Mucosal immunity induced by adenovirus-based H5N1 HPAI vaccine confers protection against a lethal H5N2 avian influenza virus challenge

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Development of effective vaccines against highly pathogenic avian influenza (HPAI) H5N1 viruses is a global public health priority. Considering the difficulty in predicting HPAI H5N1 pandemic strains, one strategy used in their design includes the development of formulations with the capacity of eliciting broad cross-protective immunity against multiple viral antigens. To this end we constructed a replication-defective recombinant adenovirus-based avian influenza virus vaccine (rAdv-AI) expressing the codon-optimized M2ex–HA–hCD40L and the M1–M2 fusion genes from HPAI H5N1 human isolate. Although there were no significant differences in the systemic immune responses observed between the intramuscular prime-intramuscular boost regimen (IM/IM) and the intranasal prime-intramuscular boost regimen (IN/IM), IN/IM induced more potent CD8+ T cell and antibody responses at mucosal sites than the IM/IM vaccination, resulting in more effective protection against lethal H5N2 avian influenza (AI) virus challenge. These findings suggest that the strategies used to induce multi-antigen-targeted mucosal immunity, such as IN/IM delivery of rAdv-AI, may be a promising approach for developing broad protective vaccines that may be more effective against the new HPAI pandemic strains.

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Introduction

The highly pathogenic avian influenza (HPAI) H5N1 virus has been a considerable problem in Asia and more recently in Europe. Repeated outbreaks of HPAI H5N1 in Southeast Asia that resulted in the death of poultry in the hundreds of millions pose a pandemic threat to human health (Li et al., 2004; Subbarao et al., 1998). Since HPAI H5N1 was first shown to infect humans in 1997, more than 417 confirmed human infections (257 resulting in death) have been reported (WHO, 2009). Even though few cases resulting from human-to-human transmission have been reported, most HPAI H5N1 infections in humans have been due to exposure to infected poultry (Ungchusak et al., 2005). Genetic assortment between human and avian influenza viruses and/or mutations in HPAI H5N1 could result in the generation of influenza strains which could result in a new pandemic-associated strain in human. Since human populations are immunologically naive to the current HPAI H5N1 isolates the design of effective vaccines is an urgent global public health priority (Hien, de Jong, and Farrar, 2004).

Currently available HPAI H5N1 vaccines are traditional egg-produced inactivated vaccines. Despite several reports showing that conventional inactivated whole H5N1 influenza virus vaccines could induce cross-protective immunity, the reported cross-protection was limited since they induced strain-specific neutralizing antibodies that were ineffective against heterologous influenza virus strains (Bublot et al., 2007; Kistner et al., 2007; Ninomiya et al., 2007). Furthermore, several disadvantages are associated with the generation of inactivated, egg-generated vaccines as follows: (i) the requirement for biosafety level 3 containment facilities; (ii) lengthy generation times, i.e., several months following the identification of new potential strains; (iii) HPAI H5N1 cannot be grown to high titers in eggs due to their high pathogenicity; and (iv) some strains of HPAI H5N1 are only modestly immunogenic (Treator et al., 2006). Generating these vaccines using traditional methods would not yield sufficient quantities for worldwide distribution for at risk populations in the event of a pandemic. Therefore, alternative vaccine strategies that can overcome these limitations are in demand.

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Several approaches, such as mammalian cell-based vaccines, viral vector vaccines, recombinant proteins, virus-like particles and DNA vaccines, have been extensively studied as alternative approaches (Horimoto and Kawaoka, 2006). Included in the list of alternative strategies are the replication-defective recombinant adenoviral vector (rAdV)-based vaccines. rAdV-based vaccines have been widely used in preclinical and clinical studies and proven to be a safe and effective vaccine vector (Bangari and Mittal, 2006; Priddy et al., 2008; Van Kampen et al., 2005). In addition, rAdV-based vaccines do not require biosafety level 3 containment facilities and are much easier to stockpile than traditional egg-produced inactivated vaccines. Furthermore, recent reports showed that a replication-defective rAdV-based avian influenza vaccines were very effective in animal models of infection (Epstein et al., 2005; Gao et al., 2006; Hoelscher et al., 2006; Hoelscher et al., 2007; Holman et al., 2008; Tompkins et al., 2007; Toro et al., 2007; Toro et al., 2008) suggesting that an rAdV-based HAPI vaccine is a logical alternative to traditional egg-derived vaccines.

Since mucosal surfaces are primary sites utilized by numerous pathogens (including influenza virus) as portals of entry, mucosal immune responses (including secretory IgA antibody [Abs] production and cytotoxic T cell responses) at mucosal sites are thought to play crucial roles in host protection against mucosal pathogens (Kilian and RM, 1999). However, current intramuscular delivery of inactivated influenza vaccines is less effective at inducing mucosal immunity (Cate et al., 1983; Clements and Murphy, 1986). Most rAdV-based influenza virus vaccines mentioned above are also delivered via intramuscular injection; however, this formulation has the capacity to induce mucosal immune responses (Baig et al. 2002) suggesting that mucosal immunization with the same formulation is likely to elicit a more potent response at this critical host–pathogen interface. Adenovirus can invade host cells via mucosal surfaces and replicate (initially at mucosal sites of the respiratory or gastrointestinal tracts) supporting the use of rAdV-based vaccines as a substitute to current vaccines.

An ideal pre-pandemic or pandemic influenza vaccine should possess the ability to generate broad protective immunity at both systemically and mucosally sites. In this study, we generated a rAdV-based influenza virus vaccine (rAdV-Al) expressing the codon-optimized M2eX–HA–hCD40L and M1–M2 fusion genes from HPAl H5N1 human isolates for the development of a broadly protective vaccine. We investigated whether the vaccination route (systemic vs. mucosal) would affect mucosal and/or systemic immune responses in addition to assessing the protection efficacy following a challenge with lethal H5N2 avian influenza (Al) virus. We demonstrated that the intranasal prime-intramuscular boost (IN/IM) vaccination regimen induced more potent anti-Al-specific mucosal immunity associated with better protection against challenge with lethal H5N2 avian influenza virus in mice compared to the intramuscular prime-intramuscular boost (IM/IM) vaccination regimen.

**Results**

**The generation of a rAdV-Al vaccine expressing multiple antigens**

To elicit broad protective immunity, a vaccine should induce both humoral and cellular immune responses to conserved target antigens, e.g., M1 and M2. M1 is highly conserved and is the influenza virus protein produced in the greatest abundance. Furthermore, the M158–66 sequence was shown to be a dominant CTL epitope in HLA-A2 αividuals and is well conserved even in HPAl H5N1 strains (Bednarek et al., 1991; Gianfrani et al., 2000; Ito et al., 1991). The sequence of the M2 24-amino acid ectodomain (M2ex) is relatively well conserved compared to hemagglutinin (HA) and neuraminidase (NA) which are the major targets of the current egg-based, inactivated vaccines. Furthermore, M2ex-specific antibody responses were shown to reduce morbidity by restricting viral replication (Fan et al., 2004; Liu et al., 2004; Zharkov et al., 2005). In addition, fusion of the extracellular domain of CD40L to antigens enhanced immune responses by targeting secreted antigens to antigen presenting cells (APCs) such as dendritic cells (DCs) via the interaction with CD40 on their surfaces (Li, 2005; Zhang et al., 2003). Finally, we used a dual-lymphocytotox regulated expression cassette using internal ribosome entry site (IRES) which is the most commonly used multiple gene transfer approach (Martinez-Salas, 1999) for the generation of multigene-expressing rAdV vaccines in a single vector (Fig. 1A). The expression of encoded proteins by rAdV-Al was verified by Western blot analysis of rAdV-Al-infected QBI293 cells. As shown in Fig. 1B, about 95 and 42 kDa bands corresponding tpa–M2ex–HA (ecd)–CD40L(ecd) and tpa–M1–M2 fusion proteins, respectively, were detected in rAdV-Al-infected QBI293 cell lysates, but not in control cell lysates.

**Comparison between systemic and mucosal rAdV-Al vaccination routes on T cell-mediated immunity in mice**

To compare the effectiveness of systemic and mucosal T cell-mediated immunity following systemic or mucosal immunization with rAdV-Al using a prime-boost regimen, HLA.A2 transgenic (Tg) and Balb/c (H-2d) mice were immunized twice at 2-week intervals with rAdV-Al via the IM/IM vaccination regimen or the IN/IM vaccination regimen. Since the M158–66 (HLA.A2-restricted epitope) and the HA149–163 (H-2Kd-restricted epitope) of human influenza virus are conserved in the HPAl H5N1 strains used for our vaccine, CD8+ T cell responses to these epitopes using splenocytes or cells from lung washes were evaluated. In HLA.A2 Tg mice, the IM/IM vaccination regimen induced higher level of M1-specific CD8+ T cell responses in spleen, compared to IN/IM vaccination regimen (Fig. 2). These results were consistent with previous reports showing that the IM immunization with rAdV encoding HA gene elicited higher systemic CD8+ IFN-γ responses than the IN vaccination (Hoelscher et al., 2006). In contrast to systemic T cell-mediated responses, the IM/IM regimen did not induce M1-specific CD8+ T cell responses at mucosal sites (Fig. 2B). While there were few infiltrating CD8+ T cells identified following vaccination via the IM/IM vaccination regimen (Fig. 2B-1, upper left panel), more lung-infiltrated CD8+ T cells were detected and 20.5% of the lung-infiltrating CD8+ T cells were M1-specific IFN-γ-secreting CD8+ T cells in the IN/IM vaccination group. Similar patterns of results were obtained in Balb/c mice (Fig. 3). The IM/IM vaccination regimen induced higher level of M1 (M1 peptide pool)- and HA (HA149–163)-specific CD8+ T cell responses in spleen in Balb/c mice (Fig. 3A). In terms of CD8+ T cell responses at mucosal site, few lung-infiltrating CD8+ T cells were detected in the IM/IM group (Fig. 3B-1, upper panel), but more lung-infiltrated CD8+ T cells were detected and ~0.82% of them were HA-specific CD8+ T cells in IN/IM group (Fig. 3B). Taken together, while the IM/IM regimen induced higher systemic CD8+ T cell responses, compared to the IN/IM vaccination regimen, the IN/IM vaccination regimen elicited more efficient T cell-mediated responses against M1 and HA at mucosal sites compared to the IM/IM vaccination regimen.

**Comparison between humoral responses following systemic or mucosal rAdV-Al vaccination**

To compare humoral immunity following either systemic or mucosal rAdV-Al immunization, sera and lung washes from Balb/c mice immunized using the two regimens were collected and analyzed by Western blot analysis of rAdV-Al-infected QBI293 cells. As shown in Fig. 1B, about 95 and 42 kDa bands corresponding tpa–M2ex–HA (ecd)–CD40L(ecd) and tpa–M1–M2 fusion proteins, respectively, were detected in rAdV-Al-infected QBI293 cell lysates, but not in control cell lysates.
Fig. 2. M1-specific CD8+ T cell responses in HLA.A2 transgenic mice. HLA.A2 transgenic mice were primed intramuscular injection (IM) or intranasal injection (IN) and boosted (IM) at 2-week interval with 5 × 10^6 pfu of rAdv-EGFP or rAdv-Al. Four weeks after the final immunization, mice were sacrificed to measure M158–66-specific CD8+ T cell responses by ELISPOT assay (A) and FACS analysis (B) using cells from lung wash and spleen (five mice per group). Data are presented as mean±SEM (A) and (B-2) and as representative density plots (B-1). Numbers in quadrants indicate the percentages of IFN-γ-secreting cells over total CD8+ T cells. Statistical significance was determined using the Student’s t-test. SFCs, spot forming cells.
and IgA antibodies. These serum antibody responses were further increased following secondary immunization (Fig. 4, 4 weeks after second immunization in serum). The levels of HA- and M2eX (H5N1)-specific IgG and IgA responses in serum following IN/IM vaccination were higher than responses observed following the IM/IM vaccination regimen.

In contrast to systemic humoral responses, mucosal anti-HA and -M2eX IgA and IgG responses in the lung following the IN/IM vaccination regimen were significantly higher than the responses observed in animals receiving the IM/IM vaccination regimen (Fig. 4, 4 weeks after second immunization in lung). Consistent with previous report (Lemiale et al., 2003), IM/IM-immunized mice did not develop potent mucosal IgA responses (Figs. 4B, D and F). Overall, these results suggested that the IN/IM vaccination regimen induced higher mucosal and systemic humoral responses than responses observed in mice immunized via the IM/IM vaccination regimen.

The effect of the rAdv-Al vaccination route on protection efficacy

To evaluate the protective efficacy of rAdv-Al in immunized Balb/c mice, 12 mice per group were immunized twice at 2-week intervals via the IM/IM or IN/IM vaccination routes followed by a challenge with a LD50 of H5N2 AI virus (A/aquatic bird/Korea/w81/2005) (IN) 4 weeks after the final immunization. Control mice immunized with buffer alone suffered substantial weight loss beginning 2 days post-challenge (dpc) and died at 5–7 dpc (Fig. 5). Although ~60% of the IM/IM-immunized mice survived, mice in this group exhibited substantial weight loss similar to that observed in the control group up to 4 dpc. In contrast, all mice in the IN/IM vaccine group were 100% protected and these animals presented with only mild and transient weight loss. The protective efficacy of this vaccination strategy correlated with the mucosal immune response, indicating that the IN/IM regimen conferred significantly better protection against challenge than the IM/IM vaccination regimen due to the ability of this vaccination strategy to induce stronger mucosal immune responses.

Despite partial (IM/IM) or complete (IN/IM) protection, the antibody responses against HA antigen of the challenge virus elicited following rAdv-Al vaccination were below detectable levels when measured by HI assay (Table 1), possibly due to sequence differences between HAs of the vaccine and the challenge virus groups (sequence identity: 93%). In contrast, in terms of HI titers against a H5N1 AI virus (A/environment/Korea/W150/2006), which has higher sequence homology (~97% of HA sequence identity), both regimens induced about ~40 HI titers (Table 1). However, IN/IM regimen induced HI titers in more mice than IM/IM regime (10/10 and 2/10, respectively).

These results suggested that even without detectable antibody levels to HA, the multi-antigen-targeted rAdv-Al vaccine induced protective immunity against antigenically distinct AI virus challenge. This protective immunity may be (in part) due to T cell responses mounted against conserved regions of viral protein antigens, e.g., the matrix region (sequence identity of M1: 96%), and specific anti-M2eX antibody responses resulting in the protection of mice from challenge as a result of viral replication inhibition. It is worth noting that M2eX-specific immune responses elicited by rAdv-Al vaccination were cross-reactive to the M2eX sequence of the challenge virus despite some
sequence differences (i.e., vaccine: SLLTEVETPTRNEWESRSSDSSD and SLLTEVETPTRNEWECRCSDSSD vs. H5N2 challenge virus: SLLTEVETPTRNGWECKCSDSSD) (Figs. 4E and F).

Discussion

In this study, we developed a replication-defective recombinant adenovirus-based avian influenza virus vaccine (rAdv-AI) encoding the codon-optimized M2eX-HA–hCD40L and the M1–M2 gene fusion from the HPAI H5N1 human isolate. We assessed the effectiveness of systemic and mucosal vaccination on immune induction in the context of protection following viral challenge. Systemic rAdv-AI intramuscular prime-intramuscular boost (IM/IM) immunization induced serum humoral and splenic T cell responses but not humoral or T cell responses at mucosal sites (Lemiale et al., 2003; Wang et al., 2004). In contrast, not only systemic but potent mucosal immune responses were induced following an intranasal prime-intramuscular boost (IN/IM) vaccination regimen (Figs. 2–4).

Furthermore, the IN/IM vaccination regimen conferred complete protection against lethal H5N2 AI virus challenge, compared to partial protection in the IM/IM vaccination group (Fig. 5). Since it is difficult to predict pandemic strains, vaccines with the capacity of eliciting broad protective immunity may be suitable. Thus, challenge with an antigenically distinct strain, such as H5N2 strain, may be a possible way to evaluate broad protective immunity of our vaccine regimen.

Previous reports using parenterally administered rAdv-based influenza virus vaccines only examined systemic immune responses (Gao et al., 2006; Hoelscher et al., 2006). Although these reports showed the induction of strong systemic immunity conferring complete protection against homologous and heterologous AI infection, mucosal immunity plays a critical role in protection against infection with many pathogens including influenza virus (Kilian and RM, 1999; Lefrançois, 2005). As shown in this study, administration of rAdv-AI using the IN/IM vaccine regimen not only induced potent mucosal immunity but also elicited systemic humoral and T cell-mediated immune responses.

In the context of protection against infections, the conventional IM/IM regimen did not confer complete protection (~60% survival rate) which is inconsistent with previous reports showing that systemic immunization with rAdv-based influenza virus vaccines expressing HA could induce complete protection against lethal AI virus challenge (Gao et al., 2006; Hoelscher et al., 2006). In addition, Hoelscher et al. (2007) showed a similar protective efficacy between IM and IN vaccination strategies. This discrepancy may be due to differences in the experimental design, challenge virus strain used and the dose of the challenge virus. In one study the immunizing and challenge virus were homologous, i.e., the HA amino acid identity between the vaccine strain and the challenge strain was ~96% (Hoelscher et al., 2007). In our study, the amino acid identity was only 93%. Furthermore, the HI titers against H5N2 challenge virus were below the detection level. Thus, our test conditions for
protective immunity may be stricter. Thus, it is still valid that IN/IM delivery may be more effective if broad protective immunity is needed. We also found that the IM/IN and IN/IN vaccination regimens conferred similar levels of protection compared to the IN/IM vaccination regimen (data not shown). The IN/IN vaccination regimen was the most effective at inducing mucosal T cell-mediated and systemic immune responses as well as mucosal humoral responses. Immunity elicited following the IM/IN vaccine regimen was comparable to the responses elicited following the IN/IM vaccine regimen. Accordingly, mucosal immunization appeared to be a suitable route for the induction of both mucosal and systemic immune responses and should be considered in the design of vaccines requiring both types of immune responses.

Interestingly, the rAdv-AI vaccine did not elicit a neutralizing humoral response against an H5N2 AI virus but provide complete protection against a lethal H5N2 Al virus challenge suggesting that T cell and M2eX-specific antibody responses played a critical role in the conferring protection. Since our rAdv-AI vaccine encodes matrix as well as HA, a possible mechanism resulting in protection against H5N2 AI virus challenge may involve M1- and HA-specific T cell-mediated responses recognizing conserved regions. This is consistent with other studies that demonstrated that M1-specific CTLs and M2eX-specific antibodies were cross-protective against different human influenza A virus subtypes (Fan et al., 2004; Jameson, Cruz, and Ennis, 1998; Liu et al., 2004; McMichael et al., 1983; Zharikova et al., 2005). In addition, two recent studies have demonstrated that protection elicited following vaccination with an rAdv-based influenza virus HA-expressing vaccine against an AI virus challenge occurred in the absence of neutralizing humoral immunity (Gao et al., 2006; Hoelscher et al., 2006; Hoelscher et al., 2007). Gao et al. have shown the presence of heterotypic H5N1 protection in the absence of neutralizing humoral responses and partial protection from homologous H5N1 challenge following vaccination with a rAdv-based HA2 vaccine (the HA2 region is known not to induce neutralizing humoral responses). Similarly, Hoelscher et al. have shown that immunized mice had neutralizing anti-HI antibodies below the levels of assay detection but that these mice were still completely protected, i.e., no loss of body weight and 100% survival. Our data also showed that mice that received rAdv-Al did not develop HI titers against H5N2 challenge virus even though amino acid sequence identity of the HA sequence between the vaccine and challenge virus was 93%. However, mice that received the rAdv-Al vaccine still were completely protected against a lethal H5N2 challenge. Taken together, these data suggested that protection against an H5N2 lethal challenge in the absence of HI titers can be facilitated by other mechanisms, namely, cell-mediated immunity or non-hemagglutinating antibodies, possibly by antibody-dependent cell-mediated cytotoxicity.

Although adenoviral vectors have proven their potency in numerous previous studies, pre-existing immunity to human adenoviruses could be a potential problem in the adenoviral vector-based vaccine field. In a recent report, however, an influenza vaccine trial found that there was no correlation between the levels of pre-existing Ad5-neutralizing antibody titers and the potency of the intranasally administered Ad5-based vaccine (Van Kampen et al., 2005). In addition, data from a phase I/II HIV vaccine trial sponsored by NIH Vaccine Research Center show that while pre-existing immunity to Ad5 does impact the performance of the Ad5-based HIV vaccine, this limitation can be overcome by increasing the dose of the vaccine (Catanzaro et al., 2006). Additionally, there is experimental evidence that vaccination through alternate routes of administration (such as oral or intranasal) (Appaiahgari et al., 2006; Xiang et al., 2003) and alternate vectors derived from rare human adenovirus or non-human adenovirus can overcome pre-existing vector immunity.

In this study, we demonstrated several advantages of the mucosal prime-systemic boost regimen using an adenoviral vector-based vaccine in terms of eliciting both mucosal and systemic immunity since this approach conferred protection against an antigenically distinct AI virus challenge. These data suggest that this strategy is a promising approach for the development of novel vaccines capable of preventing and controlling highly pathogenic H5N1 avian influenza virus infections.

Materials and methods

Peptides

M1_{58–66} (GILGFVFTL), HA_{149–163} (SACYQYKKSSFRNV), M2eX-H5N1 (SLITEVTPTRNEWRES5DSS), M2eX-H5N2 (SLITEVTPTRNG-WECKCS DSS) and overlapping peptides spanning the full length of M1 were synthesized by PEPTRON, Inc. (www.peptron.com).
Gene synthesis and replication-defective recombinant adenovirus construction

The codon-optimized tpa-M2eX3–HA–hCD40L and the tpa-M1–M2 fusion genes were synthesized by Bio S and T, Inc. (www.Biost.com). The signal sequence of TPA (tissue plasminogen activator) was fused to the 5' end of each of the fusion genes. Three copies of M2eX (2–24 amino acids of M2) sequences were linked by a Gly-Ser-Gly linker (SLTENVTPRNEWSSRDSDGGSLTENVTPRNEWSSRDSDGGSLTENVTPRNEWSSRDSDGSG) and the extracellular domain regions of the HA gene from the HPAI H5N1 human isolate, A/Hong Kong/213/2003 (HK/213/03), and the human CD40L were used. The HA gene sequence signal was also deleted. The HA gene human isolate M1 and M2 genes from A/Vietnam/1203/2004 (VN/1203/04) were linked by Gly-Ser-Gly-Ser-Gly-Ser-Gly-Ser-Gly-Ser-Ser. The two genes were linked (fused) by IRES of encephalomyocarditis virus and the final tPA–M2eX–HA–hCD40L–IRES–tpa–M1–M2 gene was cloned into a shuttle vector with tetracycline-responsive elements. As shown in Fig. 1A, an E1/E3-deleted replication-defective adenoviral vector (Qbiogene, Inc.) expressing the multi-antigens (rAdv-AI) was generated by recombination in BJS183, propagated in 293 Trex cells (Invitrogen Co.) and purified by double CsCl (discontinuous and continuous) centrifugations and dialysis according to the manufacturer’s instructions. The concentration of the resultant recombinant adenoviral vaccine was determined by the tissue culture infectious dose 50 (TCID\textsubscript{50}).

Western blot analysis

To verify the expression of the two fusion proteins (the tPA–M2eX3–HA–hCD40L and the tpa–M1–M2) QBI293 cells (Qbiogene, Inc.) which contain the E1A and E1B Ad5 viral genes were infected with rAdv-Al or rAdv-EGFP at a multiplicity of infection (MOI) of 10. At 48 h post-infection, cells were harvested, resuspended in phosphate buffered saline (PBS), mixed with an equal volume of 2× sodium dodecyl sulfate (SDS) sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.01% bromophenol blue and 10% \( \beta \)-mercaptoethanol), boiled for 10 min, separated on an 8% SDS–polyacrylamide gel electrophoresis (PAGE) gel and transferred onto nitrocellulose membranes. Detection of expressed proteins, tPA–M2eX3–HA–hCD40L and tpa–M1–M2, was confirmed by probing the blots with anti-human CD40 ligand (Santa Cruz Biotechnology, Inc.) or anti-M1 (Santa Cruz Biotechnology, Inc.) antibodies, respectively (Fig. 1B).

Immunization and challenge

Six- to 8-week-old HLA.A2 transgenic (Tg) or Balb/c mice were primed IM or intranasally IN and boosted IM at 2-week intervals with 5 × 10\(^6\) plaque forming units (pfu) (HLA.A2 Tg) or 1 × 10\(^6\) pfu (Balb/c) of rAdv-Al under anesthesia. For IM immunization, mice were injected with 50 μl of rAdv-Al into each quadriceps muscle. IN immunizations were performed by lightly anesthetizing the mice with a 200 μl intraperitoneal (IP) injection of ketamine (100 mg/kg of body weight; Yuhan Co.) and xylazine hydrochloride (10 mg/kg of bodyweight; Bayer) in PBS, and then applying 30 μl of rAdv-Al into each nostril via a micropipette. Sera were collected at 2 and 6 weeks after priming and some mice were sacrificed for mucosal and systemic immunological analysis at 6 weeks after priming. Lung washes were collected by repeated flushing using an intra-tracheal injection of 1 ml PBS at the time of sacrifice. At 4 weeks after the final vaccination, Balb/c mice were anesthetized and challenged IN with a 50% lethal dose (LD\textsubscript{50}) of antigenically heterologous avian influenza (AI) virus strain (A/aquatic bird/Korea/w81/2005, H5N2) or homologous Al virus strain (A/environment/Korea/W150/2006, H5N1). The challenge viruses were kindly provided by Dr. Young Ki Choi of Chungbuk National University.

Anti-M2eX and anti-HA antibody ELISAs

Ninety-six-well immunoplates (Nunc) were coated with 50 μl of either M2eX–H5N1 (2 μg/ml), M2eX–H5N2 (2 μg/ml) or HA protein (0.5 μg/ml) diluted in PBS. After overnight incubation at 4 °C, the plates were blocked with 5% non-fat milk in PBST (PBS–0.05% Tween-20; Sigma-Aldrich Co.) for 1 h at room temperature (RT). Sera and lung washes were serially diluted in 5% non-fat milk in PBST. Sera and lung wash dilutions were added to the coated plates and incubated overnight at 4 °C. Plates were then washed five times with PBST and incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (1:3000; Southern Biotech) or anti-mouse IgA (1:3000; Santa Cruz Biotechnology, Inc.) for 1 h at RT. Plates were washed seven times and 50 μl of TMB substrate (Sigma-Aldrich Co.) was added to each well. The reaction was stopped after 30 min by the addition of 50 μl of 2N H\textsubscript{2}SO\textsubscript{4}. The optical density at 450 nm (OD 450) was measured with an ELISA plate reader (Bio-Tek instruments). ELISA endpoint titers were expressed as the highest dilution that yielded an optical density greater than the mean plus three times standard deviation of a similarly-diluted negative control sample.

Intracellular IFN-γ staining assay

To determine the number of influenza-specific IFN-γ-producing cells, cells from spleens and lungs were washed, stimulated with the indicated peptides (10 μg/ml) for 6 h at 37 °C in the presence of brefeldin A, washed, surface-stained with anti-CD8 antibody at 4 °C and fixed/permeabilized using the fix/perm solution (FACSlyze, BD Scientific) diluted to 2× with distilled water, 0.05% Tween 20 (Sigma-Aldrich, Co.). Cells were washed once and incubated at room temperature with PE-conjugated antibodies specific for IFN-γ. Cells were washed and resuspended in PBS containing 1% paraformaldehyde. Dead cells were excluded on the basis of forward and side light scatter using a CellQuest (BD Biosciences) flow cytometer. All fluorochrome-conjugated monoclonal Abs were purchased from BD PharMingen.

IFN-γ ELISPOT assay

IFN-γ ELISPOT assays were performed as previously described (Lee et al., 2005). The indicated peptide (10 μg/ml) was used as a stimulator. IFN-γ ELISPOT responses to media controls were subtracted from the responses to the stimulators. Results are expressed as the average number of IFN-γ-secreting cells (SFCs) per indicated splenocytes.

HI assay

The HI assay was performed as described previously (Lee et al., 2009). Briefly, H5N1 virus (A/environment/Korea/W150/2006) or H5N2 virus (A/aquatic bird/Korea/w81/2005) was diluted to contain 4 agglutinating U in PBS. The diluted viruses were incubated with serial twofold dilutions of receptor-destroying enzyme-treated serum samples, starting with a 1:20 dilution at room temperature for 30 min. The antigen–antibody mixtures were tested for HA activity by the addition of 0.5% chicken red blood cells to determine the HI titers. The results are the geometric mean titers of positive sera (≥20).

Statistical analysis

All data are representative of at least more than two different experiments. To measure statistical differences between groups, a Student’s t-test was used. The difference in survival rates between