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Passive immunization with a recombinant adenovirus expressing an HA (H5)-specific single-domain antibody protects mice from lethal influenza infection

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ABSTRACT

One effective method for the prevention and treatment of influenza infection is passive immunization. In our study, we examined the feasibility of creating an antibody-based preparation with a prolonged protective effect against influenza virus. Single-domain antibodies (sdAbs) specific for influenza virus hemagglutinin were generated. Experiments in mouse models showed 100% survivability for both intranasal sdAbs administration 24 h prior to influenza challenge and 24 h after infection. sdAb-gene delivery by an adenoviral vector led to gene expression for up to 14 days. Protection by a recombinant adenovirus containing the sdAb gene was observed in cases of administration prior to influenza infection (14 d–24 h). We also demonstrated that the single administration of a combined preparation containing sdAb DNA and protein expanded the protection time window from 14 d prior to 48 h after influenza infection. This approach and the application of a broad-spectrum sdAbs will allow the development of efficient drugs for the prevention and treatment of viral infections produced by pandemic virus variants and other infections.

1. Introduction

Influenza type A virus poses a serious threat to public health and causes repeated seasonal epidemics with increased morbidity and significant economic loss. At present, there are several methods for the prevention and treatment of influenza (www.hpa.org.uk/webc/HPAwebFile/HPAweb_C/1287147812045: Health Protection Agency, 2011; Mossad, 2009). Active immunization (vaccination) is the most efficient way to protect people against this infection (Mossad, 2009), but there are some potentially severe complications that pose certain restrictions, as well as a number of conditions where administration of influenza vaccines is not allowed (Musana et al., 2004).

Passive immunization is another method for the prevention and treatment of influenza infection (Ye et al., 2012; Luke et al., 2010). Hyperimmune sera with a high titer of virus-neutralizing antibodies against a defined influenza strain obtained from immunized animals or from patients who have been subject to infection previously have been used to prevent influenza infection in recipient patients (McGuire and Redden, 1918). Currently, drugs based on hyperimmune sera are being gradually replaced by monoclonal antibodies (mAb) because of their low immunogenicity and high level of neutralizing activity and specificity (Kohler and Milstein, 1975). However, creating a low cost scheme for pharmaceutical manufacturing mAb products is difficult due to their complex structure, which puts them at a disadvantage compared with classical drugs (Shukla and Thömmes, 2010; Peterson et al., 2006). Therefore, various strategies to generate mAb fragments (Fab, scFv, VH, VL) have become more widespread (Peterson et al., 2006).

Natural serum of camelids and sharks contains smaller Abs. They lack the first constant domain of the heavy-chain (CH1), as well as the whole light chain, so they represent single-domain antibodies (sdAbs) (Hamers-Casterman et al., 1993; Vanlandschoot et al., 2011; Arbabi Ghahroudi et al., 1997; Harmsen and De Haard,

Abbreviations: Ab, antibody; mAb, monoclonal antibody; sdAb, single-domain antibody; rAd, recombinant adenovirus; Ad5, human adenovirus serotype 5; IAV, influenza A virus; HA, influenza virus hemagglutinin; HA1, membrane-distal globular domain of hemagglutinin; aHAsdAb, anti-hemagglutinin formatted sdAb; psdAb, prokaryotically (*E. coli*) expressed aHAsdAb; esdAb, eukaryotically (adenoviral vector) expressed aHAsdAb; ILZ, isoleucine zipper; VNA, virus-neutralization assay; HIA, hemagglutination inhibition assay; WB, Western blotting; BALF, bronchoalveolar lavage fluid; NS, nasal swab; Cyk8, cytokeratin 8; PEG, polyethylene glycol; scFv, single-chain variable fragment; VHH, variable fragment of a camelid antibody.

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2007; Wesolowski et al., 2009; Muyldermans et al., 2009; Ghassabeh et al., 2010; Tillib, 2011). It has been shown that these camel antigen-binding domains can be "humanized" without significant loss of their specific activity via a few amino acid substitutions (Vincke et al., 2009). The advantages of sdAbs include their smaller size, good solubility, novel structural features (i.e., better penetration into tissues and the ability to recognize epitopes inaccessible to conventional antibodies), simple genetic engineering and relatively economical production (Hamers-Casterman et al., 1993; Greenberg et al., 1996; Nuttall et al., 2001; Harmsen and De Haard, 2007; Wesolowski et al., 2009; Muyldermans et al., 2009; Ghassabeh et al., 2010; Tillib, 2011). Because the coding sequences for sdAbs are known, it is possible to produce the corresponding functionally active protein in different expression systems (prokaryotic and eukaryotic) (Harmsen and De Haard, 2007; Bazl et al., 2007). However, it should be noted that sdAbs have short half-lives *in vivo* (Harmsen et al., 2005). A promising solution is to elicit a prolonged expression of the sdAb gene in a recipient organism.

One of the most efficient methods for delivering genetic material (including genes of Abs) to target cells is viral vectors (Campana et al., 2009; Zuber et al., 2008). Adenoviral vectors are among the most commonly used tools used for delivery and expression of recombinant genes in mammalian cells (Wilson, 1996). It is known that recombinant adenoviruses are capable of efficiently transferring the genes of bacterial and viral antigens, cytokines, growth factors, and other proteins to target cells, ensuring a high level and duration of target gene expression (Lasaro and Ertl, 2009; Tani et al., 2011; Kesser et al., 2008; Kita et al., 2004). Furthermore, these vectors are safe, which has been confirmed by more than 150 clinical trials (Shirakawa, 2009).

This paper describes a novel strategy for the prevention of influenza infection through the use of a recombinant adenovirus (rAd) expressing a neutralizing camel sdAb against hemagglutinin (HA). We demonstrated that combined administration of sdAb and rAd expressing a sdAb specifically prevented lethal mouse infection for a prolonged period of time (14 d) and expanded the protection time window from 14 d prior to 2 d after influenza infection.

2. Materials and methods

2.1. Reagents

The following reagents were used in this work: Ad-Easy vector system (Stratagene, USA); bovine serum albumin (BSA), monoclonal anti-HA (aHA) antibody produced in mouse (Sigma–Aldrich, USA); recombinant influenza A virus H5N2 HA1, influenza A virus H5N2 HA/Hemagglutinin (Sino Biological Inc.) and HRP-labeled sheep anti-mouse IgG antibody (Amersham Bioscience, USA). A sdAb against mouse cytokeratin 8 (aCyk8sdAb) were obtained in our previous work (Gribova et al., 2011) and used here as a negative control.

2.2. Cell culture

The human embryonic kidney-293 (HEK-293), Madin–Darby canine kidney (MDCK) and H1299 cell lines were used in the experiments. Cells were propagated in DMEM supplemented with 10% fetal bovine serum (HyClone), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine at 37 °C in a humidified atmosphere with 5% CO_2.

2.3. Production of the cDNA clone encoding the sdAb for the specific recognition of influenza virus

The sdAb antibody capable of specifically recognizing A/Mallard duck/Pennsylvania/10218/84 (H5N2) IAV was obtained from the Tillib S.V. research group (Institute of Gene Biology, Moscow). The first stage of its generation included immunization of the Bactrian camel (*Camelus bactrianus*) with purified inactivated A/Mallard duck/Pennsylvania/10218/84 IAV (H5N2). The protein concentration of the purified virus was determined by a Bradford protein assay kit (Bio-Rad). The camel was immunized 5 times subcutaneously with 2.5 mg of virus preparation mixed with equal amounts of Freund adjuvant (complete only for the first injection). The second injection was made 3 weeks after the first and the remaining three injections were given in 10-day intervals. Blood samples (150 ml) were collected 5 d after the last injection. PBS (50 ml), heparin (100 U/ml) and EDTA (3 mM) were added to prevent clotting.

A nucleotide sequence library of sdAbs was constructed and sdAbs were selected by phage display as described (Tillib et al., 2010). Phages carrying the IAV-specific nanobodies were selected by 2 and 3 cycles of panning on the immobilized virus. Individual sequence variants of enriched nanobodies were identified by the fingerprinting-like PCR/restriction analysis ('HMR analysis') as described (Tillib et al., 2010; Arbabi Ghahroudi et al., 1997; Nguyen et al., 2001; ElsConrath et al., 2001; Saerens et al., 2004). The clones that showed high absorbance values reflecting specific binding to the immobilized IAV were considered positives. As a result, 16 different sdAbs were selected. To obtain high avidity sdAbs, initially selected sdAb sequences were modified (formatted). A special coiled-coil sequence (an isoleucine zipper domain, or ILZ (Harbury et al., 1993; Shiraishi et al., 2004), separated by a camel hinge-originated spacer sequence from the sdAb sequence was attached to the sdAb C-terminus (Tillib et al., 2010). Presumably, the ILZ domain containing peptides adopted a trimeric parallel (Harbury et al., 1993; Sorger and Nelson, 1989) conformation, which could lead to significantly increased biological activity for these peptides (Sorger and Nelson, 1989; Shiraishi et al., 2004). Two other peptidic tag sequences (HA-tag and (His)6-tag, HH) were also added to the C-terminal extremity of the formatted recombinant antibody to improve detection and purification.

cDNA sequences of selected sdAbs were subcloned (by conventional or PCR cloning) into the pHEN4 (expression plasmid (ElsConrath et al., 2001)) together with the pelB leader sequence (for periplasmic production), camel upper hinge (the longest hinge variant) and ILZ domain (Harbury et al., 1993) sequences (to obtain formatted sdAb–fsdAb), and two short tag sequences (HA-tag and (His)6-tag) at the C-terminus coding region. The plasmids were transformed into *Escherichia coli* BL21 (New England BioLabs) for bacterial expression and purification. Protein expression was induced by the addition of 1 mM isopropyl-d-1-thiogalactoside (IPTG). After 5–7 h induction at 37 °C, cells were harvested by centrifugation and the formatted sdAbs were purified from the periplasmic extract. The sdAbs were purified using Ni–NTA agarose and the QIAExpressionist purification system (QIAGEN, USA). All the purified antibodies were maintained in 1× PBS. The proteins were then sequentially concentrated (to a concentration of approximately 5 mg/ml) in Amicon 10 kDa ultrafiltration devices (Millipore, Billerica, MA), affinity purified from endotoxin using 'Detoxi-Gel Endotoxin Removing Gel' (Thermo Scientific), and sterilized by filtration using a cellulose acetate membrane filter (0.2 μm) (Nalgene Co.). Purified formatted sdAbs were stored in aliquots at 4 °C or, after addition of 50% glycerol, at −20 °C.

Degree of purity was evaluated by SDS–PAGE analysis (data not shown) and protein concentrations were determined spectrophotometrically using a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

All 16 formatted sdAbs were assessed by ELISA (binding to the immobilized IAV), erythrocyte hemagglutination inhibition (HI) and virus neutralization (VN) *in vitro* assays. The results showed that sdAbs were able to efficiently bind IAV and H5 hemagglutinin,

inhibit erythrocyte hemagglutination and neutralize IAV *in vitro*. One formatted sdAb (aHA-7), named paHAsdAb7, showed the best activity in all of our *in vitro* tests (highest HIA and VNA titers, virus neutralization at the lowest tested concentration of 100 ng/ml). Therefore, IAV neutralizing paHAsdAb7 was generated and used for construction of rAd.

2.4. Generation of recombinant adenovirus Ad5-aHAsdAb

The plasmids and the recombinant human adenovirus of serotype 5 were obtained using the aHAsdAb7 gene. The nucleotide sequence encoding the aHAsdAb7 was obtained by chemical synthesis in "Evrogen" JSC. The AdEasy Adenoviral Vector System was used to construct the Ad5-aHAsdAb according to the manufacturer's instructions. The rAd with the E1 region replaced with a transgene-free expression cassette (Ad-null) was used as a control. rAds were grown in HEK-293 and purified by double cesium chloride gradient centrifugation. The titers of Ad5-aHAsdAb and Ad5-null (2×10^{10} PFU/ml and 9×10^{9} pfu/ml, respectively) were determined by the plaque formation technique in the HEK-293 cell culture.

2.5. Western blotting (WB)

For western blotting, recombinant influenza A virus H5N2 HA1 and influenza A virus H5N2 hemagglutinin were subjected to 12% SDS–PAGE and electrotransferred to PVDF membranes (Millipore, USA). Western blotting was performed using standard methods and 10 μg/ml of aHAsdAb as the primary, 1:5000 dilution of a monoclonal anti-HA antibody as the secondary and a 1:5000 dilution of HRP-labeled sheep anti-mouse IgG antibody as the tertiary antibody. Culture media from H1299 cells infected with Ad5-aHAsdAb was also used as a primary antibody.

To obtain culture media containing aHAsdAb expressed by Ad5-aHAsdAb, the H1299 cell line was used. The cells were seeded to ~70% of the monolayer, cultivated for 24 h, and infected with the recombinant adenovirus at 100 PFU/cell. Two hours after infection, the medium was collected, the cell culture was washed, and fresh DMEM medium was added. The medium from the infected cells was collected 72 h after infection and concentrated by centrifuge ultrafiltration through a membrane with a nominally intercepted molecular weight of 10 kDa. Following thickening by a factor of 10, the supernatant was fractioned in a 10% polyamide gel and used for immune blotting analysis.

2.6. ELISA

10 mg/ml A/Mallard duck/Pennsylvania/10218/84 virus was passively adsorbed onto 96-well plates in 100 ml PBS/well overnight at 4 °C. The virus-coated plates were blocked with PBS containing 4% BSA for 1 h at 37 °C. After washing with PBST (PBS with 0.1% Tween), 50 μl of aHAsdAbs and 50 μl blocking agent (PBS with 4% BSA) were added to each well and incubated for 1 h. Binding of aHAsdAbs was detected with HRP-conjugated mouse anti-HA antibody (Sigma–Aldrich), visualized with TMB substrate (Thermo-Fisher), and quenched with 1 M H_2SO_4. The plates were read at 450 nm.

For coating the plates we also used: BSA (1 mg/ml), hemagglutinin (1 mg/ml) and HA1 (1 mg/ml) of IAV A/American green-winged teal/California/HKWF609/07 (H5N2).

2.7. Hemagglutination inhibition assay (HIA)

Tests were carried out in U-shaped microtiter plates as described (Smirnov et al., 2000). The samples (BALFs, NS, sdAbs or culture media from Ad5-aHAsdAb infected cells) were first mixed with chicken erythrocytes to absorb non-specific hemagglutinins. HI titration was then carried out in a microtiter plate against 4 HA units of the virus, 1% chicken erythrocytes and a phosphate saline (PBS, 0.01 M phosphate, 0.14 M NaCl, pH 7.2) as diluent. The results were read after the incubation at 4 °C for 60 min, whereby the titer was defined as the reciprocal value of the last serum dilution where agglutination inhibition was clearly visible.

2.8. Influenza virus

This study used an avian IAV A/Mallard duck/Pennsylvania/10218/84 (H5N2) adapted for use in mice (Smirnov et al., 2000). The virus was cultured in the allantoic fluid of chicken embryos at 37°C for 48 h. Chorioallantoic fluid derived from an embryonated eggs infected with an attenuated IAV was stored at −70 °C or subjected to ultracentrifugation at 27000 rpm at 4 °C for 1.5 h. The viral pellet was dissolved in PBS, and the virus-containing fraction was recovered by sucrose gradient centrifugation. Ultracentrifugation was then performed to remove the sucrose and obtain a purified virus solution. The titer of the virus was calculated by titration in MDCK cells using the Reed and Muench method (Reed and Muench, 1938). The 50% lethal dosage (LD_{50}) was calculated by titration in mice.

Purified IAV was inactivated with glutaric aldehyde to be used as an antigen for immunization and ELISA. The inactivation was performed by adding glutaric aldehyde to the purified virus solution to a final concentration of 0.1% and allowing the resulting mixture to equilibrate at 4 °C for 1 week.

2.9. Size exclusion chromatography

Fast protein liquid chromatography (FPLC) was performed using a Superose 12 HR 10/30 column (GE Healthcare) at a constant flow rate of 0.3 ml/min with PBS as the eluent. Cell culture media containing eaHAsdAb expressed by Ad5-aHAsdAb was prepared as described above (Section 2.5) with one modification: DMEM was changed by 293 SFMII (Invitrogen). Culture supernatants were clarified by differential centrifugation, purified by immunoprecipitation (Anti-HA Immunoprecipitation Kit, Sigma), filtered (pore size 0.22 μm), and 0.5 ml injected onto the column. Fractions (0.2 ml) were collected and analyzed by Western blotting (see above). The molecular weight of sdAbs was estimated by comparison of the elution volumes with those of standard compounds of known molecular weight (RNAse, 14 kDa; ovalbumin 43 kDa;) monitored by absorbance at 280 nm.

2.10. Challenge infection and mucosal sampling in mice

Six-week old female Balb/c mice were obtained from the Pushchino Branch of the Institute of Bioorganic Chemistry, RAS (Pushchino, Russia). The mice had free access to water and standard rodent chow and were housed in pathogen-free cages. Intranasal (i.n.) inoculations (rAds and sdAbs) and challenge infections were performed as described (Lo et al., 2008).

Mice with body weights between 18 and 20 g were injected intranasally with 50 $TCID_{50}$ of IAV in a total volume of 0.01 ml. To obtain bronchoalveolar lavage fluid (BALF), the mice were euthanized with xylazine (10–12 mg/kg IP) and ketamine (80–100 mg/kg IP), and the lungs were flushed via a tracheal catheter 3 times using 1 ml PBS. Nasal swabs (NSs) were obtained by injecting PBS though a catheter into the trachea and collecting 200 μl of flow-through from the nares. The samples were centrifuged at 500*g* for 10 min at 4 °C to remove the cell fraction, and the supernatant was stored at −80 °C.

2.11. Virus neutralization assay (VNA)

MDCK cells (2×10^4) were seeded to the wells of a 96-well microtiter plate and incubated at 37 °C in 5% CO_2 overnight. IAV A/Mallard duck/PA/10218/84(H5N2) at dose of 100TCD_{50} was added to each antibody containing sample (BALF, NS, sdAbs, culture media from Ad5-aHAsdAb infected cells). Samples were prepared by serial 2-fold dilution procedure. IAV was incubated with sdAb samples for 30 min at room temperature and then each sample was added to MDCK that had been washed with serum-free DMEM. Cells were incubated for 2 h at 37 °C in 5% CO_2 after which serum-free culture media was changed by DMEM with 5% of calf serum, 0.2% BSA and 1 μg/ml trypsin. Incubation of cultures continued for 3 days, after which neutralizing virus titer was defined.

2.12. Viral lung titer measurement

Mice were euthanized with xylazine (10–12 mg/kg IP) and ketamine (80–100 mg/kg IP). Lungs were harvested on day 4 post-infection and homogenized in sterile Hank's balanced salt solution supplemented with 0.5% lactalbumin hydrolysate (1 ml/lung). Clarified lungs were titrated for virus infectivity (EID_{50}) in 10-day-old embryonated chicken eggs from initial dilution of 1:10. The positive eggs were identified by hemagglutination test of allantoic fluid (Szretter et al., 2006). EID_{50} was calculated by using Reed–Muench method (Reed and Muench, 1938). Values are expressed as log10 EID_{50}/ml. The limit of virus detection is 1.5 $\log_{10}$ EID_{50}/ml.

3. Results

3.1. In vitro characterization of rAd-expressed anti-influenza sdAb

In the first stage of our work, initially selected aHAsdAb sequences were modified (formatted) to obtain high avidity sdAbs and improve detection and purification. A isoleucine zipper domain (ILZ), HA-tag and (His)6-tag were attached to the aHAsdAb C-terminus. Formatted aHAsdAbs were produced in bacterial periplasm (prokaryotic aHAsdAbs, paHAsdAbs). One paHAsdAb (aHA-7 or paHAsdAb7) had the best *in vitro* activity (i.e., highest HIA and VNA titers, virus-neutralization activity in the lowest tested concentration of 100 ng/ml). aHAsdAb7 gene was thus chosen for construction of rAd.

The replication-deficient rAd vector Ad5-aHAsdAb was constructed as described earlier (Gribova et al., 2011; Shmarov et al., 2010; Tutykhina et al., 2009). For the generation of aHAsdAb expressed by rAd (eukaryotic aHAsdAb, eaHAsdAb), hinge, ILZ and HA-tag sequences were included in the Ab gene. To obtain secreted eaHAsdAb, we linked the 5′-end with the nucleotide sequence of the leader peptide of secreted alkaline phosphatase (slp). The schematic for Ad5-aHAsdAb containing the aHAsdAb gene is shown on Fig. 1A.

To quantitate the concentration of eaHAsdAb in the culture media of HEK-293 cells infected with Ad5-aHAsdAb (5 pfu/cell) by ELISA, antibodies against the HA-tag were used. The concentration of eaHAsdAbs was 0.5 ± 0.2 μg/ml (Figs. 1S and 2S). The specific activity of the anti-IAV eaHAsdAbs was evaluated by HIA, VNA and WB *in vitro*. For this purpose, HEK-293 cells were infected

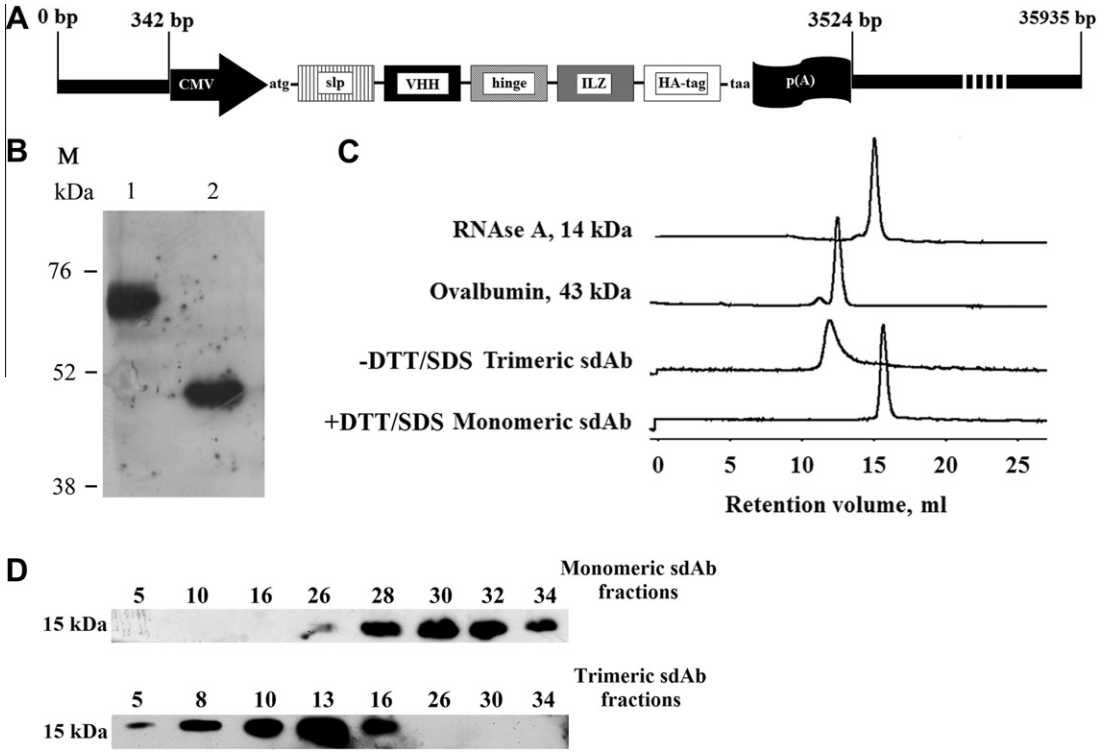

Fig. 1. Adenovirus constructed in this study. (A) The schematic of the Ad5-aHAsdAb vector. CMV – promoter of the E1 region of human cytomegalovirus; slp – leader sequences directing the secretion of the protein (seap leader peptide); VHH – aHAsdAb gene; hinge – camel IgG2 upper hinge; ILZ – coiled-coil isoleucine zipper domain; HA-tag – human influenza hemagglutinin (HA) epitope; p(A) – polyadenylation signal. (B) Western blot assay of aHAsdAb expressed by Ad5-aHAsdAb. The culture media containing eaHAsdAb was used as a primary Ab, while an Ab against the HA-tag was used as a secondary Ab. 1 – IAV hemagglutinin (A/American green-winge teal/California/HKWF609/07 (H5N2)); 2 – HA1 polypeptide of IAV hemagglutinin (A/American green-wing teal/California/HKWF609/07 (H5N2)). (C) Size exclusion chromatography profile of the eaHAsdAb. The monomeric eaHAsdAb was obtained by treatment of Ab preparation with 50 mM dithiothreitol (DTT) and 2% SDS. FPLC fractions of treated and non-treated Ab preparations were collected (from 10 ml to 17 ml of retention volume, $n = 35$ for each samples) and analyzed by WB. (D) WB was performed using standard methods and 1:5000 dilution of a monoclonal anti-HA antibody as the primary and a 1:5000 dilution of HRP-labeled sheep anti mouse IgG antibody as the secondary antibody.

with Ad5-aHAsdAb or Ad5-null (negative control) at 5 pfu/cell. Forty-eight hours after inoculation, cell culture media was collected and VNA and HIA were carried out. The results of the VNA and HIA analyses showed that the eaHAsdAb was capable of efficiently neutralizing IAV and inhibiting erythrocyte hemagglutination. The HIA antibody titer was 1/256–1/512 (negative control – <1/2), and the VNA antibody titer was 320–640 (negative control – <1/10). Based on these results, the eaHAsdAb likely bound the IAV HA1 polypeptide (membrane-distal, globular domain of hemagglutinin). Blocking by Abs then inhibited the viral hemagglutination and neutralized the viral infectivity (Khurana et al., 2011; Staneková and Varečková, 2010).

To confirm our assumption, cell culture media were prepared as described in "Section 2.5", and WB was carried out. We used IAV hemagglutinin (A/American green-winged teal/California/HKWF609/07 (H5N2)) and its HA1 polypeptide (~65 kDa and ~45 kDa, respectively) for analysis. The results of the WB analysis showed that the eaHAsdAbs bound HA1 (Fig. 1B).

To determine whether eaHAsdAb is trimer, we analyzed the culture media from cells infected with Ad5-aHAsdAb by FPLC (Fig. 1C). We used non-treated cell culture media to detect trimeric sdAb and cell culture media treated with DTT and SDS to detect monomeric sdAb. We used markers of known molecular weight (14 kDa and 43 kDa) to collect fractions containing proteins with required MW. As shown in Fig. 1C, the MW of the protein in one peak was about 45 kDa, equivalent to a trimeric form of eaHAsdAb, and that of the second, 15 kDa, was equivalent to monomeric Ab. The presence of eaHAsdAb in each fraction was monitored by WB analysis (Fig. 1D). WB analysis showed bands at 15 kDa in the fractions of two samples, because SDS–PAGE led to a shift towards lower MW (monomeric form).

3.2. In vivo characterization of Ad-expressed aHAsdAbs

To evaluate the expression level and the protective efficacy of the anti-HA1 sdAbs *in vivo*, we administered Ad5-aHAsdAb intranasally to Balb/c mice. Three doses of Ad5-aHAsdAb were tested: 10^8, 10^7 and 10^6 pfu/10 μl in each mouse. Control groups were given the same volume of recombinant adenovirus Ad5-null at a dose of 10^8 pfu/mouse and paHAsdAb7 at 50 μg/mouse (See Fig. 3S – Dose selection experiments). The NSs and BALF samples obtained were analyzed by ELISA, HIA and VNA. eaHAsdAbs that bind IAV was detected by ELISA in all BALF samples collected from mice injected with Ad5-aHAsdAb at a dose of 10^7 and 10^8 pfu/mouse. eaHAsdAbs were also detected in the NSs from the mice given the highest dose of Ad5-aHAsdAb 24–72 h after its administration (Fig. 2A). In the case of the lowest Ad5-aHAsdAb dose, sdAbs were not detected by any assay in either BALF samples or NSs (data not shown). aHAsdAbs were detected by the HIA and VNA analyses only in BALF samples from mice injected with Ad5-aHAsdAb at a dose of 10^8 pfu/mouse and with paHAsdAb7 (Figs. 2B and C).

Experiments were carried out to determine the Ad5-aHAsdAb protective dose in lethal IAV-challenged mice. The mice were divided into groups as described earlier ($N = 10$), and equal doses of Ad5-aHAsdAb, Ad5-null, paHAsdAb7 and NaCl were administered. However, paHAsdAb7s were administered 2 h prior to influenza infection. At 48 h after injections, all groups of mice were intranasally infected with IAV. This time point was chosen because the highest expression level after rAd administration was detected between 48 and 72 h (Fig. 2A). Total mortality was observed only in the group injected with NaCl, while 80% mortality was observed in Ad5-null mice. In the mice given the lowest dose of Ad5-aHAsdAb, five out of ten animals died during the survival study. There were no differences among the mice given paHAsdAb7 and Ad5-

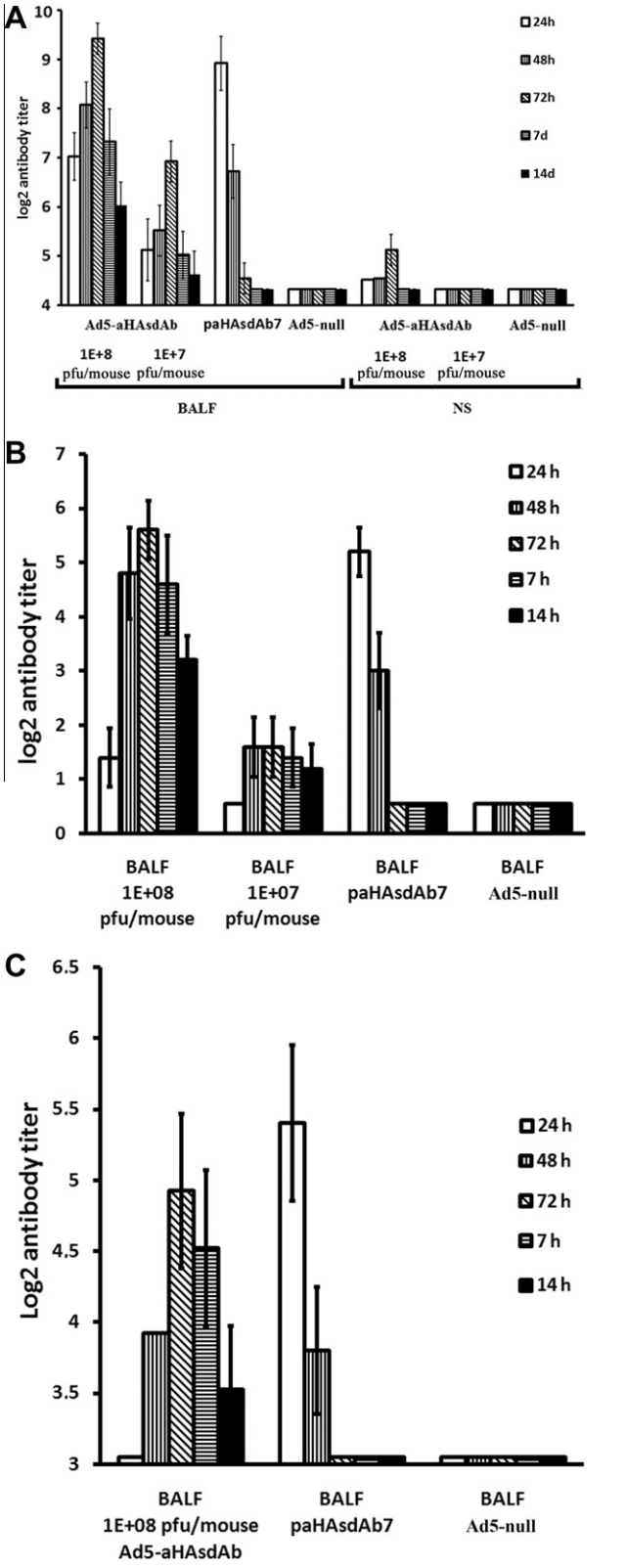

Fig. 2. Expression and activity of recombinant adenovirus-mediated aHAsdAb *in vivo*. Animals were treated with different doses of Ad5-aHAsdAb (high: 1×10^8 pfu/mouse; medium: 1×10^7 pfu/mouse; low: 1×10^6 pfu/mouse), paHAsdAb7 and negative control (Ad5-null at a dose of 10^8 pfu/mouse). BALFs and NSs were performed 24 h, 48 h, 72 h, 7 d and 14 d after viral administration. eaHAsdAb expression in BALF and NS was determined by ELISA (A). eaHAsdAb activity in BALF and NS was detected by HI assay (B) and VN assay (C).

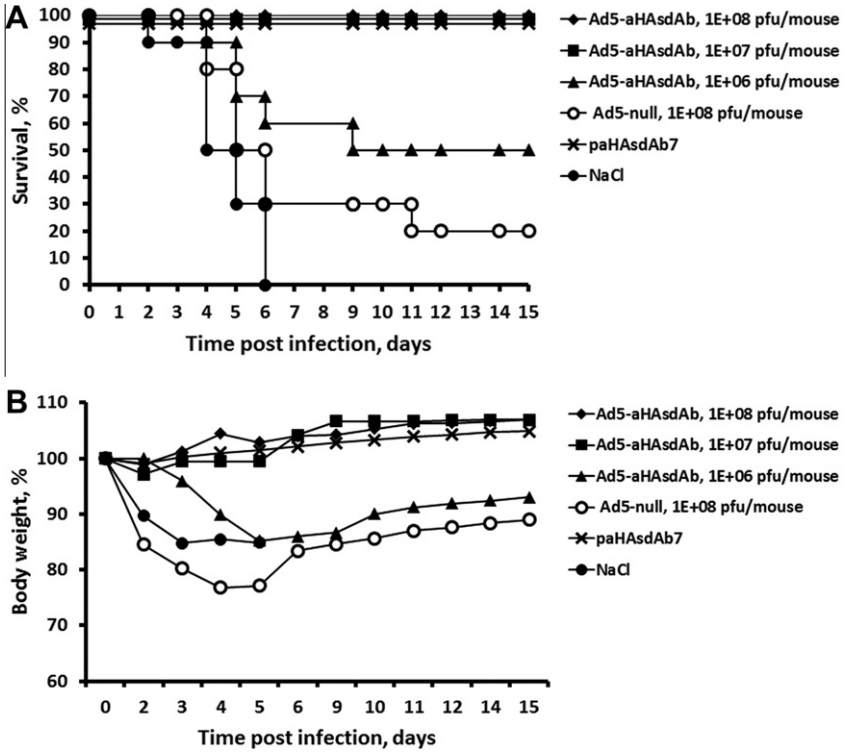

Fig. 3. Dose selection for Ad5-aHAsdAb in lethal influenza virus challenged mice. Three doses of Ad5-aHAsdAb were administered to the mice: 10^8, 10^7 and 10^6 pfu/mouse. Ad5-null at a dose of 10^8 pfu/mouse and paHAsdAb7 (50 μg/mouse) were also tested. Forty-eight hours later the animals were challenged with 50 LD_{50} of H5N2 virus. Weight loss and survival were assessed 15 d following the infection. (A) Survival rate; (B) changes in mouse body weight.

aHAsdAb at 10^8 pfu/mouse and Ad5-aHAsdAb at 10^7 pfu/mouse (Fig. 3A and B).

Based on the data obtained, the lower of the two doses of Ad5-aHAsdAb that allowed survival was selected for further tests (i.e., 10^7 pfu/mouse). To emulate the application of Ad5-aHAsdAb and paHAsdAb7 during an IAV epidemic, we administered preparations at various time points prior to and after infection. The survival rate stood at 100% for the mice treated with Ad5-aHAsdAb at each of the time points (Fig. 4), except mice that were challenged 2 h later and 24 h prior to Ad5-aHAsdAb administration (Figs. 4A and 5). Our data showed that these latter treatments resulted in 40% and 100% mortality of the animals, respectively. Thus, protection by Ad5-aHAsdAb was observed only when it was administered before influenza infection (14 d–24 h). Meanwhile, no mortality was observed in the animals that received paHAsdAb7 24 h after and 2 h and 24 h prior to influenza infection (Fig. 4). Ad5-aHAsdAb proved effective (90–100% survival) protection only from 24 h after its administration, but paHAsdAb7s only within 24 h after its administration. The combination of Ad5-aHAsdAb and paHAsdAb7 in one preparation might expand the protection window.

3.3. Protection from influenza virus challenge mediated by an Ad5-aHAsdAb and paHAsdAb combination

Ad5-aHAsdAb and paHAsdAb7 were mixed in a 1:1 ratio, and 10^7 pfu rAd was added to 50 μg of sdAb. The components in the mixture were found to be completely compatible (functionally active) (Fig. 4S). Balb/c mice were treated with the Ad5-aHAsdAb + paHAsdAb7 mix, Ad5-aHAsdAb and paHAsdAb7 at various time points prior to and after infection. During all of the observed time points the mice that received the Ad5-aHAsdAb + paHAsdAb7 mix exhibited a significant survival advantage (90–100% survivability from 14 d before to 24 h after infection) compared to mice that received only Ad5-aHAsdAb (90–100% survivability from 14 d to 24 h prior to infection) or only paHAsdAb7 (90–100% survivability from 24 h before to 24 h after infection) (Fig. 6A).

At the same time, BALF samples were collected from non-infected mice at various time points after Ad5-aHAsdAb + paHAsdAb7 mix administration. sdAb binding to IAV was detected by ELISA in all of the BALF samples (Fig. 6B). We also demonstrated a very strong correlation between the level of protection and the lung influenza virus titers (Fig. 6A and C).

An Ad5-aHAsdAb + paHAsdAb7 mix was efficient at protecting experimental animals from challenge with high lethal doses of IAV. Furthermore, single administration of this mixed preparation improved the protection window from 14 d prior to 48 h post influenza infection.

4. Discussion

Passive immunization is considered to be one of the quickest and most efficient ways of protecting against pathogens besides antibiotic therapy. Recent studies have shown that different types of recombinant antibodies (monovalent, bivalent and trivalent) are capable of fully protecting animals against specific infections (Kasuya et al., 2005; Sofer-Podesta et al., 2009; Chiuchiolo et al., 2006; Ibañez et al., 2011). For example, Hultberg et al. (2011) has shown that the usage of trivalent Ads against trimeric antigens (including IAV HA) is a very promising approach. In accordance with these data we used trivalent sdAbs in our work.

The application of sdAbs has a number of limitations, the most important being *in vivo* instability (Mabry et al., 2005). This feature is caused by the rapid antibody passage through the renal filter, resulting in the rapid blood clearance. There is a wide range of applications in which the increased antibody half-lives in serum is an essential prerequisite. One way to decrease the clearance is the fusing of sdAb to Fc fragment of IgG. Fc can provide long serum

half-lives. Additionally, Abs with Fc are able to induce antibody dependent cellular cytotoxicity and complement dependent cytotoxicity that may increase its therapeutic effect and play a critical role in the clearance of bacterial pathogens. However the interactions of the Fc with complement and Fc receptors determine the proinflammatory functions of Abs that can lead to cytokine release and associated toxic effects (Giorgini et al., 2008; Brennan et al., 2010). In addition, sdAb-Fc has a big size (approximately 80 kDa) and is not able to recognize any hardly accessible epitopes, which are more likely to be conservative, for example in narrow cavities on the surface of antigen (Holliger and Hudson, 2005).

In the various studies the instability problem was resolved through another way – the application of vectors (including adenoviral vectors) carrying the antibody gene (Kasuya et al., 2005; Sofer-Podesta et al., 2009; Chiuchiolo et al., 2006). The use of such a construct allows the generation of protective concentrations of antibodies over 2-week after administration. In our study, we examined the feasibility of creating an Ab- and rAd-based preparation with expanded protection time window (effective prior to and after infection). Our results were consistent with the results obtained by Kasuya et al. (2005). The protective effect of the rAd-expressing aHAsdAb lasted for 14 d following administration. The treatment with rAd during influenza infection development led to serious consequences (100% mortality in animal experiments). The latter fact is a considerable limitation of their use because the incubation period of influenza is usually two days, but can range from one to four days. Therefore, rAd treatment at this time may be not effective or dangerous. Combining paHAsdAbs and Ad5-aHAsdAb in a single preparation can provide a solution to this problem, which was confirmed by the results reported here. The protective period of this combined treatment may last up to 14 d, as in the case of rAd treatment. Furthermore, the application of the mixed treatment did not cause death when administered either before or after challenge.

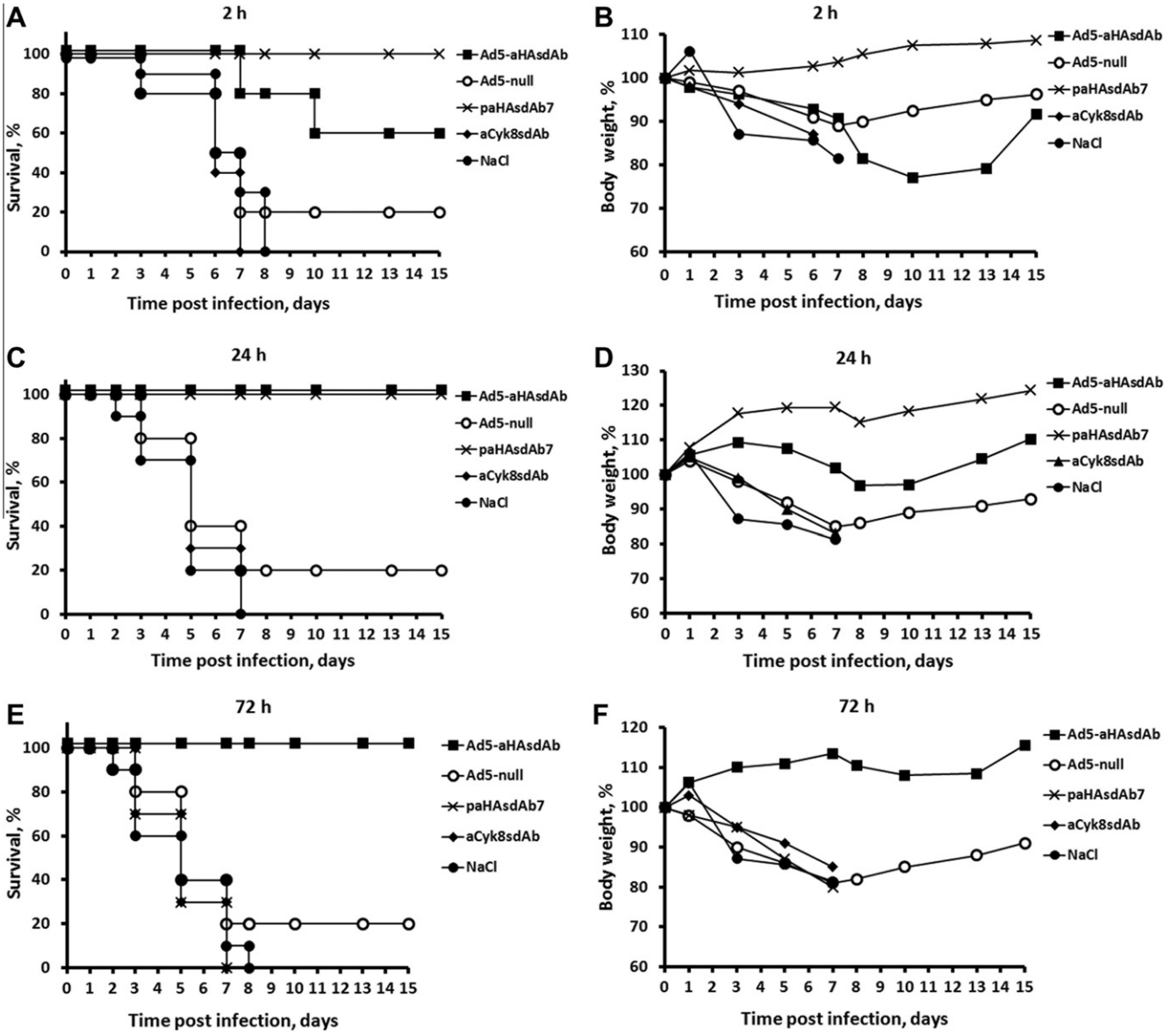

Fig. 4. Prophylactic efficacy of Ad5-aHAsdAb and paHAsdAb in mice. Mice were challenged with a lethal dose (50 LD_{50}) of A/Mallard duck/Pennsylvania/10218/84 (H5N2) 2 h (A, B), 24 h (C, D), 72 h (E, F), 7 d (G, H) and 14 d (I, J) after the introduction of Ad5-aHAsdAb at 10^7 pfu/mouse, Ad5-null at 10^7 pfu/mouse, paHAsdAb7, paCyk8sdAb as a negative control at 50 μg/mouse and 0.9% NaCl. The body weight indicated as a percentage of the initial body weight is shown at different time points during the 15-day period after challenge (B, D, F, H, and J). The survival of challenged mice was monitored for 15 d (A, C, E, G, and I). Each data point represents the average of 10 mice.

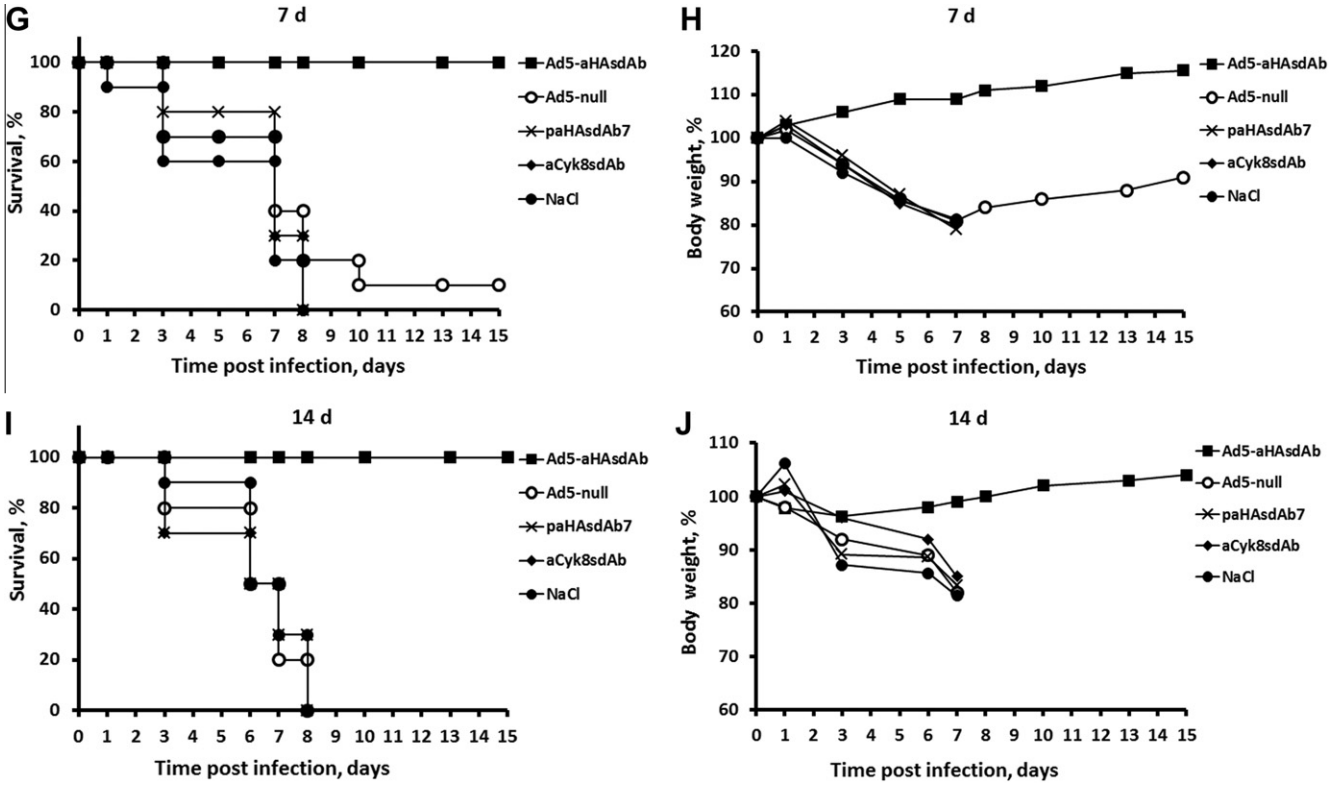

Fig. 4. (*continued*)

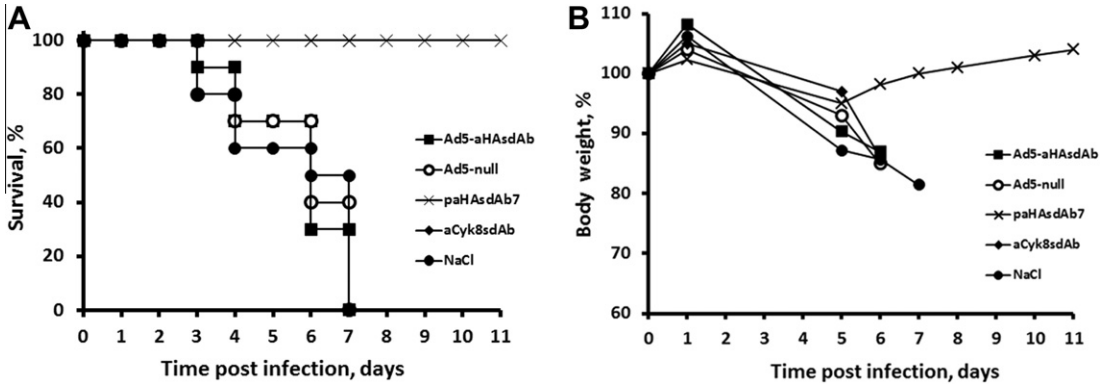

Fig. 5. Therapeutic efficacy of Ad5-aHAsdAb and aHAsdAb in mice. Ad5-aHAsdAb (10^7 pfu/mouse) and paHAsdAb7 (50 μg/mouse) were administered to mice 24 h after infection with a lethal dose (50 LD_{50}) of A/Mallard duck/Pennsylvania/10218/84 (H5N2). The survival of challenged mice was observed for 15 d after the challenge and indicates the level of protection from mortality (A). The percentage of the initial body weight after the challenge is indicated periodically during the 15-day period (B). Each data point represents the average of 10 mice.

In our studies we studied intranasally delivered Ad-vector because intranasal route of Ad-based preparations administration may help to bypass the pre-existing immune response to Ad5 (Kumaki et al., 2011; Croyle et al., 2008). Intranasal administration has some advantages in comparison with other routes: it is needle-free, noninvasive, and essentially painless, does not require medical personnel, and it can be self-administered (Weaver and Barry, 2008; Kumaki et al., 2011). Additionally, the data obtained by Croyle and co-workers have shown that single intranasal immunization with rAd5-based vaccine against Zaire Ebola lead to 100% protection in both naive mice and those with pre-existing immunity (Croyle et al., 2008). For another administration routes several other strategies to circumvent Ad (Ad5) pre-existing immunity (covalent modification of capsid proteins and Ad encapsulation, serotype switching, and usage of helper dependent Ad vectors were suggested as well (Thacker et al., 2009; Croyle et al., 2000, 2002; Fisher et al., 2001; Beer et al., 1998; Segura et al., 2008; Stone et al., 2005; Bangari and Mittal, 2006).

It should be also noted that there is another possible limitation for the application of sdAb-based preparations in medical practice. In our work, we used sdAbs against the HA1 domain of hemagglutinin. Its ability to bind the globular domain characterized by a high mutation rate was unfavorable characteristic of an anti-influenza Ab. First, the *in vivo* use of such Abs may result in the selection of resistant IAV clones (O'Donnell et al., 2012; Rudneva et al., 2012). And second, it very rarely induces cross-strain protection (narrow specificity) (Kaminski and Lee, 2011; Hensley et al., 2009). An sdAbs against conservative epitopes of different IAV proteins can be generated (Krammer et al., 2012; Okuno et al., 1994; Throsby et al., 2008; Sui et al., 2009; Staneková and Varečková, 2010).

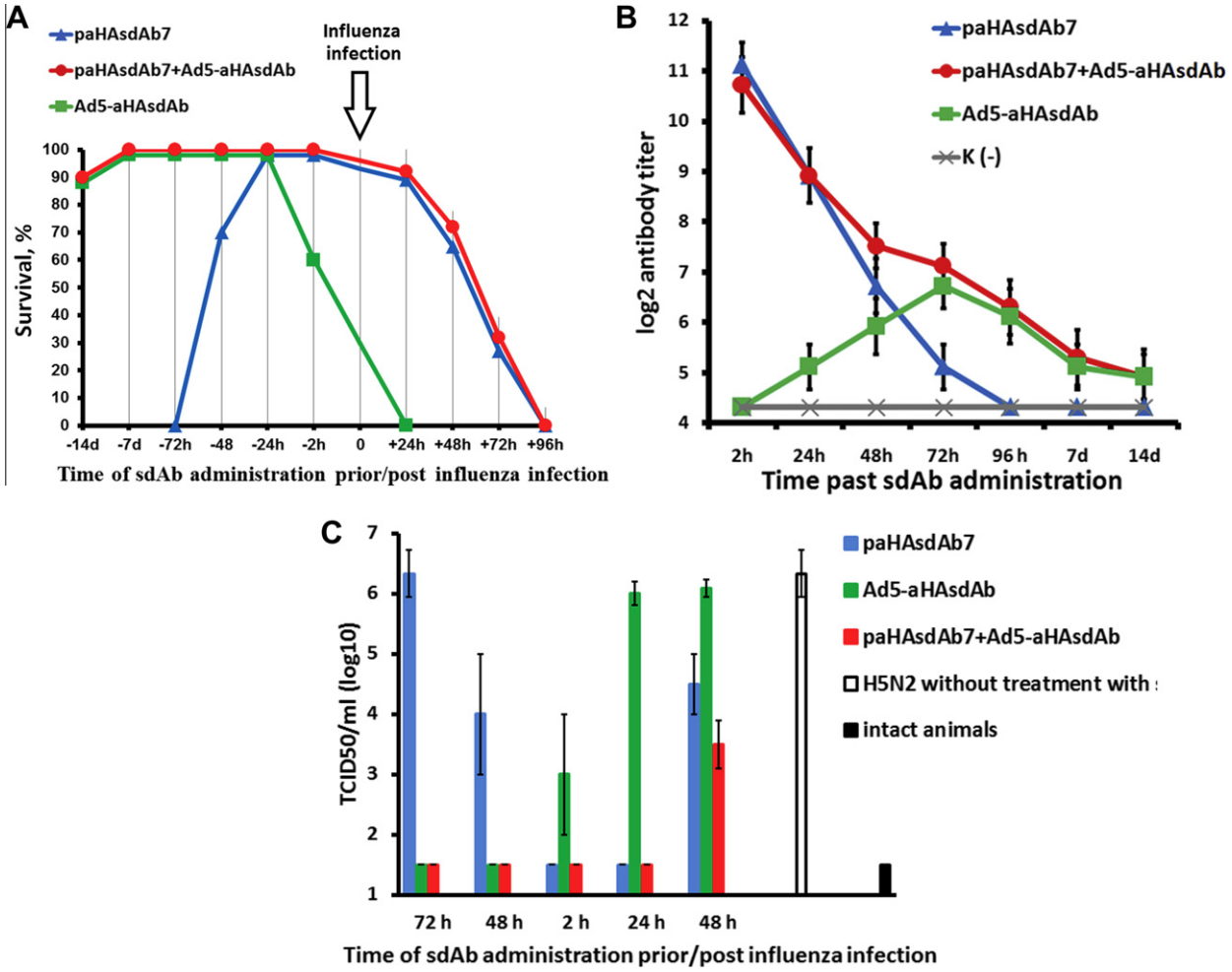

Fig. 6. Therapeutic and prophylactic efficacy of an Ad5-aHAsdAb + paHAsdAb7 mix in mice. (A) Balb/c mice were treated with the Ad5-aHAsdAb + paHAsdAb7 mix, Ad5-aHAsdAb (10^7 pfu/mouse), paHAsdAb7 (50 μg/mouse) or 0.9% NaCl. A lethal dose (50 LD_{50}) of A/Mallard duck/Pennsylvania/10218/84 (H5N2) was given at 2 h, 24 h, 48 h, 72 h, 7 d and 14 d after treatment, and 24 h, 48 h, 72 h and 96 h before. The survival of challenged mice was monitored for 15 d after the challenge and indicates the level of protection from mortality. Each data point represents the average of 10 mice. (B) Level of aHAsdAbs in BALFs after the administration of Ad5-aHAsdAb + paHAsdAb7 mix. The animals were treated with a mix of Ad5-aHAsdAb (1×10^7 pfu/mouse) and paHAsdAb7 (50 μg/mouse). BALFs were performed 24 h, 48 h, 72 h, 96 h, 7 d and 14 d after administration. aHAsdAb levels in BALFs were determined by ELISA. (C) Balb/c mice were treated as described for A. Lungs were harvested on day 4 after challenge, and virus titers were determined as described in "Section 2.12".

Presently, there is data proving that Abs against the influenza A virus M2 ion channel protein may be more universal (more cross-reactive and cross-protective) than anti-hemagglutinin and anti-neuraminidase Abs (Song et al., 2011; Gabbard et al., 2009; Wei et al., 2011).

The usage of cocktail of several antibodies targeting different unique epitopes may be very successful strategy to prevent the selection of resistant IAV clones, because it is difficult for virus to escape neutralization by adopting mutations within two or more epitopes. Also this strategy may be used for the development of broad-spectrum preparations. Adenoviral vector is suitable for the expression of several sdAb genes simultaneously. For example, Ad-vector with E1 and E3 deletions has a sufficient capacity to insert at least 5 sdAb genes.

Generating a broad-spectrum sdAbs and the application of the above strategy to prolong their protective effect would solve a range of problems, from vaccine strain selection to the prevention of new pandemic viruses and their spread.

5. Conclusions

The sdAb-based preparations reported here can be applied both for the treatment and prevention of influenza infections. Additionally, such preparations, if necessary, could be used instead of vaccination. The application of combined (protein + vector) sdAb-based preparations could widen the window of protection against influenza. This approach will allow the development of efficient prophylactics against pandemic virus variants and other infectious agents.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2012.12.021.

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