RESEARCH REPORT
SWINE HEALTH

Title: Development of a live attenuated vaccine against swine influenza by reverse genetics - **NPB #08-003,**

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Industry Summary:

Influenza A virus causes significant morbidity in swine, resulting in a substantial economic burden. Swine influenza virus also poses threat to public health, a prime example is the recent emerged swine origin H1N1 influenza virus has infected and transmitted in humans. Vaccination is the primary method for the prevention of influenza disease. Live virus vaccines provide superior immunity to that induced by conventional inactivated vaccines. Currently, there are no live vaccines available for swine influenza. The objective of this project is to develop live virus vaccine against swine influenza. We have generated two viruses which are weakened in replication. The weakened form of virus was able to grow in the tissue culture system, thus facilitating production of this virus. When tested in pigs, this virus could infect pigs without causing disease. Hence, this virus is a great candidate to serve as live vaccine. Immune response to this virus and immune protection to other swine influenza virus challenge have been tested.

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Scientific Abstract:

Influenza A viruses cause significant morbidity in swine, resulting in a substantial economic burden. Swine influenza virus (SIV) infection also poses important human public health concerns. Vaccination is the primary method for the prevention of influenza infection. Previously, we generated two elastase-dependent mutant SIVs derived from A/Sw/Saskatchewan/18789/02(H1N1): A/Sw/Sk-R345V (R345V) and A/Sw/Sk-R345A (R345A). These two viruses are highly attenuated in pigs, making them good candidates for a live vaccine. In this study, the immunogenicity and the ability of these candidates to protect against SIV infection were evaluated in pigs. We report that intratracheally administrated R345V and R345A induced antigen-specific humoral and cell-mediated immunity characterized by increased production of IgG and IgA antibodies in the serum and in bronchoalveolar lavage fluid, high levels of hemagglutination inhibition titer in serum, an enhanced level of lymphocyte proliferation and higher numbers of IFN-γ secreting cells at the site of infection. Based on the immunogenicity results, the R345V virus was further tested in a protection trial in which pigs were vaccinated twice with R345V and then challenged with homologous A/Sw/Saskatchewan/18789/02, H1N1 antigenic variant A/Sw/Indiana/1726/88 or heterologous subtypic H3N2 A/Sw/Texas/4199-2/9/98. Our data showed that two vaccinations with R345V provided pigs with complete protection from homologous H1N1 SIV infection and partial protection from heterologous subtypic H3N2 SIV infection. This protection was characterized by significantly reduced macroscopic and microscopic lung lesions, lower virus titers from the respiratory tract and lower levels of pro-inflammatory cytokines. Thus, elastase-dependent SIV mutants can be used as live vaccines against swine influenza in pigs.

Introduction:

Swine influenza virus (SIV) is the causative pathogen of swine influenza, a highly contagious, acute respiratory viral disease of swine. The mortality of SIV infected pigs is usually low, although morbidity may approach 100%. Swine influenza is characterized by sudden onset, coughing, respiratory distress, weight loss, fever, nasal discharge and rapid recovery . SIV is a member of the influenza virus A genus in the *Orthomyxoviridae* family, and the virus has a genome consisting of eight segments of negative-sense single-stranded RNA . Epithelial cells in the swine respiratory tract have receptors for both avian and mammalian influenza viruses ; thus, pigs could potentially serve as “mixing vessels” for the generation of new reassortant strains of influenza A that have pandemic capacity. There are a number of reports in which the direct transmission of influenza viruses from pigs to humans has been documented , and several of these cases have resulted in human fatalities . Consequently, effective control of SIV would be beneficial to both humans and animals.

Until 1998, classical H1N1 SIVs were the predominant isolates from pigs in the U.S and Canada . In 1997–1998, a dramatic change in the epidemiologic pattern of SIV began. Serological studies conducted by Olsen and colleagues in 1997-1998 detected a significant increase in H3 seropositive individuals, and H3N2 SIVs were isolated from pigs in both the U.S. and Canada . Furthermore, reassortment between H3N2 viruses and classical H1N1 SIV resulted in the appearance of H1N2 reassortant viruses . In addition to the isolation of H4N6 viruses, which are of duck origin, in pigs in Canada , wholly avian viruses of the H3N3 and H1N1 subtypes have also been isolated from Canadian pigs . In general, three major SIV subtypes exist - H1N1, H1N2 and H3N2 – each of which has multiple genetic and antigenic variants circulating in North American swine populations . The increased incidence of avian-like or human-like SIV reassortants raises concerns for public health and requires research devoted to the development of cross-protective SIV vaccines.

Currently available swine influenza vaccines are based on inactivated whole virus of the H1N1 and H3N2 subtypes. Application of these vaccines reduces the severity of disease but does not provide consistent protection from infection . In contrast to killed vaccines that are administered intramuscularly, intranasally administered live attenuated influenza vaccines (LAIV) induce an immune response at the site of natural infection. Therefore, an LAIV has the potential to induce broad humoral and cellular immune responses that could provide protection against antigenically different influenza viruses. LAIV against influenza viruses based on attenuation of the virus by cold adaptation are available for humans and horses . However, to date, no SIV LAIV are commercially available for use in swine in North America. Recent studies by Richt *et al.* showed that a mutant SIV with a truncated NS1 protein was highly attenuated in pigs . In addition, this SIV/NS1 LAIV was capable of stimulating a protective immune response against homologous SIVs and a partial protection against heterologous subtypic wild type (WT) SIVs . Stech *et al*. demonstrated that the conversion of a conserved

cleavage site in the influenza HA protein from a trypsin-sensitive site to an elastase-sensitive site results in *in vivo* attenuation of the influenza virus in mouse models . Furthermore, these elastase-dependent LAIV were able to induce protective systemic and mucosal immune responses. Recently, we showed that two elastase-dependent SIV viruses derived from A/Sw/Saskatchewan/18789/02 (SIV/Sk02), R345V and R345A, are attenuated in their natural host, pigs . In the current study, we addressed the immunogenic and cross-protective abilities of these mutants.

Objectives:

Objective 1: Generate a mutant swine influenza virus with a modified cleavage site within its HA, which depends on proteolytic activation by elastase. Using plasmid-based influenza reverse genetics, we will generate a recombinant virus with its HA gene manipulated so that the original trypsin-specific cleavage site Arg-Gly is replaced with the elastase-sensitive site Val-Gly. This virus has the potential to serve as a live attenuated vaccine. Where it can infect pigs and induce an immune response but is not able to cause diseases due to the inability of the virus to spread in the pigs.

Objective 2: Characterize the mutant virus. We will characterize the mutant virus *in vitro* and *in vivo*. The growth properties, genetic stability will be characterized in tissue culture. The pathogenicity of the mutant virus will be analyzed in pigs. This will tell us whether the mutant virus is stable and attenuated in pigs or not.

Objective 3: Evaluate the protective effect of attenuated live vaccine against challenge with various influenza viruses in pigs. We will investigate the potential of the mutant virus to serve as a live vaccine against swine influenza infection. Immune response to the live mutant virus will be analyzed after pigs are inoculated with the mutant virus. The protective effects of vaccination will be evaluated by challenging pigs with wild type virus A/Swine/SK/18789/02 and A/Swine/Indiana/1726/88.

Materials & Methods:

Cells and viruses. Madin-Darby canine kidney (MDCK) cells were cultured in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). The H1N1 mutant viruses SIV/Sk-R345V (R345V) and SIV/Sk-R345A (R345A) used in our study were generated as previously described . These two viruses were grown in MDCK cells in the presence of 0.5 µg/ml human neutrophil elastase (Serva Electrophoresis GmbH). Stocks of the R345V and R345A viruses reached titers of 1.7×10^8 and 2.0×10^8 PFU/ml after purification, respectively. The other SIV isolates used in the study were H1N1 A/Sw/Saskatchewan/18789/02 (SIV/Sk02), H1N1 A/Sw/Indiana/1726/88 (SIV/Ind88) and H3N2 A/Sw/Texas/4199-2/98 (SIV/Tx98). These viruses were propagated at 37ºC in the allantoic cavities of 11-day-old embryonated chicken eggs. Stock virus titers were $1.4x10^7$, $1.33x10^6$, and $1.7x10^7$ PFU/ml, respectively. Titers for all viruses used in the study were determined on MDCK cells by a plaque assay, as described previously .

Experimental design and clinical sampling. For the purpose of this study, we designed two animal trials. In the first trial (Table 1.), thirty-five five-week old SIV-negative landrace crossbred pigs were obtained from Prairie Swine Center (Floral, Saskatchewan, Canada). Pigs were randomly selected and divided into five groups with seven pigs per group. At six weeks of age (day 0), the pigs in group 1 were mock vaccinated with 4 ml of MEM, whereas the pigs in groups 2 and 4 received $4x10^6$ PFU of R345V and the pigs in groups 3 and 5 received $4x10^6$ PFU of R345A. The viruses or MEM were administered intratracheally (IT), assuring consistent infection. Three weeks after inoculation (day 21), the pigs in groups 4 and 5 received a second dose of vaccine containing the same amount of virus, whereas the pigs in groups 2 and 3 and three pigs from the control group were euthanized by intravenous administration of Euthanyl (Sodium pentobarbital 25 mg/ml) and subjected to necropsy. Ten days after the second vaccination (day 31), the pigs in groups 4 and 5 and the remaining animals in group 1 were euthanized and subjected to necropsy. At necropsy, the lungs were evaluated and scored for the presence of SIV-induced lesions, the tracheo-bronchial lymph nodes were extracted and broncho-alveolar fluid (BALF) was collected by lavaging the lungs with 20 ml of phosphate-buffered saline (PBS, 0.137 M NaCl, 2.7 mM KCl, 8 mM $NaHPO_4$, 1.47 mM KH_2PO_4, pH 7.3). Prior to the administration of tests for the presence of virus, the BALF samples were incubated at 37°C for 1 h with an equal amount of 10 mM dithiothreitol (DTT) to disrupt any mucus. Serum samples and nasal swabs were collected before and after each vaccination and at necropsy.

In the second trial (Table 2.), forty-nine four week old SIV negative pigs were assigned to seven groups with seven animals per group. The animals in groups 1 to 4 were mock vaccinated IT with 4 ml of MEM, whereas the pigs in groups 5 to 7 were vaccinated IT with $4x10^6$ PFU of R345V. Three weeks after the first vaccination, animals in groups 1 to 4 received MEM, whereas the vaccinated groups (5 to 7) received a second dose of $4x10^6$ PFU of R345V. Ten days after the second vaccination (day 31), the pigs in all groups were challenged IT with $8x10^5$ PFU of homologous or heterologous subtypic SIVs (Table 2). After the challenge, the pigs were monitored for the presence of clinical signs characteristic of SIV infection and then sacrificed on day 5 post challenge. Tissue samples from the right apical, cardiac and diaphragmatic lung lobes were taken for virus isolation and histopathology examination. Serum samples were collected prior to and after the second vaccination and after viral challenge. BALF samples were obtained at necropsy. All animal experiments were conducted at the Vaccine and Infectious Disease Organization at the University of Saskatchewan in accordance with the ethical guidelines of the University of Saskatchewan and the Canadian Council of Animal Care.

Isolation of lymphocytes from tracheo-bronchial lymph nodes. Tracheo-bronchial lymph nodes were dissected *in toto* at necropsy and stored on ice in AIM-V medium containing L-glutamine, 50 mg/ml streptomycin sulfate and 10 mg/ml gentamicin sulfate (Invitrogen, Burlington ON, Canada) supplemented with 10% FBS. Lymph node cells (LNCs) were isolated by finely mincing tissues with a scalpel, filtering the cell suspension through 40-µm nylon cell strainers (Becton Dickinson Labware, Franklin Lakes, NJ, USA) and washing the cells with AIM-V medium supplemented with 2% FBS. Prior to seeding, the cells were resuspended in AIM-V 2% FBS medium containing 50 µM of β-mercaptanol and were counted.

Detection of SIV-specific IFN-γ secreting cells by ELISPOT. Nitrocellulose UNIFILTER 350 microtiter plates (Whatman, Florham Park, NJ) were coated with mouse anti-porcine IFN-γ monoclonal antibodies (Endogen, Rockford, IL, USA) in coating buffer at a concentration of 5 µg/ml for 16 h at 4°C. The wells were washed and LNCs were seeded directly at $1x10^6$ cells/well in a final volume of 200 µl/well of AIM-V containing 2% FBS. LNCs were stimulated for 10 h at 37°C with 25 µg/ml of purified UV-inactivated SIV/Sk02 virus, 5 µg/ml of Con A (Sigma-Aldrich) or medium only. After stimulation, the plates were washed five times with PBST (PBS with 0.05% Tween 20) and incubated with rabbit anti-porcine IFN-γ (Endogen, Rockford, IL, USA) at a concentration of 2 µg/ml for 16 h at 4°C. The plates were then washed and incubated with biotinylated goat anti-rabbit IgG (H+L) (DiAMED, South San Francisco, CA, USA) at a dilution of 1:5000 for 2 h at room temperature. The wells were washed five times and incubated with streptavidin alkaline solution (Jackson ImmunoResearch, West Grove, PA, USA) at a dilution of 1:5000 for 1.5 h at room temperature. After being washed eight times with double distilled water (ddH_2O), 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (NBT/BCIP) (Sigma-Aldrich) insoluble alkaline substrate solution was added (100 µl/well), and the plates were incubated for 5 min. The plates were then washed again with ddH_2O and left to dry overnight at room temperature. Spots were counted manually under an inverted light microscope. The number of spots observed in wells stimulated only with medium was counted and subtracted as background. Data were reported as the number of IFN-γ secreting cells per 10^6 somatic cells.

Lymphocyte proliferative response (LPR) assay. LNCs were resuspended in AIM-V culture medium at a concentration of 2.5×10^5 cells/well and stimulated for 72 h at 37°C with 25 µg/ml of SIV/Sk02 antigen, 5 µg/ml of Con A or medium. Six hrs prior to the 72-h incubation, LNCs were pulsed with 0.4 µCi [5'-^{3}H]thymidine (Amersham Pharmacia, Piscataway, NJ, USA) as previously described . The cells were then harvested using standard liquid scintillation protocols, and the uptake of ^{3}H-thymidine was assessed with a beta counter (Topcount, Packard Instrument Company, Meriden, CT). The lymphocyte proliferative response (LPR) was calculated as the mean counts per minute (c.p.m.) of triplicate cultures and expressed as a stimulation index (c.p.m. in the presence of stimulus/c.p.m. in the absence of stimulus).

ELISA for antigen-specific IgG and IgA antibodies and hemagglutination inhibition (HI) assay. For antigen-specific ELISAs, 2.5 µg/ml of purified UV-inactivated SIV/Sk02, SIV/Ind88 and SIV/Tx98 antigens were coated onto 96-well Immulon-2 plates (Dynex Technology INC, Chantilly, VA, USA), which were then incubated overnight at 4°C. The plates were blocked for 1 h at room temperature with 100 µl of 1% skim milk in TBST (0.1 M Tris, 0.17 M NaCl, 0.05% Tween 20) and washed four times with PBST. Serum, nasal and BALF samples were added (100 µl/well) in triplicate at the appropriate dilutions, and the plates were incubated for 1.5 h at room temperature. Samples of previously defined positive control sera and the appropriate negative controls were run on each plate. Subsequently, the plates were incubated with mouse anti-porcine IgA monoclonal antibody (AbD Serotec) or alkaline phosphatase labeled goat anti-porcine IgG (KPL, Gaithersburg, MD, USA). The IgA ELISAs were developed by the addition of biotinylated goat anti-mouse IgG (H+L)

antibodies (DiAMED, South San Francisco, CA, USA) and streptavidin alkaline phosphatase solution (Jackson ImmunoResearch, West Grove, PA, USA). After being washed with PBS-T, the IgG and IgA ELISAs were developed by the addition of PNPP substrate [10 mg/ml p-nitro-phenyl phosphate di(tris) salt crystalline (Sigma-Aldrich), 1% diethanolamine (Sigma-Aldrich), 0.5 mg/ml $MgCl_2$, pH 9.8]. The optical density of the reaction product was measured at 405 nm (a 490 nm reference filter used to detect background, which was subtracted from the measurement reading) on a microplate reader (Molecular Devices, SpectraMax Plus 384). The titer of sample was defined as the highest dilution at which the OD of that sample was higher than the defined cutoff (the mean OD of a known negative sample plus 2 times standard deviation).

To measure the hemagglutination inhibition (HI) titers, serum samples were treated overnight with receptor-destroying enzyme (Cholera filtrate-C8772, Sigma Aldrich) at 37°C to eliminate non-specific HI factors. The viruses used in the HI assays were SIV/Sk02, SIV/Ind88 and SIV/Tx98. HI assays were performed as described elsewhere .

Histopathology evaluation. Necropsy, macroscopic examination of lungs and tissue processing for virus isolation were performed as described previously . Tissue sections of lungs were stained with hematoxylin and eosin and examined microscopically for bronchiolar epithelial changes and peribronchiolar inflammation. Lesion severity was scored by the distribution or by the extent of lesions within the sections examined as follows: 0: no visible changes; 1: mild focal or multifocal change; 2: moderate multifocal change; 3: moderate diffuse change; 4: severe diffuse change. Two independent pathologists blinded for the experimental groups scored all slides.

ELISA for IFN-α, IL-1 and IL-6 cytokines. In order to detect the IFN-α, IL-1 and IL-6 cytokines, polystyrene microtiter plates (Immulon 2; Dynex Technology INC, Chantilly, USA) were coated with the following capture antibodies: mouse anti-recombinant porcine IFN-α clone K9 (R&D # 27100-1), goat anti-recombinant porcine interleukin 6 (R&D AF 686), or mouse anti-recombinant porcine IL-1β (R&D MAB 6811) at a concentration of 1 µg/ml in coating buffer. Recombinant porcine IFN-α (Endogen rPo IFN-α; 2000 pg/ml), recombinant porcine IL-6 (R&D 686-PI-025 rPoIL-6; 5000 pg/ml) and recombinant porcine IL-1β (R&D 681-PI-010 rPo IL-1β; 10,000 pg/ml) were used as standards. Standards and homogenized lung samples were diluted in TBST-0.1% skim milk and added to the coated plates. After overnight incubation at 4°C, the detection antibodies biotinylated mouse anti-recombinant porcine IFN-α clone F17 (R&D #27105-1; 1/1000), biotinylated goat anti-recombinant porcine IL-6 (R&D BAF686; 0.2 µg/ml), and biotinylated goat anti-recombinant porcine IL-1β (R&D BAF681; 0.25 µg/ml) were added to the appropriate wells. Finally, the plates were developed, and the responses were measured as described above. Sample concentrations were calculated using Softmax Pro 5.2 version software (Molecular Devices).

Statistical analysis. Statistical analysis was performed using GraphPad Prism5 (San Diego, CA, USA) and Statistix7 (Tallahassee, FL, USA) software. Differences between the means for the two groups (vaccinated vs. unvaccinated) in each assay were determined using the Mann-Whitney nonparametric t-test. To compare the two vaccines, data from the LPR and ELISPOT assays and for the serum antibodies were transformed and one-way ANOVA for RANKSUM was applied. If the median values of at least one group differed from the others at a significance level of $P < 0.05$, the difference between the groups was considered statistically significant.

Results:

The objectives 1 and 2 have been completed in 2008 and the results were reported to the National Pork Board. Here we will report the results on objective 3.

Live attenuated viruses induced cell mediated immune responses. To determine whether the mutant viruses were immunogenic, SIV negative pigs were divided into 5 groups and intratracheally vaccinated with MEM, R345V, or R345A (Table 1).

TABLE 1. Assignment of pigs to groups for immunogenicity study

Group (n = 7)	Treatment on day:		
	0	21	31
1	MEM, 4 ml	Necropsy (n = 3)	Necropsy
2	R345V, 4 × 10^6 PFU	Necropsy	(n = 4)
3	R345A, 4 × 10^6 PFU	Necropsy	
4	R345V, 4 × 10^6 PFU	R345V, 4 × 10^6 PFU	Necropsy
5	R345A, 4 × 10^6 PFU	R345A, 4 × 10^6 PFU	Necropsy

Two groups of pigs received one immunization and were euthanized on day 21, whereas two groups of pigs received a second immunization on day 21 and were euthanized on day 31. Animals in the control group received MEM and were euthanized on either day 21 or day 31. At necropsy, tracheo-bronchial lymph nodes and BALF were collected.

To assess the ability of R345V or R345A viruses to induce cell mediated immune responses after vaccination, LNCs were isolated from vaccinated and control pigs, and antigen-specific responses were measured by IFN-γ ELISPOT and LPR assays. As shown in Fig. 1, both R345V and R345A were able to induce a significant number of antigen specific IFN-γ secreting cells after only one vaccination. A second vaccination with the same dose of the previously administered vaccines resulted in a further increase in the number of local IFN-γ secreting cells. This increase was significantly higher than the increase for the groups that received a single vaccination (p=0.002 for R345V and p=0.05 for R345A) (Figure 1A). To further measure cell-mediated responses, we conducted an LPR assay in which we assayed the antigen-specific proliferation of LNCs. Consistent with the IFN-γ ELISPOT results, the lymph node cells proliferated in response to specific antigens after the first vaccination with the median stimulation indices of 9.57 (R345V) and 38.04 (R345A). Moreover, a second vaccination resulted in a significant increase in the stimulation index (P=0.02 and P=0.05 for R345V and R345A, respectively) (Fig. 1B). Statistical analysis showed that there was a significant difference in cell mediated immune responses between the first and second vaccinations in both groups vaccinated with R345V and R345A. However, there was no statistically significant difference in the cell-mediated immune responses between the two vaccines using these two assays.

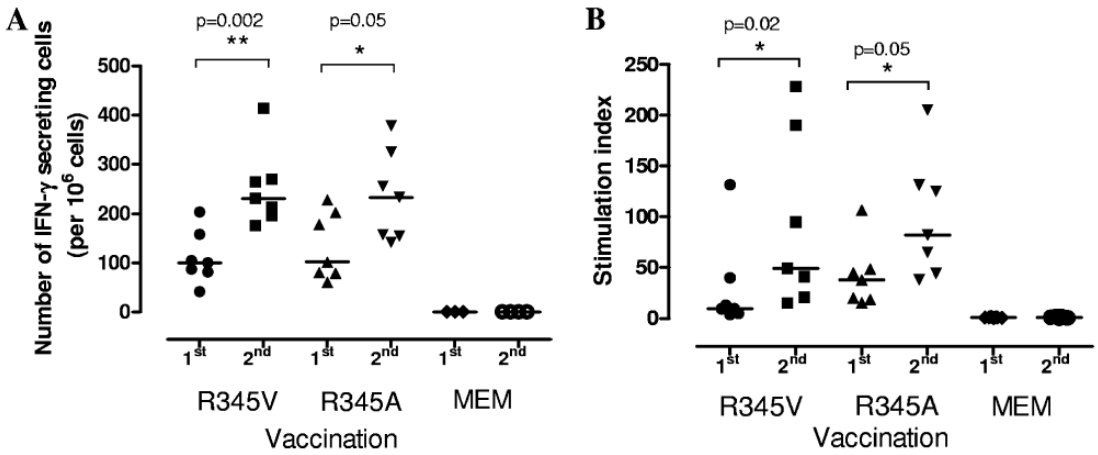

Fig.1. Cell mediated immune responses induced by mutant viruses.

Live attenuated viruses induced humoral immune responses. Sera were collected prior to the first vaccination, 21 days after the first vaccination and 10 days after the second vaccination. SIV specific antibodies were first examined by an HI assay. All pigs were negative for H1N1 SIV antibodies (HI <1:10) at the start of the experiment, and the seven unvaccinated pigs remained seronegative throughout the experiment. After the first vaccination with R345V or R345A, all pigs had low levels of antibodies against the parental H1N1 strain (HI = 20). The second vaccination resulted in a significant increase in the HI titer (HI = 160 for R345V and HI = 80 for R345A). Pigs vaccinated with R345V had significantly higher HI titers than the R345A-vaccinated pigs (P = 0.04) (Figure 2A).

Antigen-specific serum IgG and IgA levels were measured on days 21 and 31 by ELISA using inactivated H1N1 SIV/Sk02 as the capture antigen. As seen in Fig. 2B, both the R345V and the R345A viruses induced a moderate level of IgG after the first vaccination (titer = 321 for R345V and 164 for R345A). The second vaccination led to a considerable increase in the level of IgG (titer = 1611 for R345V and 955 for R345A). Similarly, the first vaccination was able to induce a detectable level of IgA (titer = 181.8 for R345V and 84.5 for R345A), and the second vaccination led to an increase in the production of IgA (titer = 746.3 for R345V and 628.7 for R345A) (Fig. 2C). At each time point, serum IgG titers were approximately two to three times higher than the corresponding IgA titers (Figure 2B and 2C).

To assess the presence of IgG and IgA antibodies specific for H1N1 SIV/Sk02 at mucosal surfaces in the upper and lower respiratory tract, nasal swabs and BALF samples from pigs in all groups were tested by ELISA. As shown in Figure 2D and 2E, the antigen-specific IgA and IgG titers in the nasal swabs remained low (<10) after the first vaccination. After the second vaccination, the IgA titer increased significantly to median titers of 80 (R345V) and 60 (R345A), whereas the IgG titers rose to just above 10.

Similarly, IgA was the dominant antibody subtype in the lower respiratory tract. In the BALF, the IgA titers were significantly higher after the second immunization compared with the titers after the first vaccination (median IgA titers = 29.0 vs 2450, $p = 0.0006$ for R345V; and 52.2 vs 2279, $p = 0.001$ for R345A) (Fig. 2F). The IgG level in the BALF also increased after the second immunization (median IgG titer = 2.3 vs 108, $p = 0.02$ for R345V; and 3.0 vs 112, $p = 0.02$ for R345A) (Fig. 2G). However, the magnitude of the increase was less than that of IgA.

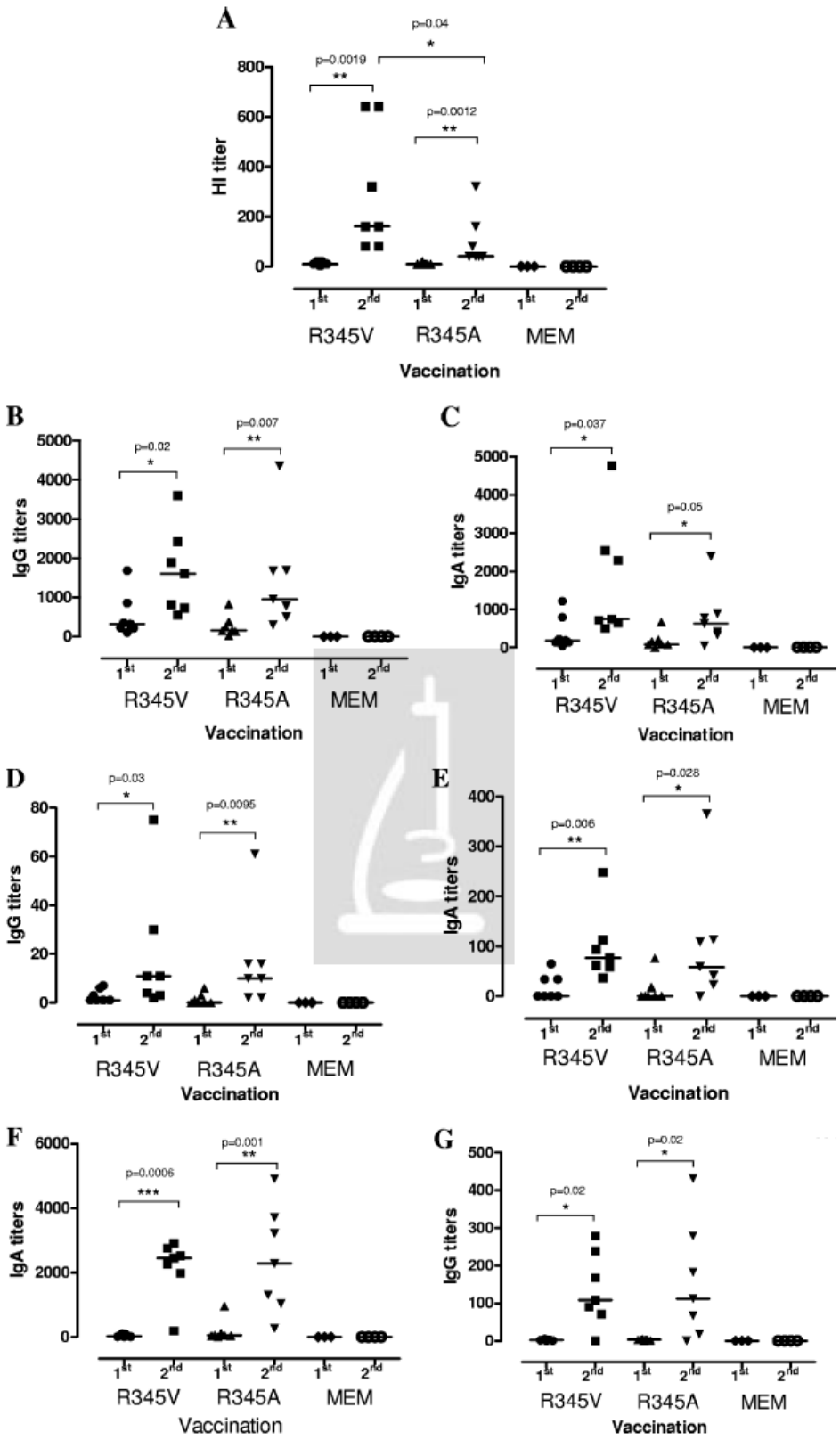

FIG. 2. SIV-specific antibodies induced by the mutant viruses.

Live attenuated virus induced cross-reactive antibodies. All pigs were negative for H1N1 and H3N2 antibodies prior to the start of the experiment as determined by an HI assay (HI titer <10). The pigs vaccinated with R345V seroconverted with respect to SIV/Ind88 H1N1 antigens during the time of study according to the HI assay (Figure 3A). The median HI titer for the SIV/Ind88 was 1:40. However, there were no detectable HI antibodies against SIV/Tx98 H3N2 after two vaccinations with R345V.

The presence of cross-reactive antibodies in the serum and the BALF samples was determined using purified UV-inactivated SIV/Ind88 and SIV/Tx98 as capture antigens in ELISA assays. Similarly to HI titers, high levels of serum IgG specific for H1N1 SIV/Ind88 were detected. Although H3N2 cross-reactive serum IgG was detectable, the level was ten times lower than IgG specific for the H1N1 antigen (Fig. 3B). In the lower respiratory mucosa (BALF), R345V vaccinated pigs had a significantly higher level of IgA antibodies and moderate levels of IgG antibodies that cross-reacted with the homologous antigenic variant H1N1 SIV/Idn88 and the heterologous subtypic H3N2 SIV/Tx98 (Fig. 3C).

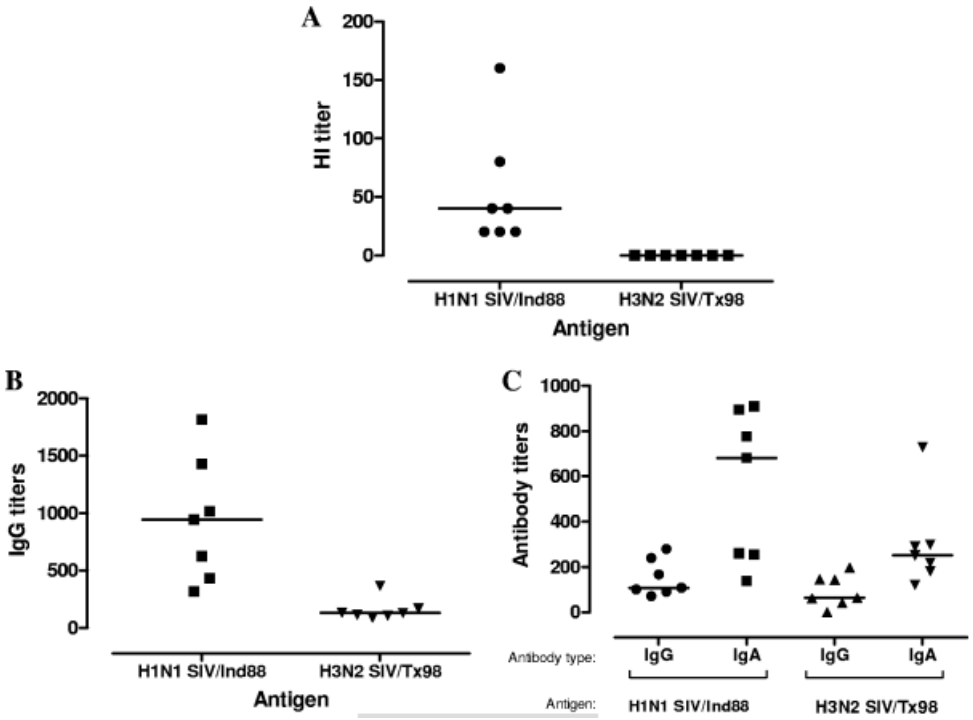

FIG. 3. Cross-reactive antibodies induced by the R345V virus.

Vaccination reduced macroscopic lung lesions after SIV challenge. The results described above indicated that (i) both viruses were immunogenic and (ii) two vaccinations were required to induce significantly high levels of immune responses. Considering that R345V exhibited slightly more consistent immune responses and induced stronger immune responses than did R345A, in the second protection trial we used the following vaccine strategy: Pigs were immunized with R345V twice within a 3 week interval (Table 2).

TABLE 2. Assignment of pigs to groups for immunoprotection study

Group (n = 7)	Vaccination		Challenge (day 31)
	1 (day 0)	2 (day 21)	
1	MEM	MEM	MEM
2	MEM	MEM	SIV/Sk02 H1N1
3	MEM	MEM	SIV/Ind88 H1N1
4	MEM	MEM	SIV/Tx98 H3N2
5	R345V	R345V	SIV/Sk02 H1N1
6	R345V	R345V	SIV/Ind88 H1N1
7	R345V	R345V	SIV/Tx98 H3N2

Ten days after the second immunization, the pigs were challenged intratracheally with homologous or heterologous subtypic SIVs and observed for 5 days. The pigs were then euthanized and necropsies were performed. During the five-day observation period, fever and mild respiratory signs such as abdominal

breathing, sneezing and nasal discharge were observed only in the unvaccinated animals challenged with the H1N1 SIV subtypes (Table 2, groups 2 and 3). Unchallenged pigs and pigs vaccinated and challenged with H1N1 SIVs did not show any clinical signs (groups 1, 5, and 6). No apparent clinical signs with respect to respiratory distress or nasal discharge were observed in H3N2 challenged pigs (groups 4 and 7). Typical SIV gross lesions are sharply demarcated, purple to plum colored, consolidated areas, and these lesions were observed in all pigs in the unvaccinated challenged groups (groups 2, 3 and 4). The lesions were most prevalent in the apical and cardiac lung lobes, whereas the diaphragmatic and intermediate lobes were less affected. The lungs of pigs vaccinated with R345V and challenged with H1N1 viruses (group 5 and 6) had no gross lung lesions and appeared similar to normal lungs. The average lung lesion scores for groups 5 and 6 were significantly lower than those for groups 2 and 3, with p=0.0017 (challenged with Sk02) and p=0.002 (challenged with Ind88) (Fig. 4). Although lung lesions were seen in vaccinated pigs challenged with heterologous subtypic H3N2 virus (group 7), the severity of these lesions was significantly less than the severity of the lesions seen in the corresponding unvaccinated challenged group (group 4); the p value of the lung lesion score between these two groups was less than 0.05 (Fig 4).

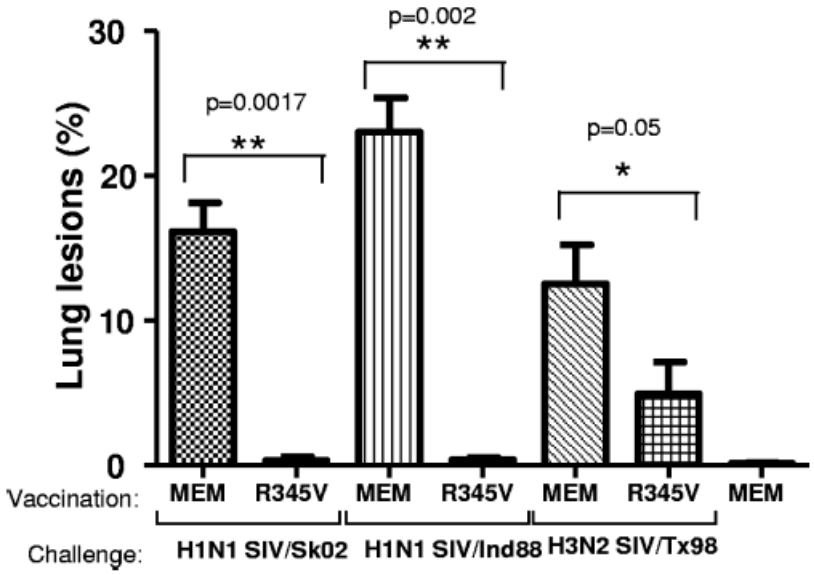

FIG. 4. Macroscopic lung lesions.

Vaccination reduced microscopic lung lesions after SIV challenge. SIV induced histopathological lung lesions are characterized by the detachment of large areas of bronchial/bronchiolar epithelium and by the accumulation of necrotic epithelial cell debris and neutrophils in the airways . Consistent with the macroscopic lung lesions observed above, unvaccinated pigs challenged with H1N1 SIV/Sk02 (group 2) or with H1N1 SIV/Ind88 (group 3) had the most severe histopathological changes characterized by severe necrotizing bronchiolitis with moderate multifocal necrosis, attenuation of surviving bronchiolar epithelium and hyperplasia of bronchial/bronchiolar mucosa (Figure 5). Severe peribronchiolar and perivascular lymphoid infiltration was accompanied by severe neutrophil infiltration in the lumen of most bronchioles. Furthermore, severe locally extensive interstitial pneumonia with atelectasis, focal alveolar necrosis and neutrophil infiltration were also observed, as well as infiltration of lymphocytes and macrophages in the alveolar walls and air spaces (panel B and D). In contrast, as seen in MEM injected pigs, very mild histopathological changes, such as mild perivascular lymphoid infiltration and occasional mild bronchiolar-associated lymphoid tissue (BALT) proliferation, were observed in vaccinated H1N1 challenged pigs (groups 5 and 6) (panel A, C and E). Focal necrosis of small bronchioles and mild, locally extensive interstitial pneumonia with atelectasis and mixed alveolar inflammatory cell infiltration were found in one vaccinated animal challenged with H1N1 SIV/Ind88 (group 6). Severe peribronchiolar and perivascular lymphoid infiltration and moderate alveolar atelectasis due to infiltration of mixed inflammatory cell proliferation in the alveolar walls and spaces were observed in all seven unvaccinated H3N2 SIV/Tx98 challenged pigs (group 4, panel F). In addition, histopathological changes, such as moderate bronchiolitis with focal necrosis, mild attenuation and moderate hyperplasia of bronchiolar epithelium, were also found in the animals in this group. In contrast, four of the seven vaccinated pigs

challenged with H3N2 SIV/Tx98 (group 7) developed mild to moderate bronchiolitis with rare epithelial necrosis, moderate bronchiolar hyperplasia and severe peribronchiolar and perivascular lymphoid infiltration and mild BALT (panel G). Lesions and epithelial damage in the remaining three pigs were similar to that of control mock-challenged pigs (groups 1, panel A).

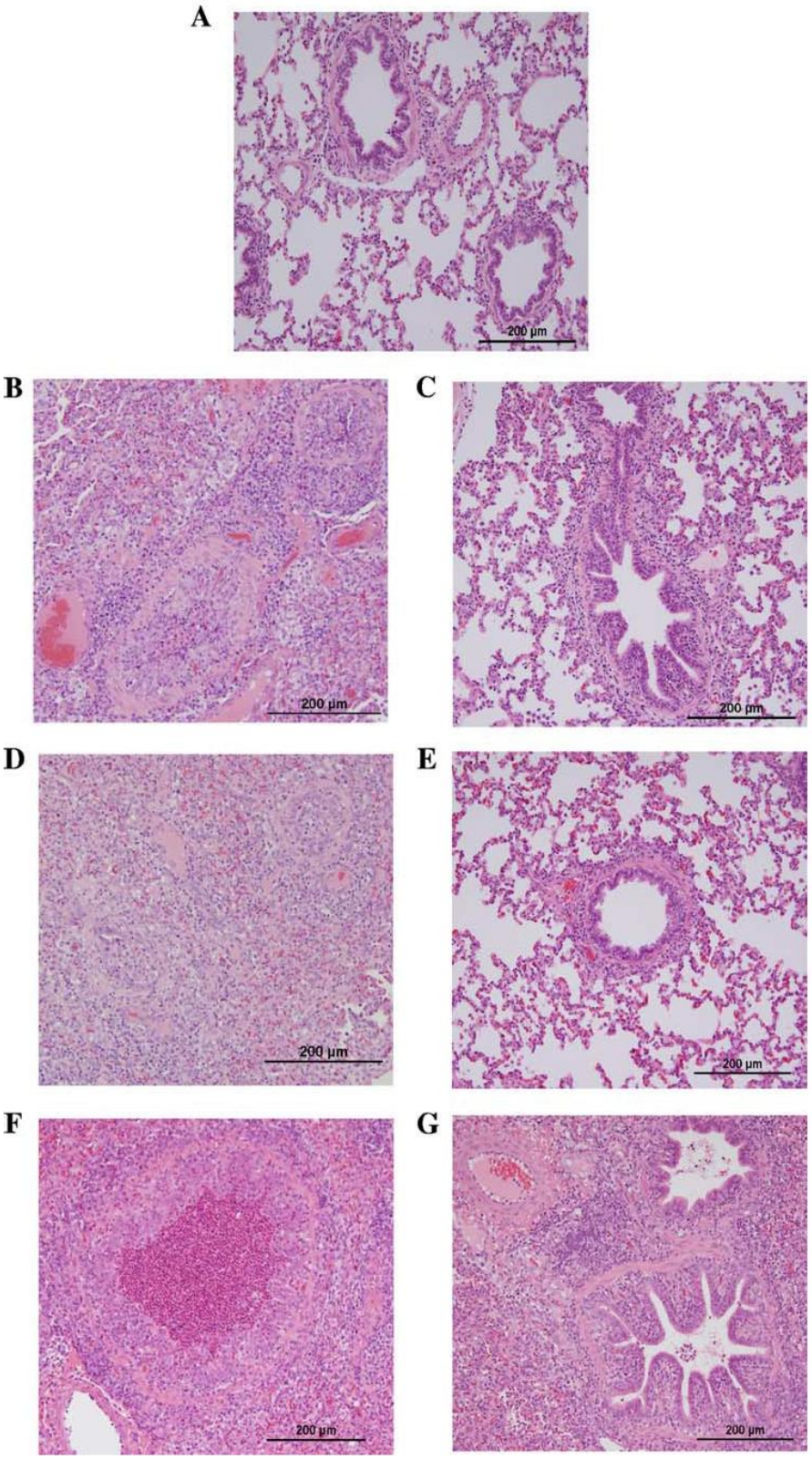

FIG. 5. Microscopic lung lesions.

Vaccination reduced virus replication in the lungs. Tissue sections from the right apical, cardiac and diaphragmatic lung lobes were used for virus isolation. Tissue processing and virus titration were done as previously described . The data are shown in Fig 6. Consistent with results obtained from necropsies and histopathology, we were able to recover viruses from all unvaccinated SIV challenged pigs, as well as from the five animals that were vaccinated and then challenged with heterologous subtypic H3N2 (group 7). The median virus titers for unvaccinated H1N1 (group 2 and 3) or H3N2 challenged (group 4) pigs were $10^{4.2}$ $TCID_{50}$/gr, $10^{3.8}$ $TCID_{50}$/gr and $10^{5.1}$ $TCID_{50}$/gr, respectively. The median virus titer for pigs vaccinated and then challenged with heterologous subtypic H3N2 (group 7) was $10^{3.1}$ $TCID_{50}$/gr. This titer was significantly lower than the virus titers for pigs in group 4 (P =0.004). No virus could be detected in the lung sections from mock-challenged or vaccinated H1N1 challenged pigs (groups 1, 5 and 6).

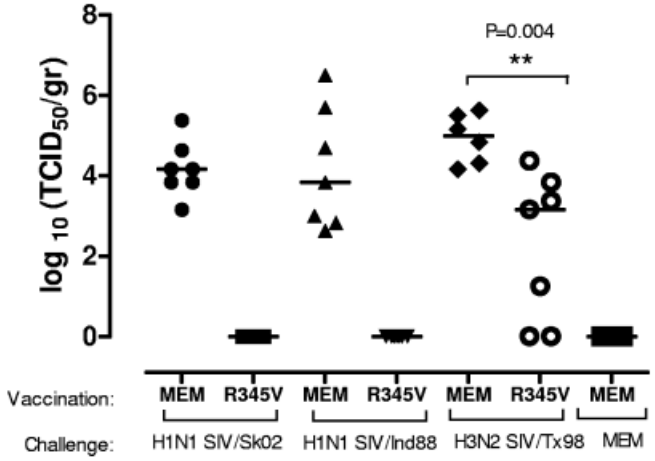

FIG. 6. Lung virus titers.

These results were published in Journal of Virology

Elastase-dependent live attenuated swine influenza A viruses are immunogenic and confer protection against swine influenza A infection in pigs.

Masic A, Booth JS, Mutwiri GK, Babiuk LA, Zhou Y.

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VI. Discussion: Explain your research results and include a summary of the results that is of immediate or future benefit to pork producers.

Attenuation of elastase-dependent LAIV is based on genetically engineering an atypical hemagglutinin cleavage site that is resistant to activation during natural infection. Replacement of the original trypsin-sensitive (Arg-Gly) cleavage site with the elastase-sensitive (Val-Gly or Ala-Gly) motif resulted in *in vivo* attenuation in pig and mouse models . Theoretically, the lack of the appropriate protease at the site of infection prevents cleavage of HA, thus disabling the fusion of progeny viruses with the endosome, which in turn blocks virus replication. Previously, we generated two elastase-dependent mutant SIVs (R345V and R345A). The two mutant viruses are genetically stable and are highly attenuated in pigs. Neither the mutant viruses nor any revertant viruses were isolated from the lungs of infected pigs , thus, these viruses may have the potential to serve as live attenuated vaccines. In this study, we first assessed the immunogenic properties of these viruses by examining the cell-mediated and humoral immune responses after one and two vaccinations. Afterward, we

tested the ability of R345V to induce protective immunity against challenge with homologous and heterologous subtypic SIVs in pigs.

It has long been believed that recovery from influenza virus infection is mediated by cellular immune responses , whereas prevention of viral infection correlates with serum and mucosal anti-influenza virus antibody titers . In addition, there is a large body of evidence showing that cell-mediated responses are an important contributor to heterologous subtypic immunity in mice and pigs . IFN-γ, produced by $CD4^+$ T helper cell type 1 (Th1) lymphocytes, $CD8^+$ cytotoxic lymphocytes and NK cells , is the major immuno-modulator that coordinates the immune responses and establishes an antiviral state of longer duration . In our study, one vaccination with R345V or R345A was sufficient to induce significant numbers of local IFN-γ secreting cells. The two-fold increase in the IFN-γ response after the second vaccination suggested that both virus candidates are capable of inducing T-cell activation (Fig. 1A). In addition, results from the LPR assay showed that one vaccination with R345V or R345A induced low proliferation of lymphocytes, whereas a second vaccination resulted in significant LNC proliferation (Fig. 1B). Previous reports on experimental infection with WT SIVs showed that a second exposure to the virus did not result in an increase in lymphocyte proliferation or IFN-γ secretion , but these results might be attributed to the multiple replication of WT SIVs. In our study, one vaccination was not sufficient to induce the maximum immune response, possibly due to the limited number of replication cycles of R345V *in vivo*.

Antibody responses to R345V and R345A in the serum and at the respiratory mucosa were measured after the first and second immunizations. Whereas one vaccination induced low levels of antigen specific IgG, IgA and HI antibodies in the serum and the BALF, secondary vaccination induced considerably higher titers of antigen-specific IgG, IgA and HI (Fig. 2). This could be attributed to the restricted replication and short antigen exposure. These data suggest that two vaccinations might be required to generate an adequate immune response and that the two vaccinations could mimic natural immunity after SIV infection and be protective against challenge with WT homologous and heterologous SIVs.

Both R345V and R345A viruses could induce cellular and humoral immunity and showed similar antigenic properties. After comparison of the statistical analyses from all performed assays, SIV mutant R345V showed enhanced serum HI and antigen-specific IgG titers, whereas serum antigen-specific IgA antibody titers, the number of IFN-γ secreting cells and the LPR stimulation index were at a similar level as in R345A vaccinated pigs. Therefore, we chose R345V as the vaccine candidate in our protection trial.

Two vaccinations via the IT route with R345V were sufficient to confer complete protection from challenge with the homologous subtypic H1N1 SIV/Sk02 and the H1N1 variant SIV/Ind88. Vaccinated challenged pigs did not show any of the clinical signs characteristic of SIV infection or elevated rectal temperatures compared with the unvaccinated challenged controls (data not shown). Virus was not detected in the lungs of any of the pigs in these groups, and macroscopic (Fig. 4) and microscopic (Fig. 5) lesions were undetectable or minimal. To demonstrate the ability of the elastase-dependant R345V SIV to induce immunity against an antigenically distinct SIV subtype, vaccinated pigs were challenged with the heterologous subtypic H3N2 SIV/Tx98 strain. At 5 days post infection, 5 of the 7 pigs in the R345V vaccinated H3N2 SIV/Tx98 challenged group had detectable virus in their lungs. However, the median virus titers were significantly lower than those for the unvaccinated H3N2 SIV/Tx98 challenged group (Figure 6). In addition, macroscopic and microscopic lesions were significantly reduced. Taken together, the data that we obtained from macroscopic and microscopic lesions, and virus titers clearly showed that two administrations of the R345V virus vaccine conferred full protection against homologous and antigenic variant H1N1 SIVs and partial protection against antigenically distinct H3N2 SIV infection.

The common objection to the use of live attenuated viruses as vaccines is the possibility of reversion to pathogenicity. The absence of the appropriate protease for cleavage of the R345V HA *in vivo* allows only a few replication cycles to occur, leading to restricted replication. An important advantage of the short and limited replication is the decreased probability of any reversion or reassortment between the vaccine virus and a WT virus. Although our vaccination requires two administrations and was delivered intratracheally (to ensure that all viruses were delivered to the respiratory tract of the pigs), the advantage of the elastase-dependent live attenuated virus is its ability to induce humoral and cell mediated immune responses. Most importantly, vaccination with this virus led to reduction in the homologous and heterologous subtypic SIV virus loads and pathogenesis. In addition, the heterologous subtypic immunity induced by the elastase-dependent live attenuated virus could have a significant impact on the epidemiology of novel SIVs emerging in the swine population by reducing viral shedding and potentially limiting the spread of novel SIVs. Currently, we are planning to test more practical and optimal routes of vaccination, such as intranasal immunization.