HHS Public Access

Author manuscript

Virology. Author manuscript; available in PMC 2017 January 01.

Published in final edited form as:

Virology. 2017 January ; 500: 209–217. doi:10.1016/j.virol.2016.10.027.

Comparative studies of infectivity, immunogenicity and cross-protective efficacy of live attenuated influenza vaccines containing nucleoprotein from cold-adapted or wild-type influenza virus in a mouse model

Irina Isakova-Sivak[a,*,1], **Daniil Korenkov**[a,1], **Tatiana Smolonogina**[a], **Tatiana Tretiak**[a], **Svetlana Donina**[a], **Andrey Rekstin**[a], **Anatoly Naykhin**[a], **Svetlana Shcherbik**[b], **Nicholas Pearce**[b], **Li-Mei Chen**[b], **Tatiana Bousse**[b], and **Larisa Rudenko**[a]

[a]Institute of Experimental Medicine, Saint Petersburg, Russia

[b]Centers for Disease Control and Prevention, Atlanta, GA, USA

Abstract

This study sought to improve an existing live attenuated influenza vaccine (LAIV) by including nucleoprotein (NP) from wild-type virus rather than master donor virus (MDV). H7N9 LAIV reassortants with 6:2 (NP from MDV) and 5:3 (NP from wild-type virus) genome compositions were compared with regard to their growth characteristics, induction of humoral and cellular immune responses in mice, and ability to protect mice against homologous and heterologous challenge viruses. Although, in general, the 6:2 reassortant induced greater cell-mediated immunity in C57BL6 mice than the 5:3 vaccine, mice immunized with the 5:3 LAIV were better protected against heterologous challenge. The 5:3 LAIV-induced CTLs also had better *in vivo* killing activity against target cells loaded with the NP_{366} epitope of recent influenza viruses. Modification of the genome of reassortant vaccine viruses by incorporating the NP gene from wild-type viruses represents a simple strategy to improve the immunogenicity and cross-protection of influenza vaccines.

Keywords

Live attenuated influenza vaccine; Nucleoprotein; Immunogenicity; Cell-mediated immunity; Cross-protection; Mouse model; Immunodominant epitope

1. Introduction

Influenza A viruses are highly contagious respiratory pathogens that continuously threaten human populations. Almost every year, there are epidemics of 3–5 million cases of severe influenza worldwide, of which at least 250,000 are fatal (Fifth meeting of National Influenza

*Correspondence to: Leading Researcher, Department of Virology, Institute of Experimental Medicine, 12 Acad. Pavlov Street, Saint Petersburg 197376, Russia., isakova.sivak@gmail.com (I. Isakova-Sivak).

[1]these authors contributed equally to this work.

Conflict of interest statement

All the authors have declared that have no conflict of interest.

Centers, 2012). The most effective tool for controlling influenza is vaccination. There are two main kinds of influenza vaccine currently in wide use: inactivated influenza vaccine (IIV) and live attenuated influenza vaccine (LAIV).

Immunization with IIV induces mainly humoral immune responses to the viral surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). However, this immunity is strain-specific and gives little protection against drifted variants of the virus. In contrast, immunization with LAIV induces a wide spectrum of immune responses, including local (mucosal) and T-cell-mediated immunity, in particular CD8+ cytotoxic T-cells (CTLs) (Tamura et al., 2005). CTL-driven immunity is cross-reactive and recognizes conserved epitopes within viral proteins, affording protection against different subtypes of influenza A virus (Kreijtz et al., 2008). One of the major targets for the CTL immune response is a molecule of viral nucleoprotein (NP), which contains multiple immunodominant CTL epitopes (Grant et al., 2013; Thomas et al., 2006).

LAIV strains are routinely prepared by either classical reassortment or reverse genetics, and usually comprise HA and NA genes derived from wild-type influenza virus (seasonal or potentially pandemic). The remaining six genes, including the NP gene, are derived from an attenuated master donor virus (MDV) (the so-called 6:2 genome composition) (Aleksandrova, 1977; Jin and Subbarao, 2015). The most widely used donor viruses – A/Leningrad/134/17/57 (Len/17) in Russia and A/Ann Arbor/6/60 (A/AA) in the USA and Europe – were isolated nearly 60 years ago. It is possible that the nucleoprotein of influenza viruses could have significantly evolved during this period, despite its relatively conserved nature, resulting in a significant change in its antigenic properties. Thus the NP-specific CTL immunity induced by vaccination with classical LAIVs may not be effective against currently circulating influenza viruses. The most straightforward way to overcome this problem would be to include the wild-type NP gene in the genome of the LAIV reassortant virus, i.e. to switch from a 6:2 to a 5:3 genome composition (Isakova-Sivak et al., 2016). Such a modification of the vaccine virus genome would require a thorough understanding of the properties of the new LAIV reassortants, including *in vitro* and *in vivo* characterization, as well as their evaluation in clinical trials.

This paper reports a comparison of H7N9 LAIV reassortants with 6:2 and 5:3 genome compositions, with regards to their growth characteristics, induction of humoral and cellular immune responses in C57BL/6 ($H2^b$) mice, and ability to protect animals against challenge with homologous and heterologous viruses. This particular subtype was selected because of its ability to induce humoral and cell-mediated immunity in C57BL/6 mice, and also because the NP of Len/17 and H7N9 virus have significant differences in the murine immunodominant epitope $NP_{366–374}$, and thus are a good model for comparative epitope-specific cell-mediated immunogenicity studies (Thomas et al., 2006).

2. Materials and methods

2.1. Materials

H7N9 LAIV reassortant virus with a 6:2 genome composition (H7N9 LAIV 6:2) was prepared by classical reassortment in eggs and was tested in preclinical and clinical trials (de

Jonge et al., 2016; Rudenko et al., 2016). This virus possesses the HA and NA genes of wild-type (*wt*) A/Anhui/1/2013 (H7N9) strain; the remaining genes come from A/Leningrad/134/17/57 (H2N2) MDV. The corresponding H7N9 LAIV 5:3 reassortant virus with the NP gene inherited from the H7N9 virus rather than the MDV was generated by means of reverse genetics. Both viruses were fully sequenced and found to be identical apart from the NP gene.

Master donor virus A/Leningrad/134/17/47 (Len/17) and its wild-type precursor A/Leningrad/134/57 (H2N2) (Len134 wt) were obtained from the repository of the Institute of Experimental Medicine (Saint Petersburg, Russia). Low-pathogenic avian influenza virus A/mallard/Netherlands/12/2000 (H7N3) was obtained from the repository of the Centers for Disease Control and Protection (CDC) (Atlanta, GA, USA). In addition, two engineered viruses were generated for challenge experiments: an H7N9-PR8 5:3 reassortant, possessing the HA, NA and NP genes of *wt* H7N9 virus and five genes from A/Puerto Rico/8/34 (H1N1) (PR8) virus; and an H1N1 7:1 virus, containing the NP gene from *wt* H7N9 virus and seven genes from the PR8 virus. All viruses were propagated in 10–11-D-old chicken embryos for two days at 33 °C and stored in aliquots at −70 °C.

C57BL6 mice of 8–10 weeks old were purchased from the laboratory breeding nursery of the Russian Academy of Sciences “Stolbovaya” (Moscow region, Russia).

$NP_{366–374}$ peptides (ASNENMDTM and ASNENMEAM) were chemically synthesized by Almabion Ltd (Russian Federation), with a purity of over 95%, as shown by high-performance liquid chromatography (HPLC). Peptides were reconstituted in dimethyl sulfoxide (DMSO) to a concentration of 1 mM and stored at −70 °C in aliquots.

2.2. Methods

2.2.1. CTL epitopes in circulating influenza A viruses—In order to predict the effectiveness of LAIV CTL immunity, we assessed the conservation of selected CTL epitopes in 757 unique NP sequences of influenza A viruses of H1N1 and H3N2 subtypes circulating in 2009–14, using the Immune Epitope DataBase (IEDB). Influenza A virus sequences were obtained from the Influenza Virus Sequence Database of the National Center for Biotechnology Information (NCBI) (Bao et al., 2008). CTL epitopes in the MDV NP sequence were screened with netCTL major histocompatibility complex I (MHCI) peptide binding and netChop proteasome processing prediction algorithms (Larsen et al., 2007). The immunogenicity of CTL epitopes was estimated using the T cell class I peptide MHC (pMHC) immunogenicity predictor algorithm (Calis et al., 2013); peptides with an immunogenicity score above 0 were assumed to be immunogenic. Conservation of immunogenic CTL epitopes was estimated by conservancy analysis, with sequence identity threshold equal to 100% (Bui et al., 2007). For murine experiments, CTL epitope–MHC binding affinity was predicted using the netMHCpan algorithm (Hoof et al., 2009).

2.2.2. Growth characteristics of H7N9 LAIV 6:2 and 5:3 reassortants in vitro—Temperature-sensitive (*ts*) and cold-adapted (*ca*) phenotypes of the studied 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. The Len/17 and Len134

wt viruses were used as control viruses possessing the opposite *ts/ca* phenotypes. Eggs were inoculated with 10-fold virus dilutions and incubated for either 48 h (at 33 °C or 38 °C) or 6 days (at 26 °C). The growth characteristics of the H7N9 LAIV viruses were analysed in Madin Darby canine kidney (MDCK) cells: cell monolayers were infected with the viruses at a multiplicity of infection (MOI) of 0.01 and 0.001 in triplicate; 150 μl of the media were collected every 12 h and stored at −70 °C prior to titration by 50% tissue culture infective dose ($TCID_{50}$). Virus titers in eggs and MDCK cells were calculated by the Reed and Muench method and expressed in terms of log_{10} 50% egg infective dose (EID_{50})/ml and $log_{10}TCID_{50}$/ml, respectively.

2.2.3. Growth characteristics of H7N9 LAIV 6:2 and 5:3 reassortants in vivo— Groups of eight female C57BL6 mice were anesthetized with ether and given 50 μl of virus suspension containing 10^6 EID_{50} by the intranasal (IN) route. The Len/17 and Len134 wt viruses were used as control viruses: the former is characterized by an attenuated (*att*) phenotype, while the latter has a *non-att* phenotype. Nasal turbinates and lungs were collected on days 3 and 6 after inoculation and stored frozen at −70 °C until used for homogenization. Tissue homogenates were prepared in 1 ml of sterile phosphate-buffered saline (PBS) containing antibiotic and antimycotic (Invitrogen, UK), using a small bead mill TissueLyser LT (QIAGEN, Germany). The clarified supernatants were used to determine virus titers by limiting dilutions in eggs, as described above. The limit of detection was 1.2 log_{10}/ml.

2.2.4. Immunogenicity and protection studies—Groups of 54 female C57BL6 mice were given two doses of 50 μl of either H7N9 LAIV 6:2 or H7N9 LAIV 5:3 virus suspension containing 10^6 EID_{50} intranasally, 21 days apart. Control animals received the same volume of PBS. Blood and bronchoalveolar lavage (BAL) samples were collected from six mice in each group on day 21 after each dose, to assess antibody immune responses. Six mice from each group were euthanized on day 7 after each dose and spleen samples were harvested for the assessment of cell-mediated immune responses. *In vivo* killing activity of CTLs was studied in six mice of each study group on day 7 after the second dose. The remaining 24 animals were used for the assessment of protective efficacy of the H7N9 LAIVs.

2.2.5. Antibody responses—Serum antibody titers were determined by hemagglutination-inhibition assay (HAI), and IgG by enzyme-linked immunosorbent assay (ELISA), as described previously (Isakova-Sivak et al., 2014) with some modifications. IgA antibodies were determined in BAL specimens collected on days 21 and 42 using 1 ml of sterile PBS.

For HAI, serum samples were treated with chicken red blood cells to remove nonspecific inhibitors and quantified against four HA units of the following viruses: (i) H7N9 LAIV 6:2; (ii) H7N9 LAIV 5:3; (iii) H7N3 wt; and (iv) H1N1 7:1. ELISA was performed with the same four antigens. (The wild-type H7N9 virus could not be used as antigen in these tests, because of the lack of a biosafety level 3 facility).

Briefly, 96-well microtitration plates (Microlon, Greiner bio-one, Germany) were coated with 100 μl of sucrose-purified virus antigen containing 16 HA units in PBS overnight at 4 °C. The plates were washed with 0.1% Tween 20 in PBS (PBST), then blocked with 200 μl of 1% bovine serum albumin (BSA) in PBS for 2 h. Twofold dilutions of sera were prepared starting from 1:2 (for IgA antibody) and 1:10 (for IgG antibody) and added to the coated wells. The plates were incubated overnight at 4 °C, then washed six times with PBST. Bound antibodies were detected with 50 μl of horseradish peroxidase-conjugated goat anti-mouse IgA or IgG (Sigma-Aldrich). Antibody titer was defined as the last dilution with an optical density (OD) at least double the mean OD in the control wells (containing all components except the serum specimens).

2.2.6. Virus/epitope-specific murine CTLs—Cytotoxic CD8+ T cells were analysed by intracellular cytokine staining (ICS) to gamma-interferon. In brief, murine splenocytes were isolated and red blood cells were lysed by ammonium-chloridepotassium lysing buffer. In some experiments, cells were cryoconserved in heat-inactivated fetal bovine serum (FBS) with 10% DMSO. For *in vitro* virus stimulation, 10^6 cells were incubated with sucrose-gradient purified viruses at an MOI of 1.0 EID_{50} per cell for one hour in 100 μl of complete RPMI-1640 without FBS in microtitration U-bottom well plates. Next, 50 μl of medium with FBS was added, to a final FBS concentration of 10%. Peptide stimulation experiments used the same procedures without addition of virus. After 16–18 h, GolgiPlug solution (Becton Dickinson, USA) – alone or with peptide (for peptide stimulation experiments) – or control solution was added and the mixture incubated for a further five hours. ICS was performed with Cytofix/Cytoperm kit (Becton Dickinson, USA) according to the manufacturer's instructions. Samples were stained with live/dead fixable stain (Invitrogen, USA), anti-CD3, anti-CD8 and anti-gamma-interferon antibody-conjugates (Becton Dickinson, USA). Samples were fixed in 1% paraformaldehyde and stored in a dark cool place prior to flow cytometric analysis. At least 300 000 events were measured using a Navios flow cytometer (Beckman Coulter, USA). The percentage of virus/peptide-specific CTLs was calculated by subtracting the gammainterferon-positive CD8+ T cells from the negative control (virus/peptide diluents probes).

2.2.7. Cytotoxic activity of CTLs in vivo—This was performed by CTL *in vivo* assay as described by Durward et al. Durward et al. (2010), with some modifications. Briefly, splenocytes from naïve C57BL6 mice were harvested and red blood cells lysed. Cells were divided into three parts (of 2×10^8 cells each) in 10 ml of complete RPMI-1640, and loaded with 1 μm peptide or an equal volume of peptide diluent (control) for one hour. Each sample was then washed and stained with either 40, 20 or 10 mM carboxyfluorescein diacetate succinimidyl ester (CFSE). The target cells were washed, filtered, and mixed with equal amounts of cells stained with each CFSE concentration. Two million target cells were administered in 100 μl of Hanks solution to anesthetized mice by retro-orbital injection. The following day, the mice were sacrificed and splenocytes were harvested and processed by flow cytometry. Cytotoxicity was represented as the ratio of the count of peptide-loaded target cells to that of control target cells.

2.2.8. Assessment of protective efficacy—To assess protection, 24 mice from each study group were divided into three sub-groups and infected intranasally with 50 μl of one of the following three challenge viruses at a dose of 10^6 EID_{50}: (i) H7N9-PR8 5:3; (ii) H7N3 *wt;* or (iii) H1N1 7:1. The first of these was a recombinant virus on a PR8 backbone with HA, NP and NA genes derived from H7N9 virus. This virus was considered as homologous challenge, since wild-type H7N9 virus could not be used. The third challenge virus was a recombinant virus based on PR8, in which the NP gene was replaced by that of the H7N9 strain (i.e. a 7:1 reassortant). Four mice in each challenge group were euthanized on days 3 and 6 after infection, and lungs and nasal turbinates were harvested for virus titration. Virus titers in tissue homogenates were determined by titration on MDCK cells by $TCID_{50}$ assay.

2.2.9. Statistical analyses—Data were analysed with the Statistica software (version 6.0; Statsoft Inc.). The statistical significance of the difference between viral titers in organs of mice was determined by the Mann-Whitney *U*-test. Differences in CTL levels and log_2-transformed HAI and ELISA antibody titers were also subjected to the Mann-Whitney *U*-test. *P* values of < 0.05 were considered significant.

2.2.10. Ethics statement—The handling of animals and chicken embryos was performed in accordance with the “Manual for laboratory animals and alternative models in biomedical research” (Manual for laboratory animals and alternative models in biomedical research Russian, 2010). Fertilized eggs used for virus propagation were discarded in an appropriate manner, according to Russian sanitary-epidemiological rules SP 1.3.2322-08 (approved 28 January 2008). Mouse experiments were reviewed and approved by the Institutional Local Ethical Committee.

3. Results

3.1. CTL epitopes in circulating influenza A viruses

Using the netCTL algorithm, we selected 138 unique 9-mer peptides from the LAIV NP with high binding affinity to the MHC of 11 human leukocyte antigen (HLA) supertypes (A1, A2, A3, A24, A26, B8, B27, B39, B44, B58, B62). Eighteen of them were found to be highly immunogenic by the T cell class I pMHC immunogenicity predictor algorithm with default mask (1, 2 and C-terminus amino acid) (Table 1). Only 8 of the 18 epitopes were highly conserved in recent strains of influenza A virus (conservancy in 90% or more of assessed sequences), suggesting that the nucleoprotein has evolved significantly during the past 60 years.

3.2. Growth characteristics of H7N9 LAIV 6:2 and 5:3 reassortants in vitro

Comparative studies of the growth characteristics of 6:2 and 5:3 H7N9 LAIV reassortants demonstrated that the NP gene had no impact on the *ts/ca* phenotypes: both the 6:2 and the 5:3 reassortants replicated poorly in eggs at 38 °C, but grew well at 26 °C (Fig. 1). These LAIVs recapitulated the *ts/ca* phenotypes of Len/17 MDV, in contrast to the wild-type virus Len134, which replicated well at 38 °C, but its growth at 26 °C was inefficient (Fig. 1). Although the titers of the 5:3 reassortant were slightly lower than those of the 6:2 counterpart in eggs at 33 °C and 26 °C (difference 0.5–0.7 $log_{10}EID_{50}$), the kinetics of virus

growth in MDCK cells at an MOI of 0.01 were identical (Fig. 2A). Only at the lower MOI of 0.001 was a slightly slower accumulation of the 5:3 virus seen in cell culture; however, the final titer of the 5:3 reassortant was higher than that of 6:2 virus (Fig. 2B).

3.3. Growth characteristics of H7N9 LAIV 6:2 and 5:3 reassortants in vivo

The two H7N9 LAIV viruses showed similar growth patterns in mouse respiratory organs: the viruses grew efficiently in the upper respiratory tract (URT), achieving titers of 4.0–4.4 $\log_{10}EID_{50}$/ml on day 3. The replication was prolonged and on day 6 the viruses were detected in the URT at titers of 3.0–3.1 $\log_{10}EID_{50}$/ml. In contrast, the growth of the viruses in the lower respiratory tract was significantly impaired and titers did not exceed 1.4 $\log_{10}EID_{50}$/ml, similar to the Len/17 MDV (Fig. 3). Interestingly, the magnitude of replication of both H7N9 LAIVs in URT was significantly higher than that of Len/17 MDV, suggesting the positive impact of H7N9 surface proteins on LAIV infectivity. These data show the classical replication pattern of attenuated influenza viruses in a mouse model, and this is in contrast to Len134 WT virus, which could grow in lung tissues up to 5.4 $\log_{10}EID_{50}$/ml and this replication was prolonged (Fig. 3).

Overall, the NP gene of wild-type H7N9 influenza virus had little or no impact on virus growth *in vitro* or *in vivo*.

3.4. Antibody responses after immunization with H7N9 LAIV 6:2 and 5:3 reassortants

The two LAIV viruses induced identical HAI antibody titers to all antigens tested, after one and two doses (Fig. 4). These data are not unexpected, since the vaccines share the same HA molecule. In contrast, serum IgG and BAL IgA antibody titers detected by ELISA differed significantly, depending on the antigen used. Thus, when H7N9 LAIV 6:2 virus was used as antigen, significantly higher IgG and IgA antibody titers were seen in mice immunized with H7N9 LAIV 6:2 reassortant than those in the H7N9 LAIV 5:3 vaccine group (Fig. 4A). However, when the H7N9 LAIV 5:3 virus was used as antigen, no differences were seen in serum IgG and BAL IgA antibody titers in the two vaccine groups (Fig. 4B). Since the two antigens differ only in the source of NP, these data reflect the impact of anti-NP antibody in overall virus immunogenicity. It is noteworthy that slightly higher serum IgG antibody titers were detected in mice immunized with two doses of 5:3 vaccine virus, when heterologous H7N3 virus was used as antigen ($p=0.049$), suggesting that the 5:3 virus induces IgG antibody that is more cross-reactive with recent H7 viruses (Fig. 4C). Some cross-reactive serum IgG and BAL IgA antibodies to heterologous H1N1 7:1 virus were observed after two doses of either H7N9 LAIV; there was no significant difference between the vaccine groups (Fig. 4D). Overall, the two H7N9 LAIVs induced similar antibody responses in mice.

3.5. Virus-specific CTL responses after immunization with H7N9 LAIV 6:2 and 5:3 reassortants

The main aim of this study was to compare NP-specific cell-mediated immunity to LAIV viruses that differ only in the source of their NP. Administration of either 6:2 or 5:3 LAIV induced CTL immunity in C57BL6 mice after each dose (Fig. 5A). A second dose of either LAIV boosted the levels of virus-specific CTLs; more dramatic induction of virus-specific CTLs was noted in mice immunized with the 6:2 virus. Interestingly, despite the higher CTL

responses in the 6:2 LAIV group, these T-cells were mainly targeted to virus containing old NP (i.e. 6:2 reassortant), while the CTL levels targeted to 5:3 LAIV were significantly lower (p=0.031). In contrast, the 5:3 LAIV induced lower levels of CTLs, but which responded equally to stimulation with 6:2 or 5:3 reassortant viruses (Fig. 5).

3.6. $NP_{366–374}$ epitope-specific CTL responses after immunization with H7N9 LAIV 6:2 and 5:3 reassortants

Fig. 5B shows the $NP_{366–374}$-specific CTL immune response in mice after administration of H7N9 6:2 or 5:3 LAIV. CTLs were induced by both vaccine viruses and were boosted by a second vaccine dose. As with whole-virus stimulation, 6:2 LAIV induced higher levels of epitope-specific CTLs than 5:3 LAIV. While both viruses induced cross-reactive epitope-specific CTLs, the proportion of CTL clones targeted to the NP epitope from H7N9 virus in relation to those targeted to the epitope from Len/17 was significantly (more than 30- fold) higher in the 5:3 LAIV group. These data suggest that the two mutations in the NP_{366} epitope had an impact on the cross-reactivity of the epitope-specific CTLs.

3.7. In vivo cytotoxic activity of CTLs induced by 6:2 and 5:3 H7N9 LAIVs

Fig. 6 shows the results of the *in vivo* assay to estimate the ability of CTLs of LAIV-immunized mice to kill $NP_{366–374}$-loaded target cells. As expected, Len/17 NP_{366}-loaded targets were more effectively killed in mice immunized with 6:2 LAIV than in those immunized with 5:3 LAIV. The proportion of peptide-loaded to control cells in mock-immunized mice was 1.2 compared with 0.8 in mice immunized with 6:2 LAIV-immunized, and 1.14 in 5:3 LAIV-immunized mice (Fig. 6A). In contrast, the proportion of H7N9 NP_{366}-loaded cells to control cells was significantly lower in 5:3 LAIV-immunized mice (1.4) than in mock-immunized mice (1.7), while the proportion of peptide-loaded cells in the 6:2 LAIV group (1.5) was not significantly different than that for mock-immunized mice (Fig. 6B). This suggests that 5:3 LAIV-immunized mice should be better protected against influenza viruses carrying more recent NPs.

3.8. Protection against homologous and heterologous influenza viruses

Induced immunity in both LAIV groups was sufficient to fully protect mice from challenge with H7N9 virus: no infectious virus was detected in the lungs of immunized animals, while high pulmonary titers were observed in the control group (Fig. 7A).

After challenge with the second virus, a natural avian influenza isolate, lung titers in the 5:3 LAIV-immunized group on day 3 were significantly lower than those in the control group (p=0.024); titers in the 6:2 LAIV-immunized animals were lower than in the control group, but the difference was not significant (p=0.077) (Fig. 7B). Although the difference between the study groups was not significant (p=0.19), these data suggest that the 5:3 reassortant gives better cross-protection than the 6:2 counterpart. Importantly, both vaccines resulted in faster clearance of the challenge virus: no virus was detected six days after challenge.

The third challenge virus, an H1N1 7:1 reassortant, in contrast to the PR8 virus, was not lethal for mice (data not shown). However, it replicated efficiently in the mouse lower respiratory tract. Strikingly, the protection profile in this challenge group was similar to that

for H7N3: though the difference between the LAIV groups was not statistically significant (p=0.29), mice immunized with 5:3 LAIV shed the virus at significantly lower levels than mock-immunized mice three days after challenge (p=0.024), while virus titers in 6:2 LAIV-immunized mice were not significantly different from controls (p=0.11) (Fig. 7C). Overall, the immune responses induced by 5:3 H7N9 LAIV reassortant were more cross-protective than those induced by classical 6:2 LAIV.

4. Discussion

Live attenuated influenza vaccine has historically been generated by reassortment of genes from currently circulating or potentially pandemic wild-type influenza A virus with genes from an MDV. The current 6:2 genome structure was initially proposed because it was postulated that the HA and NA glycoproteins were the main targets for adaptive immune responses and should come from the wild-type parental virus, while the remaining genes determine the biological properties of the LAIV vaccine strain (high yield, *ts/ca* and attenuated phenotype) and should be derived from the MDV. However, more recently, studies of influenza-related immune responses have revealed that internal and non-structural proteins of influenza virus have an important impact on cell-mediated immune responses. Apparently, this type of immunity constrains the number of infected cells and reduces virus yield, but does not prevent the dissemination of free virions (Janeway et al., 2001; Bender et al., 1992; Moskophidis and Kioussis, 1998). CTL immunity is targeted to a number of influenza proteins, predominantly epitopes of internal proteins, such as matrix protein and nucleoprotein (Grant et al., 2013; Moskophidis and Kioussis, 1998; Chen et al., 2014). Those proteins are relatively conserved as a result of sequence functional constraints and CTL immunity against them is cross-reactive between influenza subtypes (Saha et al., 2006; Rimmelzwaan et al., 2009).

Recently Machkovech et al. (2015) have shown evidence of selective pressure on nucleoprotein CTL epitopes in human influenza A virus. Another study demonstrated the possibility of generating CTL immune- escaped influenza virus variants within an immune-compromised patient persistently infected with an influenza virus (Valkenburg et al., 2013). Moreover, there is a risk that CTL immunity after human viruses exposure will be ineffective against zoonotic influenza viruses with diversified CTL epitope sequences, such as H7N9 avian-origin virus (De Groot et al., 2013).

Russian LAIVs contain NP from an H2N2 human viral strain that originated in 1957. We found that only 44% of the CTL epitopes of the Len/17 MDV nucleoprotein are conserved in currently circulating influenza A H1N1 and H3N2 viruses. The Len/17 NP-specific CTLs induced after immunization with classical LAIV might therefore be relatively inefficient in protecting against recent viruses. Modification of the genome composition of LAIV strains to include the NP of the wild-type strain may overcome this problem.

Since humanized mice were not available for our studies, we searched for a good virus subtype to study NP-specific CTL response in C57BL6 mice, a mouse model for which the T-cell immune response to influenza infection has been well characterized (Thomas et al., 2006). The H7N9 subtype was considered a good model virus because H7N9 LAIV

replicated well in the C57BL6 mouse inducing strong antibody and cellular immune responses (our unpublished data, confirmed in this study), and also due to significant differences in the murine immunodominant epitope $NP_{366–374}$. Earlier studies indicated that while the primary CTL response to PR8 virus was driven by the two viral determinants ($PA_{224–233}$ and $NP_{366–374}$), the $NP_{366–374}$-specific CD8+ T cells dominated the secondary response to PR8 challenge, accounting for up to 80% of the influenza-specific CTLs (Thomas et al., 2006; Belz et al., 2000). In our study, we used a two-dose immunization schedule because it better reflects the situation with humans who are usually primed to some extent due to previous exposure to influenza infection or immunization, whereas mice are immunologically naïve and two vaccine doses are required to induce substantial levels of antibody and cell-mediated immunity.

Our studies found that the NP of H7N9 virus had only a slight impact on the growth characteristics of the LAIV reassortant strain, in eggs, cell culture, and mouse respiratory organs. The infectivity of the 5:3 reassortant allowed functional antibody and cell-mediated immunity to be established after vaccination. In general, the 6:2 LAIV reassortant induced more pronounced ELISA antibodies and cell-mediated immune responses than the 5:3 LAIV virus, when measured against 6:2 LAIV virus (containing Len/17 NP). However, this superior immunogenicity disappeared when antibodies and CTLs were measured against viruses containing recent NPs. It was interesting that ELISA could detect different levels of IgG antibody against 6:2 and 5:3 LAIV antigens within one serum sample. Since these viruses differ only in the source of NP, it can be speculated that ELISA can also detect a subset of anti-NP antibody. Although NP is not normally found on the virion surface and theoretically anti-NP antibody should not be detected using whole-virion antigens, the use of detergent in some steps of our ELISA protocol might contribute to the binding of the anti-NP antibody to the NP protein of the coating virions. The limitation of our study is the lack of purified nucleoproteins to measure NP-specific antibodies directly. The experimental set-up did not allow the protective role of anti-NP antibody against heterologous challenge viruses to be distinguished from that of NP-specific CTLs. It should be noted that the protective effect of anti-NP antibody in C57BL6 mice against lethal challenge could be seen only with high concentrations of this antibody in mouse sera (Lamere et al., 2011).

The main purpose of this study was to determine whether the incorporation of *wt* NP into the genome of LAIV virus would be advantageous in terms of inducing cross-reactive CTLs and protecting mice against antigenically divergent viruses. As mentioned above, we used C57BL6 mice in which the CTL response is mainly restricted by the $NP_{366–374}$ epitope (Thomas et al., 2006; Belz et al., 2000; Belz et al., 2000, 2001; Kedzierska et al., 2005; Young et al., 1994). This fragment differs in the 7th and 8th positions in Len/17 and H7N9 NPs. These are the substitutions in TCR facing residue 7 (from acidic Asp to acidic Glu) and in MHC I anchoring residue 8 (from hydroxyl Thr to aliphatic Ala). These differences might be not critical for peptide-MHC I complex formation, because both peptides preserve high predicted binding affinity to H-2Db molecules by NetMHCpan 2.8 algorithm (data not shown). Unfortunately, the epitope processing prediction algorithms are not well fitted for murine machinery to utilize in this experiment. Nevertheless, both of them could be immunogenic but able to induce the CTL clones with different TCR recognition moieties. Through *in vitro* measurement of CTL cross-reactivity to $NP_{366–374}$ epitopes, we estimated

that these amino acid substitutions were critical to pMHC-complex recognition by T-cell receptors. Thus, despite the higher CTL response specific for NP_{366}Len/17 epitope in the 6:2 LAIV-immunized mice, the proportions of NP_{366}H7N9-specific CTLs relative to NP_{366}Len/17-specific CTLs were 30 times lower in this vaccine group than in the 5:3 LAIV group. These results demonstrate the excessive induction of CTLs targeted to old irrelevant epitopes by classical 6:2 LAIV, unnecessarily loading the immune system. Moreover, the 5:3 LAIV-immunized mice had the greatest specific NP_{366}-targeted killing activity and protection against challenge viruses containing recent NPs. These experiments demonstrated the clear advantages of LAIVs with up-to-date CTL epitopes in inducing a relevant T-cell immune response, rather than producing a large immunological reaction to irrelevant antigens. Although we did not assess the other major immunodominant epitopes outside of NP protein, previous studies indicate that in mice infected with viruses from which dominant CTL epitopes have been eliminated the expansion of T cells reactive against subdominant epitopes might occur (Webby et al., 2003). Of note, the second major immunodominant epitope $PA_{224–233}$ differs by one amino acid between Len/17 MDV and the two viruses used in the challenge studies: PR8 and H7N3 (Cys-2-Ser), therefore the expansion of CD8+ T cells against other conserved subdominant epitopes, such as $PB1_{703–711}$, $NS2_{114–121}$, $M1_{128–135}$ might have contributed to the observed enhanced cross-protection of the 5:3 LAIV. The fact that both 6:2 and 5:3 LAIVs efficiently protected mice against heterologous H1N1 viral challenge is in line with other studies demonstrating the heterosubtypic protection of LAIVs based on different backbones (Jang and Seong, 2013). This cross-protective potential of LAIVs is mostly driven by the CTLs targeted to conserved viral proteins, and in our studies we attempted to further improve this mode of protection afforded by LAIVs.

The NP gene of Len/17 MDV does not bear any *ts* loci associated with the *ts/att* phenotypes of the Russian LAIV; these phenotypes are controlled by specific mutations located in PB2 (V478L) and PB1 (K265N; V591I) (Isakova-Sivak et al., 2011; Kuznetsova et al., 2015). Therefore the Len/17 NP gene can be easily replaced with that of wild-type virus without compromising the biological properties of the LAIV virus. In contrast, the US A/AA-based LAIV contains a mutation in the viral NP (D34G) that, together with other mutations in the viral PB2 (N265S) and PB1 (K391E; E581G; A661T) are responsible of the *ts* phenotype of the MDV (Jin, 2003). However the other studies found that the four *ts* loci in PB2 and PB1 proteins are sufficient to impart *ts/att* phenotypes to the genome of wild-type virus, suggesting that NP protein of A/AA MDV also can be substituted with WT NP without affecting important viral characteristics (Zhou et al., 2012; Jin et al., 2004).

The results of our study may also have implications for the improvement of inactivated influenza vaccines. In recent years, numerous studies have demonstrated the establishment of functional influenza- virus-specific CD8+ and CD4+ T cells after both intranasal and intramuscular immunization with IIV (Wang et al., 2015; Keijzer et al., 2014; Ann et al., 2014). It would therefore also be important to reconsider the genome composition of reassortant viruses for IIVs. These viruses are usually prepared using highly egg-adapted virus A/ PR/8/34 (H1N1), which dates back to the 1930s, and the CTL epitopes of recent viruses have also significantly evolved since then.

As a conclusion, we strongly recommend incorporating wild-type NP into the genome of seasonal LAIV and IIV reassortant viruses to improve cell-mediated immune responses.

Acknowledgments

Funding

This study was supported by Russian Scientific Foundation Grant No 14-15-00034. Writing assistance in the preparation of this manuscript, in the form of editing by Pat Butler, was funded by the World Health Organization.

References

1. Aleksandrova GI. Use of the genetic recombination method for obtaining vaccinal strains of the influenza virus. Vopr. Virus. 1977; (4):387–395. [PubMed: 335659]
2. Ann J, et al. Adjuvanted inactivated influenza A(H3N2) vaccines induce stronger immunogenicity in mice and confer higher protection in ferrets than unadjuvanted inactivated vaccines. Vaccine. 2014; 32(43):5730–5739. [PubMed: 25173481]
3. Bao Y, et al. The influenza virus resource at the national center for biotechnology information. J. Virol. 2008; 82(2):596–601. [PubMed: 17942553]
4. Belz GT, et al. A previously unrecognized H-2D(b)-restricted peptide prominent in the primary influenza A virus-specific CD8(+) T-cell response is much less apparent following secondary challenge. J. Virol. 2000; 74(8):3486–3493. [PubMed: 10729122]
5. Belz GT, Xie W, Doherty PC. Diversity of epitope and cytokine profiles for primary and secondary influenza a virus-specific CD8+ T cell responses. J. Immunol. 2001; 166(7):4627–4633. [PubMed: 11254721]
6. Bender BS, et al. Transgenic mice lacking class I major histocompatibility complex-restricted T cells have delayed viral clearance and increased mortality after influenza virus challenge. J. Exp. Med. 1992; 175(4):1143–1145. [PubMed: 1552285]
7. Bui HH, et al. Development of an epitope conservancy analysis tool to facilitate the design of epitope-based diagnostics and vaccines. BMC Bioinforma. 2007; 8:361.
8. Calis JJ, et al. Properties of MHC class I presented peptides that enhance immunogenicity. PLoS Comput Biol. 2013; 9(10):e1003266. [PubMed: 24204222]
9. Chen L, et al. Immunodominant CD4+ T-cell responses to influenza A virus in healthy individuals focus on matrix 1 and nucleoprotein. J. Virol. 2014; 88(20):11760–11773. [PubMed: 25078703]
10. De Groot AS, et al. Low immunogenicity predicted for emerging avian-origin H7N9: implication for influenza vaccine design. Hum. Vaccin. Immunother. 2013; 9(5):950–956. [PubMed: 23807079]
11. Durward M, Harms J, Splitter G. Antigen specific killing assay using CFSE labeled target cells. J. Vis. Exp. 2010:45.
12. Fifth meeting of National Influenza Centres. WHO Western Pacific and South-East Asia Regions. Wkly Epidemiol Rec. 2012; 87(7):61–64. [PubMed: 22355834]
13. Grant E, et al. Nucleoprotein of influenza A virus is a major target of immunodominant CD8+ T-cell responses. Immunol. Cell Biol. 2013; 91(2):184–194. [PubMed: 23399741]
14. Hoof I, et al. NetMHCpan, a method for MHC class I binding prediction beyond humans. Immunogenetics. 2009; 61(1):1–13. [PubMed: 19002680]
15. Isakova-Sivak I, et al. Genetic bases of the temperature-sensitive phenotype of a master donor virus used in live attenuated influenzavaccines: a/leningrad/134/17/57 (H2N2). Virology. 2011; 412(2): 297–305. [PubMed: 21315402]
16. Isakova-Sivak I, et al. Characterization of reverse genetics-derived cold-adapted master donor virus A/Leningrad/134/17/57 (H2N2) and reassortants with H5N1 surface genes in a mouse model. Clin. Vaccin. Immunol. 2014; 21(5):722–731.
17. Isakova-Sivak I, Korenkov D, Rudenko L. Reassortant viruses for influenza vaccines: is it time to reconsider their genome structures? Expert Rev. Vaccin. 2016; 15(5):565–567.

18. Janeway, CJ.; Travers, P.; Walport, M., et al. Immunobiology: The Immune System in Health and Disease. 5th. New York: Garland Science; 2001. T cell-mediated cytotoxicity. Available from: ⟨http://www.ncbi.nlm.nih.gov/books/NBK27101/⟩. 2001
19. Jang YH, Seong BL. Cross-protective immune responses elicited by live attenuated influenza vaccines. Yonsei. Med. J. 2013; 54(2):271–282. [PubMed: 23364956]
20. Jin H. Multiple amino acid residues confer temperature sensitivity to human influenza virus vaccine strains (flumist) derived from cold-adapted a/ann arbor/6/60. Virology. 2003; 306(1):18–24. [PubMed: 12620793]
21. Jin H, et al. Imparting temperature sensitivity and attenuation in ferrets to A/Puerto Rico/8/34 influenza virus by transferring the genetic signature for temperature sensitivity from cold-adapted A/Ann Arbor/6/60. J. Virol. 2004; 78(2):995–998. [PubMed: 14694130]
22. Jin H, Subbarao K. Live attenuated influenza vaccine. Curr. Top. Microbiol Immunol. 2015; 386:181–204. [PubMed: 25059893]
23. de Jonge J, et al. H7N9 live attenuated influenza vaccine is highly immunogenic, prevents virus replication, and protects against severe bronchopneumonia in ferrets. Mol. Ther. 2016; 24(5):991–1002. [PubMed: 26796670]
24. Kedzierska K, et al. Contribution of T cell receptor affinity to overall avidity for virus-specific CD8+ T cell responses. Proc. Natl. Acad. Sci. USA. 2005; 102(32):11432–11437. [PubMed: 16061802]
25. Keijzer C, et al. Inactivated influenza vaccine adjuvanted with bacterium-like particles induce systemic and mucosal influenza A virus specific T-cell and B-cell responses after nasal administration in a TLR2 dependent fashion. Vaccine. 2014; 32(24):2904–2910. [PubMed: 24598720]
26. Kreijtz JH, et al. Cross-recognition of avian H5N1 influenza virus by human cytotoxic T-lymphocyte populations directed to human influenza A virus. J. Virol. 2008; 82(11):5161–5166. [PubMed: 18353950]
27. Kuznetsova SA, et al. Effect of Point Mutations in the Polymerase Genes of the Influenza A/PR/8/34 (H1N1) Virus on the Immune Response in a Mouse Model. Vopr. Virus. 2015; 60(2):25–30.
28. Lamere MW, et al. Regulation of antinucleoprotein IgG by systemic vaccination and its effect on influenza virus clearance. J. Virol. 2011; 85(10):5027–5035. [PubMed: 21367900]
29. Larsen MV, et al. Large-scale validation of methods for cytotoxic T-lymphocyte epitope prediction. BMC Bioinforma. 2007; 8:424.
30. Machkovech HM, et al. Positive selection in CD8+ T-Cell epitopes of influenza virus nucleoprotein revealed by a comparative analysis of human and swine viral lineages. J. Virol. 2015; 89(22):11275–11283. [PubMed: 26311880]
31. Karkishchenko, N.; Grachev, S., editors. Manual for laboratory animals and alternative models in biomedical research [Russian]. Moscow, Russia: Profile-2S; 2010. p. 358
32. Moskophidis D, Kioussis D. Contribution of virus-specific CD8+ cytotoxic T cells to virus clearance or pathologic manifestations of influenza virus infection in a T cell receptor transgenic mouse model. J. Exp. Med. 1998; 188(2):223–232. [PubMed: 9670035]
33. Rimmelzwaan GF, et al. Influenza virus CTL epitopes, remarkably conserved and remarkably variable. Vaccine. 2009; 27(45):6363–6365. [PubMed: 19840674]
34. Rudenko L, et al. H7N9 live attenuated influenza vaccine in healthy adults: a randomised, double-blind, placebo-controlled, phase 1 trial. Lancet Infect. Dis. 2016; 16(3):303–310. [PubMed: 26673391]
35. Saha S, et al. A fused gene of nucleoprotein (NP) and herpes simplex virus genes (VP22) induces highly protective immunity against different subtypes of influenza virus. Virology. 2006; 354(1):48–57. [PubMed: 16945400]
36. Tamura S, Tanimoto T, Kurata T. Mechanisms of broad cross-protection provided by influenza virus infection and their application to vaccines. Jpn J. Infect. Dis. 2005; 58(4):195–207. [PubMed: 16116250]
37. Thomas PG, et al. Cell-mediated protection in influenza infection. Emerg. Infect. Dis. 2006; 12(1):48–54. [PubMed: 16494717]

38. Valkenburg SA, et al. Acute emergence and reversion of influenza A virus quasispecies within CD8+ T cell antigenic peptides. Nat. Commun. 2013; 4:2663. [PubMed: 24173108]
39. Wang Z, et al. Establishment of functional influenza virus-specific CD8(+) T cell memory pools after intramuscular immunization. Vaccine. 2015; 33(39):5148–5154. [PubMed: 26277069]
40. Webby RJ, et al. Protection and compensation in the influenza virus-specific CD8+T cell response. Proc. Natl. Acad. Sci. USA. 2003; 100(12):7235–7240. [PubMed: 12775762]
41. Young AC, et al. The three-dimensional structure of H-2Db at 2.4 A resolution: implications for antigen-determinant selection. Cell. 1994; 76(1):39–50. [PubMed: 7506996]
42. Zhou B, et al. Engineering temperature sensitive live attenuated influenza vaccines from emerging viruses. Vaccine. 2012; 30(24):3691–3702. [PubMed: 22449422]

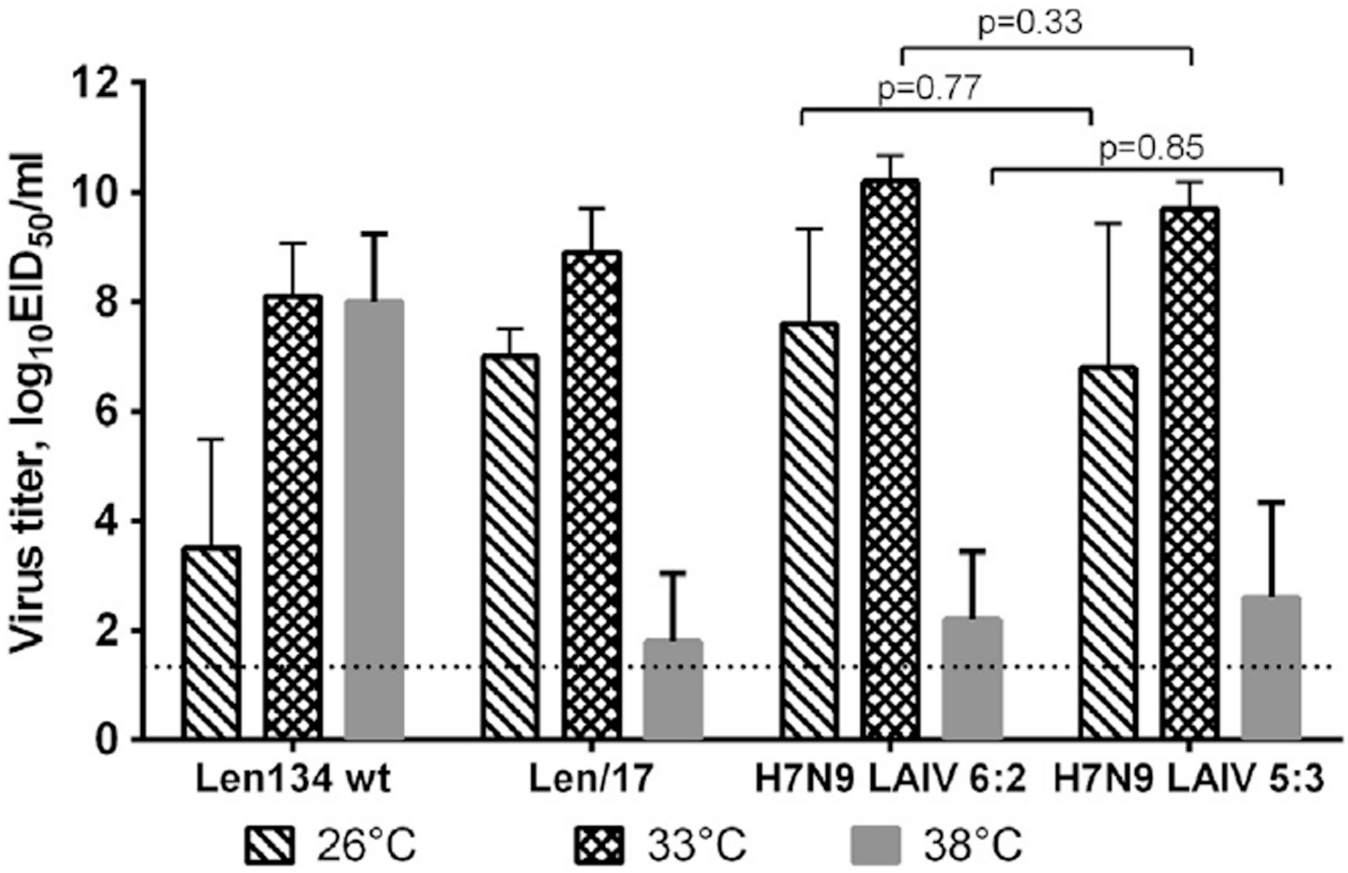

Fig. 1.

Replication of H7N9 LAIV influenza viruses and the control wild-type and cold-adapted H2N2 viruses in eggs at different incubation temperatures. Viruses stocks propagated in eggs at the permissive temperature (33 °C) were titrated by end-point dilutions at the permissive or non-permissive temperatures (26 °C and 38 °C). The bars represent virus titers at indicated temperature ± standard deviations (SD) (T lines). P values are shown for the two H7N9 LAIVs being compared in this study (Mann-Whitney *U* test). Dotted line indicates the assay limit of detection (1.2 $\log_{10}EID_{50}$/ml).

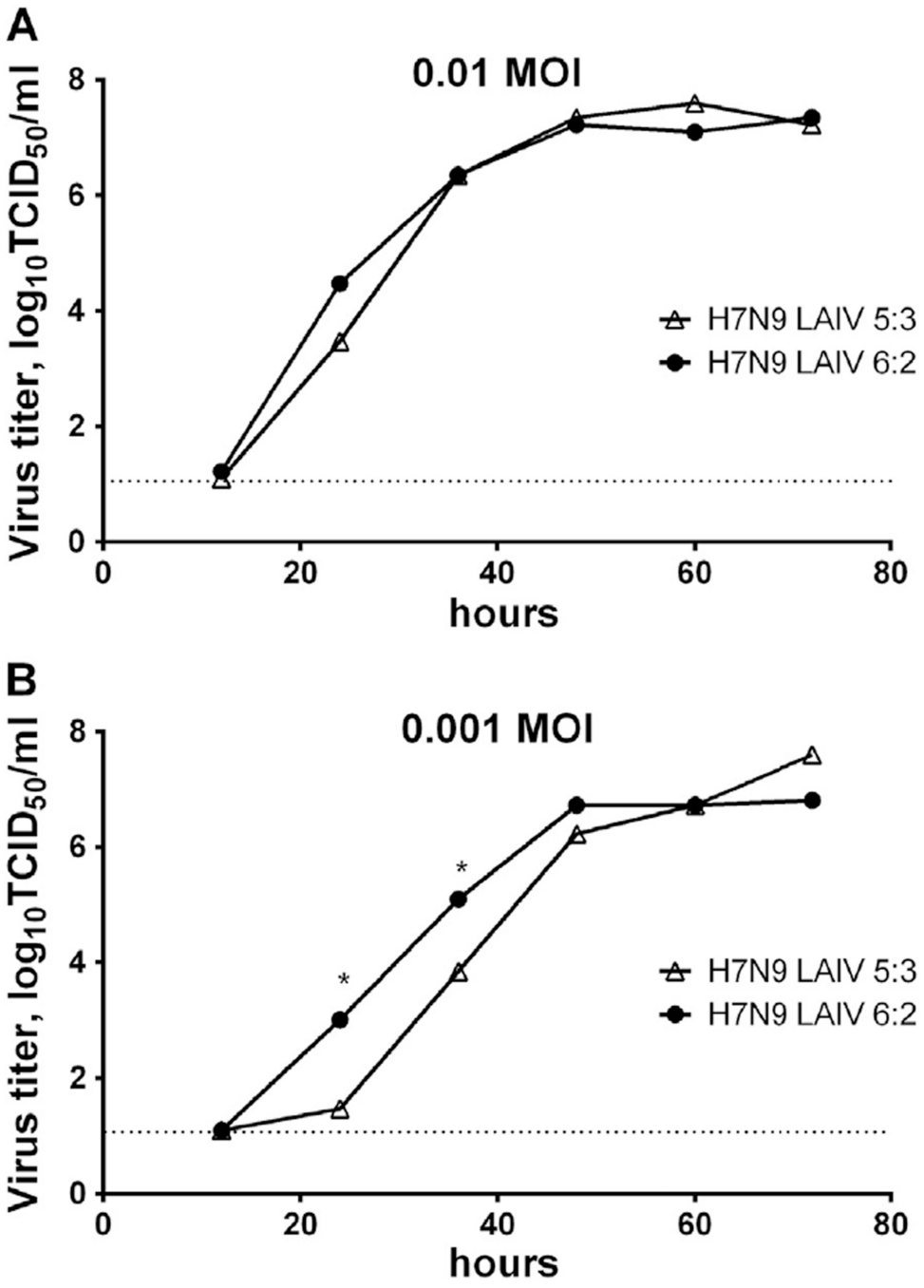

Fig. 2.

Replication of H7N9 LAIV 6:2 and 5:3 reassortant viruses in MDCK cells. Cell monolayers were infected with studied viruses at a multiplicity of infection (MOI) of 0.01 and 0.001 in triplicates and incubated at the permissive temperature (33 °C). Culture supernatants were collected every 12 h and stored at −70 °C prior to titration by 50% tissue culture infective dose (TCID50). Statistically significant differences between studied viruses are indicated with an asterisk ($p < 0.05$, Mann-Whitney *U* test). Dotted line indicates the assay limit of detection (1.1 $\log_{10}TCID_{50}$/ml).

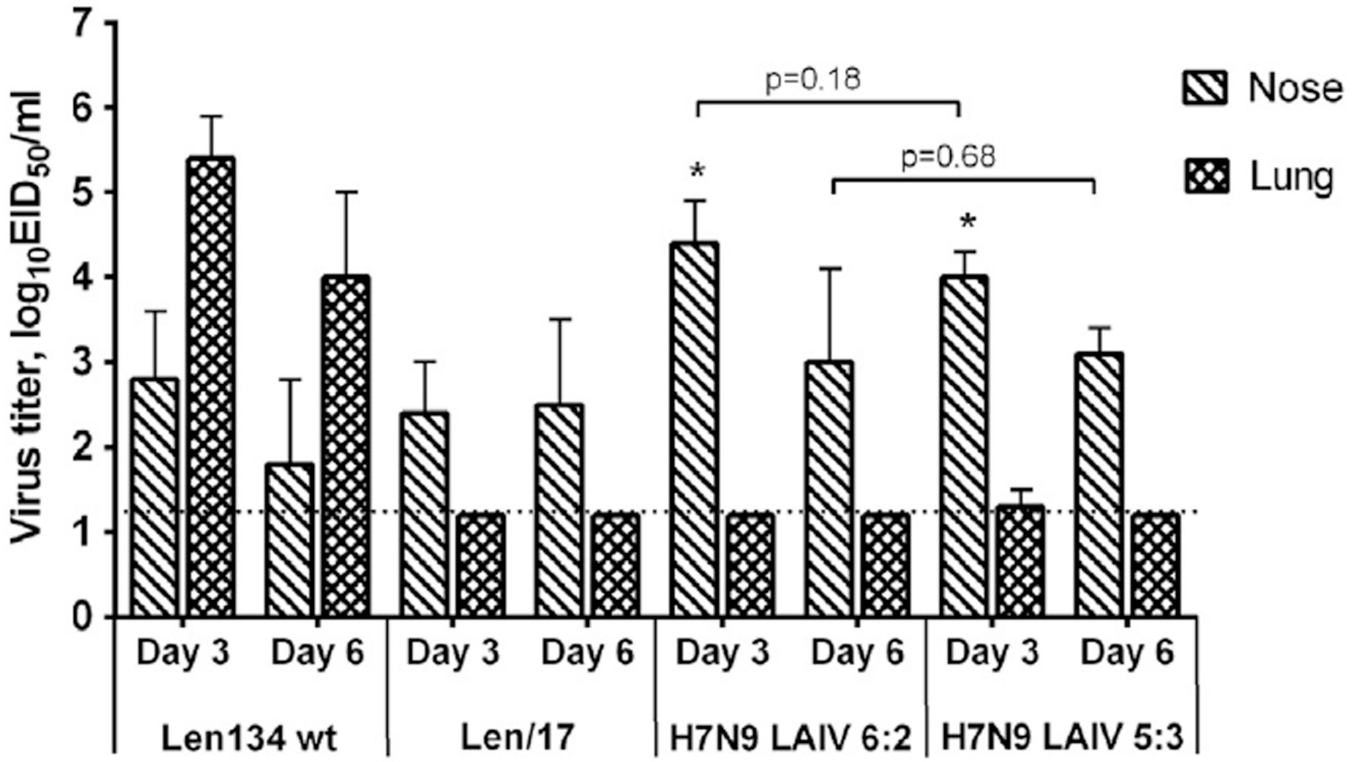

Fig. 3.

Replication of H7N9 LAIV influenza viruses and the control wild-type and cold-adapted H2N2 viruses in respiratory organs of C57BL6 mice. Groups of 8 mice were inoculated i.n. with 10^6 EID_{50} of each virus; four mice from each group were euthanized on either day 3 or day 6 p.i.. Mouse respiratory tissues were collected and homogenized, and viral titers were determined by end-point titration in eggs. The virus titers are expressed as the mean $log_{10}EID_{50}$/ml ± SD (T lines). The limit of virus detection was 1.2 $log_{10}EID_{50}$/ml, indicated by a dotted line. Asterisks indicate significant differences in titers of H7N9 LAIV viruses compared to Len/17 MDV ($p < 0.05$, Mann-Whitney *U* test).

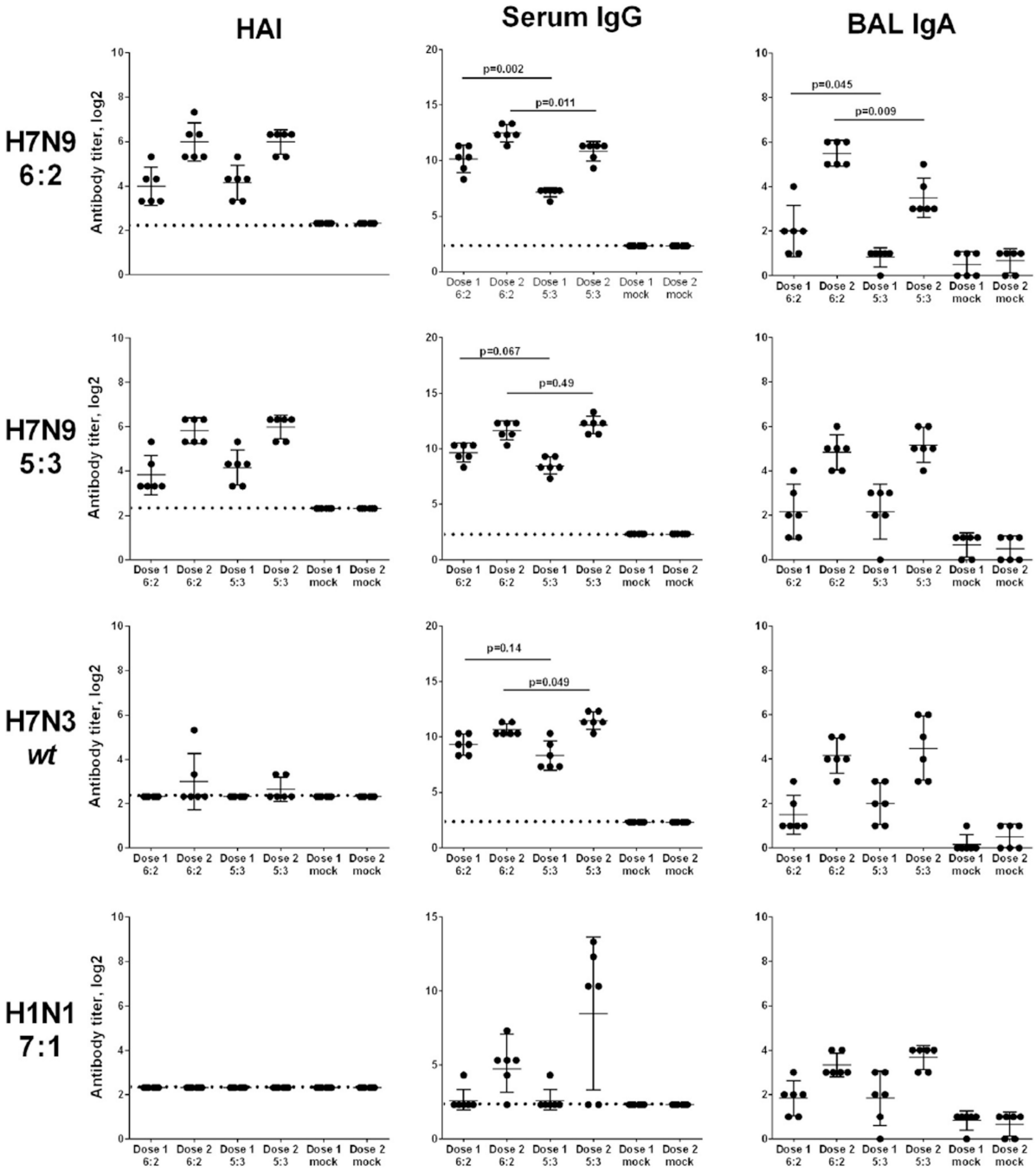

Fig. 4.

Antibody immune responses in mice immunized with H7N9 LAIV 6:2 and 5:3 reassortants. Groups of 12 mice were inoculated i.n. with two doses of 10^6 EID_{50} of each LAIV virus 21 days apart or mock-vaccinated. Mice sera and bronchoalveolar lavage (BAL) were collected from 6 mice in each group 21 days after the first dose and 21 days after the second dose. HI and ELISA tests were performed using egg-grown whole viruses as antigens (left panel indicates the antigens used in HAI and ELISA tests). Bars represent geometric mean with 95% confidence interval calculated from log_2-transformed HI and ELISA titers. Statistical

significance of differences between the vaccine groups was estimated by the Mann-Whitney test. Dotted line indicates the assay limit of detection.

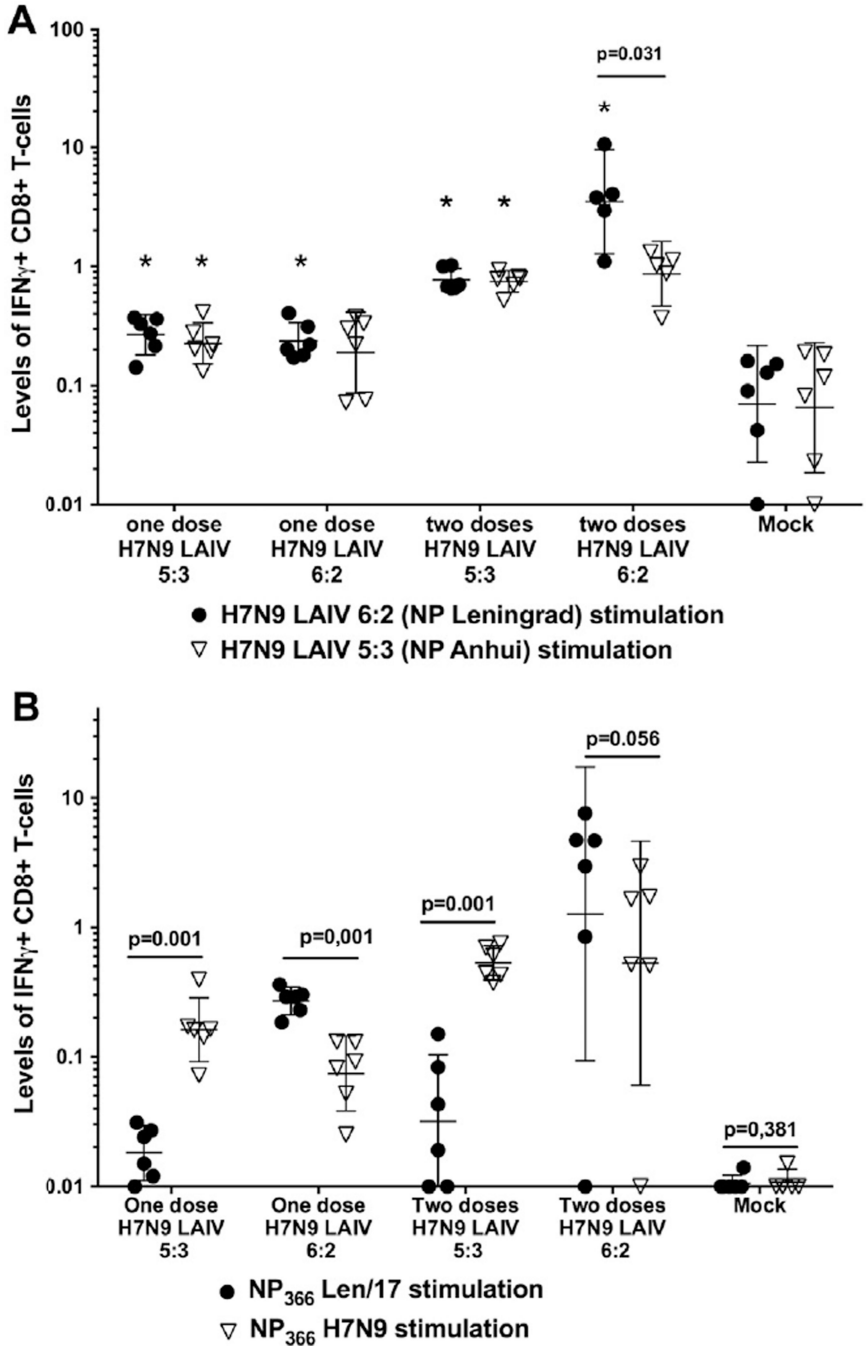

Fig. 5.

A. CTL immune responses in mice immunized with H7N9 LAIV 6:2 and 5:3 reassortants. Groups of 12 mice were inoculated i.n. with two doses of 10^6 EID_{50} of each LAIV virus 21 days apart or mock-vaccinated. Mice splenocytes were collected from 6 mice in each group 7 days after the first dose and 7 days after the second dose. Levels of IFNγ-secreting CD8+ T-cells were determined after whole-virus stimulation (**A**) or after stimulation with NP_{366} peptide (**B**). Bars represent geometric mean with 95% confidence interval. Statistical significance of differences was estimated by the Mann-Whitney *U* test. Significant

differences between immunized and control groups are marked by asterisks ($p < 0.01$). The exact p-value is shown for in-group differences.

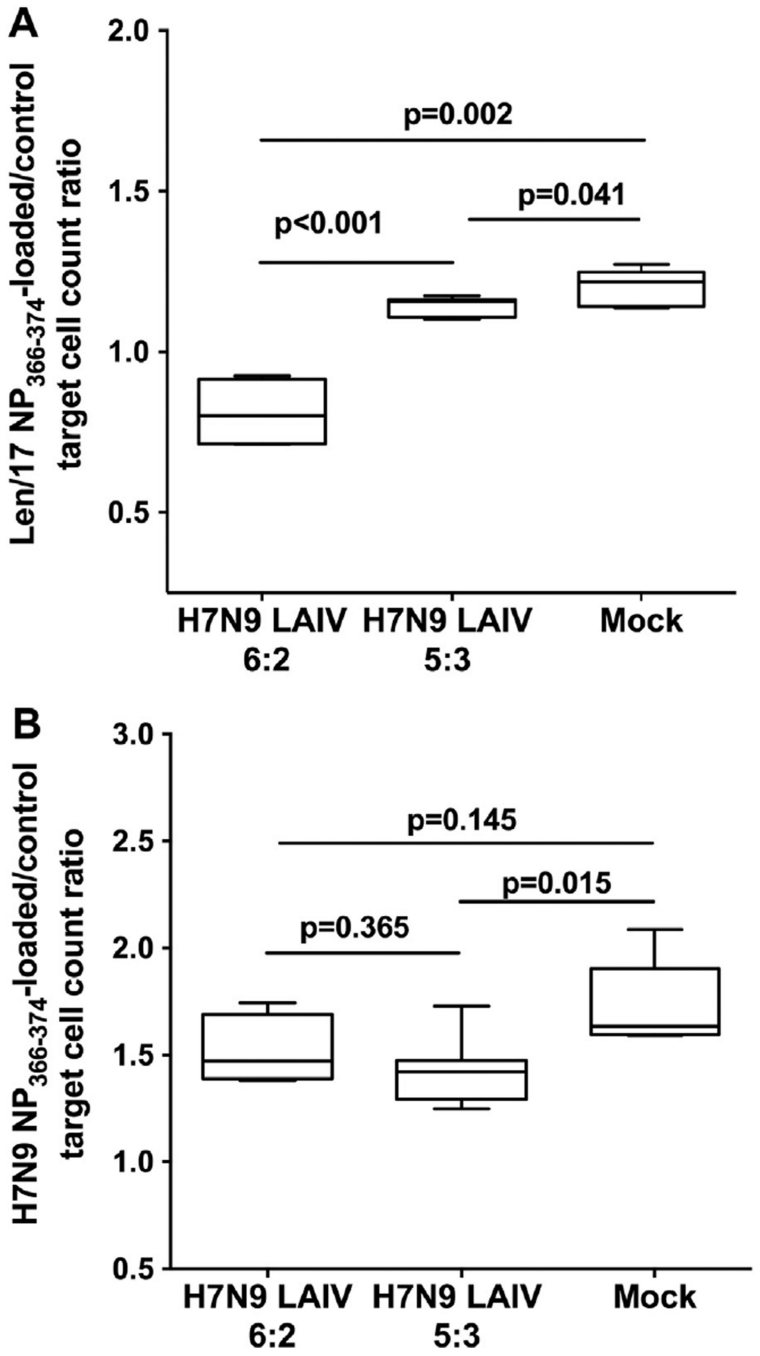

Fig. 6.

In vivo cytotoxic activity of CTLs induced by 6:2 and 5:3 H7N9 LAIVs. Groups of 6 mice were inoculated i.n. with two doses of 10^6 EID_{50} of each LAIV virus 21 days apart or mock-vaccinated. Splenocytes from naive C57BL/6 mice were loaded with either Len/17 (**A**) or H7N9 (**B**) $NP_{366–374}$ peptides and adoptively transferred by retro-orbital injection to vaccinated mice on day 7 after the second dose. The next day, specific cytotoxicity was measured and represented as the ratio of the count of peptide-loaded target cells to that of

control target cells. Bars represent medians and minimum to maximum intervals. Statistical significance of differences was estimated by Mann-Whitney *U* test.

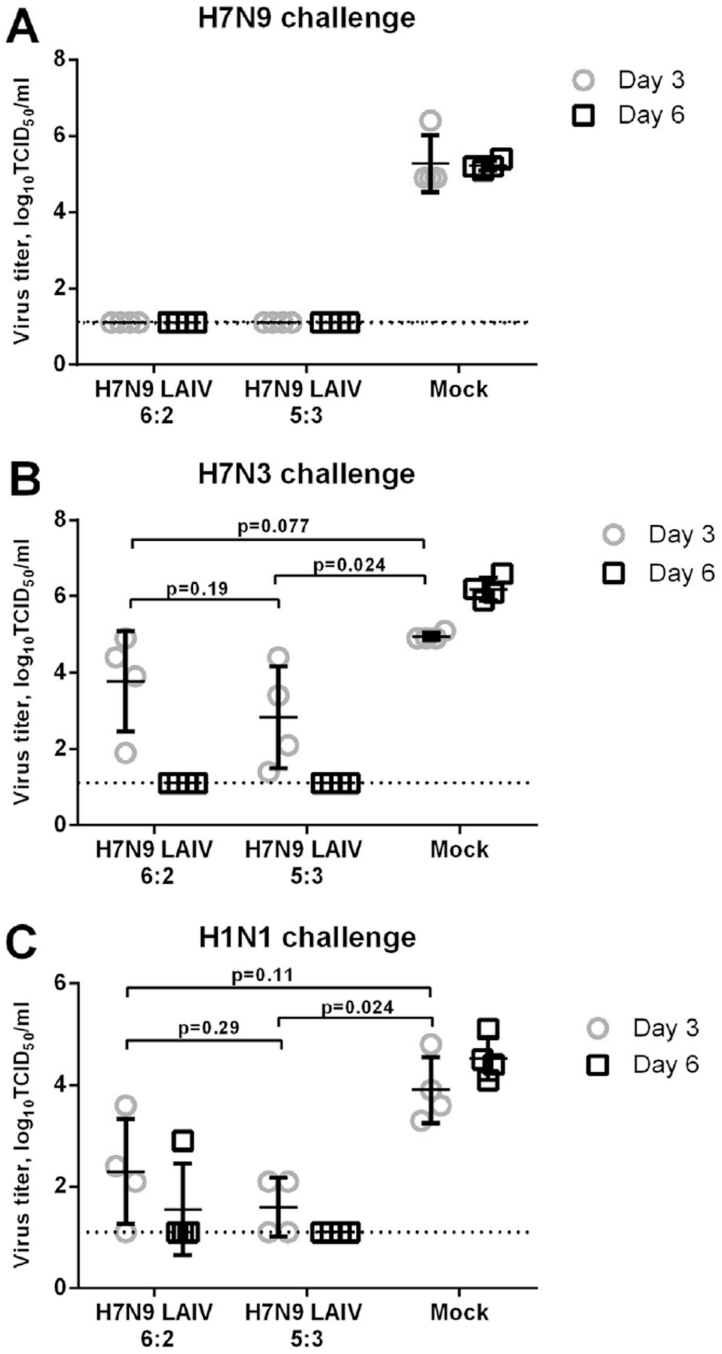

Fig. 7.

Infectious viral titers in immunized mice challenged with homologous and heterologous viruses. Groups of 24 mice were inoculated i.n. with two doses of 10^6 EID_{50} of each LAIV virus 21 days apart or mock-vaccinated. Three weeks after second vaccination 8 mice from each group were challenged with either H7N9-PR8 5:3 reassortant virus (**A**) or H7N3 wt.virus (**B**) or H1N1 7:1 reassortant virus (**C**). Three and six days post challenge lungs were collected from 4 mice from each group, and viral titers in homogenized tissues were determined by 50% tissue culture infective dose ($TCID_{50}$).. The virus titers are expressed as

the mean $\log_{10}TCID_{50}$/ml ± SD (T lines). The limit of virus detection was 1.1 $\log_{10}TCID_{50}$ml, indicated by a dotted line. Tissues in which no virus was detected were given a value of 1.1 $\log_{10}$ to calculate the mean titer. *P* values are calculated by Mann-Whitney *U* test.

Table 1

Conservancy of the nucleoprotein CTL epitopes of MDV A/Leningrad/134/17/57 (H2N2) in influenza A viruses of H1N1 and H3N2 subtypes circulating in 2009–14.

Human CTL epitopes of the influenza virus A/Leningrad/134/17/57 (H2N2) nucleoprotein				CTL epitope conservancy (%)[a]	Predicted T-cell immunogenicity score[b]
No. of epitope	Position, amino acid	HLA supertype restriction	Sequence		
84, 136	198–206	A24, B27	KRGINDRNF	98.6%	0.20
177	199–207	B58	RGINDRNFW	98.6%	0.17
31	200–208	A3	GINDRNFWR	98.4%	0.29
141	174–182	B27	RRSGAAGAA	98.4%	0.11
38	317–325	A3	RPNENPAHK	97.9%	0.13
138, 142	245–253	B27, B39	SRNPGNAEI	97.8%	0.11
52	66–74	A3	MVLSAFDER	97.4%	0.06
11	250–258	A1	NAEIEDLIF	92.9%	0.35
167	114–122	B44	EEIRRIWRQ	33.0%	0.49
50, 165	23–31	A3, B44	TEIRASVGK	27.7%	0.03
100	439–447	A26	DMRAEIIRM	27.6%	0.42
51	438–446	A3	SDMRAEIIR	27.6%	0.32
49	30–38	A3	GKMIDGIGR	27.0%	0.26
119	276–284	B8	LPACVYGPA	5.8%	0.02
159	17–25	B44	GERQNATEI	4.9%	0.02
145	125–133	B39	NGDDATAGL	4.4%	0.16
89, 206	211–219	A26, B62	NGRKTRIAY	4.2%	0.02
134	213–221	B27	RKTRIAYER	4.0%	0.29

[a] Conservancy in 757 unique NP sequences estimated with sequence identity threshold equal to 100% in IEDB.

[b] Estimated by T cell class I pMHC immunogenicity predictor algorithm in IEDB. Epitopes with a score above zero were assumed to be immunogenic.