Self-adjuvanting influenza candidate vaccine presenting epitopes for cell-mediated immunity on a proteinaceous multivalent nanoplatform

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**Abstract**

 We exploit the features of a virus-like particle, adenoviral dodecahedron (Ad Dd), for engineering a multivalent vaccination platform carrying influenza epitopes for cell-mediated immunity. The delivery platform, Ad Dd, is a proteinaceous, polyvalent, and biodegradable nanoparticle endowed with remarkable endocytosis activity that can be engineered to carry 60 copies of a peptide. Influenza M1 is the most abundant influenza internal protein with the conserved primary structure. Two different M1 immunodominant epitopes were separately inserted in Dd external positions without destroying the particles’ dodecahedric structure. Both kinds of DdFluM1 obtained through expression in baculovirus system were properly presented by human dendritic cells triggering efficient activation of antigen-specific T cells responses. Importantly, the candidate vaccine was able to induce cellular immunity *in vivo* in chickens. These results warrant further investigation of Dd as a platform for candidate vaccine, able to stimulate cellular immune responses.

Keywords: virus-like particle, influenza vaccine, M1 immunodominant epitopes, adenoviral dodecahedron, cell-mediated immunity

List of abbreviations: aa, amino acid, Ad, adenovirus; Ad3, adenovirus serotype 3; APC, antigen-presenting cells; CTL, cytotoxic T lymphocyte; Dd, dodecahedron; FCS, fetal calf serum; HA, hemagglutinin; HLA, human leucocyte antigen (a group of the most important antigens responsible for tissue compatibility); MoDC, monocyte-derived dendritic cells; NA, neuraminidase; Pb, penton base protein; PBMC, peripheral blood mononuclear cells; pDC, plasmacytoid dendritic cells**;** PE, Phycoerythrin; PMA/Iono, phorbol 12-myristate 13-acetate/ionomycin; rwtDd, recombinant wild-type Dd; SPF, specific pathogen-free; VLP, virus-like particle.

**Introduction**

 Virus-like particles (VLP) are naturally occurring biodegradable nanomaterials that incorporate such viral features as repetitive surfaces, particulate structures and induction of innate immunity through activation of pathogen-associated molecular-pattern recognition receptors. They carry no genetic information and can be often easily produced on a large scale. VLPs are being developed as safe and effective vaccine platforms for inducing potent B- and T-cell responses. The prophylactic human vaccines based on VLPs that have been registered include ~~the~~ vaccines against [human papillomavirus](http://en.wikipedia.org/wiki/Human_papillomavirus) (HPV) and against hepatitis B. These examples show the VLP vaccines constructed to protect against their virus of origin; however, VLPs can also be used to present foreign epitopes to the immune system. In the design of our vaccine the VLP platform built from a protein derived from the adenovirus (Ad) is used for carrying the epitopes of another virus, influenza, with the goal of establishing immunity against influenza and not against Ad infection.

The adenoviral dodecahedron (Ad Dd) is composed solely of 12 copies of a pentameric viral protein, penton base (Pb), one of two Ad proteins responsible for virus cell entry [[1](#_ENREF_1)]. Dd attaches to receptors recognized by Pb within an Ad particle, but, in addition, it recognizes heparan sulphates, which do not serve as receptors for the Ad of origin [[2](#_ENREF_2)]. Dd has an extraordinary propensity for intracellular entry - up to 300 000 of Dd can be seen in one cell in culture [[3](#_ENREF_3)]. Due to its polyvalency, the vector is able to deliver several millions of foreign molecules to one cell [[3](#_ENREF_3)]. Importantly, it is efficiently taken up by human DC and induces their maturation [[4](#_ENREF_4)]. Dd can be stored frozen and/or lyophilized and shows remarkable stability at temperatures up to 45oC [[5](#_ENREF_5)]. The crystallography and site-directed mutagenesis combined with biochemical analysis showed that the N-termini of the Pbs, Dd building blocks, interlock within the particle through strand exchange, thus creating the major stabilization element of the dodecahedral particle [[6](#_ENREF_6)].

The humoral response to currently used influenza vaccines is based on the induction of neutralizing antibodies mainly against the influenza surface glycoprotein, hemagglutinin (HA). Such neutralizing Abs block entry of the influenza virus but they are protective only against virus with closely matching HA. Antibody response builds up rather fast and declines slowly afterwards [[7](#_ENREF_7)], while the cell-mediated immunity that does not involve [Abs](http://en.wikipedia.org/wiki/Antibodies) seems to be lasting longer [[8](#_ENREF_8)]. The current influenza vaccines containing killed influenza virions do not induce CTL responses, because they are inefficiently processed for MHC class I presentation [[9](#_ENREF_9)]. In addition, due to the high mutability of influenza surface proteins (antigenic drift), the current vaccines have to be produced new every year following the WHO recommendations. It is thought that the cross-reactive CTL responses may have protective potential against variants of influenza viruses that are not neutralized by antibodies [[10](#_ENREF_10)].

The matrix protein M1 (252 aa residues) is the most abundant influenza internal virion protein with the most conserved primary structure among influenza virus proteins [[11](#_ENREF_11)], suggestive of its utility in induction of cellular immunity. Mice immunized with matrix protein were not protected after challenge but they showed enhanced clearance of the virus from the lungs after infection, signifying involvement of cell-mediated immunity [[12](#_ENREF_12)]. Indeed, M1 is able to elicit CTL activity in mice and generates specific CD8+ T cells response in humans [[13-15](#_ENREF_13)].

 Recently, an *ex vivo* analysis of cross-reactive CD4+ and CD8+ memory T cell response to overlapping peptides spanning the full proteome of two influenza strains has been performed in healthy individuals [[16](#_ENREF_16)]. M1 and nuclear protein turned out to be the immunodominant targets of cross-recognition. Those identified M1 epitopes that were recognized by human T cell repertoires are used here for the construction of a vaccine that should induce heterosubtypic T cell-mediated immunity conferring broad protection against avian and human influenza A viruses.

 We exploit the features of a VLP, the adenoviral dodecahedron, for engineering a vaccination platform carrying epitopes derived from the influenza M1 protein for induction of cell-mediated immunity. A vaccine in form of Dd bearing M1 epitopes was constructed, expressed in the baculovirus system and purified. It was efficiently internalized, processed and presented by the HLA class II and cross-presented by the HLA class I molecules triggering CD4+ and CD8+ T cell responses. Importantly, upon chicken vaccination with Dd carrying the M1 epitopes both cellular and humoral immune responses were elicited in the absence of an adjuvant. Our data show that the proposed vaccine is able to induce strong and possibly long-lasting cell-mediated immunity.

**Material and Methods**

**Construction of Dd bearing M1 epitopes.** For the cloning of FluM140-57 epitope (EALMEWLKTRPILSPLTK) into the variable loop of penton base (Pb), the 54-long sequences of forward and reverse primers encoding the FluM140-57 epitope along with the fragment of Ad3 Pb were obtained from Oligo.pl (Poland). The primers (Suppl. Table 1) were amplified by PCR, yielding the fragment of 90 nucleotides. In the two subsequent steps the first PCR product was amplified using as a matrix pFastBacDual containing the Ad3 Pb sequence, with primers permitting insertion of restriction enzymes‘ recognition sites. In the last step the final PCR product was obtained with deleted nucleotide fragment specifying the 157VTVND Pb sequence. The 1414 bp-long insert was then cloned into pFastBacDual using BamHI and PstI cloning sites. The genes encoding FluM155-72 epitope (LTKGILGFVFTLTVPSER) either in the variable Pb loop or at the N-terminus of the Pb were purchased from GeneArt GmbH (Germany). Three different constructs were designed. In the first one the 157VTVND sequence from the variable loop was replaced by the FluM155-72 epitope. Two others contained the same epitope at the N-terminus of Pb devoid of 8 or 46 amino acids (Figure 1A). The provided sequences were cloned into pFastBacDual under the control of polyhedrin promotor. The correct cloning was confirmed by sequencing.

**Dd expression and purification**. Dd and Dd bearing M1 epitopes were expressed in the baculovirus expression system [[5](#_ENREF_5)]. Expressing cells were collected, lysed and the lysates’ supernatants were fractionated by ultracentrifugation on a sucrose gradient, followed by ion-exchange chromatography [[2](#_ENREF_2)]. Concentration of purified proteins was measured with Nanodrop microspectrophotometer and confirmed by SDS-PAGE analysis in the presence of a known amount of albumin. Protein analysis was done under denaturing conditions, whereas the assembly status of purified Dd and Dd-M1 was analyzed by native agarose gel electrophoresis [[2](#_ENREF_2)]. After electrophoresis proteins were stained with Coomassie Brilliant Blue (CBB) or analyzed by Western blot with primary rabbit anti-Ad3 Dd, anti-FluM140-57 or anti-FluM155-72 antibodies (Suppl. data). ECL detection system (Amersham Biosciences) was used throughout this work.

**Vaccine internalization by dendritic cells.** MoDC (1x106/ml) cells were resuspended in RPMI medium. Dd, Dd-FluM140-57, Dd-FluM155-72 (10µg,) or molar equivalent (350ng) of M140-57 or M155-72 peptides (NeoMPS, France) in RPMI were applied onto cells. After 90min incubation cells were rinsed with cold PBS and fixed in 100% cold methanol for 10min. MoDC were then transferred onto poly-D-lysine-coated glass coverslips (12mm, BD Biosciences) and left for 3h at RT until dry. Coverslips placed into wells of 24-well plate were incubated for 1h at 37°C with the primary anti-Dd or anti-FluM140-57 or anti-FluM155-72 antibodies, rinsed with PBS and incubated with the secondary antibody Alexa Fluor 488 chicken anti-rabbit IgG, and finally with DAPI (Applichem, 1µg/µl, 5min at RT). Images were collected with EZ-C1 Nikon CLSM attached to inverted microscope Eclipse TE2000 E using oil immersion objective ×60, Plan Apo 1.4NA (Nikon). DAPI and Alexa Fluor fluorescence was excited at 408 and 488nm, and emission was measured at 430-465 and 500-530nm, respectively. All images were collected at 512×512 resolution and zoom 2.0. Figures were processed with EZ-C1 Viewer and Photoshop 6.0.

**Generation of anti-influenza specific CD8+ T lymphocytes and cross-presentation of FluM1 by MoDC.** Anti-FluM1 CTLs were generated as previously described [[17](#_ENREF_17)]. Briefly, GEN2.2 plasmacytoid DC loaded with 1µM FluM158-66 (GILGFVFTL) peptide and irradiated with 30Gy (Eckert & Ziegler gamma irradiator, BEBIG, France) were co-cultured with PBMC from HLA-A\*0201+ donor at a 1:10 ratio in RPMI-1640 Glutamax/10% FCS. After one week cell specificity was controlled with iTAgTM HLAA\*0201 FluM158-66 tetramer assay (Beckman Immunomics).

 For presentation assay, MoDC at 106cells/ml were pulsed for 18h with DdFluM155-72 (30 µg/ml), Dd (30µg/ml) or peptides (FluM158–66 or FluM155-72 orHIVpol476-484, each at10µM). Cells incubated with medium alone (non-stimulated) or with 5ng/ml PMA and 500ng/ml ionomycin were used as negative and positive controls, respectively. Loaded MoDC were washed before use and IFN-γ secretion by tetramer+ CD8+ T cells was analyzed as follows. T cells were first labelled with iTAg HLA-A\*0201 Flu M158-66 tetramer-PE for 30min at RT, washed and restimulated with loaded MoDC (10:1 ratio) for 5.5h and with 1µl/ml brefeldin A (BD Biosciences) during the last 3h. Cells were then surface-labelled with anti-CD3-PC7 and anti-CD8-APC antibodies (Beckman Coulter) and submitted to IFN-γ intracellular staining (BD Biosciences). IFN-γ staining was analyzed on tetramer+ CD8+ T cells on a FACS Canto II BD Biosciences flow cytometer.

**Analysis of anti-influenza specific CD4+ T lymphocytes induction and stimulation by DdFluM1.** *In vitro* stimulation of PBMC was performed by 7 days incubation in the presence of DdFluM140-57, DdFluM155-72, Dd, 30µg/ml each or 10µM M1 peptides. Cells were then restimulated with the same amount of antigen for 8h and with brefeldin A at 1µl/ml during the last 5h. Cells were next surface-labeled with anti-CD3-PC7 (Beckman Coulter) and anti-CD4-PerCPCy5.5 (BD Biosciences) antibodies and submitted to IFN-γ intracellular staining (BD Biosciences). CD4+ T cells were analyzed for IFN-γ staining by flow cytometry.

 **Immunization.** Mixture ofDdFluM140-57 and DdFluM155-72 at 1:1 (molar ratio), called DdFluM1 candidate vaccine, was used in animal studies. In the first experiment one-week-old birds (n=10) were immunized subcutaneously or intramuscularly with 10 or 50µg in 100µl PBS of Dd-FluM1 vaccine or Dd and boosted two weeks later using the same amount of candidate vaccine (or Dd) with and without complete Freund’s adjuvant, with non-vaccinated chickens as controls. Four weeks later the chickens were humanely sacrificed and the blood and spleen were harvested.In the second experiment one-week-old birds (n=25) were vaccinated subcutaneously with 50µg of Dd or DdFluM1 and boosted two weeks later with the same amount of protein. Blood sera and spleens of 5 vaccinated and control birds were collected for analysis at 3, 4 and 5 weeks after the first vaccination. At 5 weeks the remaining chickens were boosted again with Dd-FluM1 and immune responses were analyzed three weeks later.

 **Cell-mediated immune responses.** The secretion of chicken interferon-γ (chIFNγ) was measured in spleens from immunized or control chickens using capture ChELISA [[18](#_ENREF_18)]. Splenocytes were isolated as previously described [[18](#_ENREF_18)], seeded on 96-well plates (106 cells/well) and stimulated for 72h at 37°C in 5% CO2 with DdFluM1 at 20µg/ml. Medium alone was used as a negative control and pokeweed (PWE, 10µg/ml) or PMA/Iono (1µg/ml) (both from Sigma-Aldrich) were used as positive controls. For ChIFN-γELISAtheMaxiSorp Nunc-Immuno F96 microwell plates were coated for 1h at 37◦C with homemade mouse Ig anti-chicken IFN-γ 1E12 (2µg/ml) in PBS and then blocked for 30min at 37◦C with 2.5% casein in PBS. Supernatants of splenocytes stimulated as above were subsequently added and plates were incubated for 1h at RT. After this, the biotin-labelled mouse antibody 1D12 directed against chIFN-γ (1:10000; SouthernBiotech) was added, followed by 1h incubation with streptavidin–horseradish peroxidase conjugate (1:20000; Biosource Europe). After six washes, peroxidase activity was revealed with TMB peroxidase substrate (Sigma-Aldrich, USA) before stopping the reaction with 1M H3PO4. The optical density was determined at 450-560nm with an ELISA reader.

**Results**

 **FluM1 epitopes on dodecahedric platform.** Two immunodominant M1 epitopes have been identified through the examination of the virus-specific CD4+ and CD8+ memory T cell responses to the human H3N2 influenza A virus proteome [[19](#_ENREF_19)]. These epitopes, cross-recognized by healthy individuals, were FluM140-57 - EALMEWLKTRPILSPLTK (CD8+) and FluM155-72 - LTKGILGFVFTLTVPSER (CD4+ and CD8+). The FluM155-72 contains shorter FluM158-66 (GILGFVFTL) epitope identified many years ago [[20](#_ENREF_20)]. We decided to engineer both epitopes separately into the Dd structure. These epitopes have been inserted in the Dd structure in the variable loop of Ad3 Pb (a building block of Dd) or as an extension of the Pb N-terminal domain (Figure 1A and 1B and Suppl. data).

**DdFluM140-57 and DdFluM155-72 expression and purification.** The dodecahedric VLPs bearing FluM1 epitopes were expressed in baculovirus system (of note, expression in bacteria yields free pentameric penton bases only, unpublished results). Expressing cells were lysed and the lysate was fractionated on a 15-40% sucrose gradient (Figure 1C). The Dd-FluM140-57 and DdFluM155-72 monomers migrated on the SDS-PAGE at 63-kDa and 59-kDa band size, respectively. Similarly as for rwtDd, the majority of pentameric Pbs-FluM1 assembled into symmetrical particles of ~3.5 MDa made up of 12 pentameric Pbs-FluM1. However, only FluM140-57 in the variable Pb loop and FluM155-72 at the N-termini of Pb-46aa were expressed with high yield and these were further used. Since heavy sucrose fractions containing VLPs were contaminated with Pbs, cellular proteins, lipids and nucleic acids, final Dds-FluM1 purification was achieved by ion-exchange chromatography on a Q-Sepharose column where Dd-FluM1 eluted at 380mM NaCl. The presence of FluM1 epitopes in the Dd structure was confirmed by western blot (Figure 2) and mass spectrometry analysis, with 88 and 90% covering, respectively. Negative stain EM analysis showed that insertion of FluM1 epitopes either into the Dd variable loop or at the N-terminus of Dd did not affect the vector assembly (Figure 2D). Thus, the final material consisted of Dd particles each carrying 60 copies of respective M1 epitope. DdFluM140-57 and DdFluM155-72 yield was approximately 4 and 5mg of purified particles, respectively, from 100ml culture.

 **Internalization of Dd-FluM1 into human dendritic cells.** Both DdFluM140-57 and DdFluM155-72 efficiently penetrated MoDC (Figure 3). The entry potential of Dd did not seem to be affected by the insertion of FluM1 epitopes; furthermore at 1.5h post application all cells looked similar.

 **Presentation of FluM1 epitopes to specific T cells.** We observed the lack of cytotoxicity of Dd in human PBMC exposed for 24h to Dd (Figure 4A and 4B). Then, we analyzed whether FluM1 epitopes on Dd were efficiently cross-presented by MoDC to M1-peptide-specific T cells. The epitope FluM140-57 hasbeen shown to induce CD8+ T cells while FluM155-72 was able to induce both CD8+ and CD4+ T cells [[19](#_ENREF_19)]. For measuring the frequency of anti-FluM1 CD8+ T cells, T cells were generated from PBMC obtained from HLA-A\*0201 healthy volunteers stimulated by a pDC line loaded with the FluM158-66 peptide. After a 7-day co-culture the cells were characterized by tetramer labeling (Figure 3C, upper dot plots), to measure the frequency of specific CD8+ T cell, effectors of cell-mediated adaptive immunity. Anti-FluM158-66 specific T cells represented 8.7 to 25.5% of CD8+ T lymphocytes. Secretion of IFN-γ by these anti-Flu tetramer+ CTLs was then used as a read-out to measure the cross presentation of Dd bearing the FluM155-72 epitope by MoDC (Figure 4C). The spontaneous background of IFN-γ released by CD3+CD8+ HLA-A2 FluM158-66 tetramer+ was then used as reference to measure the fold changes in IFN-γ produced following stimulation (Figure 4D). When autologous MoDC loaded with the FluM158-66 peptide were used as APC, 4.2 to 11.5% (mean 8.1%) of the anti-FluM158-66 T cells secreted IFN-γ. As expected, these specific CTLs were not stimulated by the negative control HIV pol476-484 peptide. The MoDC loaded with the free FluM155-72 peptide activated only few specific CD8+ T cells as anticipated. This peptide being too long to bind directly to HLA class I molecules, after internalization by the MoDC should be processed to generate the FluM158-66 peptide presented by HLA-A\*0201 molecules to the specific T cells. When MoDC were loaded with the Dd-FluM155-72, 1.8 to 8.2% (mean 4.1%) of the anti-FluM1 T cells secreted IFN-γ (Figure 4D), close to previously reported observations [[21](#_ENREF_21)]. This result demonstrates that the DdFluM155-72 was internalized and processed by the MoDC to generate the FluM158-66 peptide, that was efficiently cross-presented in the context of HLA-A\*0201 molecules, allowing for secretion of IFN-γ by anti FluM158-66 peptide specific CTLs.

We then evaluated whether the Dd-FluM1 could trigger stimulation of anti-FluM1 CD4+ T cell. For this, the PBMC from HLA-A\*0201 healthy volunteers were cultured in the presence of, respectively, free FluM1 peptides (each separately) or Dd-FluM1. After a 7-day culture the cells were re-stimulated with the antigens, and IFN-γ secretion of the CD4+ T cells was measured by flow cytometry to quantify the number of generated anti-Flu specific CD4 T cells (Figure 5). None of free M1 peptides was able to stimulate CD4+ helper T cells - following re-stimulation no T lymphocytes secreted IFN-γ. However, when these peptides were presented on the dodecahedral platform, an amplification of CD4+ T cells was observed; for the 2 donors, we obtained 2.6 and 1.6% of anti FluM155-72, and 1.2 and 1.4% of anti FluM140-57 CD4+ T cells, confirming that this vector is able to activate CD4+ responses at levels comparable to pandemic vaccine [[22](#_ENREF_22)]. Free FluM1peptides applied at concentrations about 20-fold exceeding those of Dd-FluM1 showed no immunogenicity, while the peptides associated with the Dd were efficiently taken-up, processed and presented by the HLA class II and cross-presented by the HLA class I molecules, triggering CD8+ and CD4+ T cell responses.

 **Characterization of Dd-FluM1 candidate vaccine application in the context of adjuvant presence.** For the determination of conditions suitable for vaccine administration, two different doses (10 and 50µg) of candidate vaccine were applied subcutaneously or intramuscularly with and without Freund’s complete adjuvant to specific pathogen-free (SPF) chickens. For analysis of the cellular response, chIFN-γ secretion by immune cells isolated from immunized chicken spleens was measured two weeks after the last immunization and *ex vivo* restimulation with M1 peptides (Figure 6A and 6B). Subcutaneous application of Dd alone resulted in mitigated chIFNγ production. Significantly higher secretion of chIFN-γ was observed for all groups of chickens vaccinated with DdFluM1 in comparison to birds not vaccinated or vaccinated with unmodified particle. Even that all vaccination regimes with DdFluM1 elicited similar IFNγ production, the best result was observed for the chickens vaccinated without Freund’s adjuvant with 50µg of DdFluM1 administred subcutaneously. Also some weak anti-FluM1 peptide humoral response was observed in sera of immunized chickens (Suppl. Figure 1), which was 2-3 times lower than the response level against unmodified particle. It is relevant that M1 protein is known to induce mainly cellular immune responses [[23](#_ENREF_23)] while its contribution to humoral immunity is less significant [[24](#_ENREF_24), [25](#_ENREF_25)]. For both kinds of immune response at the given dose the presence of adjuvant turned out to be dispensable. The 50µg dose of candidate vaccine applied subcutaneously without adjuvant was selected for the subsequent experiments.

 **The kinetics of immune responses.** Secretion of chIFN-γ by splenocytes as well as the presence of M1-specific antibodies in serum was analyzed in vaccinated and non vaccinated chickens during 8 weeks (Figure 6C). At second week after the first boost, robust chIFN-γ secretion by splenocytes was detected. It was followed by high and constant IFN-γ level persisting until the end of the experiment, suggesting strong activation of T cells and showing the duration of this immunity (Figure 6D and 6E), portentous of potential long-term protection *in vivo*. Additionally, a weak induction of anti-M1 humoral response was observed; an increasing with time level of anti-M1 IgG was noted in the sera of vaccinated chickens (Suppl. Figure 1).

**Discussion**

 We describe here the design, production and properties of a novel candidate influenza vaccine composed of a VLP, adenoviral dodecahedron, carrying two different immunodominant M1 epitopes inserted in two external sites of Dd without affecting either Dd integrity or cell entry capacity. Our candidate vaccine induces cell-mediated immunity (CTL response). A CTL response directed toward the internal conserved proteins of the virus will not prevent the disease but might reduce its severity. It might conceivably elicit a state of immunological memory that permits the [immune system](http://www.ncbi.nlm.nih.gov/books/n/imm/A2528/def-item/A2921/) to respond more rapidly and effectively to encountered pathogens.

 It appears that the immune-stimulatory adjuvant molecule potentiate the cell-mediated responses [[26](#_ENREF_26), [27](#_ENREF_27)]. Since soluble proteins are poorly cross-presented, the delivery of antigens in a particulate form is considered critical for inducing robust CD8+ T cell activation [[28](#_ENREF_28), [29](#_ENREF_29)]. Here comes the utility of VLPs for vaccine construction. Their multiprotein nanostructure that mimics the organization and conformation of authentic native viruses but does not contain the viral genome potentially yields safer and cheaper vaccine candidates than whole viruses. And for polyvalent noninfectious VLP vaccines manufactured in cell culture systems, the chemical inactivation (as used in production of the current influenza vaccine) is not required. Some evidence suggest a superior immunogenicity of influenza VLPs over recombinant HA protein-based subunit vaccine [[30](#_ENREF_30)]. VLPs, unlike single proteins, have the ability to bind and enter cells using appropriate surface receptors. They present antigens in a structured repetitive way, which might allow efficient induction of immune responses [[31](#_ENREF_31)]. Some prophylactic VLP-based vaccines are currently on the market, against hepatitis B virus and human papillomavirus (HPV). However, both these vaccines contain adjuvants added to increase the immune response to them, aluminium hydroxide or AS04 (GlaxoSmithKline) or aluminum hydroxyphosphate sulphate [[32-35](#_ENREF_32)]. The GlaxoSmithKline sponsored studies did not reveal any AS04 toxicity in rats [[36](#_ENREF_36)]. However, aluminum, the most commonly used vaccine adjuvant, is a demonstrated neurotoxin and a strong immune stimulator, conceivably apt to induce neuroimmune disorders (see [[37](#_ENREF_37)] and the references therein).

Importantly, VLPs are thought to be effective immunogens in stimulating innate immune cells, particularly MoDC [[30](#_ENREF_30)]. Our vaccination platform alone is a potent activator of DC; application of adenoviral Dd on human MoDC induced formation of motile cytoplasmic veils and up-regulation of expression of immune co-stimulatory molecules, indicating maturation and activation of MoDC [[4](#_ENREF_4)]. This suggests that the use of adenoviral Dd as a vaccination platform might eliminate the necessity of inclusion of potentially harmful immune adjuvants. Indeed, the results presented in Figure 5B confirm this hypothesis since the absence of Freund’s adjuvant did not thwart the M1 immune responses in chickens.

 In our vaccine the M1 epitopes are engineered into the penton base (Pb) protein, the Dd building block, one epitope as an N-terminal extension and another inserted into the variable Pb loop. This vaccine spontaneously formed upon expression in the baculovirus system contains the Dd nanoparticle composed of twelve pentameric Pbs, with each Dd bearing a different M1 epitope in 60 copies. Our *in vitro* tests showed that FluM1 epitopes delivered on a Dd platform are efficiently captured, processed and presented in the context of HLA class II and cross-presented by the HLA class I molecules, activating both CD8+ memory T cells and CD4+ T cells that are known to support the generation and maintenance of CD8+ memory T cells. This is an important result, as an effective influenza vaccine of new generation should elicit specific CTLs that could provide protection across heterologous strains by targeting conserved regions of viral proteins. The establishment of memory T cells will not prevent infection, but can promote viral clearance, reducing the severity of illness. We also demonstrated the duration of this immunity following boost vaccination, which suggests that the proposed vaccine may confer long-lasting protection. It seems that VLP vaccine containing M1 in addition to HA are more efficacious than those with HA alone; when chickens were vaccinated either with VLPs composed of HA alone (HA-VLP) or with VLPs containing HA and M1 (HAM-VLP), upon challenge with highly pathogenic avian influenza virus 75% of HA-VLP vaccinated chickens showed the presence of replicating virus while none of the HAM-VLP vaccinated birds showed traces of virus replication [[38](#_ENREF_38)].

 The epitopes recognized by human T cell repertoire are used here for the construction of a vaccine, which was applied to chickens. Recently H7N1 isolate A/chicken/Italy/1067/99 derived CD8+ T-cell epitopes were identified [[39](#_ENREF_39)]. Among 18 predicted MHC-restricted epitopes, twice was identified the sequence C-terminally located in peptide FluM140-57 and twice the sequence N-terminally located in peptide FluM155-72, which means that these epitopes can be presented via chicken MHC.

 It is plausible to think that the pre-existing immunity to human Ad3 will not compromise the activity of our candidate vaccine. Ad3 is not very infectious serotype in humans and even less in animals; after five Dd applications to rats we observed a rather slow build-up of anti-Dd antibodies (unpublished results). Of note, the penton base, a building block of Dd, is one of 11 structural proteins of adenovirus; hence the eventual humoral immune response after administration of dodecahedric vector composed of only Pbs should be of lesser concern than after administration of adenovirus vector, which is already in use.

 In conclusion, taking into consideration the efficient activation of cell-mediated immunity by Dd-FluM1 both *in vitro* and in the animal model, and the fact that the epitopes carried by Dd are strongly conserved among different strains of influenza, our approach could be a good starting point in construction of a vaccine expressing also the major protective antigen of influenza, HA, to provide complete defense against influenza strains.

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