza VLPs. Left panel — EM of negatively stained A/Anhui/1/2013 (H7N9) VLP, assembled from HA and NA proteins expressed from separate baculovirus vectors in insect cells. After Smith *et al*. (2013). Right panel — influenza VLPs were produced in a baculovirus system with the sequences of the genes for HA, NA, and M1 of the 1918 pandemic virus. After Perrone *et al*. (2009).

VLP VACCINES

When VLP is used as a platform for the presentation of foreign epitopes, in majority of cases this is achieved through modification of the VLP gene sequence, so that fusions of VLP components with a foreign epitope are assembled into VLPs during expression. When large protein domains are needed as an antigen, they might be more difficult to display on VLPs, as such insertions may be incompatible with the VLP assembly. In that case, a chemical conjugation can be a solution.

Some VLP vaccines have been licensed and commercialized, others have entered clinical development, while many are in the proof-of-hypothesis stage. Prophylactic human vaccines, based on VLPs that have been registered and are in use protect against hepatitis B virus (HBV) and human papilloma virus (HPV) infections.

Hepatitis B vaccine. Hepatitis B virus infection is one of the most common human diseases. Each year over one million people die from HBV-related chronic liver diseases. Hepatitis B vaccine became the first recombinant protein-based vaccine for humans, approved by the

Federal Drug Administration (USA) in 1986. It is based on a recombinant HBV surface antigen (HBsAg), which upon production in yeast or mammalian cells forms 22-nm spherical VLPs that are adsorbed on an aluminum hydroxide gel (Greiner *et al*., 2012). These particles are composed of host cell-derived lipids (30–50%) and about 70 copies of the S protein (226 amino acid residues, ~25 kDa) and are stabilized by intra- and intermolecular disulfide bonds. Contrary to mammalian-derived HBsAg particles, yeast-derived particles contain unglycosylated S protein. Highly hydrophobic S protein in HBsAg VLPs is in tight association with lipids that have been shown to be responsible for the antigenic properties of HBsAg particles, stabilizing their structure and protein conformation. Thus, these VLPs exhibit a lipoprotein-like structure with an ordered and rather rigid lipid interface and a more hydrophobic and fluid inner core. Protein molecules included in each particle display a protruding part and another one deeply inserted into the lipid core (Greiner *et al*., 2010). Loss of protein protrusions due to particle heating at 60ºC modifies the organization of both the lipid core and lipid membrane surface, followed by a loss of HBsAg antigenicity (Greiner *et al*., 2014).

A course of 2–3 vaccine injections is given intramuscularly, the second injection at least one month after the first one and the third injection being administered up to six months after the first one. The induced anti-HBV antibodies and immune system memory provide immunity to hepatitis B infection. The common brands available are Recombivax HB (Merck), Engerix-B (GlaxoSmith-Kline Biologicals), Elovac B (Human Biologicals Institute), Genevac B (Serum Institute), Shanvac B (Shanta Biotechnics in India), etc. Engerix-B contains purified surface antigen (HBsAg) of HBV expressed in yeast, and although it is not glycosylated, it is immunogenically and physically similar to the antigen isolated from the plasma of chronic carriers (Drug master file at http://www.fda.gov/BiologicsBloodVaccines/Vaccines/ApprovedProducts/ucm110102.htm). Protection lasts for at least 25 years in cases of adequate initial response to vaccination, but some guidelines now recommend a single booster after 5 years. The hepatitis B vaccine was found to be generally safe although the Engerix B vaccine appeared to triple the risk of CNS inflammatory demyelination in infant boys (Mikaeloff *et al*., 2009). A possible culprit is thiomersal, a mercury-containing vaccine preservative that is currently being phased out in many countries.

HPV vaccine. About 30 to 40 types of HPV can be transmitted through sexual contact, *via* the anogenital region. Persistent infection with high-risk HPV types — different from the ones that cause skin warts — may progress to precancerous lesions and invasive cancer. Cervical cancer is an important public health problem worldwide, especially in developing countries. HPV vaccines are virus-like particles assembled from 72 pentamers of HPV major capsid protein — L1. Two kinds of prophylactic HPV vaccines exist: a bivalent vaccine (Cervarix) that offers protection against HPV serotypes 16 and 18, and a quadrivalent vaccine (Gardasil) that protects against infection with serotypes 6, 11, 16, and 18. The GlaxoSmithKline-manufactured Cervarix contains 20 μg each of HPV16 L1, and HPV18 L1 proteins expressed in a baculovirus system, while the Merck-manufactured Gardasil contains 20 μg of HPV6 L1, 40 μg of HPV11 L1, 40 μg of HPV16 L1, and 20 μg of HPV18 L1 proteins; these proteins are expressed in yeast, purified, and combined to make the vaccine. These vaccines contain in addition adjuvants such as alumina (aluminum hydroxide) and AS04 with monophosphoryl lipid A (MPL) in Cervarix, and amorphous aluminum hydroxyphosphate sulfate in Gardasil. Such vaccines are highly efficacious if given before exposure to HPV, i.e. to adolescent girls between 9 and 13 years of age in a three-dose schedule. The current duration of protection is 8.4 years with the bivalent vaccine and 5 years with the quadrivalent vaccine. A development of a nine-valent prophylactic HPV vaccine (HPV6/11/16/18/31/33/45/52/58) is currently being undertaken. Research is also directed towards the development of a prophylactic L2 vaccine and therapeutic vaccines (active after infection).

EXPERIMENTAL VLP VACCINES

A large number of VLP-based vaccine candidates is undergoing a clinical evaluation. The site *clinicaltrials.gov* lists 88 studies for VLP vaccines, created predominantly against viral infections. Among them, there are 29 completed trials for HPV L1 VLP vaccines, three trials for Norwalk (norovirus) VLP vaccines, one for Chikungunya VLP vaccine, etc. An interesting case is a completed trial for a therapeutic anti-HIV vaccination with p17/p24:Ty-VLP, which investigates the immunity of VLP made of yeast Ty protein and decorated with HIV p24 protein. However, this vaccine, although it elicited antibodies against p24 (and Ty) was not able to slow the progression of HIV-1 disease (Lindenburg *et al*., 2002). There is also a trial listed on a non-viral vaccine, against essential hypertension, which consists of angiotensin II (Ang II), a naturally occurring octapeptide, coupled to the surface of Qβ bacteriophage VLP. This vaccine decreased the blood pressure of Ang II-induced hypertensive mice (Chen *et al*., 2013).

Some clinical trials concern investigation of anticancer VLP vaccines. Melan-A VLP and CYT004-MelQbG10 vaccines for melanoma patients consist of a melanocyte differentiation antigen Melan A (also called MART-1) encapsulated in Qβ VLP together with short immunostimulatory oligonucleotides (CpGs) or other adjuvants. Upon administration, this vaccine may activate the immune system to exert a specific cytotoxic T lymphocyte (CTL) response against cancer cells expressing Melan A antigen that is upregulated in most melanomas. Indeed, Melan A vaccination resulted in an increase of T-cells at the injection site (Goldinger *et al*., 2010).

INFLUENZA VLPS VACCINES

Commercial influenza vaccines are either conventional vaccines made of killed virus or "split" or subunit vaccines that require an influenza virus that grows well enough in eggs or cultured mammalian cells to produce sufficient amounts of the essential vaccine antigen, hemagglutinin (HA). Split vaccines are produced in the same way as whole virus vaccines, but virus particles are disrupted using detergents. Subunit vaccines consist of purified HA and NA proteins, with the other viral components removed. Growing and preparation of such vaccines is rather long and expensive. Development of recombinant HA-based vaccines might help to overcome these limitations, but some doubts persist concerning the effectiveness of vaccines based only on HA. Presumably they are not able to elicit cell-mediated immunity and thus to immunize against different influenza strains. Influenza VLPs composed of more than one influenza protein could conceivably provide a solution to these shortcomings. For this purpose, corresponding influenza genes are usually cloned into the baculovirus vectors in order to produce multipro-

tein influenza VLPs in insect cells. Assembled VLPs are then secreted as enveloped, pleomorphic particles (Fig. 1) resembling influenza virions.

Already in 2005 it has been shown that VLPs built of the HA, NA, and M1 proteins of H9N2 influenza virus induced a protective immune responses in mice (Pushko *et al.*, 2005). Somewhat later similar results were obtained for H5N1 VLPs in mice and H1N1 VLPs in humans (Bright *et al.*, 2008; Lopez-Macias *et al.*, 2011). These vaccines were shown to display conformation-dependent antigenic epitopes associated with HA oligomers and induced robust anti-HA and anti-NA antibody responses in clinical studies.

The site *clinicaltrials.gov* lists human trials with influenza VLPs vaccines, namely for: trivalent seasonal VLP vaccine (Novavax), H5N1 VLP (pandemic influenza), H1N1 2009 VLP vaccine and seasonal VLP vaccine (Novavax trivalent and quadrivalent vaccines). Interesting cases are represented by three plant-made vaccines — H5 VLP vaccine (alum-adjuvanted plant-made VLPs containing the HA protein of H5N1 influenza, Landry *et al.*, 2010), H1 VLP vaccine (single non-adjuvanted dose of the H1 VLP influenza vaccine), and H5-VLP + GLA-AF vaccine. In the latter case the vaccine consists of recombinant H5 and N1 proteins produced in a plant-based expression system and assembled into virus-like particles together with the adjuvant glucopyranosyl Lipid A (GLA-AF) or with the alum adjuvant. None of the influenza VLP vaccines has been approved so far.

It appears that in these trials, apart from investigating the immune response, emphasis is placed on the use of appropriate adjuvants in order to obtain a more pronounced response of the immune system. This is surprising since it has been demonstrated that VLPs display excellent adjuvant properties, capable of inducing innate and cognate immune responses. However, it is plausible that since polymorphous, asymmetric influenza VLPs do not contain repetitive high-density viral surface proteins like the homogenous symmetrical VLPs, they are not capable of presenting epitopes in a way required for eliciting a strong immune response. Indeed, it has been shown that when the cytotoxic T lymphocyte epitope, a peptide derived from influenza A virus, was presented on a symmetrical platform formed of SV40 VLP, influenza-specific CTLs were induced, and heterosubtypic protection against influenza A viruses was achieved without the need of adjuvants (Kawano *et al.*, 2014).

ADENOVIRAL DODECAHEDRON AS VACCINE PLATFORM

Vaccines against human papillomavirus and hepatitis B are VLP vaccines constructed to protect against their virus of origin. However, VLPs can also be used to present foreign epitopes to the immune system (chimeric VLPs). When constructing a novel influenza vaccine, we used the VLP platform built from a protein derived from the adenovirus (Ad) for carrying the epitopes of influenza virus, with the goal of establishing immunity against influenza and not against Ad infection (Szurgot *et al.*, 2013).

Adenoviral dodecahedra are non-enveloped symmetrical VLPs built from 12 pentons, which are non-covalent complexes composed of pentameric penton bases and trimeric fiber proteins, both proteins being responsible for intracellular penetration of the virus (Fig. 2). These VLPs, smaller than the virus of origin, are generated during the life cycle of certain adenovirus serotypes, including human adenovirus serotype 3 (Ad3), where they participate in spread of progeny virus through loosening of tight junctions (Fender *et al.*, 2005; 2012; Lu *et al.*, 2013). These so called dodecahedra-fibre (DFs) of 4.8 MDa and diameter of about 50 nm can also be produced upon expression of Ad3 pentons in the baculovirus system. Formation of these particles is solely due to penton base interactions, as attested, upon expression of the penton base protein alone, by the formation of dodecahedra devoid of fibers, with molecular weight of 3.6 MDa and diameter of 28 nm (called dodecahedra-base DB, hereafter referred to as Dd, Fig. 2A). Stability of the dodecameric structure does not depend on disulfide bridges or cations as in other VLPs (Simon *et al.*, 2014). Instead, the major mechanism of stability lies in interlocking of the 60 N-terminal domains derived from 12 pentameric penton bases, which results in formation of a strong net stabilizing the VLP (Szolajska *et al.*, 2012). Dds retain integrity under different physicochemical conditions, which enables their convenient storage as well as attachment of therapeutic agents (Zochowska *et al.*, 2009).

Dds efficiently penetrate cellular plasma membrane and access the cytoplasm, whereupon up to 300 000 particles can be observed in one cell *in vitro* (Garcel *et al.*, 2006). This extraordinary internalization capacity makes Dd a very attractive delivery tool. Ad3 Dd inventors first proposed the use of adenoviral dodecahedron for human gene therapy as an alternative to whole adenovirus (Fender *et al.*, 1997). Later, both DF and Dd have been used as vectors for direct intracellular delivery of anti-cancer agents covalently attached to the vector surface or as facilitators of drug delivery to tumors (Fender *et al.*, 2003; Zochowska *et al.*, 2009; Wang *et al.*, 2011a; Wang *et al.*, 2011b; Beyer *et al.*, 2011; Wang *et al.*, 2013; Zochowska *et al.*, 2014). Intracellular delivery, important in both therapeutic and fundamental applications, faces two major challenges: efficient cellular uptake and avoiding endosomal sequestration. It appears that adenoviral Dd displays properties of the virus of its origin that ensures a remarkably efficient penetration without endosomal sequestration.

Another possible application of dodecahedron, typical for VLPs, is in vaccine construction as a delivery platform for foreign antigens. Importantly, VLP size suggests that it will be able to target not only dendritic cells at the injection site, but also lymph node-resident DC. In order to attach proteins of interest to Dd, initially a system was designed that takes advantage of the interaction of proline-rich PPVY motifs in the N-terminus of penton base (VLP building block) with cellular structural domains called WW. The WW modules served as adaptors, on average enabling delivery of more than ten million active protein molecules per cell (Garcel *et al.*, 2006). This attachment mode was also used for delivery of a model antigen, ovalbumin (OVA), with DF. Immunization with WW-OVA/DF induced integrated humoral and antigen specific T-cell responses which resulted in effective prophylactic and therapeutic protection against the highly aggressive B16-OVA melanoma in mice (Villegas-Mendez *et al.*, 2010). Thus, antigen delivery by a dodecahedric vector seems to be a promising novel strategy for development of oncological vaccines.

EXPERIMENTAL INFLUENZA VACCINE CONSTRUCTED ON AN ADENOVIRAL DODECAHEDRON PLATFORM

We exploit the features of the adenoviral dodecahedron for engineering a multivalent vaccination platform

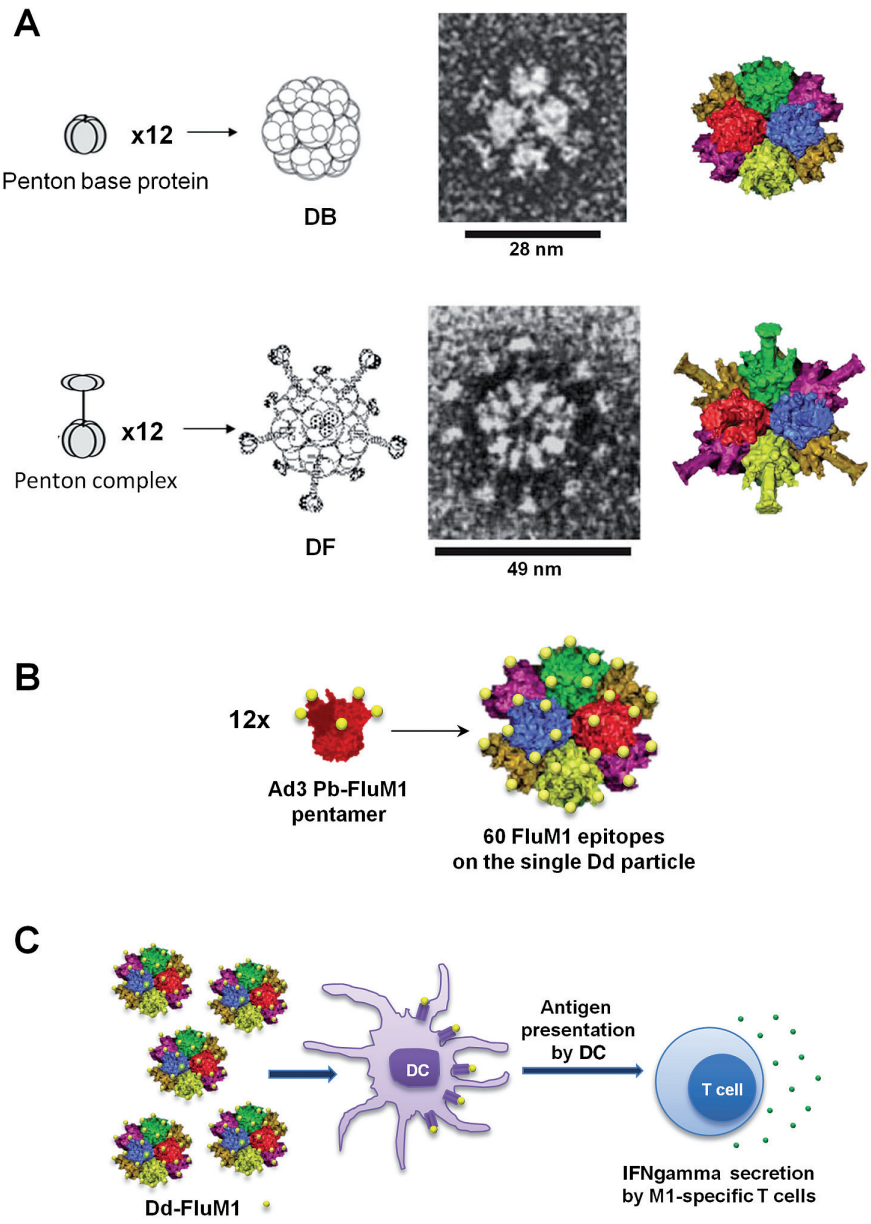

Figure 2. Adenoviral dodecahedron.
(**A**) Adenoviral dodecahedra. Left panels depict schematically the assembly of Dd (dodecahedron base, DB) and DF from adenovirus penton bases or pentons, respectively. Middle panels show cryo-electron microscopy of Dd and DF (from Fender *et al.*, 1997), while right panels show Dd and DF structure at 9Å resolution (from Fuschiotti *et al.*, 2006). (**B**) Diagram of Dd formation bearing multiple M1 epitopes. (**C**) Schematic view of vaccine interaction with dendritic cells resulting in epitope presentation to T lymphocytes and inducing secretion of gamma interferon, a marker of cellular immunity.

carrying two different influenza antigens - matrix protein M1 and hemagglutinin. This kind of vaccine should be able to induce both humoral and cell-mediated immunity, possibly providing long lasting protection against different strains of influenza virus. As described earlier, viral surface protein, hemagglutinin (HA, 568 residues), is the key antigen of influenza virus able to induce antibodies neutralizing viral penetration (Knossow & Skehel, 2006). However, due to the high mutation rate, traditional vaccines mainly based on HA confer protection only against the immunizing and closely related virus strains and are ineffective against strains with serologically distinct HA. In contrast, an internal matrix protein M1 (252 aa residues), the most conserved influenza virus protein (Ito *et al.*, 1991), is capable of stimulating cellular immunity of the infected hosts, eliciting cytotoxic T lymphocytes activity in mice and generating specific CD8+T response in humans (Webster & Hinshaw, 1977; Garigliany *et al.*, 2010; Lee *et al.*, 2008). Thus, the use of M1 as an antigen could increase effectiveness of the vaccine, in addition to protection afforded by anti-HA.

At first, we constructed a vaccine composed of Dd as a platform for delivery and presentation, decorated with multiple copies of the complete matrix protein M1 (Naskalska *et al.*, 2009) using yeast-derived WW domains as adaptors. The M1 protein was expressed in bacteria with a GST-tag at the N-terminus and with a WW linker at the C-terminus. N-lauroylsarcosine-solubilized GST-M1WW protein was purified on Glutathione-Sepharose and subsequently incubated with Dd, which allowed formation of Dd-M1WW VLP decorated with approximately 10 copies of the M1 protein. Successful internali-

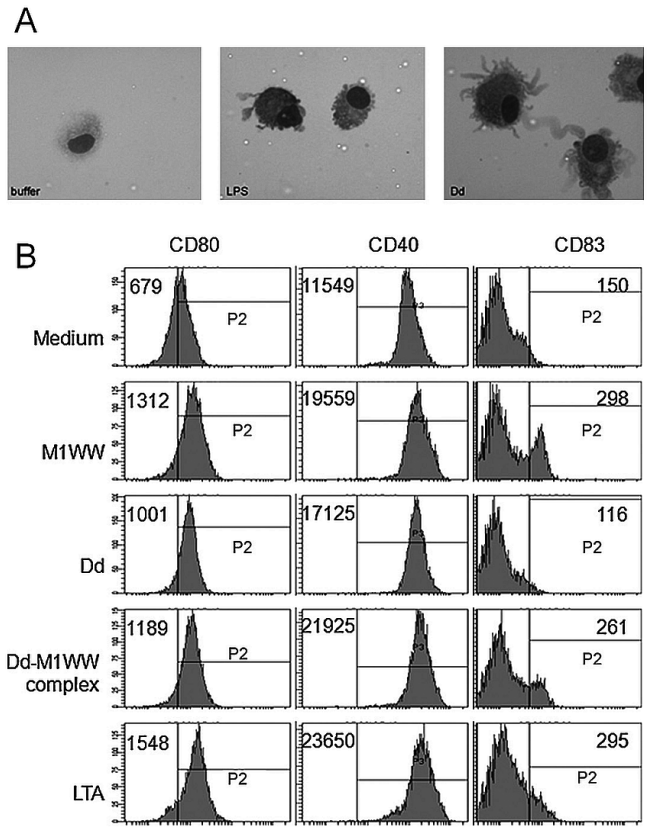

Figure 3. Human myeloid dendritic cells (MDC) maturation upon Dd uptake. (**A**) Optical microscopy images of MDC incubated with a buffer (negative control), Dd or lipopolysaccharide (LPS, positive control). (**B**) Expresssion of DC activation markers shown with flow cytometry after 48h culture of DC in the presence of Dd or M1WW alone, or with the Dd-M1WW complex and with lipoteichoic acid (LTA, positive control). Maturation was observed by upregulation of CD40 and CD80. After Naskalska *et al.* (2009).

zation of Dd-M1WW into myeloid dendritic cells (MDC, the most important antigen presenting cells, APC) was demonstrated by flow cytometry, with no internalization of GST-M1WW alone. Functional analyses demonstrated that the Dd-M1WW vaccine activated MDC and efficiently triggered specific cell-mediated responses. Indeed, formation of motile cytoplasmic veils (Fig. 3) and upregulation of specific activation markers, CD40 and CD80, demonstrated maturation and activation of DC. Moreover, Dd carrying M1WW was efficiently presented by MDC to M1-specific CD8+ T lymphocytes, and anti-M1 T lymphocytes that were in contact with DC primed with the Dd-M1WW complex secreted IFN gamma, indicating efficient capture, processing and presentation of the antigen by the dendritic cells.

To avoid problems with solubility of the M1WW we applied an alternative approach, which involved the use of specific epitopes derived from the M1 protein. We based our strategy on comprehensive ex vivo analysis of cross-reactive CD4+ and CD8+ memory T cell response to overlapping peptides spanning the full proteome of two influenza strains (Lee *et al.*, 2008). This analysis showed that the key targets of cross-recognition were M1 and nucleoprotein (NP). In addition, the earlier investigation demonstrated that M1-derived peptides were able to recall memory CTL responses from human peripheral blood mononuclear cells (PBMC) (Gotch *et al.*, 1987; Gianfrani *et al.*, 2000; Plotnicky *et al.*, 2003). For construction of a vaccine that should induce heterosubtypic T cell-mediated immunity we engineered two immunodominant M1 epitopes $FluM1_{40–57}$ — EALMEWLKTRPILSPLT(CD8+) and $FluM1_{55–72}$ —LTKGILGFVFTLTVPSER (CD4+ and CD8+) into the Dd structure. The epitopes have been inserted either in the variable loop of Ad3 Pb or as an extension of the Pb N-terminal domain without destroying the particles' dodecahedric structure (Fig. 2B). The final vaccine consisted of a 1:1 mixture of two kinds of Dd nanoparticles, each bearing the M1 epitope in 60 copies.

Electron microscopy showed that insertion of the FluM1 peptides into two different external sites of Dd did not affect the vector assembly, as well as its entry potential; both $DdFluM1_{40–57}$ and $DdFluM1_{55–72}$ efficiently penetrated MDC, which was confirmed by confocal microscopy (Fig. 4B). In vitro assays demonstrated that our candidate vaccine exhibited no cytotoxic effect on human PBMC isolated from healthy donors. Importantly, FluM1 epitopes delivered on a Dd platform were efficiently captured by MDC, processed and presented by HLA class II and cross-presented by the HLA class I molecules, activating both CD8+ memory T cells and CD4+ T cells. In contrast, free FluM1 peptides applied at concentrations exceeding those in Dd-FluM1 complex by about 20-fold showed no immunogenicity. These are important results, as an effective influenza vaccine should elicit specific CTLs that could provide protection against heterologous strains by targeting conserved viral proteins. Establishment of memory T cells will not prevent the disease, but can promote viral clearance, reducing illness severity (Pushko *et al.*, 2005).

Functionality of the Dd-FluM1 vaccine was verified in chickens. For this, IFNγ secreted by immune cells isolated from immunized chicken spleens was measured and *ex vivo* restimulated with the M1 peptides. It appeared that 50 μg of DdFluM1 administered subcutaneously acted as an efficient vaccine. High and constant IFNγ level persisted until the 8th week after vaccination, suggesting strong and long lasting activation of T cells (Fig. 4C). Significantly, presence of an adjuvant turned out to be dispensable, which suggests that the use of adenoviral Dd as a vaccination platform might eliminate the necessity of addition of potentially harmful substances.

We wished to include in the vaccine a major influenza antigen, hemagglutinin (HA), to provide a complete defense against influenza infection. At first, we attempted to construct a vaccine with the HA protein attached externally to Dd through a previously described adaptor containing WW domains (Naskalska *et al.*, 2013). In the following approach the use of the WW linker was abandoned as the linker alone (over 100 amino acid residues) might elicit an immune response. Instead, we decided to use a fragment of trimeric Ad3 fiber protein as a linker to Dd. As described above, in the dodecahedra-fibers produced upon expression of Ad3 pentons (complexes of penton base and fiber, see Fig. 2A), the N-terminus of a fiber nests in the cavity on the penton base surface (Cao *et al.*, 2012), which results in formation of the DF bearing 12 trimeric fibers. Therefore, hemaggluti-

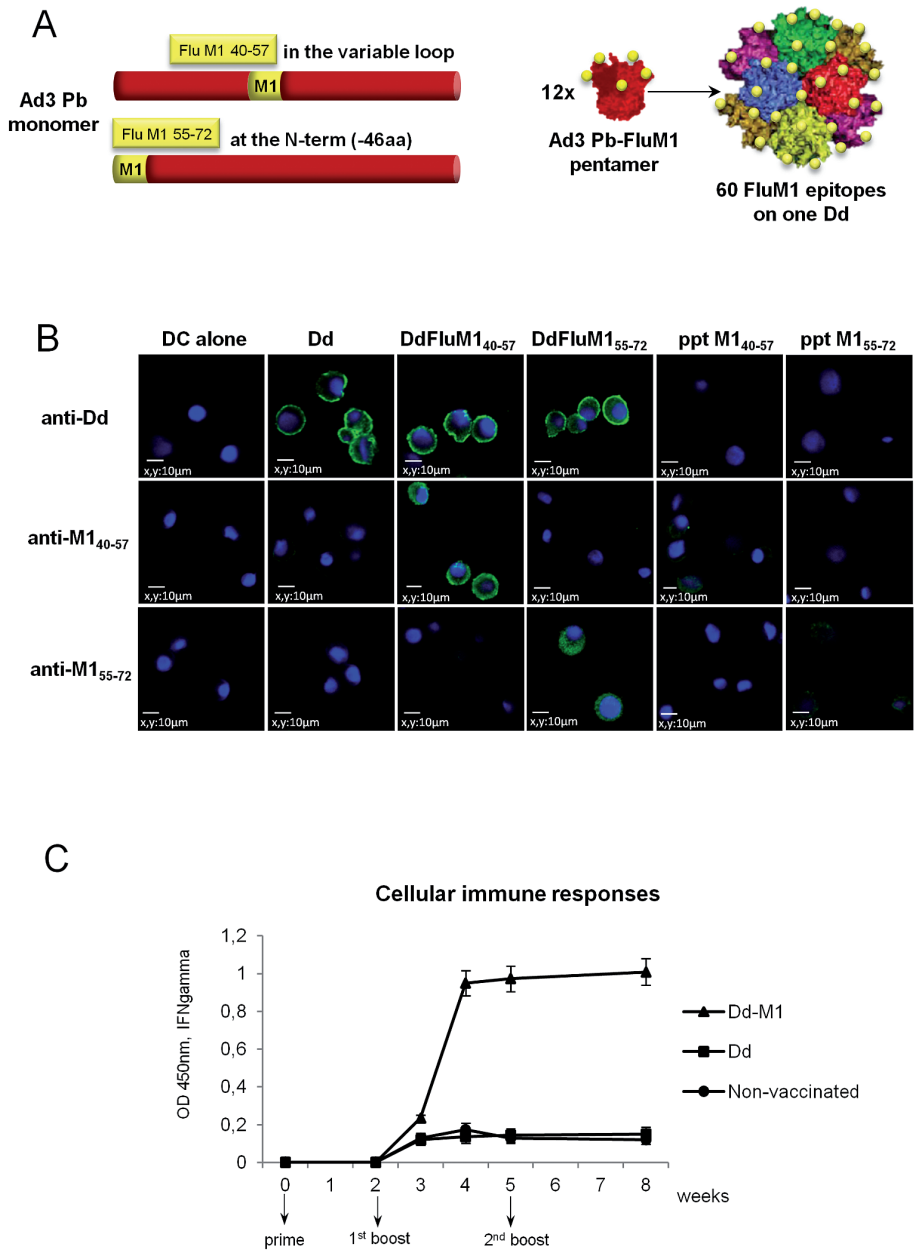

Figure 4. Influenza vaccine constructed on a platform of adenoviral dodecahedron bearing epitopes derived from influenza M1 protein.
(**A**) Left side — schematic view of constructs with M1 epitopes inserted into monomers of Ad3 penton base (Pb) protein that is a building block of Dd. Right side — diagram of vaccine formation. (**B**) Dd-M1 internalization into human dendritic cells, visualized by confocal microscopy using anti-Dd, anti-FluM1$_{40-57}$ and anti- FluM1$_{55-72}$ antibodies. Cell nuclei were counterstained with DAPI (blue signal). The last two columns show lack of internalization of free epitopes (ppt). (**C**) Chicken immunization with Dd-M1 vaccine. The graph shows secretion of IFN-γ (IFN gamma) by splenocytes of immunized or control chickens at the indicated time points (average values). One of three groups of chickens (n=25) was vaccinated with 50µg of the vaccine, while non-vaccinated birds and those immunized with Dd vector (50µg) served as controls. Isolated splenocytes were restimulated with the 1:1 mixture of M1$_{40-57}$ and M1$_{55-72}$ peptides and secretion of IFN-γ was measured by ELISA. After Szurgot *et al.* (2013).

nin devoid of TM, CT and SP was cloned in fusion with a fragment of the fiber protein, which contained a region responsible for its attachment to the penton base prolonged by a fragment of trimeric shaft, yielding a fusion protein called Fi-HA. Sequences encoding Dd-FluM1 and Fi-HA were expressed simultaneously from one recombinant baculovirus. This approach resulted in production of soluble complexes containing Dd with attached Fi-HA. This material will be used in an influenza vaccine preparation and will be tested for its vaccination properties in the chicken model.

CONCLUSIONS

Virus-like particles are multimeric nanostructures assembled from viral structural proteins responsible for cell penetration by the virus, ensuring efficient cell entry and thus tissue-specific targeting. It is important for vaccinology that VLPs present repetitive high-density displays of epitopes and display excellent adjuvant properties capable of inducing innate and cognate immune responses. VLPs provide delivery systems that combine good safety profiles with strong immunogenicity and

constitute a safe alternative to inactivated infectious viruses. They present both, high-density B-cell epitopes for antibody production and intracellular T-cell epitopes, thus inducing, respectively, potent humoral and cellular immune responses. Uptake of VLPs by antigen-presenting cells leads to efficient immune responses resulting in keeping pathogenic microorganism infections under control.

Acknowledgements:

This work was partially supported by grants: N N302 505738 and UMO2013/09/B/NZ3/02327 of the Polish National Science Centre.

REFERENCES

Beyer I, van Rensburg R, Strauss R, Li Z, Wang H, Persson J, Yumul R, Feng Q, Song H, Bartek J (2011) Epithelial junction opener JO-1 improves monoclonal antibody therapy of cancer. *Cancer Res* **15**: 7080–7090.

Beyer I, Cao H, Persson J *et al.* (2012) Coadministration of epithelial junction opener JO-1 improves the efficacy and safety of chemotherapeutic drugs *Clin Canser Res* **18**: 3340–3351.

Beyer T, Herrmann M, Reiser C, Bertling W, Hess J (2001) Bacterial carriers and virus- likeparticles as antigen delivery devices: role of dendritic cells in antigen presentation. *Curr Drug Targets Infect Disord* **1**: 287–302.

Bright RA, Carter DM, Crevar CJ, Toapanta FR, Steckbeck JD, Cole KS *et al.* (2008) Cross- clade protective immune responses to influenza viruses with H5N1 HA and NA elicited by an influenza virus-like particle. *PLoS ONE* **3**: e1501.

Cao C, Dong X, Wu X, Wen B, Ji G, Cheng L, Liu H (2012) Conserved fiber-penton base interaction revealed by nearly atomic resolution cryo-electron microscopy of the structure of adenovirus provides insight into receptor interaction. *J Virol* **86**: 12322–12329.

Chen X, Qiu Z, Yang S, Ding D, Chen F, Zhou Y, Wang M, Lin J, Yu X, Zhou Z, Liao Y (2013) Effectiveness and safety of a therapeutic vaccine against angiotensin II receptor type 1 in hypertensive animals. *Hypertention* **61**: 408–416.

Fender P, Ruigrok RW, Gout E, Buffet S, Chroboczek J (1997) Adenovirus dodecahedron, a new vector for human gene transfer. *Nat Biotechnol* **15**: 52–56.

Fender P, Schoehn G, Foucaud-Gamen J, Gout E, Garcel A, Drouet E, Chroboczek J (2003) Adenovirus dodecahedron allows large multimeric protein transduction in human cells. *J Virol* **77**: 4960–4964.

Fender P, Boussaid A, Mezin P, Chroboczek J (2005) Synthesis, cellular localization, and quantification of penton-dodecahedron in serotype 3 adenovirus-infected cells. *Virology* **340**: 167–173.

Fender P, Hall K, Schoehn G, Blair GE (2012) The impact of human adenovirus type 3 dodecahedron on host cells and its potential role in viral infection. *J Virol* **86**: 5380–5385.

Fuschiotti P, Schoehn G, Fender P, Fabry CM, Hewat EA, Chroboczek J, Ruigrok RW, Conway JF (2006) Structure of the dodecahedral penton particle from human adenovirus type 3. *J Mol Biol* **356**: 510–520.

Garcel A, Gout E, Timmins J, Chroboczek J, Fender P (2006) Protein transduction into human cells by adenovirus dodecahedron using WW domains as universal adaptors. *J Gene Med* **8**: 524–531.

Garigliany MM, Habyarimana A, Lambrecht B, Van de Paar E, Cornet A, vanden Berg T, *et al.* (2010) Influenza A strain-dependent pathogenesis in fatal H1N1 andH5N1 subtype infections of mice. *Emerg Infect Dis* **16**: 595–603.

Gianfrani C, Oseroff C, Sidney J, Chesnut RW, Sette A (2000) Human memory CTL response specific for influenza A virus is broad and multispecific. *Hum Immunol*; **61**: 438–452.

Goldinger SM, Imhof L, Willers J, French LE, Dummer R (2010) P30 Phase II clinical trial using Virus-Like Particle (VLP) vaccine including a melan-A analogon and imiquimod. *Melanoma Research* **20**: e56 (MELANOMA III: Medical Therapy).

Gotch F, McMichael A, Smith G, Moss B (1987) Identification of viral molecules recognized by influenza-specific human cytotoxic T lymphocytes. *J Exp Med* **165**: 408–416.

Greiner VJ, Egelé C, Oncul S, Ronzon F, Manin C, Klymchenko A, Mély Y (2010) Characterization of the lipid and protein organization in HBsAg viral particles by steady-state and time-resolved fluorescence spectroscopy. *Biochimie* **92**: 994–1002.

Greiner VJ, Ronzon F, Larquet E, Desbat B, Estèves C, Bonvin J, Gréco F, Manin C, Klymchenko AS, Mély Y (2012) The structure of HBsAg particles is not modified upon their adsorption on aluminium hydroxide gel. *Vaccine* **30**: 5240–5245.

Greiner VJ, Manin C, Larquet E, Ikhelef N, Gréco F, Naville S, Milhiet PE, Ronzon F, Klymchenko A, Mély Y (2014) Characterization of the structural modifications accompanying the loss of HBsAg particle immunogenicity. *Vaccine* **32**: 1049–1054.

Guu TS, Liu Z, Ye Q, Mata DA, Li K, Yin C, Zhang J, Tao YJ (2009) Structure of the hepatitis E virus-like particle suggests mechanisms for virus assembly and receptor binding. *Proc Natl Acad Sci U S A* **106**: 12992–12997.

Ito T, Gorman OT, Kawaoka Y, Bean WJ, Webster RG (1991) Evolutionary analysis of the influenza A virus M gene with comparison of the M1 and M2 proteins. *J Virol* **65**: 5491–5498.

Kawano M, Morikawa K, Suda T, Ohno N, Matsushita S, Akatsuka T, Handa H, Matsui M (2014) Chimeric SV40 virus-like particles induce specific cytotoxicity and protective immunity against influenza A virus without the need of adjuvants. *Virology* **448**: 159–167.

Knossow M, Skehel J (2006) Variation and infectivity neutralization in influenza. *J Immunol* **119**: 1–7.

Landry N, Ward BJ, Trépanier S, Montomoli E, Dargis M, Lapini G, Vézina LP (2010) Preclinical and clinical development of plant-made virus-like particle vaccine against avian H5N1 influenza. *PLoS One* **5**(12): e15559.

Lee LY, Ha L A, Simmons C, de Jong M D, Chau N V, Schumacher R, Peng YC, McMichael AJ, Farrar JJ et al (2008) Memory T cells established by seasonal human influenza A infection cross-react with avian influenza A (H5N1) in healthy individuals. *J Clin Invest* **118**:
3478–3490.

Lindenburg CE, Stolte I, Langendam MW, Miedema F, Williams IG, Colebunders R, Weber JN, Fisher M, Coutinho RA (2002) Long-term follow-up: no effect of therapeutic vaccination with HIV-1 p17/p24:Ty virus-like particles on HIV-1 disease progression. *Vaccine* **20**: 2343–2347.

Lopez-Macias C, Ferat-Osorio E, Tenorio-Calvo A, Isibasi A, Talavera J, Arteaga-Ruiz O *et al.* (2011) Safety and immunogenicity of a virus-like particle pandemic influenza A (H1N1) 2009 vaccine in a blinded: randomized, placebo-controlled trial of adults in Mexico. *Vaccine* **29**: 7826–7834.

Lu ZZ, Wang H, Zhang Y, Cao H, Li Z, Fender P, Lieber A (2013) Penton-dodecahedral particles trigger opening of intercellular junctions and facilitate viral spread during adenovirus serotype 3 infection of epithelial cells. *PLoS Pathog* **9**: e1003718.

Mikaeloff Y, Caridade G, Suissa S, Tardieu M (2009) Hepatitis B vaccine and the risk of CNS inflammatory demyelination in childhood. *Neurology* **72**: 873–880.

Naskalska A, Szolajska E, Chaperot L, Angel J, Plumas J, Chroboczek J (2009) Influenza recombinant vaccine: Matrix protein M1 on the platform of the adenovirus dodecahedron. *Vaccine* **27**: 7385–7393.

Naskalska A, Szolajska E, Andreev I, Podsiadla M, Chroboczek J (2013)Towards a novel influenza vaccine: engineering of hemagglutinin on a platform of adenovirus dodecahedron. *BMC Biotechnol* **13**: 1–9.

Nilsson J, Miyazaki N, Xing L, Wu B, Hammar L, Li TC, Takeda N, Miyamura T, Cheng RH (2005) Structure and assembly of a T=1 virus-like particle in BK polyomavirus. *J Virol* **79**: 5337–5345.

Perrone LA, Ahmad A, Veguilla V, Lu X, Smith G, Katz JM, Pushko P, Tumpey TM (2009) Intranasal vaccination with 1918 influenza virus-like particles protects mice and ferrets from lethal 1918 and H5N1 influenza virus challenge. *J Virol* **83**: 5726–5734.

Plotnicky H, Cyblat-Chanal D, Aubry JP, Derouet F, Klinguer-Hamour C, BeckA, et al. (2003) The immunodominant influenza matrix T cell epitope recognized inhuman induces influenza protection in HLA-A2/K(b) transgenic mice. *Virology* **9**: 309: 320.

Pushko P, Tumpey TM, Bu F, Knell J, Robinson R, Smith G (2005) Influenza virus-like particles comprised of the HA, NA, and M1 proteins of H9N2 influenza virus induced protective immune responses in BALB/c mice. *Vaccine* **23**: 5751–5759.

Simon C, Klose T, Herbst S, Han BG, Sinz A, Glaeser RM, Stubbs MT, Lilie H (2014) Disulfide linkage and structure of highly stable yeast-derived virus-like particles of murine polyomavirus. *J Biol Chem* **289**: 10411–10408.

Smith GE, Flyer DC, Raghunandan R, Liu Y, Wei Z, Wu Y, Kpamegan E, Courbron D, Fries LF 3rd, Glenn GM (2013) Development of influenza H7N9 virus like particle (VLP) vaccine: homologous A/Anhui/1/2013 (H7N9) protection and heterologous A/chicken/Jalisco/CPA1/2012 (H7N3) cross-protection in vaccinated mice challenged with H7N9 virus. *Vaccine* **31**: 4305–4313.

Szolajska E, Burmeister WP, Zochowska M, Nerlo B, Andreev I, Schoehn G, Andrieu JP, Fender P, Naskalska A, Zubieta C, Cusack S, Chroboczek J (2012) The structural basis for the integrity of adenovirus Ad 3 dodecahedron. *PLoS One.* **7**: e46075.

Szurgot I, Szolajska E, Laurin D, Lambrecht B, Chaperot L, Schoehn G, Chroboczek J (2013) Self-adjuvanting influenza vaccine presenting epitopes for cell-mediated immunity on a proteinaceous multivalent nanoplatform. *Vaccine* **31**: 4338–4346.

Thuenemann EC1, Meyers AE, Verwey J, Rybicki EP, Lomonossoff GP (2013) A method for rapid production of heteromultimeric pro-

tein complexes in plants: assembly of protective bluetongue virus-like particles. *Plant Biotechnol J* **11**: 839–846.

Villegas-Mendez A, Garin MI, Pineda-Molina E, Veratti E, Bueren JA, Fender P, Lenormand JL (2010) *In vivo* delivery of antigens by adenovirus dodecahedron induces cellular and humoral immune responses to elicit antitumor immunity. *Mol Ther* **18**: 1046–1053.

Wang H, Li Z, Liu Y, Persson J, Beyer I, Möller T, Koyuncu D, Drescher MR, Strauss R, Zhang XB, Wahl JK 3rd, Urban N, Drescher C, Hemminki A, Fender P, Lieber A (2011a) Desmoglein 2 is a receptor for adenovirus serotypes 3, 7, 11 and 14. *Nat Med* **17**: 96–104.

Wang H, Li Z, Yumul R, Lara S, Hemminki A, Fender P, Lieber A (2011b) Multimerization of adenovirus serotype 3 fiber knob domains is required for efficient binding of virus to desmoglein 2 and subsequent opening of epithelial junctions. *J Virol* **85**: 6390–6402.

Wang JW, Roden RB (2013) Virus-like particles for the prevention of human papillomavirus-associated malignancies. *Expert Rev Vaccines* **12**: 129–141.

Wang H, Yumul R, Cao H, Ran L, Fan X, Richter M, Epstein F, Gralow J, Zubieta C, Fender P, Lieber A (2013) Structural and functional studies on the interaction of adenovirus fiber knobs and desmoglein 2. *J Virol* **87**: 11346–11362.

Webster RG, Hinshaw VS (1977) Matrix protein from influenza A virus and its role in cross-protection in mice. *Infect Immun* **17**: 561–566.

Zeltins A (2013) Construction and characterization of virus-like particles: a review. *Mol Biotechnol* **53**: 92–107.

Zinkernagel RM (2014) On the role of dendritic cells versus other cells in inducing protective CD8+ T cell responses. *Front Immunol* **5**: 30 (10; 5: 30. eCollection 2014).

Zochowska M, Paca A, Schoehn G, Andrieu JP, Chroboczek J, Dublet B, Szolajska E (2009) Adenovirus dodecahedron as a drug delivery vector. *PLoS One* **4**: e5569.

Zochowska M, Piguet A-C, Jemielity J, Kowalska J, Szolajska E, Dufour J-F, Chroboczek J (2014) Virus-like particle-mediated intracellular delivery of mRNA cap analog with in vivo activity against hepatocellular carcinoma. *Nanomedicine: Nanotechnology, Biology, and Medicine*, pii: S1549-9634(14)00419-5.