**Supplementary Data**

**Cells**. All cultures were carried out at 37oC under 5% CO2 atmosphere. GEN2.2 plasmacytoid dendritic cells (pDC) were cultivated on a MS-5 irradiated feeder layer in RPMI-1640 Glutamax supplemented with 10% FCS as previously described [1].

This study was conducted under a procedure approved by the French Blood Agency Institutional Review Board. All donors signed informed consent forms. Peripheral blood mononuclear cells (PBMC) were purified by Ficoll-Hypaque density gradient centrifugation (Eurobio). Cells were cryopreserved in liquid nitrogen in RPMI-1640 supplemented with 20% FBS and 10% dimethyl sulfoxide (Sigma). All experiments were performed on subsequently thawed samples. Monocyte-derived dendritic cells (MoDC) were differentiated from adherent blood monocytes using 500U/ml GM-CSF and 10ng/ml IL-4 (TEBU Peprotech) for 5 days. Anti-human mouse monoclonal fluorescent-conjugated antibodies were used to control MoDC differentiation: anti-CD14 (Beckman Coulter) and CD209, CD11c and isotype controls (BD Biosciences).

**Materials.** The synthetic peptides FluM158-66 - GILGFVFTL, FluM140-57 - EALMEWLKTRPILSPLTK and FluM155-72 - LTKGILGFVFTLTVPSER and HIVpol476-484 – ILKEPVHGV have been purchased from NeoMPS, France.

The following antibodies have been used: rabbit polyclonal anti-Ad3 Dd (prepared in the laboratory) at 1:40000, rabbit polyclonal anti-HA (Influenza National WHO Laboratory, France) at 1:200, anti-rabbit-horseradish peroxidase (Sigma) at 1:100000, rabbit anti-FluM140-57 and anti-FluM155-72 (EZBiolab USA) used at1:20000 for WB analysis and at 1:500 for internalization assays, and Alexa Fluor 488 chicken anti-rabbit IgG (Invitrogen, Carlsbad CA, USA) at 1:500.

**Electron microscopy.** Samples at approximately 0.1 mg protein/ml were applied to the clean side of carbon on mica (carbon/mica interface) and negatively stained with 1% sodium silicotungstate, pH 7. Micrographs were taken under low-dose conditions with a Jeol 1200 EX II microscope at 100 kV and a nominal magnification of 40000.

**Chickens.** Specific pathogen-free (SPF) White Leghorn chickens were hatched from eggs provided by Lohmann Valo (Cuxhaven, Germany). After hatching, all birds were kept in biosecurity level 3 (BSL-3) isolators and animal experiments were authorized and supervised by Biosafety and Bioethics Committees at the Veterinary and Agrochemical Research Institute in Brussels, following national and European regulations.

**Dd cytotoxicity.** Fresh PBMC were cultured in RPMI-10% FCS alone or in the presence of Dd overnight and analyzed by flow cytometry. The conventional 7AAD exclusion by living cells and annexin V staining of apoptotic cells were used to evaluate viable cells according to the manufacturer’s instructions (Beckman Coulter). Average values were obtained from triplicate measures.

**Humoral immune responses.** Chicken blood sera were collected at the indicated timepoints. MaxiSorp Nunc-Immuno F96 microwell plates were coated overnight at 37◦C with FluM140-57 or FluM155-72 synthetic peptides mixed 1:1 (v/v) at 5µg/ml each. Wells were blocked for 30min at 37◦C with 200µl of 2.5% casein in PBS. Serial dilutions of sera in PBS containing 0.1% Tween 80, 5% NaCl and 4% BSA were added to the wells and incubated for 2h at RT. Plates were then treated for 1h with biotin-labeled mouse antibody anti-chicken IgG (SouthernBiotech) at 1:10000, followed by 1h incubation with streptavidin-peroxidase polymer (1:20000). Plates were washed thoroughly with PBS 0.1% Tween-20 between each step. Peroxidase activity was measured using TMB substrate (Lab-Systems) and after blocking the reaction by adding 1M H3PO4 optical densities were read at 450-560nm.

Results

**FluM1 epitopes on dodecahedric platform.** The Ad Pb contains two external loops thought not to be involved in VLP stability [3]. The RGD loop (310-348 aa in Ad3 Pb) contains the RGD motif, required for interactions with cellular integrins, one of Ad3 receptors. Since we did not want to tinker with Dd internalization, therefore the two 18-aa M1 epitopes were separately inserted into the second loop (the variable loop) in place of the internal 157VTVND sequence between residues 150-169 in Ad3 Pb (Figure 1A). The Dds bearing these epitopes were expressed in the baculovirus system, with weak expression observed for Dd containing internally positioned FluM155-72 (Figure 1C). This may be due to the hydrophobic nature of this epitope (its GRAVY index is 0.706). Insertion of 18 aa long hydrophobic sequence might have affected Dd synthesis and folding. Much higher expression was obtained for Dd with the loop-inserted epitope FluM140-57; its GRAVY index is negative (-0.122) suggesting a more hydrophilic character.

The FluM155-72 epitope was then introduced as an extension of the Pb N-terminal domain. The rwtDd is heavily proteolyzed at the N-termini, in particular before Gly9 and before Ala38 [2]. In addition, we have shown that Ad3 Pb N-termini can be shortened by 41 aa without affecting Dd stability; further shortening results in gradually diminishing Dd yield, while the removal of 46 N-terminal aa results in Dd disassembly to free Pbs [4]. Therefore, in order to prevent removal of the FluM1 epitope by Dd proteolysis while retaining dodecahedric structure of the vector, Dd shortened at the N-termini either by 8 or 46 aa residues (Dd-8aa and Dd-46aa) was used for N-terminal extension with the epitope   
FluM155-72. This resulted in Pb proteins that were 11 residues longer or 27 residues shorter, respectively, than the wild type protein. Relatively high yield was obtained for Dd-46aa whereas only weak expression was observed for the construct containing FluM155-72 at the N-terminus of Dd-8aa (Figure 1C). This suggests that the extension of N-termini above the native length possibly affects Dd stability. Mass spectrometry analysis of Dd-FluM140-57 in the variable loopand Dd-46 FluM155-72 confirmed the presence of correct M1 epitopes at the appropriate sites. These two proteins were chosen for further experiments.

**Anti-M1 humoral immune response.** During initial elaboration of vaccination conditions, similarly as for cell-mediated immune response humoral response was elicited in SPF chickens in the absence of adjuvant (Suppl. Figure 1A). This weak response was 2 – 3 times lower than reaction against Dd alone. When using the vaccination regimen presented in Fig. 6C, an increasing level of anti-M1 IgG was noted in the sera of vaccinated chickens (Suppl. Figure 1B). It is relevant that contribution of M1 protein to humoral immunity is not very significant [[5](#_ENREF_23), 6].

Figure legend

**Figure 1**. Humoral immune anti-M1 responses. Sera from chickens were isolated at the indicated timepoints and assayed by ELISA. (A) Initial characterization of vaccination regimen. Eight groups of chickens (n=10) were vaccinated via different routes with different doses of Dd-M1 vaccine composed of DdM140-57 and DdM155-72 mixed 1:1 (w/w), with or without complete Freund’s adjuvant, boosted two weeks later with the same vaccines and analyzed two weeks later (see Fig. 6). Birds non-vaccinated or vaccinated subcutaneously (SC) with Dd alone served as controls. Negative - non-vaccinated chickens, Dd - chickens vaccinated with the vector, SC - subcutaneous vaccination, IM - intramuscular vaccination, adj IM - vaccination with complete Freund’s adjuvant. Bars represent the average values for each group of chickens. (B) Kinetics of humoral anti-M1 immune responses. Chicken immunization with Dd-M1 vaccine composed of DdM140-57 and DdM155-72 mixed 1:1. One of three groups of chickens (n=25) was vaccinated with 50µg of mixed vaccine in total volume of 100 µl. Non-vaccinated birds and those immunized with Dd vector (50µg, 100µl) served as controls. Average values for each group of chickens at the indicated timepoints are shown.

Table 1. Oligonucleotides used to generate Dd bearing influenza M140-57 epitope.

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| **Step 1** |
| Forward 5´-AGAAAAGCTCCTGAAGGTGAAGCCTTGATGGAATGGTTGAAAACGAGGCCGATT- 3’  Reverse 5´-CTCTTTATGATCATAGGTTTTCGTCAACGGCGACAAAATCGGCCTCGTTTTCAA- 3´ |
| **Step 2, 3, 4** |
| Forward 5’-TTGGGTTGAATTAAAGGTC- 3’  Reverse 5’-CTGCGGTAGGCTGTGTTGAT- 3’ |

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