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Heterosubtypic protection against influenza A induced by adenylate cyclase toxoids delivering conserved HA2 subunit of hemagglutinin

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ABSTRACT

The protective efficacy of currently available influenza vaccines is restricted to vaccine strains and their close antigenic variants. A new strategy to obtain cross-protection against influenza is based on conserved antigens of influenza A viruses (IAV), which are able to elicit a protective immune response. Here we describe a vaccination approach involving the conserved stem part of hemagglutinin, the HA2 subunit, shared by different HA subtypes of IAV. To increase its immunogenicity, a novel strategy of antigen delivery to antigen presenting cells (APCs) has been used. The HA2 segment (residues 23–185) was inserted into a genetically detoxified adenylate cyclase toxoid (CyaA-E5) which specifically targets and penetrates CD11b-expressing dendritic cells. The CyaA-E5–HA2 toxoid induced $HA2_{93-102}$, $HA2_{96-104}$ and $HA2_{170-178}$-specific and Th1 polarized T-cell responses, and also elicited strong broadly cross-reactive HA2-specific antibody response. BALB/c mice immunized with three doses of purified CyaA-E5–HA2 without any adjuvant recovered from influenza infection 2 days earlier than the control mock-immunized mice. More importantly, immunized mice were protected against a lethal challenge with $2LD_{50}$ dose of a homologous virus (H3 subtype), as well as against the infection with a heterologous (H7 subtype) influenza A virus. This is the first report on heterosubtypic protection against influenza A infection mediated by an HA2-based vaccine that can induce both humoral and cellular immune responses without the need of adjuvant.

1. Introduction

Influenza viruses are highly contagious pathogens that remain a major medical problem and serious threat to human health. The major drawback of current vaccines is that they induce virus-neutralizing (VN) antibodies targeted mostly against the immunodominant HA1 subunit of hemagglutinin (HA), which is highly variable. The VN antibodies prevent infection by blocking virus attachment to the cell surface. However, the narrow specificity of VN antibodies makes current vaccines ineffective against drifted influenza A viruses and against new emerging influenza virus variants with a pandemic potential. Since T-cell immunity plays an important role in clearance of the virus and contributes to the milder course of infection, the design of new vaccines inducing also T cell responses is desirable in order to achieve a broader control of the influenza infection. Vaccines based on the conserved antigens which would elicit both antibody and T cell responses are likely to provide a universal strategy for the control of unexpected flu outbreaks (Wang and Palese, 2009).

The evolution of influenza viruses have resulted in 17 HA subtypes that are further divided into two major phylogenetic groups: group 1 (subtypes H1, H2, H5, H6, H8, H9, H11, H12, H13) and group 2 (subtypes H3, H4, H7, H10, H14 and H15) (Tong et al., 2012; Nabel et Fauci, 2012). Recent studies suggest that the conserved stem region of HA2 glycoprotein (HA2 gp) represents a promising candidate for the preparation of a universal flu vaccine (Gocník et al., 2008; Wang et al., 2010a,b; Steel et al., 2010; Bommakanti et al., 2010). The influenza virus HA is a polypeptide synthesized as a precursor (HA0) that trimerizes in the endoplasmic reticulum and is transported to the cell surface via the Golgi apparatus. The HA0 is post-translationally cleaved by host proteases into two subunits HA1 and HA2, which remain linked by a single

Abbreviations: IAV, influenza A virus; HA, hemagglutinin; HA2, the light chain of hemagglutinin; APCs, antigen presenting cells; CyaA, adenylate cyclase; CyaA-E5, adenylate cyclase toxoid; CyaA-E5–OVA, adenylate cyclase toxoid with inserted OVA epitope; CyaA-E5–HA2, adenylate cyclase toxoid with inserted HA2 epitopes; VN antibodies, virus neutralizing antibodies; CTL, cytotoxic T cells; A/Miss, influenza virus A/Mississippi/1/85 (H3N2); A/Chick, influenza virus A/Chicken Germany/34 (H7N1); LD_{50}, lethal dose of virus at which 50% of infected mice died; DCs, dendritic cells; PBS, phosphate buffered saline; EHA2, ectodomain of HA2 aa 23–185; NP, influenza A nucleoprotein; IFN, interferon.

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disulphide bond (Skehel and Wiley, 2000). Unlike HA1, the HA2 subunit is relatively well conserved among various influenza strains. HA2 is responsible for the fusion of viral and cell membranes in the endosomes, thus allowing the release of a ribonucleoprotein complex of the virus into the cytoplasm (Gerhard et al., 2006). It was shown that HA2-specific antibodies derived from mice (Okuno et al., 1993; Varečková et al., 2003a), humans (Kashyap et al., 2008; Corti et al., 2010) or recombinant libraries (Ekiert et al., 2009; Sui et al., 2009; Throsby et al., 2008; Lim et al, 2008) are broadly neutralizing against group 1 and group 2 of influenza viruses (Varečková et al., 2008; Stropkovská et al., 2009; Wang et al., 2010a,b; Corti et al., 2011). These studies suggest that epitopes in the stem region of HA are accessible to antibodies that can prevent the fusion step and inhibit a viral replication *in vitro* as well as *in vivo*, thus contributing to an earlier recovery from the influenza infection (reviewed in Staneková and Varečková, 2010). Moreover, it was shown that vaccines designed to elicit HA2-specific antibodies confer protection against a lethal influenza infection (Gocník et al., 2007, 2008; Wang et al., 2010b; Steel et al., 2010; Bommakanti et al., 2010).

In the present study, we have exploited a genetically detoxified *Bordetella pertussis* adenylate cyclase toxin (CyaA) for the development of a cross-protective vaccine carrying the stem part of the HA, HA2 gp. The CyaA toxoid has been previously shown to specifically target and penetrate into cytosol of human and animal myeloid phagocytic cells expressing the $\alpha_M\beta_2$ integrin serving as complement receptor 3 (CR3), and known also as CD11b/CD18 or Mac-1 (Guermonprez et al., 2001). This receptor is expressed on professional antigen presenting cells (APCs), such as the myeloid dendritic cells (DC), neutrophils/granulocytes, macrophages, natural killer cells (NK), as well as on a restricted subset of B and NKT lymphocytes (Guermonprez et al., 2001, 2002; Simsova et al., 2004). The capacity of CyaA to selectively penetrate $CD11b^+$ APCs was recently exploited for the development of an antigen delivery platform that enables targeting of passenger antigens into the cytosol of APCs for processing and presentation on MHC class I molecules (www.genticel.com). In parallel, however, a fraction of the cell-bound CyaA molecules is endocytosed with the receptor and reaches the compartment in which processing and loading of antigens onto MHC II molecules occurs. Therefore CyaA-based vaccines could be used for simultaneous induction of strong antigen-specific $CD4^+$ T helper, as well cytotoxic $CD8^+$ T lymphocyte (CTL) responses. CyaA-based vaccines have already been shown to be effective in protecting vaccinated animals against a lethal viral challenge or against growth of transplanted tumors (Sebo et al., 1995; Sakamoto et al., 1992; Guermonprez et al., 1999; Fayolle et al., 1996, 1999; Saron et al., 1997; Loucka et al., 2002; Schlecht et al., 2004; Préville et al., 2005; Berraondo et al., 2007). Furthermore, the use of CyaA vector allowed to induce and also potentiate a long-lasting antigen-specific humoral immune response (Mascarell et al., 2005).

Here we used the genetically detoxified CyaA as a tool for delivery of the conserved stem portion of the HA2 protein (amino acids 23–185) originated from the human H3 subtype virus (A/Aichi/2/68 (H3N2). Vaccination of BALB/c mice with this immunogen elicited both, HA2-specific $CD8^+$ and $CD4^+$ T cell responses, as well as, a strong HA2-specific antibody response, which cross-protected mice against lethal infection with the human-H3 or the highly pathogenic avian-H7 influenza A virus types, respectively.

2. Material and methods

2.1. Viruses

All virus stocks used in this work originated from the collection of viruses of the Institute of Virology, Slovak Academy of Sciences, Bratislava, Slovak Republic. Virus stocks were propagated in 10-day-old embryonated chicken eggs for 48 h at 35 °C. Infectious allantoic fluid was aliquoted and stored at −80 °C. The virus titer was evaluated by micro-hemagglutination assay using guinea pig erythrocytes.

2.2. Purified viruses

A/PR/8/34 (H1N1); A/Beijing/262/95 (H1N1); A/Texas/36/91 (H1N1), A/Dunedin/4/73 (H3N2); A/NT/60/68 (H3N2); A/Mississippi/1/85 (H3N2); Ab4 (H3N2) mutant virus derived from A/Aichi/2/68 (H3N2); A/Wyoming/3/03 (H3N2); A/Sydney/5/97 (H3N2); A/Duck/Czech/56 (H4N6); A/Chicken/Germany/34 (H7N1) were purified from infectious allantoic fluid by sucrose gradient centrifugation as described previously (Russ et al., 1974).

2.3. Preparation of mouse adapted viruses

Mouse adapted human influenza virus A/Mississipi/1/85 (H3N2) (A/Miss) and mouse adapted avian influenza virus A/Chicken/Germany/34 (H7N1) – Rostock Fowl PlagueVirus (A/Chick) were prepared in mouse lungs as follows. Two 6-week old BALB/c mice were infected intranasally under the light anesthesia with 40 μl of allantoic fluid containing wild-type H3N2 or H7N1 virus. Their lungs were harvested 2 days after the infection. Lung cells were homogenized in 1 ml of PBS, pH 7.2. Cell debris was sedimented and supernatant (40 μl) was used for further infection. After six passages, the virus was propagated in 10-day old chicken embryos for 48 or 36 h (for FPV). Infectious dose was estimated by the titration of mouse adapted virus on mice and the median lethal dose (LD_{50}) was determined by the Reed and Muench method. All experiments with highly pathogenic avian influenza virus were conducted under BSL-3 containment, including work with animals.

2.4. Synthetic peptides

H-2Kd-restricted synthetic peptide $CD8^+$ T-cell epitope $HA2_{93-102}$ (SYNAELLVAL), I-Ad restricted $CD4^+$ T-cell epitopes: $HA2_{96-104}$ (AELLVALEN), $CD4^+$ T-cell epitope $HA2_{170-178}$ (RFQIKGVEL) and H-2b restricted synthetic peptide $CD8^+$ T-cell epitope $NP_{366-374}$ (ASNENMETM) were provided by Proimmune (UK).

2.5. Mice

Six week-old BALB/c female mice (Faculty of Medicine, Masaryk University, Brno, Czech Republic) were used in animal experiments. In all experiments presented in this paper, animals were treated according to the European Union standards and the fundamental ethical principles including animal welfare requirements were respected.

2.6. Recombinant HA_{23-185} antigen and CyaA-E5–HA2 toxoids

Free recombinant $HA2_{23-185}$ protein, corresponding to residues 23 to 185 of HA2gp of the recombinant X-31 virus (derived from A/Aichi/2/68 (H3N2) was produced in *Escherichia coli* BL21 cells using the pLM-1 plasmid kindly provided by Prof. D.C. Wiley and Dr. J. Chen, Harvard University, Boston, USA). The HA2 protein was purified as described previously (Chen et al., 1999). The sequence encoding residues 76 to 130 of HA2 was PCR amplified from the plasmid PLM-1, using primers 5′-GACGACGACAAGATGAGAATTCAGGACC TCGAGAAA (forward) and 5′-GAGGAGAAGCCCGGTCAAGCATTTTCCCTCAGTTG (reverse). The amplified DNA was cloned into pTriEx™-4 (Novagen) to generate pTriEx–$HA2_{76-130}$.

For construction of the pT7CTACT-E5–HA2 plasmid, the $HA2_{23-185}$ encoding sequence was amplified from plasmid PLM1

$HA2_{23-185}$ and subcloned into pGem®-T ease vector (Promega) to yield pGem$HA2_{23-185}$ used for HA2 insert preparation. The PCR primers were designed (a) to allow cloning of the $HA2_{23-185}$ insert into a unique *Bsr*GI restriction site located between codons 232 and 233 of the CyaA open reading frame on a pT7CTACT1 vector (Jelinek et al, 2012); (b) to introduce an *Eco*RI restriction site for rapid identification of clones carrying the insert; (c) to introduce stop codons interrupting CyaA-E5–HA2 synthesis when inserted in an inverted orientation and (d) to introduce positively charged flanking amino acid residues towards the termini of the inserted polypeptide sequence, in order to compensate for the negatively charged amino acid residues present at the HA2-processing sites (Karimova et al., 1998). To allow monitoring of the delivery of the AC domain with inserted $HA2_{23-185}$ into the MHC class I pathway, the CyaA-E5–HA2 construct was further tagged by insertion of the $OVA_{257-264}$ epitope SIINFEKL, as described previously (Osicka et al., 2000). The CyaA-E5–HA2 and CyaA-E5–OVA constructs were genetically detoxified by ablating the catalytic adenylate cyclase (AC) enzyme activity by placing a GlySer dipeptide insert between residues 188 and 189, thereby disrupting the ATP binding site of the AC enzyme (Osicka et al., 2000). Orientation and exact sequences of all cloned inserts were verified by DNA sequencing.

The plasmids were transformed into *E. coli* BL21/pMM100 ($lacI^q$) cells and CyaA toxoids were produced and purified by a combination of ion exchange and hydrophobic chromatography (Karimova et al., 1998; Osicka et al., 2000). In the Phenyl-Sepharose chromatography step, the resin with bound CyaA was repeatedly washed with several bed volumes of 60% isopropanol (Franken et al., 2000). This allowed to reduce bacterial endotoxin content below 200 U/mg of the total purified protein, as determined by the quantitative chromogenic Limulus amebocyte lysate assay (QCL-1000; Cambrex).

2.7. Generation of bone marrow-derived dendritic cells

Bone marrow-derived dendritic cells (DCs) were generated according to Lutz et al. (1999). Briefly, DCs were grown in RPMI 1640 medium supplemented with 10% FCS (Life Technologies), 0.1 mg/ml streptomycin, 1000 U/ml penicillin and 0.25 μg/ml amphotericin (Sigma–Aldrich), 50 μM 2-mercaptoethanol, 1% non-essential amino acids (Biochrom), 1 mM sodium pyruvate, 2 mM glutamine and 200 U/ml granulocyte-macrophage colony-stimulating factor (GM-CSF). Bone marrow cells (2×10^6), flushed from femurs and tibias of mice, were seeded in 100-mm dishes in 10 ml of medium. During the cultivation, 10 ml of fresh medium was added (day 3) or changed (day 6), respectively. Slightly attached cells were used for experiments at days 6–8. Prior addition of CyaA, the RPMI medium used for cultivation of DC was replaced by Dulbecco's modified Eagle's medium (DMEM medium; 1.9 mM Ca^{2+}) containing 200 U/ml GM-CSF.

2.8. In vitro assay for $OVA_{257-264}$ epitope delivery for MHC class I presentation

Bone marrow-derived DCs were used for *in vitro* presentation of antigens to the B3Z hybridoma $CD8^+$ T cells that recognize the OVA 257–264 peptide SIINFEKL on the murine Kb MHC class I molecule (Karttunen et al., 1992). Presentation of the OVA peptide SIINFEKL on MHC class I K^b molecules on DCs was determined as production of β-galactosidase in B3Z cells, in which T cell receptor binding to SIINFEKL/K^b complex on DCs activated expression of the *lacZ* reporter gene under control of the IL-2 promoter NF-AT elements (Karttunen et al., 1992). DCs were seeded in 96-well plate and incubated with triplicates test samples at various concentrations of CyaA toxoids in DMEM for 4 h at 37 °C in a humid 5% CO_2 atmosphere. The cells were then washed with PBS and further cultured with B3Z hybridoma (10^5 cells/well) in a 200 μl final volume of complete medium (RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 100 μM 2-mercaptoethanol, 100 U/ml of penicillin, 100 μg/ml of streptomycin). After 18 h of incubation at 37 °C, cells were lyzed by addition of 100 μl/well of PBS containing 100 μM 2-mercaptoethanol, 9 mM $MgCl_2$, 0.125% Nonidet P-40 and 0.15 mM chlorophenol red-ß-D-galactopyranoside (CPRG) to determine ß-galactosidase activity. After incubation at 30 °C for 2 h, 50 μl/well of stop buffer (1 M glycine) was added and the absorbance at 570 nm was determined using a microplate reader (Safire², Schoeller Instruments).

2.9. Cell transfection

MDCK cells (5×10^4 cells/well) were grown in 24-well plates to 70–80% confluence. Transfection of plasmid pTriEx–$HA2_{76-130}$ was performed using TurboFect™ *in vitro* Transfection Reagent (Fermentas) according to the manufacturer's instruction and after 24 h post-transfection (p.t.), the samples were used in further experiment.

2.10. Immunization and challenge of mice

Six week-old female BALB/c mice were immunized intraperitoneally (i.p) on days 0, 20, 40 with 50 μg CyaA-E5–$HA2_{23-185}$ or CyaA-E5–OVA (control group) in phosphate-buffered saline (PBS). Mice were challenged intranasally (i.n.) on day 60 with a lethal dose of homologous A/Miss ($2LD_{50}$) or heterologous A/Chick ($2LD_{50}$) influenza viruses in a volume of 40 μl. Mice were monitored daily for clinical symptoms and for mortality. All challenge experiments with the highly pathogenic avian A/Chick influenza virus were conducted at an animal biosafety level 3 facility.

2.11. Sera collection

Blood was collected from facial vein of 5 mice of each experimental group before the first immunization and after each immunization dose.

2.12. Analysis of antibody responses by indirect ELISA

Individual mice sera were tested for the presence of HA2-specific antibodies by enzyme-linked immunosorbent assay (ELISA). In brief, 96-well microplates were coated overnight with EHA2 (30 ng/100 μl) or purified influenza viruses (300 ng/100 μl) in PBS at 4 °C. Before adding serum samples the adsorbed purified virus was treated with buffer pH 5 or pH 7 for 30 min. Serum samples in 2-fold dilutions in PBS containing 0.5% ovalbumin (starting from dilution 1:100) were added to coated plate wells for 90 min of incubation at room temperature and the microplates were repeatedly washed with PBS containing 0.02% Tween-20, before horseradish peroxidase-conjugated goat anti-mouse IgG, IgG1, IgG2a sera (AbCam®, USA) were added, followed by addition of o-phenylenediamine with 0.03% hydrogen peroxide (Sigma) as substrate. The reaction was stopped by adition of 3 M HCl and absorbance at 492 nm was measured in a Multi-Mode Microplate ELISA Reader (BioTek, Synergy HT). The titers of specific antibodies were calculated as the reciprocal values of serum dilution at the point where the regression line drawn through the three points of the titration curve crossed the cut-off value. Cut-off was estimated as the average value of 3 preimmune serum samples ± 3 standard deviations.

2.13. Indirect immunofluorescence

MDCK cells were transfected with plasmid DNA using Turbofect (Fermentas) according to the manufacturer's protocol. In brief,

MDCK cells were grown on glass cover slips to 60–70% confluence. Twenty four hours after transfection the cells were fixed with 3% paraformaldehyde (Sigma) in PBS for 10 min and permeabilized with 1% Triton X-100 (Koch-Light) for 60 s. Cell samples were washed three times with PBS, incubated with the mixture of the primary antibodies for 1 h at room temperature, washed repeatedly and incubated for 1 h with anti-mouse IgG antibody conjugated to FITC (Dako). After the final wash, the cell samples were labeled with DAPI (4′,6-diamino-2-phenylindole) in mounting medium (Santa Cruz Biotechnologies). Fluorescence of transfected cells was evaluated in a fluorescent microscope (Leica CTR 600).

2.14. Microneutralization assay

Virus (A/Miss-150 pfu/well or Ab4-250 pfu/well) were incubated with preimmune or immune serum from mice immunized with CyaA-E5–HA2 or CyaA-E5–OVA (final dilution 1:100) at 37 °C/30 min. Monolayers of MDCK cells in 96-well microplate were infected with the mixture of virus–serum (100 μl/well) and incubated at 37 °C for 18 h. After the incubation, cells were fixed with cold methanol and replicated virus was detected with a nucleoprotein-specific monoclonal antibody 107L (1.5 μg/ml) (Varečková et al., 1995) and goat anti-mouse IgG conjugated with a horseradish peroxidase (Bio-Rad). After adding of substrate solution containing 3-amino-9-ethylcarbazole (Sigma, A6926) with 0.03% H_2O_2, red stained infected cells were evaluated. The antibody-mediated inhibition of virus replication was estimated as the reduction of number of infected cells per well by serum antibodies of immunized and control group of mice.

2.15. Estimation of infectious virus titer in mouse lungs

Lungs of immunized mice challenged with H3N2 (A/Miss) or H7N1 (A/Chick) were collected in two day-intervals (lungs from 2 mice/group for each interval) and viral load was measured. Lungs were homogenized in 1 ml of PBS and the titer of the infectious virus in lung suspension was estimated by a rapid culture assay on MDCK cell monolayer in the presence of trypsin, as described previously (Tkáčová et al., 1997). Briefly, a confluent MDCK cell monolayer grown in a 96-well microtitre plate was infected with 2-fold dilutions (in PBS, pH 7.2) of lung homogenate (100 μl/well) and incubated for 45 min at room temperature. Cells were then incubated for 18 h in serum-free Ultra-MDCK medium containing 0.5 μg TPCK trypsin (Sigma) at 37 °C in 5% CO_2 atmosphere. After 18 h the cells were fixed with methanol and the virus was visualized using a sandwich of the monoclonal antibody 107L (1.5 μg/ml), specific for nucleoprotein of influenza A virus (Varečková et al., 1995), using a horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad) and substrate solution containing 3-amino-9-ethylcarbazole (Sigma, A6926) with 0.03% H_2O_2. Infected cells were scored positive as differentiated visibly red stained cells by light microscopy. The titer of infectious virus was determined as the reciprocal value of the highest dilution of the tested lung homogenate at which infected cells were still detected.

2.16. Detection of vRNA in lungs of infected mice

Viral RNA (vRNA) in mouse lungs was detected by RT-PCR. Total RNA from lung-cell homogenate (200 μl) was isolated with the RNA Instapure System (Eurogentec) according to manufacturer's instructions. The final RNA precipitate was resuspended in 20 μl RNase/DNase-free H_2O supplemented with 2 U RNase inhibitor (Fermentas) μl^{-1}. The reaction mixture for reverse transcription (60 min at 42 °C) contained 5 μl total RNA, 1 mM each dNTP, 5× RT reaction buffer (Fermentas), 0.2 μg random heptamer (Invitrogen), 200 U Moloney murine leukemia virus reverse transcriptase (Fermentas) and H_2O up to a final volume of 20 μl. Amplification of cDNA was carried out using oligonucleotide primers specific for the influenza A nucleoprotein (NP). Primers specific for NP (H3 subtype) 5′-GTGAGGATGCAACAGCTGGTCTAAC (forward) 5′-TACCCCTCTTTTTCGAAGTCGT AC (reverse) and NP (H7 subtype) 5′-ATCCATCATTGCTCGTTGTG (forward), 5′-GCATCTGTTGGGAGAATGGT (reverse) were designed. The β-actin primers 5′-AGGTCATCACTATTGGCAAC (forward) and 5′-ATCTTGATCTTCATGGTGCT (reverse) were used as normalization control.

2.17. IFN-γ ELISPOT assay

Interferon (IFN-γ) ELISPOT was performed using the Elispot kit (eBioscience) according to manufacturer's instructions. Briefly, mice were immunized i.p. with 50 μg CyaA-E5 –$HA2_{23–185}$ or CyaA-E5–OVA in PBS. Spleens were removed at day 9 after the first immunization (three mice per group) and mechanically dissociated into single cell suspensions by 70 μm cell strainers (Falcon). The splenocyte suspensions were stimulated in the presence of the 10 μM synthetic peptide ($HA2_{93–102}$, $HA2_{96–104}$, $HA2_{170–178}$) in 96-multiscreen filtration plates coated with anti-mouse IFN-γ antibody for 24 h at 37 °C and in 5% CO_2 in RPMI media containing 10% FCS, antibiotics penicillin (100 U/ml) and streptomycin (100 mg/ml), as described previously (Jackson et al., 1994; Saikh et al., 1995). IFN-γ secreting cells were detected with a sandwich of biotinylated anti-mouse IFN-γ antibody with streptavidin peroxidase using hydrogen peroxide and 3-amino-9-ethylcarbazole (Sigma) as substrates in 0.1 M sodium acetate (pH 5.0). The numbers of spot-forming cells (SCF) per well were counted with the aid of a dissecting microscope and expressed as the mean number of triplicates of IFN-γ SCF per 2×10^6 cells with the standard deviation. Two negative controls for splenocytes from mice immunized with CyaA-E5–$HA2_{23–185}$ were used. Cells were incubated with irrelevant peptide $NP_{366–374}$ (C57BL6, H-2^b) as well as without any peptide. As another negative control splenocytes from mice immunized with CyaA-E5–OVA were incubated with relevant or irrelevant peptide, or without any peptide, respectively.

3. Results

3.1. CyaA toxoid delivers the inserted OVA-tagged $HA2_{23–185}$ peptide into cytosol of antigen presenting cells

To enable targeting of the $HA2_{23–185}$ antigen into cytosol of CD11b-expressing antigen presenting cells (APCs) for processing and subsequent presentation on MHC class I molecules to $CD8^+$ T cells, we inserted an $OVA_{257–264}$ epitope-tagged $HA2_{23–185}$ polypeptide segment between the residues 232 and 233 of the AC domain of genetically detoxified CyaA. The resulting CyaA-E5–HA2 toxoid and its mock CyaA-E5–OVA variant were produced in *E. coli* BL21/pMM100 cells and purified close to homogeneity (not shown). Prior the use for immunization of mice, the capacity of toxoids to bind APCs and to deliver the antigenic cargo into their cytosol for processing and presentation was assessed *in vitro*. It has been repeatedly shown that only toxoids capable of translocating their OVA epitope-tagged AC domain into cytosol of APCs elicit a SIINFEKL peptide-specific stimulation of co-incubated B3Z $CD8^+$ T hybridoma cells that recognize the epitope in complex with K^b MHC I molecules on APCs (Holubova et al., 2012).

As shown in Fig. 1, DC were incubated with the increasing concentrations of CyaA-E5–HA2 or CyaA-E5–OVA toxoids for 4 h before being mixed with the $OVA_{257–264}$-specific $CD8^+$ B3Z hybridoma T cells. Starting from a 5 nM toxoid concentration (1 μg/ml), a comparable efficiency of $OVA_{257–264}$ epitope (SIINFEKL) presentation *in vitro* on DC to B3Z T cells was detected for

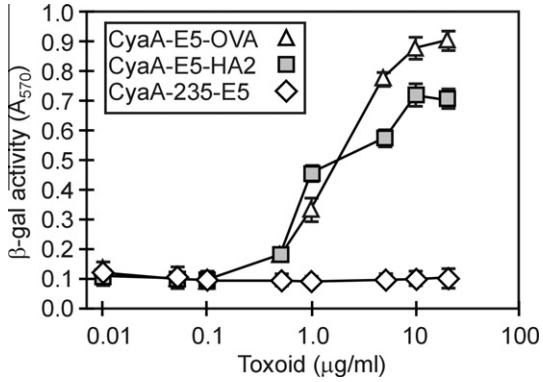

Fig. 1. Antigen delivery capacity of the CyaA-E5–HA2 construct *in vitro*. Bone marrow-derived dendritic cells were incubated with indicated concentrations of the CyaA-E5–HA2 and CyaA-E5–OVA toxoids for 4 h before the B3Z CD8$^+$ T hybridoma cells (K^b restricted) were added and incubation was continued for 18 h. Stimulation of B3Z cells by the SIINFEKL epitope of ovalbumin presented on K^b MHC I molecules was measured as the amount of β-galactosidase enzyme accumulated in B3Z cells (Karttunen et al., 1992). Mock CyaA-233-E5 toxoid was used as a negative control (CyaA-233-E5) 1 μg/ml corresponds to 5 nM toxoid concentration.

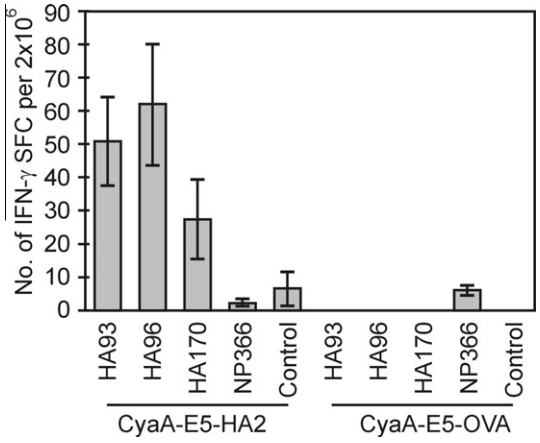

Fig. 2. Immunization with CyaA-E5–HA2 induces antigen-specific T cell response. Three BALB/c mice per group were immunized i.p. with a single dose of 50 μg of CyaA-E5–HA2 or of CyaA-E5–OVA (negative control). The presence of HA2-specific IFN-γ-secreting CD4$^+$ and CD8$^+$ T cells in mouse spleens was assessed on day 9 after immunization by ELISPOT assay upon *in vitro* restimulation with the indicated peptides. Numbers of IFN-γ-secreting cells represent the average values plus standard deviations from the pooled triplicate values from two independent experiments ($n = 6$). Three types of negative controls were used: (i) "Control" means that cells were not stimulated with any peptide, (ii) NP366 means that corresponding peptide was used as an irrelevant peptide, (iii) splenocytes from mice immunized with CyaA-E5–OVA incubated with relevant or irrelevant peptides as well as cells not stimulated with any peptide, all represent further negative controls. Baseline corresponds to a negative reaction (no spots).

both CyaA-E5–HA2 or CyaA-E5–OVA toxoids, when assessed as an IL-2-dependent expression of ß-galactosidase in stimulated B3Z cells. In contrast, no unspecific B3Z stimulation was observed with DC incubated with the control CyaA-233-E5 toxoid lacking the $OVA_{257–264}$ epitope. These results show that the insertion of the 170 residue long $OVA_{257–264}$-tagged $HA2_{23–185}$ antigen polypeptide did not greatly affect the capacity of the resulting CyaA-E5–HA2 toxoid to translocate its AC domain into APC cytosol for processing by proteasome and subsequent presentation of the delivered $OVA_{257–264}$ epitope on K^b MHC class I molecules.

3.2. CyaA-E5–HA2 induces HA2-specific T and B cell responses in BALB/c mice

The $HA2_{23–185}$ antigenic peptide of the H3 subtype comprises several conserved T and B-cell epitopes of IAV. Therefore, we evaluated its capacity to induce specific T and B cell immune responses in mice when delivered in a form of the CyA-E5–$HA2_{23–185}$ toxoid. BALB/c mice were immunized i.p. with 50 μg of CyaA-E5–HA2 or the mock CyaA-E5–OVA toxoid in PBS without any adjuvant. Induction of HA2-specific CD8$^+$ and CD4$^+$ T cell immune responses was analyzed by IFN-γ ELISPOT assays using splenocytes of mice sacrificed 9 days post immunization. For the detection of specific responses splenocytes were restimulated *in vitro* with synthetic peptides corresponding to the CD8$^+$ T cell *H-2d*-restricted peptide $HA_{93–102}$ (SYNAELLVAL), the CD4$^+$ T cell *I-Ad*-restricted peptides $HA_{170–178}$ (RFQIKGVEL) and $HA_{96–104}$ (AELLVALEN) of HA2, respectively, as these epitopes are known to be cross-reactive among IAV subtypes (Saikh et al., 1995; Jackson et al., 1994). The specificity of stimulation was verified by the use of an irrelevant H-*2b*-restricted peptide of the $NP_{366–374}$ epitope (ASNENMETM). As shown in Fig. 2, *in vitro* restimulation with the specific peptides corresponding to the CD8$^+$ T cell epitope $HA_{93–102}$ and CD4$^+$ T cell epitopes $HA_{96–104}$ and $HA_{170–178}$ elicited a specific IFN-γ production by T cells that were present in splenocyte suspensions from CyaA-E5–HA2-immunized mice. No IFN-γ production was detected after restimulation of splenocytes from the control group of mice immunized with CyaA-E5–OVA.

CyaA toxoid has been previously shown to deliver epitopes via endocytosis into the endosomal compartment for MHC class II-restricted presentation to CD4$^+$ T helper cells, which subsequently also increased the specific antibody response (Loucka et al., 2002; Mascarell et al., 2005). Therefore, we further examined whether HA2-specific antibodies were effectively induced by the immunization of mice with the CyaA-E5–HA2 toxoid. Mice were immunized i.p. at days 0, 20 and 40 with 50 μg of CyaA-E5–HA2 in PBS without adjuvant and the control group received CyaA-E5–OVA (50 μg). Another group of mice was immunized i.p. at the same intervals with 5 μg of free $HA2_{23–185}$ protein, which on a molar basis equaled the amount of antigen delivered by the 10-times higher amount of the approximately 10-times larger CyaA-E5–HA2 toxoid (163 residues of $HA2_{23–185}$ compared to the 1898 residue-long CyaA-E5–HA2). The presence of HA2-specific IgG antibodies in the sera of immunized mice was then analyzes by ELISA in serum samples after each immunization. As shown in Fig 3, no anti-HA2 antibodies were detected after the first i.p. immunization (priming), while an about 11-fold higher titer of HA2-specific antibodies was detected after the first booster immunization in the sera of mice immunized with CyaA-E5–HA2, as compared to the sera of mice that received only a HA2 protein. The antibody response was strongly enhanced after the second booster immunization, where an HA2-antibody titer of 71,593 was determined for the sera of mice receiving CyaA-E5–HA, as compared to the titer value of 42,378 for the sera of mice immunized with 5 μg of a purified HA2 protein (Fig 3). Antibody titer was calculated on the basis of reactivity of tested sera in ELISA binding test as described in methods. It represents a reciprocal value of serum dilution, at the point where the regression line drawn through the three points of the titration curve crossed the cut-off value. These results showed that delivery of $HA2_{23–185}$ by the CyaA-E5–HA2 toxoid vector also enhanced the specific antibody response against $HA2_{23–185}$, as compared to that induced by a protein antigen only.

As shown in Fig. 4, immunization with CyaA-E5–HA2 resulted in the induction of antibodies of qualitatively different isotype composition when compared with the antibody composition induced by a HA2 protein alone. CyaA-E5–HA2 induced simultaneous production of IgG1 (56.6%), IgG2a (35%) and IgG2b (8.4%) antibody isotype responses against $HA2_{23–185}$, suggesting that a mixed Th1 and Th2-polarized immune response was induced. In

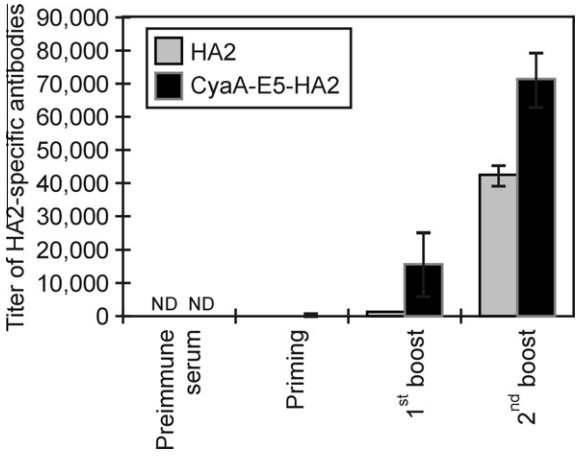

Fig. 3. Immunization with CyaA-E5–HA2 elicits anti-HA2 antibody response. Five BALB/c mice per group were immunized at days 0, 20, and 40 i.p. with 50 μg of CyaA-E5–HA2 or CyaA-E5–OVA, or with 5 μg of purified HA2 protein in PBS without adjuvant. HA2-specific IgG antibody titers were determined in sera of mice after each immunization step, i.e. at days 19 (priming), 39 (first boost), and 59 (second boost). Titer of antibodies specific to HA2 gp (*Y* axis) represents the reciprocal value of serum dilution at the point where the regression line drawn through the three points of the titration curve crossed the cut-off value. Cut-off was estimated as the average value of 3 preimmune serum samples ± 3 standard deviations. *Note:* ND means not detectable level of antibodies.

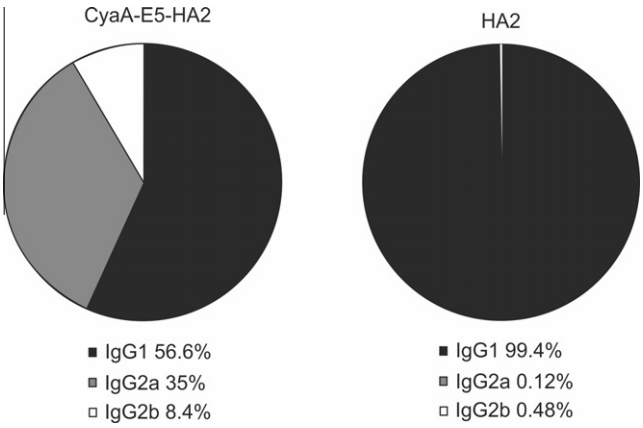

Fig. 4. Immunization with CyaA-E5–HA2 induces a mixed IgG isotype profile of HA2-specific antibodies. BALB/c mice (5 per group) were immunized i.p. with 50 μg of CyA-E5–HA2 or with 5 μg of purified HA2 protein and blood was drawn from mice 14 days after the second immunization boost (i.e. at day 59 of immunization). Individual sera were analyzed for titers of HA2-specific antibodies of the IgG1, IgG2a or IgG2b isotypes by ELISA.

contrast, nearly 100% IgG1 antibody response was detected in the sera of mice immunized i.p. with a HA2 protein alone, which indicates Th2 polarization.

3.3. Immunization with CyaA-E5–HA2 elicits broadly cross-reactive antibodies

It was important to assess how broadly cross-reactive was the antibody response elicited by CyaA-E5–HA2. The reactivity of antibodies induced after the second booster immunization with influenza A viruses of different HA subtypes as antigens was therefore assessed by ELISA. HA2-specific antibodies induced by CyaA-E5–HA2 (Fig. 5) were cross-reactive with influenza A viruses of the H3, H4 and H7 subtypes, albeit the determined antibody titers against H4 (titer 10.7 ± 1.2 $\log_2$) and H7 (titer: 11.1 ± 1.8 $\log_2$) subtypes were several-fold lower than those against the best recognized A/NT H3N2 virus (16.7 ± 0.8 $\log_2$). No significant differences in reactivity were detected among viruses of H3 and H7 subtypes. However, CyaA-E5–HA2 induced antibodies preferentially bound to pH5 form of HA (Fig. 6). Lower antibody binding was measured with prefusion form of HA, i.e. with the native conformation of HA, though the cross-reactivity pattern of the reactivity was similar to that of pH 5 form.

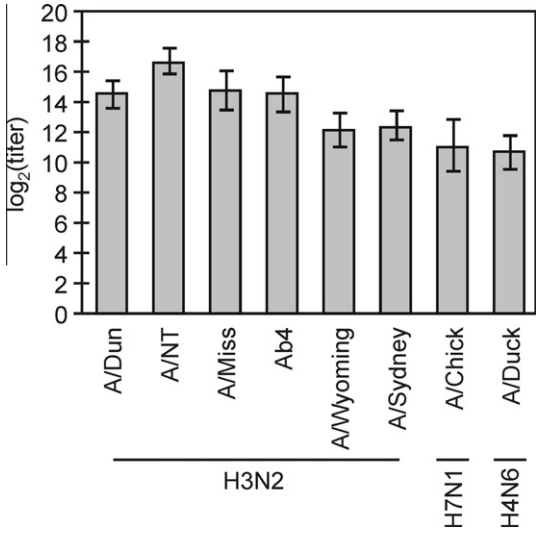

Fig. 5. Immunization with CyaA-E5–HA2 induces IAV subtype cross-reactive HA2-specific antibodies. Titers of HA2-specific antibodies reactive with pH5-treated purified influenza viruses of the indicated subtypes were determined by ELISA. Three individual sera were analyzed for each group of mice after the second booster immunization (day 59). Titer of non-immune serum represents baseline (0). There was not statistically significant difference in the reactivity among IAV of H3 and H7 subtypes in relation to the homologous strain A/Miss (A/Dun $p = 0.9284$, A/NT $p = 0.1378$, Ab4 $p = 0.8509$, Wyoming $p = 0.1014$, Sydney $p = 0.1117$, H7 subtype FPV-R $p = 0.0662$). Significant difference of reactivity in relation to A/Miss was observed with IAV of H4 subtype A/Duck ($p = 0.0290$).

3.4. CyaA-E5–HA2-induced antibodies recognize the broadly protective $HA2_{76–130}$ region

To corroborate the analysis, we examined whether the induced antibodies can recognize the $HA2_{76–130}$ fragment consisting of residues 76 to 130, which was recently described as a target of the broadly neutralizing 12D1 monoclonal antibody (Wang et al., 2010a,b). Towards this aim, the $HA2_{76–130}$ polypeptide was expressed in pTriEx–HA2-transfected MDCK cells and its recognition by sera from three mice after the 2nd booster immunization with CyaA-E5–HA2 or CyaA-E5–OVA (control) were evaluated by immunofluorescence. As documented in Fig. 7, the serum from CyaA-E5–HA2-immunized mouse showed a positive staining of MDCK cells transfected with pTriEx–HA2, while no reaction was observed with the preimmune or control sera of mice immunized with CyaA-E5–OVA, respectively. Therefore, it can be concluded that the antibody response induced with CyaA-E5–HA2 comprised antibodies specific to the $HA2_{76–130}$ region. Even though this has not been tested, we cannot exclude that also a population of neutralizing antibodies of the 12D1 like specificity was induced.

3.5. Vaccination with CyaA-E5–HA2 accelerates virus elimination from lungs of mice infected with H3 and H7 influenza viruses

To investigate the cross-protective potential of the vaccination with CyaA-E5–HA2, BALB/c mice were immunized with CyaA-E5–HA2 as described above and challenged with a $2LD_{50}$ dose of homologous A/Miss (H3) or heterologous A/Chick (H7) virus sub-

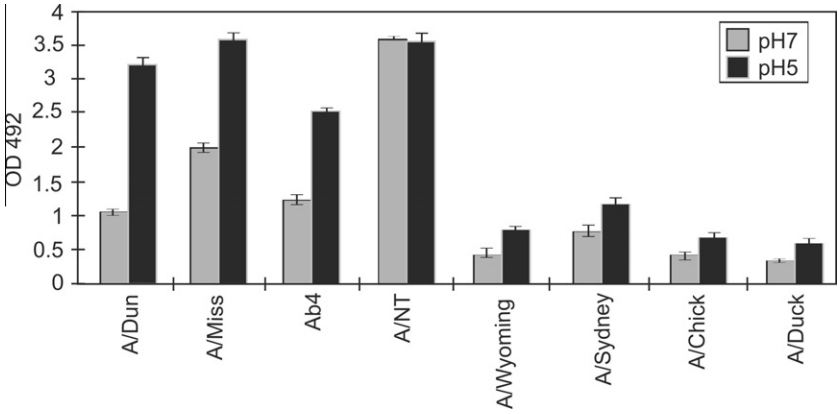

Fig. 6. Reactivity of sera with viruses of native and low pH conformation of HA by ELISA. Purified viruses of appropriate subtypes were used as antigens in native or in pH5 treated form for detection of specific antibodies. The values are corrected for reactivity with non-immune serum.

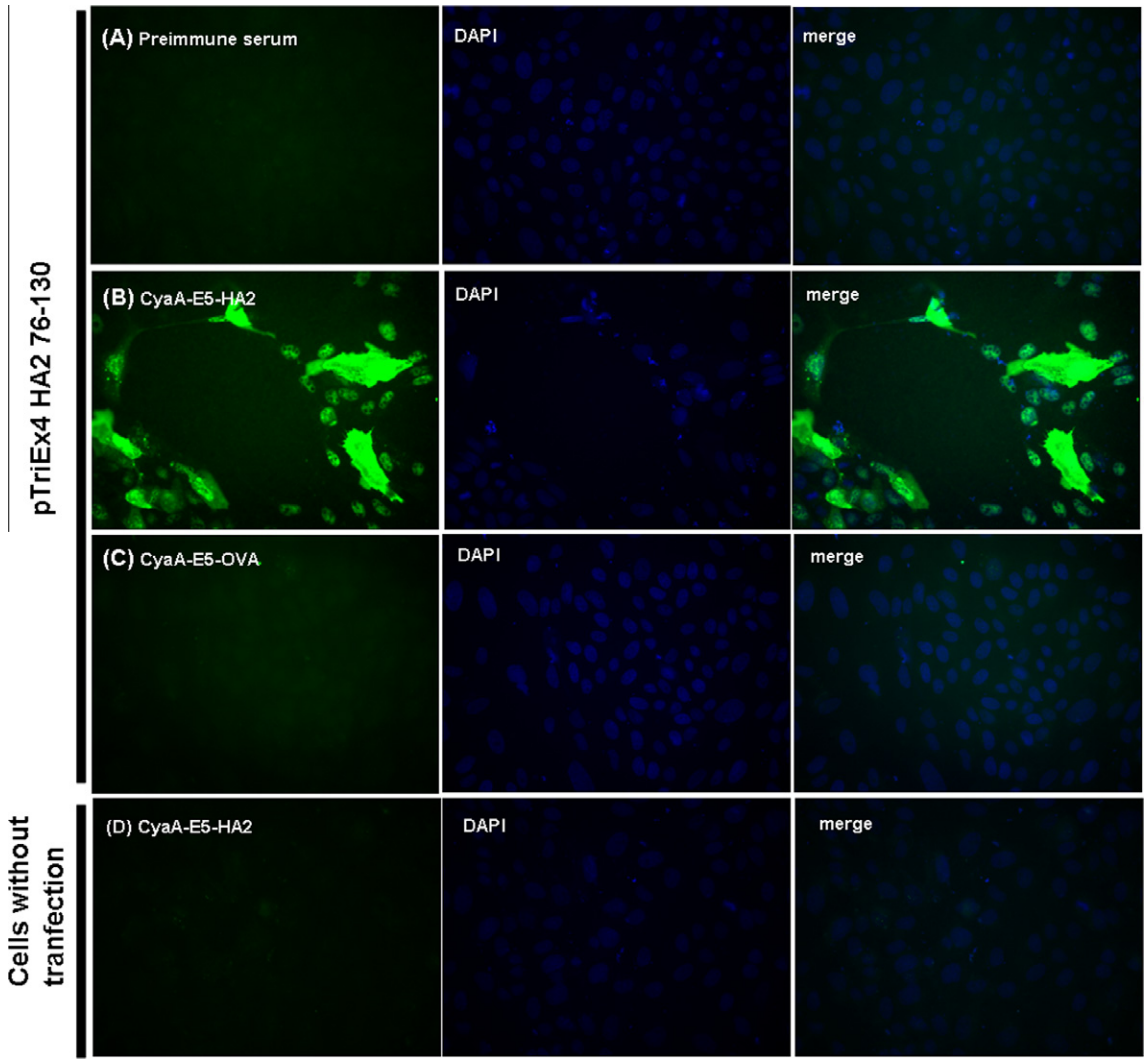

Fig. 7. CyaA-E5–HA2-induced antibodies recognize the conserved $HA2_{76-130}$ part of HA2. MDCK cells were transfected with pTriEx–$HA2_{76-130}$ (A-C) or with mock pTriEx plasmid (D). Cells were stained with preimmune mouse serum (A), with immune serum taken after the second booster immunization with CyaA-E5–HA2 (B) and a control serum of mice immunized with the mock toxoid CyaA-E5–OVA (C), respectively. MDCK cells transfected with empty pTriEx plasmid (D) were used as control for unspecific background staining with immune serum of mice immunized with CyaA-E5–HA2.

types, respectively. The course of the infection, i.e. survival, as well as virus titers in the lungs were followed on days 2, 4, 6, 8, and 10 after infection and compared with mock-immunized mice that received CyaA-E5–OVA or PBS, only. As shown in Fig. 8a, the viral

titers in lungs of CyaA-E5–HA2-immunized mice, challenged with homologous A/Miss virus, reached the maximum ($17 \pm 0.5 \log_2$ units) on day 4 and decreased to $10.6 \pm 1.0 \log_2$ units on day 6, with no infectious virus or vRNA being detectable already on day 8 post infection. In contrast, infectious virus and vRNA were still detected on day 8 post infection in lungs of the control mice that were mock-immunized with CyaA-E5–OVA.

When mice were challenged with the heterologous mouse-adapted A/Chick virus, the viral titers in lungs of CyaA-E5–HA2 immunized mice reached a maximum on the day 2 and were progressively decreasing. On the day 8 p.i., the virus could not be detected anymore. The similar course of the infection has been observed in the control group of mice (Fig. 8b). However, the A/Chick virus vRNA was detected 8 days p.i. in the lungs of both vaccinated and control mice, while the last detectable viral titer determined on the 6th day p.i. was lower in lungs of immunized mice ($6.6 \log_2$ units) than in lungs of control mice ($9.9 \log_2$ units), in correlation with the intensity of corresponding vRNA bands detected by RT-PCR.

These results showed that immunization with CyaA-E5–HA2 enabled accelerated clearance of influenza virus from the lungs of mice infected with the homologous A/Miss virus and resulted in lower infectious titers of the heterologous A/Chick influenza A virus.

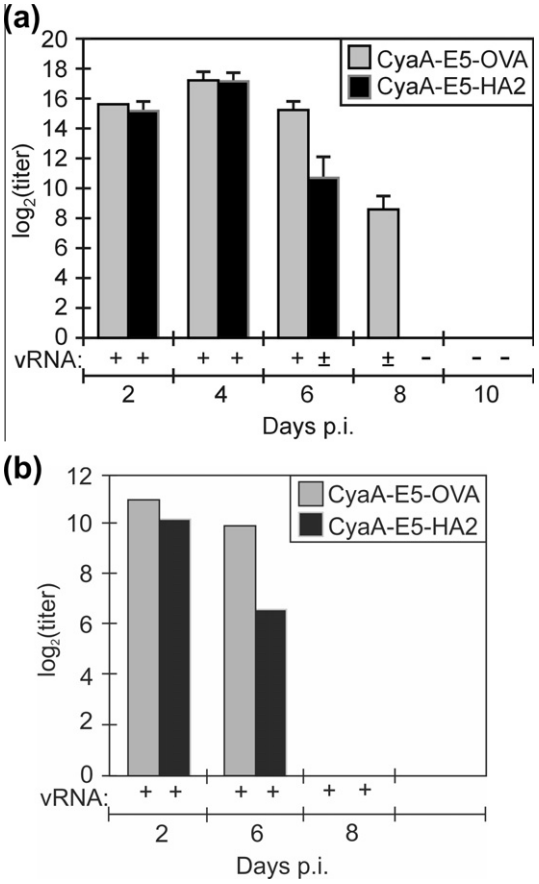

Fig. 8. Immunization with CyaA-E5–HA2 accelerates the drop of vRNA load and clearance of influenza virus in lungs of mice infected with A/Miss (H3N2) virus. Upon i.p. immunization with 3 doses of 50 μg of CyaA-E5–HA2 or CyaA-E5–OVA toxoids, two mice per group were infected on day 59 with a $2LD_{50}$ dose of A/Miss (H3N2) (a) or A/Chick (H7N1) (b) influenza virus. Viral titers and vRNA loads were determined in homogenates of mice lungs at indicated dates post infection by rapid culture and by RT-PCR assays. The titer of infectious virus was determined as the reciprocal value of the highest dilution of sample (lung homogenate) that still infected cells. vRNA was detected in lung homogenates using primers specific for influenza A virus NP. Means of duplicate values from two experiments are given. For H7N1 virus, lungs from only one mouse in each interval was analyzed.

3.6. Immunization with CyaA-E5–HA2 confers cross-protection against a lethal infection of mice with IAV of H3 and of H7 subtypes

To examine the degree of protection resulting from vaccination with the CyaA-E5–HA2 toxoid, we followed the survival of the immunized mice that were challenged on day 60 with a $2LD_{50}$ of homologous (A/Miss, H3N2), or the heterologous (highly pathogenic) avian influenza virus (A/Chick, H7N1). Immunization with the CyaA-E5–HA2 conferred a full protection and 100% of survival of mice against a lethal challenge with A/Miss (H3N2) influenza virus when compared to mock-immunized mice treated with CyaA-E5–OVA ($p = 0.0006$) or PBS-treated mice that all died by the day 10 after infection ($p = 0$) (Fig. 9a). Moreover, the immunization with CyaA-E5–HA2 conferred on mice also a very high level of protection (80%, $p = 0.0007$) against a lethal challenge with the highly pathogenic heterologous A/Chick (H7N1) virus that killed all mock-immunized mice (Fig. 9b). Furthermore, in both challenge studies the course of infection was noticeably milder in the CyaA-E5–HA2-vaccinated mice than in the control group, as judged also from monitoring of clinical symptoms, such as a low activity and an appearance of scrubby fur.

Based on these results it can be concluded that the immunization with CyaA-E5–HA2 conferred an inter-subtype protective immunity against a lethal infection with influenza A viruses. In our previous *in vivo* experiments, mice were fully protected against the homologous virus after the immunization with purified EHA2 applied with Freund adjuvants. However, only a partial and statistically non-significant protection from the infection with the virus of heterologous H7 subtype was achieved (Janulíková et al., 2012). The immunization of mice with EHA2 protein increased the survival of experimental animals from 30% in the control non-immunized mice, to 56%. More effective cross-protection after the immunization with CyaA-E5–HA2 is likely to be due to induction of specific T-cells, which recognize conserved regions on HA2 gp (Saikh et al., 1995; Jackson et al., 1994). Furthermore, it should be noted that cross-protection was achieved by immunization with CyaA-E5–HA2 without the need of any added adjuvant. This suggests that the CyaA toxoid exhibited an adjuvanting capacity itself and can be used for enhancement of HA2 immunogenicity.

3.7. CyaA-E5–HA2 induced antibodies reduce the virus replication

To understand the role of antibodies induced after immunization of mice with CyaA-E5–HA2, their activity was examined in microneutralization test. The cca 50% reduction of virus replication was observed in RCA (Fig. 10) with homologous A/Miss virus as well as with heterologous virus Ab4 of conformationally changed HA of the same H3 subtype.

4. Discussion

We report here that vaccination of mice with a conserved stem part of HA2 of influneza A virus delivered by the *B. pertussis* adenylate cyclase toxoid (CyaA) has a potential for development of HA2-based cross-protective influenza vaccines. This observation is of a particular interest in the light of the currently ongoing phase I clinical trials with cGMP batches of CyaA toxoids that carry the human papillomavirus 16 and 18 antigen E7, aimed for tumor immunotherapy (www.genticel.com), thus paving the way to clinical use of the CyaA-based antigen delivery approach.

One of the drawbacks of currently available influenza vaccines is their incapacity to induce a broadly cross-protective humoral

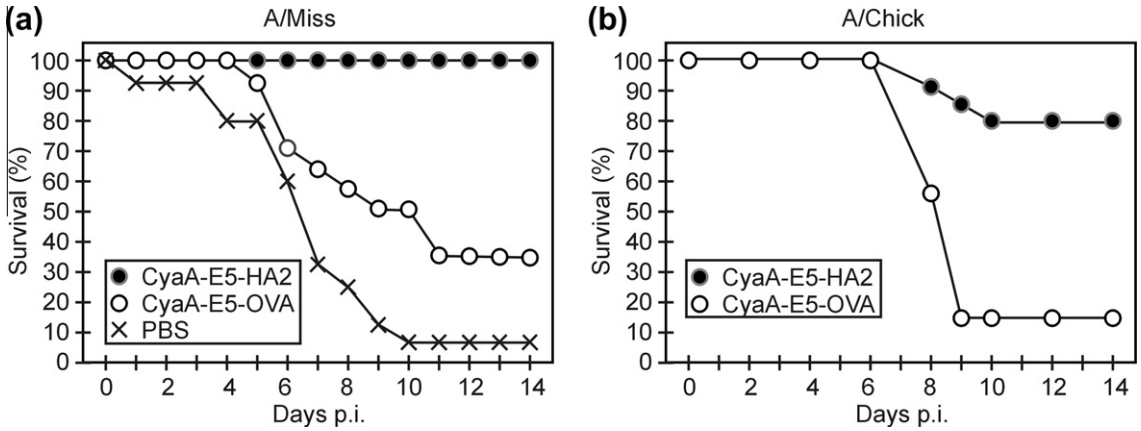

Fig. 9. Immunization with CyaA-E5–HA2 fully protects mice against lethal challenge with the homologous A/Miss (H3N2) virus and confers high level of protection against heterologous challenge with A/Chick (H7N1) virus. Groups of 14 to 15 immunized, or control mice were infected on day 59 with $2LD_{50}$ dose of (a) A/Miss (H3N2) or (b) of A/Chick (H7N1) virus and mice survival was monitored until 14 days post infection. The significance of survival was evaluated using Fisher's exact test. For mice infected with A/Miss (H3N2) the difference in survival between mice treated with CyaA-E5–HA2 ($n = 14$) and CyaA-E5–OVA ($n = 14$) was at the $p = 0.0006$ level. The difference in survival between mice immunized with CyaA-E5–HA2 and control group that received PBS only was at $p = 0.0000$. For mice infected with A/Chick (H7N1) virus the difference in survival between mice immunized with CyaA-E5–HA2 and CyaA-E5–OVA mock-immunized mice was at $p = 0.0007$.

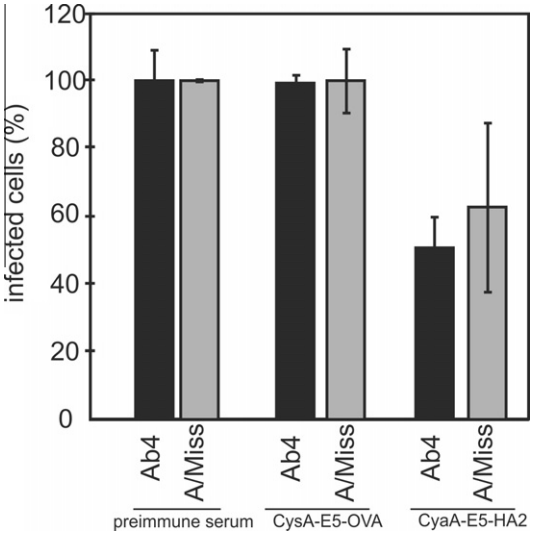

Fig. 10. Antibodies induced with CyaA-E5–HA2 reduce virus replication. MDCK cells were infected with homologous virus A/Miss (H3N2) or with Ab4 virus with conformationally different HA of the same subtype. Axis *Y* represents the percent of infected cells in the presence of preimmune (100%) and HA2-specific serum at dilution 1:100.

and cell-mediated immune response against a variety of influenza A virus subtypes. Several studies have shown that the conserved part of hemagglutinin, its HA2gp stalk, is a promising candidate for development of broadly-protective influenza vaccines (Gocník et al., 2008; Steel et al., 2010; Bommakanti et al., 2010; Wang et al., 2010b). It was observed that HA2-specific antibodies can reduce replication of influenza virus (Lim et al., 2008; Varečková et al., 2003b; Edwards and Dimmock, 2000, 2001; Okuno et al., 1993), improve the recovery from influenza infection and provide protection against a lethal influenza infection (Varečková et al., 2003b; Gocník et al., 2007, 2008; Prabhu et al., 2009). Recently, broadly neutralizing antibodies specific to the HA2 stem of HA were identified, which exhibited activity against both, group 1 and group 2 influenza viruses (Kashyap et al., 2008; Throsby et al., 2008; Sui et al., 2009; Wei et al., 2010; Wang et al., 2010a,b, 2011; Stropkovská et al., 2010; Nabel and Fauci, 2010; Ekiert et al., 2011). These antibodies could be used as a therapeutic cocktail for vaccination against influenza A viruses of different HA subtypes and represent thereby the inspiration for new immunization strategies that would efficiently elicit HA2-specific antibodies.

By fusing the conserved HA2 stalk region segment to a carrier targeting antigen-presenting cells, we developed here an experimental influenza vaccine that exhibited an enhanced cross-protective potential and immunogenicity of HA2 for efficient stimulation of both humoral and cellular immunity. These results go well with previous reports on the use of the adenylate cyclase toxoid as an antigen delivery tool used for induction of both humoral (Mascarell et al., 2005) and cell-mediated immune responses (Guermonprez et al., 1999; Fayolle et al., 1996; Loucka et al., 2002; Schlecht et al., 2004). Of particular interest appears to be the fact that no adjuvant was needed to achieve efficient immunization with CyaA-E5–HA2 that elicited high levels of HA2-specific antibodies and protection against viral challenge up to $6LD_{50}$. This is of particular importance in the light of the quite limited choice of adjuvants approved for use in humans and the recent controversial reports on potential negative side effects of adjuvants used in some commercial flu vaccines (Stropkovská et al., 2010).

Moreover, we have also observed that HA2-specific antibodies induced by the vaccination with CyaA-E5–HA2 were cross-reactive within group 2 HA proteins (H3, H4, H7). However, their reactivity with subtypes of H1 belonging to group 1 was weak (data not shown). Our results showed further that the immunization with CyaA-E5–HA2 induced also antibodies recognizing $HA2_{76–130}$ which might exhibit a similar specificity as the antibody 12D1 that was recently described as broadly neutralizing (Wang et al., 2010a,b). In addition, the vaccination with a synthetic peptide corresponding to residues 76 to 130 of HA2 (the binding region of 12D1) was reported to provide a protection in mice against influenza viruses of phylogenetically distinct subtypes (Wang et al., 2010a,b). This goes well with the here-observed cross-protective potential of the vaccination with CyaA-E5–HA2.

It has been previously shown that the vaccination with adenylate cyclase toxoid-based vaccines allows the induction of Th1-polarized immune responses (Dadaglio et al., 2000; Mascarell et al., 2005). Analysis of anti-HA2 specific immunoglobulin isotypes indicated that i.p immunization with CyaA-E5–HA2 induced a mixed Th1 and Th2 type of immune response. In contrast, prevailing Th2-polarized immune response (Abs of IgG1 isotype), with negligible amount (0.6%) of IgG2a and IgG2b isotypes, was

obtained uppon the vaccination with HA2 protein alone. The HA2-specific immune response obtained after i.p vaccination of mice with CyaA-E5–HA2 comprised antibodies of the IgG1, as well as of IgG2a and IgG2b isotypes, the latter beeing shown to be important mediators of ADCC in mice (Kipps et al., 1985; Denkers et al., 1985). It is, therefore, plausible to speculate that HA2-specific antibodies elicited by the vaccination with CyaA-E5–HA2 might contribute to the protection against IAV also by an indirect mechanism, such as the Ab-dependent cytotoxicity (ADCC) via the Fc region recognition or the FcR-mediated phagocytosis (Huber et al., 2001; Staneková and Varečková, 2010). Inhibition of virus fusion with host cell membranes might be another possible mechanism by which HA2-specific antibodies could neutralize the influenza infection (Varečková et al, 2003b; Gocník et al., 2007; Wang et al., 2010a,b), We showed here that antibodies induced by the immunization with CyaA-E5–HA2 reduced the virus replication. Therefore, we can speculate that they might contribute directly, at least partially, to the milder course of the influenza infection in immunized mice.

It was shown that induction of both $CD4^+$ T cells and $CD8^+$ T cells plays an important role in the recovery from the influenza infection (McMurry et al., 2008). In CyaA-E5–HA2-immunized mice we observed, indeed, an increase in the number of influenza virus-specific IFNγ-secreting splenocytes after restimulation with HA2-specific $CD4^+$ and $CD8^+$ T cell peptides. The induction of HA2-specific $CD8^+$ and $CD4^+$ T cells response was achieved already after the first immunization dose. Two of these epitopes recognized by T cells (the $CD8^+$ epitope $HA2_{93-102}$ and the $CD4^+$ epitope $HA2_{96-104}$) are located in the conserved region 76_{-130} of HA2 that was shown to induce antibody mediated protection against distinct viral subtypes (Wang et al., 2010a,b). Therefore, it can be supposed that the cross-protective potential of this region of HA2 might be enhanced by stimulation of T-cell immune response when CyaA is used as a tool for HA2 antigen delivery. We report here for the first time that a HA2-based vaccine can induce not only a protective humoral response, as shown previously (Gocník et al., 2008; Steel et al., 2010; Wang et al., 2010a,b; Bommakanti et al., 2010), but also induce a $CD8^+$ and $CD4^+$ T cell response that may enhance the protective immunity. These results suggest that the use of adenylate cyclase toxoids as antigen carrier is a valid approach for stimulation of both arms of protective immunity against influenza virus infection.

This conclusion is underpinned by our observation that vaccination with CyaA-E5–HA2 provided earlier clearance of influenza virus from mice lungs and protected mice not only against a lethal infection with homologous, human influenza virus A/Miss H3N2, but also conferred protection against a heterologous and highly pathogenic avian influenza virus A/Chick H7N1. The latter is, indeed, sporadically transmitted from birds to humans and exhibits the potential to start a new influenza pandemic.

It is particularly noteworthy that this report is the first demonstration of the cross-protection mediated by HA2-based vaccine without the need for any adjuvant. It should be stressed in this respect that the T-cell and specific antibody responses were induced by a non-replicating vector, CyaA-E5–HA2, which could previously be reached only after vaccination with live attenuated influenza vaccines, the application of which in humans has some restrictions (in USA high risk groups comprising the old people, newborns as well as pregnant women and immune-deficient patients are excluded) (Stropkovská et al., 2010).

It remains to be explored how readily the here-developed CyaA-E5–HA2-based vaccine can be modified so as to broaden its protective efficacy also against other circulating IAV strains, particularly the H1N1, or the highly pathogenic avian influenza viruses H5N1 and H9N2, respectively. The here selected $CD8^+$ (HA_{93-102}) and $CD4^+$ (HA_{96-104}) T-cell epitopes are highly conserved not only within viruses of group 2 (H3, H4), but conservancy analysis showed also their high homology with viruses of group 1 (H1 – 80% and H5 – 70% vs. H3 subtypes). We assume that after considering the differences in the region $HA2_{23-185}$ derived from HA of any influenza virus within group 1 and upon selection of a suitable combination of the HA2 peptides from each group (e.g. HA2 gp from H1 subtype (group 1) and HA2 gp from H3 subtype (group 2), a protection against influenza viruses from both phylogenetic groups 1 and 2 could be achieved. Such a recombinant CyaA–HA2 based vaccine would represent an alternative to currently available influenza vaccines and might help to minimize the devastating effects of newly emerging highly pathogenic influenza A viruses.

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