Sequential immunization with universal live attenuated influenza vaccine candidates protects ferrets against a high-dose heterologous virus challenge

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Abstract

The development of universal influenza vaccines, i.e. vaccines that can provide broad protection against seasonal and potentially pandemic influenza viruses, has been a priority for more than 20 years. Several approaches have been proposed that redirect the adaptive immune responses from immunodominant hypervariable regions to low-immunogenic but highly conserved regions of viral proteins. Here we induced broadly reactive anti-hemagglutinin (HA) stalk antibody by sequential immunizations with live attenuated influenza vaccines (LAIVs) expressing chimeric HA (cHA). These vaccines contained the HA stalk domain from H1N1pdm09 virus but antigenically unrelated globular head domains from avian influenza viruses H5N1, H8N4 and H9N2. In addition, the source of the viral nucleoprotein (NP) of the LAIV strains was changed from A/Leningrad/17 master donor virus (MDV) to wild-type (WT) H1N1pdm09 virus, in order to induce CD8 T-cell immune responses more relevant to current infections. To avoid any difference in protective effect of the various anti-neuraminidase (NA) antibodies, all LAIVs were engineered to contain the NA gene of Len/17 MDV. Naïve ferrets were immunized with three doses of (i) classical LAIVs containing non-chimeric HA and NP from MDV (LAIVs (NP-MDV)); (ii) cHA-based LAIVs containing NP from MDV (cHA LAIVs (NP-MDV)); and (iii) cHA-based LAIVs containing NP from H1N1pdm09 virus (cHA LAIVs (NP-WT)). A high-dose challenge with H1N1pdm09 virus induced significant pathology in the control, non-immunized ferrets, including high virus titers in respiratory tissues, clinical signs of disease and histopathological changes in nasal turbinates and lung tissues. All three vaccination regimens protected animals from clinical manifestations of disease: immunized ferrets did not lose weight or show clinical symptoms, and their fever was significantly lower than in the control group. Further analysis of virological and pathological data revealed the following hierarchy in the cross-protective efficacy of the vaccines: cHA LAIVs (NP-
WT) > cHA LAIVs (NP-MDV) > LAIVs (NP-MDV). This ferret study showed that prototype universal
LAIVs that combine the two approaches of inducing anti-HA stalk antibody and more relevant CD8 T-
cell immune responses are highly promising candidates for further clinical development.

**Key words:** universal influenza vaccine, chimeric hemagglutinin, nucleoprotein, live attenuated
influenza vaccine, sequential immunization, ferret model.

### 1. Introduction

Influenza viruses are highly contagious respiratory pathogens that pose a constant threat
throughout the world. In addition to annual epidemics, influenza A viruses can potentially cause
pandemics, when the new virus differs antigenically from previously circulating variants, so that the
human population is immunologically naïve. The global spread of various subtypes of influenza A
viruses in birds provides the preconditions for interspecies transmission: the past two decades have seen
an increase in the number of cases of human infection with avian influenza viruses H5, H7 and H9 [1-3].
One of the most important initiatives to prepare for an influenza pandemic is focused on developing and
evaluating appropriate vaccines. Different approaches and platforms have been used to develop vaccines
against potentially pandemic influenza viruses and, over the past few years, a large body of data has
been accumulated on the safety and immunogenicity of these vaccines. The latest summary of clinical
trials of potential pandemic vaccines can be found on the WHO website [4].

This pandemic preparedness plan uses a strain-specific approach, in which candidate vaccine
viruses are prepared against the pathogens thought most likely to cause the next pandemic. However, as
was seen in 2009, such predictions are not always accurate, and fundamentally new approaches are
required to develop vaccines that can provide protection against both seasonal and newly emerging
potentially pandemic strains. Over the past 20 years, research has been carried out in many parts of the
world to develop a universal influenza vaccine that induces broadly reactive and long-lasting immune
responses. The general principle of this work is to redirect the adaptive immune response from
immunodominant hypervariable regions to low-immunogenic, highly conserved regions of viral proteins
[5-9]. Influenza virions contain multiple conservative domains that, because of their functional
significance, are rather weak immunogens; classical approaches to immunization are not capable of
inducing robust immune responses to these sites [10].

One such conservative antigen region is the stalk domain of the viral hemagglutinin (HA)
molecule, which contains epitopes that are highly conserved among influenza A viruses belonging to the
same phylogenetic group. Group 1 influenza viruses are those with H1, H2, H5, H6, H8, H9, H11, H12,
H13, H16, H17, or H18 HA, while group 2 comprises those with H3, H4, H7, H10, H14, and H15 HA
[11]. Several strategies have been proposed to increase the immunogenicity of the HA stalk domain; the
most promising one involves sequential immunization with vaccines expressing chimeric HA molecules.
(cHA) that contain an identical stalk domain (for example, from the H1N1 virus), but in which the globular domains vary significantly in antigenicity, i.e. belong to different subtypes of influenza A viruses [12,13]. Several recent studies have demonstrated that live attenuated influenza vaccines (LAIVs) expressing chimeric HAs are capable of inducing cross-reactive HA stalk-specific antibodies, either when used as a priming vaccine followed by inactivated influenza vaccine (IIV) [14,15], or when several cHA-based LAIVs are administered sequentially to mice. With sequential immunization with two or three doses of LAIV, mice were better protected against heterologous challenge infection when the vaccines had the same HA stalk domain, compared with vaccines that contained intact HA [16]. It was, therefore, considered important to test the same vaccination regimen in a ferret model, the most suitable animal model for studying influenza infection [17].

Another promising approach for designing broadly protective influenza vaccines is the development of immunogens that induce strong T-cell immunity, which is known to be cross-reactive among antigenically different viruses [18-20]. Although T-cell immunity does not prevent influenza infection, there is evidence that T cells can recognize the conserved epitopes of viral proteins, reduce viral load and alleviate symptoms in animals and humans after infection with heterologous influenza viruses [21-24]. LAIVs are believed to be good inducers of virus-specific cytotoxic CD8+ T-cell immunity [25-29]. Therefore, sequential immunization with cHA-containing LAIVs should not only induce broadly protective stalk-specific antibodies, but also stimulate cross-reactive T-cell immunity.

However, the cytotoxic T-cell (CTL) immunity induced by licensed LAIVs might be suboptimal in protecting against currently circulating influenza viruses, because the nucleoprotein (NP) and M1 protein, known as the most immunodominant targets for CTL immunity, originate from master donor virus isolated over 60 years ago [30,31]. Even though these proteins are relatively conserved among different subtypes of influenza A viruses, they are still subject to slow evolutionary changes [32]. Over the past 20 years, a large number of studies have indicated that certain immunodominant T-cell epitopes have disappeared as the influenza viruses have evolved [33-35]. The mutations in the epitopes mean that CTLs targeted to the original, non-mutated epitopes will be inefficient in detecting virus-infected cells [36]. This problem can be partially solved by replacing the NP gene in the genome of the LAIV virus by one from a currently circulating influenza virus. Indeed, recently we showed that engineering of LAIV reassortants with 5:3 genome compositions induced CTL responses that were more relevant to current infections [36-38].

Continuing this research, in the present study, we combined both the above-mentioned strategies by engineering a panel of universal LAIV candidate viruses that express chimeric HA molecules and NP of a wild-type virus. The sequential immunization regimen was tested in a non-primed ferret model to assess the ability of the vaccines to protect against heterologous virus challenge.
A high dose of challenge virus was used in order to evaluate the vaccines’ ability to reduce influenza virus-induced pathology.

2. Materials and Methods

2.1. Viruses and proteins

The LAIV reassortant viruses used in this study are shown in Table 1. All vaccine viruses were generated by standard plasmid-based reverse genetics (RG) on the basis of cold-adapted master donor virus (MDV) A/Leningrad/134/17/57 (H2N2) (Len/17) [39]. RG plasmids bearing chimeric HA genes containing the stalk domain of A/California/7/2009 (pH1N1) or A/South Africa/3626/2013 (pH1N1) virus and the head domains of A/Vietnam/1203/2004 (ΔH5N1) (polybasic cleavage site deleted), A/mallard/Sweden/24/02 (H8N4) and A/quail/Hong Kong/G1/1997 (H9N2) were generated as described previously [16]. RG plasmids bearing control non-chimeric full-length HAs of the H5N1, H8N4 and H9N2 viruses were also generated as in the previous study [16]. The three groups of LAIV viruses were rescued by electroporation of Vero cells using the Neon transfection system (Invitrogen, USA): (i) classical LAIVs containing non-chimeric HA and NP from MDV; (ii) cHA-based LAIVs with NP from MDV; and (iii) cHA-based LAIVs with NP from pH1N1 virus. Importantly, to avoid any effect of anti-NA antibody on the protection, all nine LAIV strains had the same NA gene from Len/17 H2N2 virus. All LAIV viruses were propagated in 10-day-old embryonated chicken eggs for 3 days at 33 °C. Virus stocks were harvested, clarified by low-speed centrifugation and stored in aliquots at –70 °C.

Table 1. Overview of the Len/17-based LAIV viruses used in this study

<table>
<thead>
<tr>
<th>Virus designation</th>
<th>Origin of viral genes*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HA head domain</td>
</tr>
<tr>
<td>H5N2 (NP-MDV)</td>
<td>H5N1</td>
</tr>
<tr>
<td>H8N2 (NP-MDV)</td>
<td>H8N4</td>
</tr>
<tr>
<td>H9N2 (NP-MDV)</td>
<td>H9N2</td>
</tr>
<tr>
<td>cH5/1N2 (NP-MDV)</td>
<td>H5N1</td>
</tr>
<tr>
<td>cH8/1N2 (NP-MDV)</td>
<td>H8N4</td>
</tr>
<tr>
<td>cH9/1N2 (NP-MDV)</td>
<td>H9N2</td>
</tr>
<tr>
<td>cH5/1N2 (NP-WT)</td>
<td>H5N1</td>
</tr>
<tr>
<td>cH8/1N2 (NP-WT)</td>
<td>H8N4</td>
</tr>
<tr>
<td>cH9/1N2 (NP-WT)</td>
<td>H9N2</td>
</tr>
</tbody>
</table>

* The remaining five genes originated from MDV A/Leningrad/134/17/57 (H2N2).

MDV, master donor virus; WT, wild-type.
The A/South Africa/3626/2013 (pH1N1) virus was obtained from the influenza virus repository of the National Institute for Biological Standards and Control (NIBSC) (London, UK).

Recombinant proteins, including cH6/1 (containing HA head domain of A/mallard/Sweden/81/02 (H6N1) and HA stalk domain of pH1N1), H2 (from A/Albany/1/1968), H3 (from A/Perth/16/2009), H6 (from A/mallard/Sweden/81/02) and H9 (from A/chicken/Hong Kong/G9/1997), were kindly provided by Professor F. Krammer (Mount Sinai School of Medicine, New York, USA).

2.2. In vitro studies

Temperature-sensitive and cold-adapted (ts/ca) phenotypes of the LAIV viruses were determined by titration at different temperatures in eggs: 38 °C compared with 33 °C for the ts phenotype and 26 °C compared with 33 °C for the ca phenotype. Eggs inoculated with 10-fold virus dilutions were incubated for either 72 hours (for 33 °C and 38 °C) or 6 days (for 26 °C). In addition, LAIV virus growth was analysed in Madin-Darby canine kidney (MDCK) cells to determine the 50% tissue culture infectious dose (TCID\(_{50}\)) on day 4 after inoculation. Virus titers in eggs and MDCK cells were calculated using the Reed and Muench method and expressed in terms of log\(_{10}\) 50% egg infectious dose (EID\(_{50}\))/ml and log\(_{10}\)TCID\(_{50}\)/ml, respectively. A virus was considered to be temperature-sensitive (ts phenotype) if the infectious titers at 33 °C were at least 5.0 log\(_{10}\)EID\(_{50}\) greater than at 38 °C. A virus was considered to be cold-adapted (ca phenotype) if the infectious titers at 26 °C were not more than 3.0 log\(_{10}\)EID\(_{50}\) lower than at 33 °C [40].

2.3. Animals

Male ferrets (Mustela putorius furo), aged 5–6 months and weighing 1.1–1.9 kg at the beginning of the experiment, were supplied by Scientific-Production Organization House of Pharmacy JSC (St Petersburg, Russia). They were prescreened by routine hemagglutination inhibition (HAI) test to ensure that they were negative to circulating human influenza viruses and the viruses being tested. Prior to infection, ferrets were randomly selected and housed individually in isolation units with free access to food and water. All animal experiments were conducted using protocols approved by the Local Bioethical Committee of the Institute of Preclinical Research Ltd (St Petersburg, Russia) (Protocol #BEC 2.12/18 authorized on 28 February 2018). All inoculations, nasal washes and blood sample collections were performed with the animal under short-term anesthesia induced by intramuscular injection of Zoletil 100, 12.5 mg/kg of body weight; every effort was made to minimize suffering. At the end of the study, animals were euthanized with an overdose of Zoletil-xylazine combination.

2.4. Ferret immunization and challenge
Four groups of seven ferrets were inoculated intranasally with three doses of LAIV viruses or phosphate-buffered saline (PBS) placebo at 21-day intervals, as illustrated in Figure 1. The vaccine viruses were administered in a dose of 7.0 log10EID50 and an inoculum of 0.5 ml.

Nasal fluid wash (NFW) specimens were collected on days 1 and 3 after each vaccine dose. Blood samples for serum preparation were collected 14 days before vaccination and on day 63 to assess immunogenicity. Seven days after the last vaccine dose (day 49), three ferrets from each group were euthanized and tissues were collected for evaluation of the safety of the vaccines tested. The remaining four animals from each group were challenged intranasally with 6.0 log10EID50 of A/South Africa/3626/2013 (pH1N1) virus on day 63, after which NFW samples were collected every day until day 67. On that day, all animals were sacrificed and various tissues were collected to assess viral replication and pathological changes as observed on gross pathology and histopathology.

Figure 1. Overview of study design (A) and designation of the study groups (B).

2.5. Virological methods

Virus replication in the ferrets’ respiratory tract was assessed by endpoint titration of NFW, lung and trachea specimens in embryonated chicken eggs. Tenfold sample dilutions were inoculated into 10–
11-day-old eggs and incubated at 33 °C for 72 hours. Virus titers were expressed as log_{10}EID_{50}/ml for NFW and log_{10}EID_{50}/gram for trachea and lung specimens.

2.6. Clinical signs and morbidity outcomes

Ferrets were observed daily for clinical signs (body weight, level of activity, nasal discharge, and sneezing). Nasal symptoms were scored as follows: 1 – nasal rattling could be heard or the ferret sneezed during transport from its cage to the evaluation area; 2 – there was evidence of nasal discharge on the external nares; 3 – the animal exhibited mouth breathing; 0 – the animal exhibited none of these symptoms. Activity level was scored over a range from zero to three according to the extent that the animal could be induced to play: 0 – the animal was fully playful; 1 – the animal responded to play overtures but did not initiate any play activity; 2 – the animal was alert but not at all playful; 3 – the animal was neither playful nor alert. Scores were summed for each ferret and group medians calculated.

Body temperature was measured using temperature data loggers (Star-Oddi, Reykjavik, Iceland) implanted into the peritoneal cavity and programmed to record body temperature every 30 minutes. The effect of vaccination or virus infection on the body temperature was expressed as the area under the curve (AUC) of the body temperature increase after immunization or challenge (AUC delta T) and as the maximum temperature increase (max delta T). The body temperature increase was calculated by subtracting the baseline temperature (average of the temperatures recorded in periods between vaccinations) from the temperature recorded at any time during the vaccination or challenge phase, excluding the periods of sedations, where a sharp temperature decrease was registered. The AUC was calculated using the trapezoidal rule and the same baseline temperature as mentioned above.

2.7. Assessment of immune responses

Antibody immune responses were assessed by determining serum antibody titers in an HAI assay; IgG antibody was quantified by enzyme-linked immunosorbent assay (ELISA), as described elsewhere [41] with some modifications. For HAI, serum samples were pretreated with receptor-destroying enzyme (RDE) (Denka Seiken, Tokyo, Japan) to remove non-specific inhibitors and quantified against four HA units of the following viruses: (i) A/South Africa/3626/2013 (pH1N1); (ii) A/Tokyo/3/67 (H2N2); (iii) H5N2 (NP-MDV); (iv) A/herring gull/Sarma/51/2006 (H6N1); (v) H8N2 (NP-MDV); (vi) H9N2 (NP-MDV). For ELISA, high-sorbent 96-well plates (Greiner, Germany) were coated with 100 µl of either 16 HA units of sucrose-purified H5N2 LAIV virus (positive control) or 2 µg/ml of the following recombinant HA proteins: cH6/1, H2, H3, H6, and H9 in carbonate-bicarbonate buffer in a volume of 100 µl per well overnight. Twofold dilutions of sera were prepared starting from 1:10 and added to the coated wells, which were then incubated with anti-ferret IgG conjugates to horseradish peroxidase supplied by Sigma (USA). The antibody titers were defined as the last dilution
with optical density (OD) values more than 3 SD greater than the mean OD measured in control wells (all components except for the serum specimens). A fourfold or more rise in antibody titer after vaccination was considered a reliable indicator.

2.8. Pathology studies

At the time of necropsy, a complete macroscopic (gross pathology) examination was performed. The nasal turbinates (NT), trachea and lungs were studied in detail, and the abdominal and pelvic cavities were examined. Tissue sections of nasal turbinates, trachea and left cranial and left caudal lung lobes were taken from each sacrificed animal on day 4 post-challenge and used for histological analysis.

After fixation in 10% buffered formalin, lungs were embedded in paraffin and prepared for histopathological analysis. For nasal turbinates and trachea, after fixation in formalin the specimens were decalcified in Gooding-Stewart fluid (a mixture of 25% concentrated formic acid, 5% formalin solution and 75% distilled water) for 14 days and then embedded in paraffin and prepared for histopathological analysis. Tissue sections of 5 µm were prepared and stained with Alcian blue at pH 2.5, followed by hematoxylin and eosin staining to reveal goblet cells on microscopic examination. For the assessment of fibrin deposition, sections of lungs were stained with picro-Mallory stain [42]. Infection pathology was evaluated as described elsewhere [43], including indicators of damage to the epithelial lining and acute inflammation. The damage to lung tissues was scored using epithelial hypertrophy, epithelial hyperplasia, pseudosquamous epithelium, and epithelial necrosis of bronchi and bronchioles, as well as hypertrophic pneumocyte type 2, hyperemia septa, alveolar emphysema, and alveolar hemorrhages. Acute inflammation in lungs was assessed in terms of bronchitis, bronchiolitis, peribronchitis, peribronchiolitis, interstitial infiltrate, alveolitis, vasculitis, and perivasculitis. The infection damage to nasal turbinates was assessed as epithelial damage, hyperemia, lymphocytic infiltrate, exudate, and edema. Each of these parameters was scored as: 0 absent; 1 minimal; 2 slight; 3 moderate; 4 strong; and 5 severe. The sum of the parameter scores for damage and inflammation was used for statistical analyses to estimate the degree of vaccine-induced protection. At least six microscopic fields were scored for each lung specimen. The percentage of affected tissue was estimated from the sections as a measure of the extent of damage.

2.10. Statistical analyses

Data were analyzed with Statistica software (version 6.0; Statsoft Inc.). The statistical significance of the differences in pathology estimates, as well as in viral titers in eggs, MDCK cells, NFW specimens and tissues of ferrets was assessed using the Mann-Whitney U-test. Differences in log2-transformed HI and ELISA antibody titers were also subjected to the Mann-Whitney U-test. P values of <0.05 were considered significant.
3. Results

3.1. Virological characterization of LAIV viruses used in the study

The phenotypic properties of the recombinant LAIV viruses containing cHAs and/or wild-type nucleoprotein generated for this study were compared with those of the analogous LAIVs containing classical full-length HA. LAIV viruses are characterized by ts/ca phenotypes, i.e. they actively replicate at low temperature (26 °C), but their replication at high temperature (38 °C) is impaired. The replacement of the HA in classical LAIVs with cHA, as well as the replacement of Len/17 NP with that of pH1N1 virus, did not affect the ts phenotype: all cHA LAIVs replicated poorly at high temperature in eggs (Table 2). Five of six cHA LAIVs also preserved the ca phenotype; the cH5/1N2 (NP-WT) candidate that contained both chimeric HA and wild-type NP replicated poorly at 26 °C; the difference in titer compared with growth at 33 °C was 4.5 log10EID50. Interestingly, the analogous cH5/1N2 containing the NP from MDV preserved its ca phenotype (the difference in viral titer between 33 °C and 26 °C did not exceed 3.0 log10EID50). These data suggest that the source of the NP gene in the LAIV virus could affect the ca phenotype. However, the effect is strain-specific, as the other stalk-based LAIVs – cH8/1N2 (NP-WT) and cH9/1N2 (NP-WT) – replicated efficiently at low temperature in eggs (Table 2).

Importantly, all engineered LAIV candidates replicated efficiently in eggs in optimal conditions, though for one virus, cH5/1N2 (NP-WT), the endpoint titer was significantly lower than that of the classical counterpart. Since the analogous cHA-containing LAIV with MDV NP had a titer similar to that of the control virus, it is likely that the wild-type NP was responsible for the lower viral titers in eggs. The replication profile of the LAIV viruses in MDCK cells was affected by the origin of both the HA stalk domain and the NP gene. Both LAIV candidates that had the cH5/1 HA molecule showed significantly lower virus titers than their control LAIV expressing natural HA (Table 2). These results indicate that the inclusion of an irrelevant stalk domain in H5 molecules can reduce the ability to replicate in mammalian cells, without affecting growth activities in eggs. The effect of the pH1N1 stalk domain was strain-specific, since cH8/1N2 (NP-MDV) and cH9/1N2 (NP-MDV) LAIVs had similar titers in MDCK cells as the H8N2 and H9N2 LAIVs, respectively. An effect of wild-type NP on virus titer in MDCK cells was noted for all three pairs of LAIV viruses. However, the effect varies: for the cH5/1- and cH9/1-containing LAIVs, the WT NP decreased virus titer (7.3 vs 6.4 log10TCID50/ml, \( P=0.049 \), and 8.2 vs 5.2 log10TCID50/ml, \( P=0.044 \), respectively); for the cH8/1-containing LAIVs, the WT NP increased viral replication in mammalian cells (5.8 vs 7.0 log10TCID50/ml, \( P=0.029 \)).

Table 2. Virological characteristics of LAIV viruses used in this study
### LAIV virus

<table>
<thead>
<tr>
<th>LAIV virus</th>
<th>Virus titer in eggs, log$<em>{10}$EID$</em>{50}$/ml</th>
<th>Phenotype</th>
<th>Virus titer in MDCK cells, log$<em>{10}$TCID$</em>{50}$/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>33 °C</td>
<td>38 °C</td>
<td>26 °C</td>
</tr>
<tr>
<td>H5N2 (NP-MDV)</td>
<td>9.2±0.3</td>
<td>2.7±0.5</td>
<td>6.5±0.7</td>
</tr>
<tr>
<td>H8N2 (NP-MDV)</td>
<td>7.6±0.8</td>
<td>1.6±0.2</td>
<td>5.5±0.4</td>
</tr>
<tr>
<td>H9N2 (NP-MDV)</td>
<td>8.4±0.5</td>
<td>1.7±0.6</td>
<td>5.5±0.8</td>
</tr>
<tr>
<td>cH5/1N2 (NP-MDV)</td>
<td>8.6±0.3</td>
<td>2.2±0.5</td>
<td>6.3±0.1</td>
</tr>
<tr>
<td>cH8/1N2 (NP-MDV)</td>
<td>7.7±0.7</td>
<td>1.2±0.2</td>
<td>5.1±0.9</td>
</tr>
<tr>
<td>cH9/1N2 (NP-MDV)</td>
<td>8.9±0.4</td>
<td>2.0±0.6</td>
<td>6.5±0.7</td>
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<tr>
<td>cH5/1N2 (NP-WT)</td>
<td>7.5±0.4†</td>
<td>1.5±0.4</td>
<td>3.0±0.3</td>
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<tr>
<td>cH8/1N2 (NP-WT)</td>
<td>8.1±0.7</td>
<td>2.2±0.8</td>
<td>5.2±0.7</td>
</tr>
<tr>
<td>cH9/1N2 (NP-WT)</td>
<td>7.8±0.7</td>
<td>2.1±0.4</td>
<td>5.2±0.8</td>
</tr>
</tbody>
</table>

*†P<0.05 compared to the corresponding control LAIV virus with classical HA and NP from MDV; ‡P<0.05 compared to the cH8/1N2 (NP-WT) LAIV.*

#### 3.2. Safety testing of universal LAIV candidates in ferrets

In the three groups of ferrets given vaccine, no significant differences were observed in clinical scores or percentage body weight loss at any time. Fluctuations in body temperature over the immunization phase were similar in all the study groups (Figure 2). The vaccines were considered to be safe and well tolerated,
Figure 2. Safety of sequential immunization of ferrets. A. Percentage body weight change. B. Area under the curve of delta T relative to baseline temperature for each ferret over the immunization phase. C. Scores for clinical signs of influenza infection in ferrets throughout the immunization period. No significant differences were observed for any of the parameters.

3.3. Shedding and immunogenicity of universal LAIV candidates in ferrets

All nine LAIV reassortant viruses replicated in the upper respiratory tract of intranasally immunized ferrets. While the mean viral titers detected in NWF specimens varied between test groups, the difference was not statistically significant (Figure 3). Importantly, the vaccine viruses replicated efficiently after the second and third immunizations, indicating that the immunity induced to the previous vaccination(s) was not sterile, which allowed LAIV viruses with heterologous HA head domains to overcome this immunity.
Figure 3. Replication of LAIV viruses in the upper respiratory tract of immunized ferrets after each vaccine dose.

On day 63, HAI antibody titers against a panel of influenza A viruses were low (Figure 4A). The only significant increase was seen for the anti-H9N2 HAI antibody in the LAIVs (NP-MDV) group. In contrast, much higher titers of serum IgG antibodies were detected. Thus, when an H5N2 LAIV whole virus was used as antigen, the IgG antibody titers exceeded 1:1024 in all three vaccine groups, indicating that the immunizations were successful (Figure 4B). Significant rises in IgG titers were also seen for the recombinant H8 and H9 protein antigens, which also indicates successful immunization, as all the vaccine groups included reassortant viruses containing the HA head domains from H8N4 and H9N2 viruses. Although the H2N2 hemagglutinin belongs to the same group as the H5, H8 and H9 HAs, there was no significant increase in anti-H2 antibody titers in the sera of immunized ferrets (Figure 4B). Importantly, significant titers of IgG antibodies reacting with chimeric cH6/1 protein were detected only in the two immunization groups given cHA-containing LAIVs (Figure 4B). These data clearly indicate that the source of stalk domain in LAIV virus significantly affects the ability to induce stalk HA-reactive antibody after sequential immunization.
Figure 4. Serum antibody immune responses in immunized ferrets. A. HAI antibody titers, measured against a panel of LAIV viruses expressing natural HAs. B. Serum IgG titers measured in ELISA against H5N2 LAIV virus and a panel of recombinant HA proteins. *indicates significant difference with the mock-vaccinated group. The dashed line indicates the limit of detection.

3.3. Protective efficacy of universal LAIV candidates against a high-dose H1N1pdm09 viral challenge

Challenge virus shedding

Challenge virus was detected on day 4 after infection in all respiratory tissues tested: lungs, NT and trachea. However, all vaccinated animals shed challenge virus in the URT at significantly lower levels than the mock-vaccinated group (Figure 5A). There was no significant difference between the
different vaccination regimens in virus shedding in the URT. However, virus titers in the trachea were significantly lower in animals from the two LAIV groups expressing cHAs, but not for classical LAIVs, compared with the mock group (Figure 5B). Strikingly, a significantly lower pulmonary virus titer was observed only for the animals immunized with cHA LAIVs that contained WT NP. These data indicate that the replacement of MDV NP with that of WT virus can further improve protection against recent wild-type influenza virus.

**Figure 5.** Replication of H1N1pdm09 challenge virus in respiratory tract of immunized ferrets. A. Virus titers in nasal wash fluids. with Mock group is indicated for each vaccine group: # significant difference between cHA LAIVs (NP-MDV) group and mock vaccinated group; * significant difference between cHA LAIVs (NP-WT) group and mock-vaccinated group; † significant difference between LAIVs (HA-MDV) group and mock-vaccinated group. B. Virus titers in ferret tissues. Significant differences are indicated (Mann-Whitney U test).

**Body weight loss and clinical manifestations of disease**
All vaccinated animals were protected from body weight loss and clinical signs of disease, as compared with the mock-immunized ferrets (Figure 6). Only the control animals displayed clinical symptoms characteristic of influenza infection, such as sneezing and nasal discharge.

Figure 6. Clinical signs of influenza infection in immunized ferrets after challenge. A. Percentage change in body weight. # significant difference between cHA LAIVs (NP-MDV) group and mock-vaccinated group; * significant difference between cHA LAIVs (NP-WT) group and mock-vaccinated group; † significant difference between LAIVs (HA-MDV) group and mock-vaccinated group. B. Scores for clinical signs for each ferret during the four days after the challenge. * Significant difference from the mock-vaccinated group (Mann-Whitney U test).

Body temperature

The body temperature of the ferrets in the challenge phase is shown in Figure 7A. There was a significant difference between all three vaccine groups and the control group in the maximal delta T parameter (Figure 7B), suggesting that the vaccinated animals were protected against severe fever. However, in terms of the area under the curve, the only significant difference was between the cHA LAIVs (NP-WT) group and the controls (Figure 7C). These data suggest that, while all vaccine regimens reduced fever similarly, the duration of fever was significantly shorter in animals that received LAIVs containing chimeric HA and WT NP.
Figure 7. Body temperature of immunized ferrets after challenge. A. Variation in body temperature over the challenge phase. The several sharp decreases in temperature are the result of sedation during challenge (D63) and collection of nasal wash fluids. B. Maximal increase in body temperature over the challenge phase. C. Area under the curve of delta T relative to baseline temperature for each ferret over the challenge phase. Statistical differences with mock-vaccinated group are indicated (Mann-Whitney U test); ns = not significant.

Histopathological analysis of upper and lower respiratory tract

Analysis of pathological changes in URT tissues demonstrated that all vaccinated groups were protected from damage to respiratory epithelium after H1N1pdm09 challenge: mucus-producing goblet cells (stained with alcian blue stain) were preserved in NTs of LAIV-vaccinated ferrets, whereas these cells were absent in the mock-vaccinated group (Figure 8A). However, semi-quantitative analysis of pathological changes in nasal turbinates, and estimation of the percentage of affected tissue, revealed significant protection only in the two groups given LAIV that expressed chimeric HAs (Figure 8B, C). Interestingly, only the cHA LAIVs (NP-WT) group was significantly protected in terms of both the percentage of affected tissue and the total pathology score (Figure 8B, C). A semi-quantitative analysis of the infection pathology of individual NT specimens is given in Supplement Table 1.
Histological examination of ferrets’ trachea showed few or no pathological changes. Minimal to moderate glandular, subepithelial and intraepithelial mononuclear infiltrates were defined. Rarely damage to cilia, epithelial hypertrophy and hyperplasia were found; however, there were no significant differences between the groups.

Figure 8. Pathology of the upper respiratory tract of immunized ferrets four days post-challenge. A. Gross pathology (GP) of nasal turbinates (upper panel). Histopathology of NT (middle panel) and trachea (lower panel). B. Percentage affected nasal turbinate tissues. C. Semi-quantitative analysis of NT infection pathology. H&E – haematoxylin and eosin. AB – alcian blue. Statistical differences with mock-vaccinated group are indicated (Mann-Whitney U test).
The lungs of the control ferrets showed severe pathological changes, characterized by moderate to strong bronchopneumonia and moderate interstitial and alveolar pneumonia (Figure 9A). There was regularly defined hyperemia of the alveolar septa with large hemorrhages, alveolar emphysema, necrosis and denudation of bronchial and bronchiolar epithelium. Alveolar and bronchial spaces were often filled with edema, debris, and large numbers of polymorphonuclear cells. Vasculitis and perivasculitis were defined.

The lungs of ferrets from the groups given cHA LAIVs (NP-MDV) and LAIVs (NP-MDV) had macroscopically visible emphysema foci and alveolar hemorrhages of varying severity. Exudate was observed in the lumen of the bronchi and bronchioles, in which lymphocytes and mononuclear cells predominated. Mild to moderate signs of catarrhal bronchitis, peribronchitis, bronchiolitis, perivasculitis and vasculitis were seen. Damage to pulmonary tissue was manifested as moderate lymphocytic and mononuclear infiltration in the interstitial tissue of the respiratory department, hyperemia of alveolar septa and alveolitis, shown as thickening of alveolar septa, and diffuse inflammatory infiltration. Focal hemorrhages and alveolar emphysema were defined (Figure 9A).

In the cHA LAIVs (NP-WT) group, morphological changes included minimal or slight thickening and hyperplasia of bronchial and bronchiolar epithelium, often without a microscopic picture of bronchitis and bronchiolitis. No exudate was detected in the lumen of the bronchi and bronchioles. In peribronchial areas, interstitial and alveolar tissues, there was predominantly minimal to moderate lymphocytic and mononuclear infiltration. Less frequently, there was necrotic damage to the bronchial epithelium, accompanied by moderate or severe lymphocytic infiltration (Figure 9A). Picro-Mallory staining of the lungs showed fibrin deposits in the lumen of the bronchi and the alveolar tissue. Detection of a large amount of fibrin and severe inflammatory infiltration of lung tissue in animals of the control group indicated the development of severe exudative fibrous bronchitis and alveolitis. In all vaccinated animals, fibrin was determined less frequently or was absent in the foci of inflammation (Figure 9A).

In conclusion, all groups of vaccinated animals showed fewer pathological changes and a lower percentage of affected tissue in the lungs than the control group. However, differences in the pathology score and percentage of affected tissue were statistically significant only for the cHA LAIVs (NP-WT) group, most probably because of the low number of animals analyzed (Figure 9B, C). A semi-quantitative analysis of the infection pathology of individual lung specimens is given in Supplement Table 2.

Overall, the universal LAIV candidates expressing cHAs and WT NP were the most effective of the LAIVs tested in this trial against a high-dose heterologous virus challenge.
Figure 9. Pathology of the lower respiratory tract of immunized ferrets four days post-challenge. **A.** Gross pathology (GP) of ferrets’ lungs (upper panel). Histopathology of lungs with different dyes (middle and lower panels). **B.** Percentage affected lung tissues. **C.** Semi-quantitative analysis of lung infection pathology. H&E – haematoxylin and eosin. P-M – picro-Mallory stain. Statistical differences with the mock-vacciated group are indicated (Mann-Whitney U test).
Discussion

Because influenza A viruses are constantly evolving, vaccination with classical influenza vaccines is not always fully effective. Circulating strains are often antigenically significantly different from the seasonal vaccine components [44,45]. In addition, the causative agent of the next influenza pandemic cannot be predicted with complete certainty, meaning that the population may be unprotected against the new virus. As a result, the development of broadly protective influenza vaccines against different subtypes of influenza A virus has been a priority for some 20 years [6].

There have been various studies showing that influenza vaccines or other immunogens, such as recombinant proteins, that contain chimeric HA proteins (i.e. identical stalk domains and different globular head domains) are able to elicit significant HA stem-reactive antibody levels. As a result, such vaccines provide significant heterosubtypic protection, thus serving as a prototype universal influenza vaccine [46-51]. Recently, Nachbagauer and co-authors demonstrated in a ferret model that a cHA-based LAIV was a better priming vaccine for inducing high levels of cross-reactive HA stalk antibody after boost with inactivated influenza vaccine, regardless of the LAIV platform used [14,15]. We have previously demonstrated that sequential immunization with two or three doses of cHA-based LAIVs can induce HA stalk-reactive antibody that enhances heterosubtypic protection in mice, compared with heterologous prime-boost immunization with classical LAIVs [16]. In the present study, we assessed the cross-protective efficacy of such cHA-based LAIVs in a ferret model. We also improved the performance of the LAIV by replacing the NP from the Len/17 MDV by the NP from the current H1N1pdm09 virus.

The rationale for including recent NP in the LAIV genome composition has been discussed in the context of our previous studies in a mouse model [37,38], and in in vitro study of human donor peripheral blood mononuclear cells [36]. In this ferret study, for the first time the two strategies for enhancing the cross-protective potential of traditional LAIVs were combined, and sequential immunizations with the vaccines expressing cHAs and WT NP were compared with classical LAIVs and with cHA-based LAIVs with classical NP.

In vitro characterization of the LAIV viruses showed that they all preserved the ts phenotype, which is important for the maintenance of attenuated phenotype of the vaccine viruses. However, the inclusion of chimeric HA and/or WT NP genes in some cases affected the ca phenotype and/or their ability to replicate efficiently in MDCK cells. Similar effects have been demonstrated previously for the engineered cHA-based LAIVs [16], as well as for the 5:3 reassortant viruses bearing WT NPs [38]; in all the studies these effects were strain-specific. Despite these variations in virological characteristics, all LAIV candidates used in the mouse studies were attenuated, i.e. did not grow in the lungs, but were able to replicate in the upper respiratory tract, inducing protective immunity. In the current ferret study, we could not assess LAIV virus replication in the lower respiratory tract because of the limited number of...
animals. However many previous studies of Len/17-based LAIV viruses have confirmed the attenuated phenotype of these viruses [43,52,53]. The vaccine viruses were able to replicate in the upper respiratory tract, inducing humoral immune responses, but no clinical signs of disease were registered after immunization with any of the LAIVs tested. These findings indicate that the three-dose immunization schedule is safe, regardless of the LAIV virus composition tested in the trial.

In contrast to our previous mouse study [16], the ferrets developed lower HAI titers to the homologous viruses used for immunization. In mice, a significant increase in anti-H5N1 HAI antibody was seen in all groups that received H5 HA-containing LAIVs as the first dose [16]. In the ferret study, anti-H5N1 HAI antibodies were undetectable, and anti-H5N1 serum IgG antibodies were much lower than in the mouse study. Most probably, in ferrets a single dose is not sufficient to induce homologous antibody to avian influenza viruses [52], while the other LAIVs bearing heterologous HA head domains were unable to boost the HAI antibody. Nevertheless, the most important immunological result is that only cHA-containing LAIVs were able to induce HA stalk-reactive IgG antibody, which is in agreement with our previous findings in mice [16] and with reports of other authors [14,15].

The main purpose of this trial was to determine whether the replacement of the HA and NP genes in classical LAIVs with chimeric HA and WT NP genes enhanced the cross-protective efficacy of the vaccines. Our previous comparative ferret study of H3N2 LAIVs containing NP from MDV or wild-type virus did not detect significant differences in the cross-protective efficacy of the vaccines, although there was a slightly lower challenge virus titer in the URT in WT NP LAIV group. This was probably because the challenge virus failed to replicate in lungs and did not cause significant pathology [54]. In contrast to that study, here we used a high-dose viral challenge that induced significant pathology and clinical manifestations of disease in the control animals, allowing the protective potential of the vaccines to be evaluated through a number of parameters.

Overall, all three vaccination regimens protected animals from clinical manifestations of disease: immunized ferrets did not lose weight and did not present clinical symptoms such as sneezing and change in behavior, and their maximal temperature increase over the challenge phase was significantly lower than in the control group. These findings confirm previous findings that even classical LAIVs afford partial protection against heterologous/heterosubtypic influenza virus challenge [55,56]. Despite this protection against clinical symptoms, the immune responses induced by the sequential immunizations were not sterile, i.e. challenge virus was isolated from the upper and lower respiratory tract of challenged animals. Although challenge virus replication in the URT was significantly lower than in the mock-vaccinated group for all three vaccine groups, only the cHA-containing LAIVs were able to significantly reduce viral titer in the trachea. Strikingly, only the LAIVs containing both cHA and WT NP protected against pulmonary virus replication. It should be noted that all three vaccine
groups showed reduced pulmonary virus titers, and the fact that the decreases in the other two vaccine groups were not significant may be related to the small number of ferrets in each group.

A recent study by Nachbagauer et al. [14,15] found that a cHA LAIV prime followed by a cHA IIV boost induced a broad antibody immune response that fully protected ferrets from replication of heterologous virus in lungs. In that study, in addition to HA-reactive antibody, the protection could have been afforded by anti-NA antibody, since the viruses contained the NA of H1N1pdm09 virus. In our study, all LAIV candidates contained NA from an irrelevant strain, Len/17 H2N2 virus, to avoid any impact of anti-NA antibody on protection. The inability of our vaccination regimens to fully protect ferrets against challenge virus pulmonary replication might be related to the lower serum IgG antibody titers and the higher dose of the challenge virus. Overall, the virological findings suggest the following hierarchy in the protective efficacy of the vaccine groups: cHA LAIVs (NP-WT) > cHA LAIVs (NP-MDV) > LAIVs (NP-MDV).

In line with the virological findings, other observations also suggest that simultaneous inclusion of chimeric HA and wild-type NP in LAIV viruses provides better protection against pathological changes after challenge with high-dose heterologous virus. The most straightforward and objective clinical observation is temperature increase over the challenge phase. This was registered with implanted temperature recorders, which measure body temperature every 30 minutes. The resulting data array allows precise assessment of temperature increase using the AUC delta T parameter. Strikingly, despite similar Max delta T values for all three vaccine groups, the AUC values differed significantly between groups, with the lowest value being observed for the cHA LAIVs (NP-WT). These data suggest that, while fever was similarly reduced in all test groups, the duration of fever was significantly shorter in animals that received LAIVs containing both cHA and WT NP gene; this may be associated with CTL immunity. These findings were further confirmed by histopathological analysis of upper and lower respiratory tract tissues of challenged ferrets: a significant reduction in pathology scores, relative to the control group, was detected only for group given cHA-containing LAIVs, with maximal protection observed in the cHA LAIVs (NP-WT) vaccine group.

The primary limitation of this study is that T-cell immunity was not analyzed for the immunized ferrets. This was because of a lack of well-defined reagents and protocols for this animal model. Although several recent studies have established protocols and suggested the use of specific antibodies, the results are still controversial. In recent years, great strides have been made in characterizing T-cell immune responses to influenza infection in ferrets, and some reagents and standardized procedures have been established [57-59]. On the basis of this study, we plan to analyze cell-mediated immunity in the ferret model using the most promising reagents and protocols.

In summary, we designed for the first time universal influenza vaccine candidates on the basis of licensed LAIVs by increasing their ability to induce cross-reactive anti-HA stalk antibody and CTL
immunity targeted to relevant T-cell epitopes within the viral nucleoprotein. Comprehensive analysis of safety, immunogenicity and protective efficacy of these prototype universal LAIVs in a ferret model provided evidence that such vaccines are highly promising for further clinical development.


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