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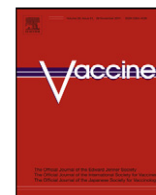

Vaccination with NS1-truncated H3N2 swine influenza virus primes T cells and confers cross-protection against an H1N1 heterosubtypic challenge in pigs

Matthew A. Kappes [a,b,1], Matthew R. Sandbulte [b,1], Ratree Platt [b], Chong Wang [c], Kelly M. Lager [a], Jamie N. Henningson [a], Alessio Lorusso [a], Amy L. Vincent [a], Crystal L. Loving [a], James A. Roth [b], Marcus E. Kehrli Jr. [a,*]

[a] Virus and Prion Diseases Research Unit, National Animal Disease Center, USDA-ARS, 1920 Dayton Ave, PO Box 70, Ames, IA 50010, USA
[b] Department of Veterinary Microbiology and Preventive Medicine, Iowa State University, Ames, IA, USA
[c] Department of Veterinary Diagnostic and Production Animal Medicine, Iowa State University, Ames, IA, USA

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ABSTRACT

The diversity of contemporary swine influenza virus (SIV) strains impedes effective immunization of swine herds. Mucosally delivered, attenuated virus vaccines are one approach with potential to provide broad cross-protection. Reverse genetics-derived H3N2 SIV virus with truncated NS1 (NS1Δ126 TX98) is attenuated and immunogenic when delivered intranasally in young pigs. We analyzed T-cell priming and cross-protective efficacy in weanling piglets after intranasal inoculation with NS1Δ126 TX98 versus wild type TX98. *In vivo* replication of the truncation mutant was minimal compared to the wild type virus. T-cell responses were greater in magnitude in pigs infected with the wild type virus in *in vitro* restimulation assays. According to the expression of activation marker CD25, peripheral T cell recall responses in NS1Δ126 TX98 infected pigs were minimal. However, intracellular IFN-γ data indicate that the attenuated virus induced virus-specific $CD4^+CD8^-$, $CD4^+CD8^+$, $CD4^-CD8^+$, and γδ T cells within 28 days. The IFN-γ response appeared to contract, as responses were reduced at later time points prior to challenge. $CD4^+CD8^+$ cells isolated 5 days after heterosubtypic H1N1 challenge (day 70 overall) showed an elevated CD25 response to virus restimulation. Pigs previously infected with wild type TX98 were protected from replication of the H1N1 challenge virus. Vaccination with NS1Δ126 TX98 was associated with significantly lower levels of Th1-associated cytokines in infected lungs but provided partial cross-protection against the H1N1 challenge. These results demonstrate that NS1Δ SIV vaccines can elicit cell-mediated cross-protection against antigenically divergent strains.

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1. Introduction

Pigs have been recognized as a natural host of influenza A virus since the virus was first isolated in 1930. After a relatively slow evolution of classical H1N1 swine influenza virus (SIV) in the North American swine population, a new reassortant H3N2 lineage emerged and was established around 1998 [1]. Compared to the classical swine H1N1, this SIV lineage has shown a propensity for frequent gene reassortment and rapid antigenic drift [2–5]. Variants of this reassortant lineage, which have been isolated routinely in recent years, possess H3N2, H1N1, and H1N2 serotypes (reviewed by Vincent et al. [6]). Segments of the "triple reassortant internal gene" (TRIG) cassette were acquired from swine, avian, and human influenza A lineages [1]. Occasional reassortants have emerged in swine with novel surface glycoproteins with the TRIG [5,7]. Five of the six gene segments which encode internal proteins in the 2009 pandemic H1N1 virus were derived from this North American TRIG lineage [8]. The emergence of this virus illustrates the risk to public health as influenza A virus genes of diverse origins are shuffled together in the backbone of the mammalian-adapted triple reassortant virus.

Additional antigenically distinct reassortant viruses could potentially emerge from swine lineages into the human population [9]. New variants may also have great economic costs to the swine industry. Therefore, there is a strong rationale for investigations into the immunological relationships among recent and emerging subtypes or genotypes of swine-origin reassortant influenza viruses. Experimental evaluation of modern vaccine technology for SIV in the swine host is important for achieving greater control of the various strains in swine populations and limiting the risk of transmission to humans. The antigenic diversity of influenza viruses circulating and becoming established in global swine populations suggests that veterinary vaccination programs are unlikely

* Corresponding author: Tel.: +1 515 337 7254.
E-mail address: marcus.kehrli@ars.usda.gov (M.E. Kehrli Jr.).
[1] These authors contributed equally to the study.

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to produce neutralizing humoral immunity against all emerging strains. Therefore, an important consideration in evaluating candidate vaccines should be the degree to which they elicit cross-reactive cell-mediated immunity. Although influenza-specific T cells do not provide sterilizing immunity, they protect more broadly than neutralizing antibodies against heterologous and heterosubtypic strains through viral clearance [10,11]. Broadly cross-reactive T cell epitopes are typically located in internal proteins of influenza viruses such as the nucleoprotein (NP) and matrix (M1) segments, which are more conserved than the surface hemagglutinin (HA) and NA glycoproteins [12–14]. Cellular immunity to influenza in the absence of cross-reactive antibody can markedly reduce viral replication in humans and pigs [15,16].

Live virus vaccines are considered to be more effective than inactivated or non-replicating vaccines as inducers of cellular immunity, particularly for MHC class I-restricted T cells, but all licensed SIV vaccines in the US are based on inactivated virus antigens. Molecular approaches have been used to construct mutated SIV genomes which confer attenuated replication properties, including reduced NS1 suppression of type I interferon, dependence on the enzyme elastase for HA cleavage, and temperature-sensitive mutations in polymerase genes [17–19]. Truncation of NS1 protein in triple reassortant H3N2 strain A/Sw/Texas/4199-2/98, from a length of 230 amino acids to 126, produced a mutant with restricted replication in the swine respiratory tract but strong immunogenic properties [20]. Intranasal inoculation of pigs with this virus (NS1Δ126 TX98) resulted in robust protection against homologous challenge and significantly reduced viral replication and clinical signs upon challenge with a drift variant strain [21]. One likely factor in the partial heterologous protection was the production of mucosal IgA antibodies that had significant cross-reactivity against the drift variant. No investigation was made into the priming of T cells subsets in the NS1Δ126 TX98 immunized pigs, but we hypothesize that cross-protection was mediated at least in part by the cell-mediated immune (CMI) response. A theoretical concern with live SIV vaccines, such as NS1Δ126 TX98, is the possibility that they would undergo reassortment with circulating strains and produce variants with altered virulence, transmissibility, or host range. This vaccine does not possess any novel genetic elements to contribute to the current circulating pool of influenza viruses as the parental H3N2 TRIG background from which the NS1Δ126 TX98 vaccine strain was generated continues to circulate widely in the US swine population, and therefore concerns about the swine host acting as a mixing vessel in this context should be minimal [6]. Attenuated H1N1 SIVs made by introducing elastase dependent mutations also protected pigs against homologous and heterologous H1N1 challenge while eliciting significant T-cell responses and lung IgA titers against both strains [22].

In the present investigation we analyzed the CMI response following NS1Δ126 TX98 or wild type virus vaccination by measuring *ex vivo* responses of each major T cell subset from vaccinated or unvaccinated pigs to attenuated or wild-type virus. Serum and peripheral blood mononuclear cells (PBMC) were collected at multiple time points following vaccination, the last of which was 5 days after heterologous H1N1 virus challenge. The heterologous challenge virus was a reassortant H1N1 (rH1N1) subtype isolated after classical swine H1N1 surface glycoproteins reassorted with H3N2 SIV [23]. The heterosubtypic challenge strain carries the TRIG cassette, which is common to the TX98-derived vaccine candidates (Supplemental Table 1). Immunization with either attenuated or wild type virus elicited antigen-specific responses by peripheral T cells. After heterologous challenge of the immunized animals, T-cell sensitivity to viral stimulation was augmented again in both groups, but the phenotypic characteristics of responding cells were shifted markedly.

2. Materials and methods

2.1. Viruses

The H3N2 isolate A/swine/Texas/4199-2/1998 (TX98) was propagated in allantoic cavities of 10-day old embryonated chicken eggs to produce an inoculum for vaccination. To limit recall responses to non-viral endogenous egg antigens, the IA04 H1N1 challenge viral stock was propagated in Madin-Darby canine kidney (MDCK) cells. The same strains were grown in MDCK cell cultures to generate recall antigen for *ex vivo* stimulation of T cells. Live influenza A virus NS1Δ126 TX98 was generated by reverse genetics, as previously described [17], and propagated in allantoic cavities of 10-day old embryonated chicken eggs to produce an inoculum for vaccination. Sequences of IA04 were generated by 454 genome sequencing technology, complemented by Sanger sequencing to fill gaps, as described previously [24]. Sequence data covering portions of each TX98 gene segment were accessed from the NCBI database.

2.2. Experimental design

Twenty-four three-week old pigs were treated with ceftiofur crystalline antibiotic (EXCEDE™, Pharmacia & Upjohn Company) per manufacturer's recommended dose and randomly separated into groups of eight pigs to be housed for 1 week prior to immunization. All pigs were confirmed seronegative for SIV by hemagglutination inhibition (HI) assays against H1N1 and H3N2 antigens and free from detectable influenza infection by virus isolation from nasal swabs collected on the day of vaccination. At day 0, inocula were prepared by dilution in sterile phosphate buffered saline (PBS) and administered intranasally by slowly dripping 2 ml into the nasal cavity of each pig. Pigs in the non-vaccinated group received an intranasal sham inoculation. The second group received NS1Δ126 TX98 at a 50% tissue culture infectious dose ($TCID_{50}$) of 10^6 per ml. The third group received wild type TX98 at a dose of 10^6 $TCID_{50}$/ml. At day 65 post vaccination (dpv), all subjects were challenged with the wild type H1N1 influenza strain IA04 at a dose of 10^6 $TCID_{50}$/ml. Pigs were observed for clinical signs for 5 days after challenge. Nasal swabs were obtained 0, 2, 4, 6, 65, 68, 70 dpv and serum samples were collected 0, 14, 28, 49, 56, 65, 70 dpv. Anticoagulated whole blood for isolation of mononuclear cells to assess CMI was collected 28, 49, 56, and 70 dpv. Pigs were euthanized and necropsied 5 days post challenge. Bronchoalveolar lavage fluid (BALF) was collected for virus titration as described previously, using 50 ml Minimum Essential Medium per lung lavage [21]. Viral titers were analyzed in nasal swab and BALF samples by a standard tissue culture infectious dose assay on MDCK cell monolayers. Fifty percent endpoint titers were computed by the Reed–Meunch method [25]. The presence of macroscopic lung lesions for all seven lung lobes was examined and scored as previously described [26]. All animal studies were conducted in accordance with the National Animal Disease Center's Institutional Animal Care and Use Committee.

2.3. Serology

Serum samples were heat inactivated at 56 °C for 30 min and treated with a 20% kaolin suspension (Sigma–Aldrich, St. Louis, MO) for 30 min, followed by adsorption with 0.5% turkey red blood cells to remove nonspecific agglutinins. HI tests were performed with 0.5% turkey red blood cells and MDCK-propagated TX98 virus, using standard techniques [27].

2.4. Histopathology

Sections of lung tissue were collected from each animal and fixed in a 4% buffered formalin solution at time of necropsy for

histopathologic examination. A pathologist blinded to the study scored all tissues. Lung sections were examined for the percentage of airways with lesions, percentage of airways with epithelial changes, and percentage of airways with inflammation (1 = 0–25%, 2 = 26–50%, 3 = 51–75%, 4 = 76–100%); severity of airway epithelial changes (0 = none, 1 = early, 2 = mild, 3 = moderate, 4 = severe); interstitial pneumonia (0 = none, 1 = mild, 2 = moderate, locally extensive, 3 = moderate, multifocal to coalescing, 4 = severe); as well as edema and neutrophilic infiltration (0 = none, 1 = mild, 2 = moderate, 3 = severe). Epithelial changes graded on a 5-point scale were as follows: 0, no lesions; 1, early epithelial changes characterized by focal to multifocal loss of cilia and epithelial degenerative changes; 2, mild epithelial flattening with loss of cilia and goblet cells; 3, moderate epithelial flattening with decreased thickness of respiratory epithelium, loss of cilia and goblet cells; 4, flattened epithelium with areas of mucosa covered by a single layer of cuboidal epithelium and epithelial loss (necrosis). Mean scores across all parameters were calculated for each pig.

2.5. Influenza-specific isotype ELISA

Influenza antigen and isotype-specific ELISAs were performed as previously reported [3]. Briefly, TX98 and IA04 virus supernatants were purified by ultracentrifugation and diluted to 100 HA units/50 μL. Immulon-2 96-well plates were coated with 200 HA units per well overnight at room temperature. BALF samples collected the day of necropsy were treated with 10 mM dithiothreitol for 1 h at 37 °C to disrupt mucous and were assayed in triplicate for each strain and for each antibody isotype (IgG or IgA). Optical density (O.D.) readings were measured at 405 nm using an automated ELISA reader and the mean O.D. values were calculated for each triplicate reading.

2.6. In vitro T-cell stimulation assays

Whole blood samples were collected in vacutainer cell preparation tubes with sodium citrate (CPT™, BD Diagnostics, Franklin Lakes, NJ) on 28, 49, 56, 70 dpv. Tubes were centrifuged at 1500 × g for 30 min at room temperature, and peripheral blood mononuclear cells (PBMCs) were collected. Remaining red blood cells were lysed by adding 2 volumes of buffered water (deionized water with 0.15 M Na_2HPO_4 and 0.15 M KH_2PO_4) for 90 s followed by 1 volume of 3× PBS. PBMC were pelleted, washed an additional time with PBS, and resuspended in RPMI++ (RPMI 1640 with L-glutamine and 25 mM HEPES), supplemented with 15% fetal bovine serum (Atlanta Biologicals, Norcross, GA), penicillin (150 I.U./ml)/streptomycin (150 μg/ml)/amphotericin B (0.38 μg/ml) solution (Mediatech, Inc., Herndon, VA), and 1.5 μg/ml gentamicin (Invitrogen, Grand Island, NY). Each PBMC suspension (10^6 cells in 200 μL) was dispensed into multiple wells of a 96-well flat-bottomed tissue culture plate. One well received 50 μL RPMI++ media as negative control. One well received 50 μL concanavalin A (25 μg/mL) as a mitogen control. Other wells received 50 μL each of live or UV-inactivated wild type TX98 or live NS1Δ126 TX98, each at a titer of 10^6 $TCID_{50}$/mL. Plates were incubated in 5% CO_2 at 37 °C. After 4 days, PBMCs were transferred to 96-well round-bottomed tissue culture plates with 50 μL protein transport inhibitor brefeldin A (BD Biosciences Pharmingen, San Diego, CA) per well and incubated for an additional 4 h. PBMC were washed once with PBS++ (PBS with 0.5% bovine serum albumin and 0.1% sodium azide). The primary antibody mix for surface antigens (50 μL) was added and incubated at room temperature (RT) for 15 min. The primary antibody mix consisted of mouse anti-swine CD4 (IgG2b), mouse anti-swine CD8 (IgG2a), mouse anti-swine γδ TCR (IgG1, directly conjugated with fluorescein isothiocyanate), and mouse anti-swine CD25 (IgG1, directly conjugated with phycoerythrin) in PBS++. All monoclonal antibodies were purchased from VMRD (Pullman, WA; cat # 74-12-4, 76-2-11, PGBL22A and PGBL25A, respectively) and titrated to optimal dilutions for labeling. Direct conjugation of fluorochromes to monoclonal antibodies was performed by Chromaprobe (Maryland Heights, MO). PBMC were washed twice with PBS++ and 50 μL of secondary antibody mix was added to all wells and incubated at RT for 15 min. The secondary antibody mix consisted of goat anti-mouse IgG2b-PE-Cy-7 (cat# 1090-17, Southern Biotech, Birmingham, AL) and goat anti-mouse IgG2a-PE-TR (cat# M32217, Caltag Laboratories, Burlingame, CA) diluted in PBS++ at optimal dilutions determined by titration (dilution factors are available upon request). PBMC were washed 3 times with PBS++.

For intracellular staining, PBMC were incubated with 100 μL BD cytofix-cytoperm solution (BD Biosciences Pharmingen) at RT for 30 min. The primary antibody mix (50 μL), consisting of rabbit anti-swine IFN-γ (cat # AT-3072, MBL International, Woburn, MA) and mouse anti-swine IL-10 biotin (IgG1, cat # 3075, MBL International, Woburn, MA) in BD perm-wash solution, was added to PBMC's and incubated at RT for 15 min, followed by 2 washes with BD perm-wash solution. The secondary antibody reagent mix, consisting of goat anti-rabbit IgG-AF700 (Alexa Fluor 700, cat# A21038, Invitrogen) and streptavidin-PerCP-Cy5.5 (cat# 551419, BD Biosciences Pharmingen) in BD perm-wash solution, was added and incubated at RT for 15 min. Plates were washed 3 times with BD perm-wash solution. A 1% solution of ultrapure formaldehyde (Polyscience, Warrington, PA) in PBS (250 μL) was added to all wells followed by transfer of treated cells to flow tubes (Falcon, BD Labware) and kept in the dark at 4 °C until flow cytometric analysis. The FACSCanto cytometer (BD Biosciences, San Jose, CA) was used to analyze the samples at the Flow Cytometry Facility, Iowa State University. Data were collected from at least 10,000 live cells per sample, based on light scatter properties and were analyzed using FlowJo software (Tree Star Inc, Ashland, OR). Four T-cell subsets were defined by surface markers as follows: $CD4^+CD8^-\gamma\delta\ TCR^-$, $CD4^+CD8^+\gamma\delta\ TCR^-$, $CD4^-CD8^+\gamma\delta\ TCR^-$, and $CD4^-CD8^-\gamma\delta\ TCR^+$. Expression indices (EI) were calculated for the CD25, IFN-γ, and IL-10 responses of virus antigen-stimulated T-cell subsets. Each EI was computed, using CD25 as the target for example, (%CD25 positive cells × geometric mean fluorescence intensity) for cells stimulated with virus, divided by the same target for control media-treated cells of the same T-cell subset of the same animal.

2.7. Cytokine analysis

A 1 ml aliquot of BALF was centrifuged at 300 × g for 10 min at 4 °C to pellet cellular debris. The cell-free BALF was used to assay for levels of IL-1β, IL-8, IL-6, TNF-α, IL-2, IL-4, IL-12p70, IFN-γ, and IL-10 by SearchLight multiplex ELISA performed according to the manufacturer's recommendations (Aushon Biosystems, Billerica, MA). The average of duplicate samples for each sample was used for statistical analysis.

2.8. Statistics

Lung lesion scores, $\log_2$-transformed HI titers, and $\log_{10}$-transformed viral titers data were analyzed using a mixed linear model for repeated measures (Proc Mixed, SAS for Windows, Version 9.2, SAS Institute Inc., Cary, NC, USA). Covariance structures within pigs across time were tested and modeled using the REPEATED statement to determine the optimal covariance structure. Linear combinations of the least squares means estimates were used in *a priori* contrasts after testing for a significant ($p < 0.05$) treatment group effect. Comparisons were made between each group at each time-point using a 5% level of significance ($p < 0.05$) to assess statistical differences. Cytokine concentrations were analyzed by one-way ANOVA in JMP software (SAS Institute, Cary,

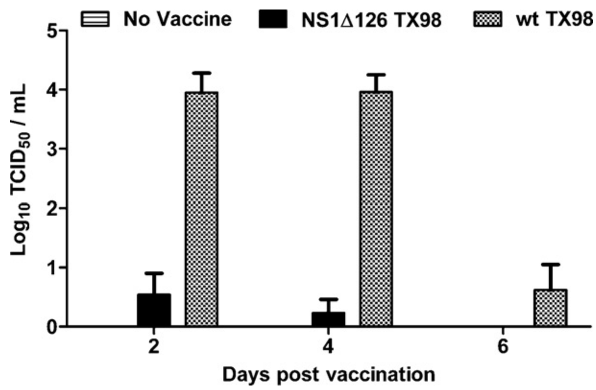

Fig. 1. Nasal shedding of virus following primary immunization. Nasal swabs collected 2, 4, and 6 days after inoculation with wild type TX98 (H3N2), NS1Δ126 TX98, or sham inoculum were titrated by infectivity on MDCK cell monolayers. Titers were computed from results of replicate dilutions by the Reed–Muench method. Values are reported as group mean ± SE.

NC) and isotype ELISA data were analyzed by two-way ANOVA (v5.04 GraphPad Prism, La Jolla, CA). Where ANOVA showed significant differences among groups, the Tukey post-test was applied for pairwise comparisons. *In vitro* T-cell EI values were natural log-transformed and analyzed by repeated measures ANOVA and controlled pairwise comparisons, using the Glimmix procedure in SAS. Data from 49 and 56 dpv assays (representing secondary responses immediately preceding challenge infection) were pooled as a single prechallenge data point for the repeated measures analysis.

3. Results

3.1. Vaccine virus replication in naïve pigs

TX98 mutants with truncated NS1 replicated much less extensively in the upper respiratory tract than wild type TX98 (Fig. 1). The wild type virus, administered intranasally, replicated sufficiently to produce nasal swab viral titers of approximately 10^4 $TCID_{50}$/ml through day 4. The mean titer dropped below 10 $TCID_{50}$/ml on day 6. In pigs vaccinated with the attenuated NS1Δ126 TX98 mutant, the mean viral titers did not exceed 10 $TCID_{50}$/ml on days 2 or 4, and no virus was detected on day 6. This disparity in wild type TX98 and NS1Δ126 TX98 replication *in vivo* is consistent with the attenuated phenotype in previously reported results [17].

3.2. Immune responses to vaccine or primary infection

Hemagglutination inhibition titers indicated that pigs seroconverted in response to NS1Δ126 TX98 vaccination, but were below the conventional protective threshold of 40 HI units (Fig. 2A). Whereas the wild type TX98 elicited geometric mean HI titers greater than 80 at day 14 and thereafter, the geometric mean HI titers of NS1Δ126 TX98 vaccinates peaked at 20 HI Units. The negative control group had no detectable HI antibodies throughout the study. Therefore, while NS1Δ126 TX98 elicited only modest levels of HI antibody, the results demonstrate measurable peripheral antibody responses to the vaccine.

While the wild type TX98 immunization did generate significantly higher serum HI antibody titers, antibody isotype ELISAs performed with BALF samples demonstrated no statistical difference between the NS1Δ126 TX98 and the wild type TX98 groups irrespective of isotype (IgG or IgA) or antigen (TX98 or IA04) (Fig. 2B). These data indicate that, at least in lungs, NS1Δ126 TX98 vaccine elicited a mucosal antibody response equivalent to that

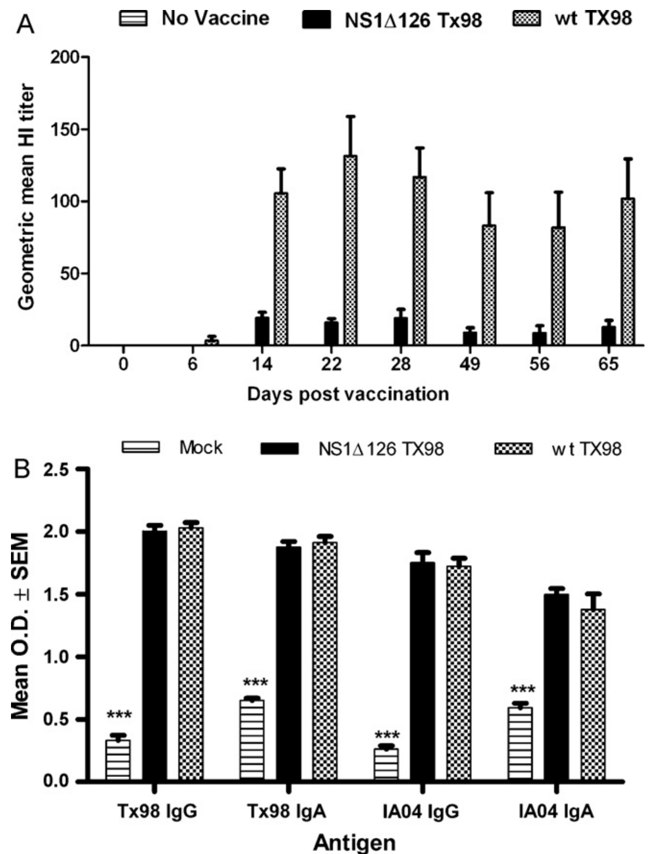

Fig. 2. Antibody response to vaccination. (A) Geometric mean serum hemagglutination inhibition (HI) titers against the H3N2 vaccine backbone, TX98, in samples collected through the day of heterologous challenge infection (d65). HI titration was performed with turkey red blood cells, beginning with a 10-fold dilution of serum. No positive titers were measured from the non-vaccinated control animals. The wild type TX98 treatment group was statistically different from all other treatment groups starting on day 14 post vaccination and continuing throughout the end of the study ($p < 0.01$). (B) BALF collected at time of necropsy [5 dpi, 70 dpv] was assayed in triplicate for levels of influenza specific IgG and IgA antibodies against purified whole virus antigens, either homologous TX98 or heterologous IA04 (H1N1). Values are reported as group mean ± SE O.D. values for each treatment group.

induced by wild type TX98 virus, including comparable levels of cross-reactive antibodies to the H1N1 virus.

We monitored induction of CMI after vaccination by *in vitro* T cell stimulation assays. Flow cytometry was used to measure changes in cell surface expression of the activation marker CD25 (IL-2 receptor α-chain) and the production of the cytokines interferon-γ (IFN-γ) and interleukin-10 (IL-10) in cells isolated from peripheral blood. As with the serological data, results of these assays indicate an increase in magnitude of T cell priming following wild type TX98 inoculation compared to NS1Δ126 TX98 (Fig. 3). Antigen-driven CD25 upregulation of all four major T cell subsets was low-to-undetectable after vaccination with NS1Δ126 TX98 and before virus challenge (Fig. 3A). Wild type TX98 inoculation, in contrast, primed for statistically significant increases in CD25 responses of $CD4^+CD8^+$ T cells on day 28. In the time period that immediately preceded heterosubtypic challenge (day 46/56), the wild type TX98 group continued to show a statistically significant increase in CD25 EI in all subsets except $CD4^+CD8^-$ cells.

However, intracellular IFN-γ data from day 28 demonstrate that an antigen-specific T-cell response was primed by NS1Δ126 TX98 vaccination. For all four subsets of T cells, elevated virus-specific IFN-γ responses were statistically significant in groups primed with NS1Δ126 TX98 or wild type virus (Fig. 3B). Later, in the day 49/56 time period, only pigs primed with wild type virus had significant

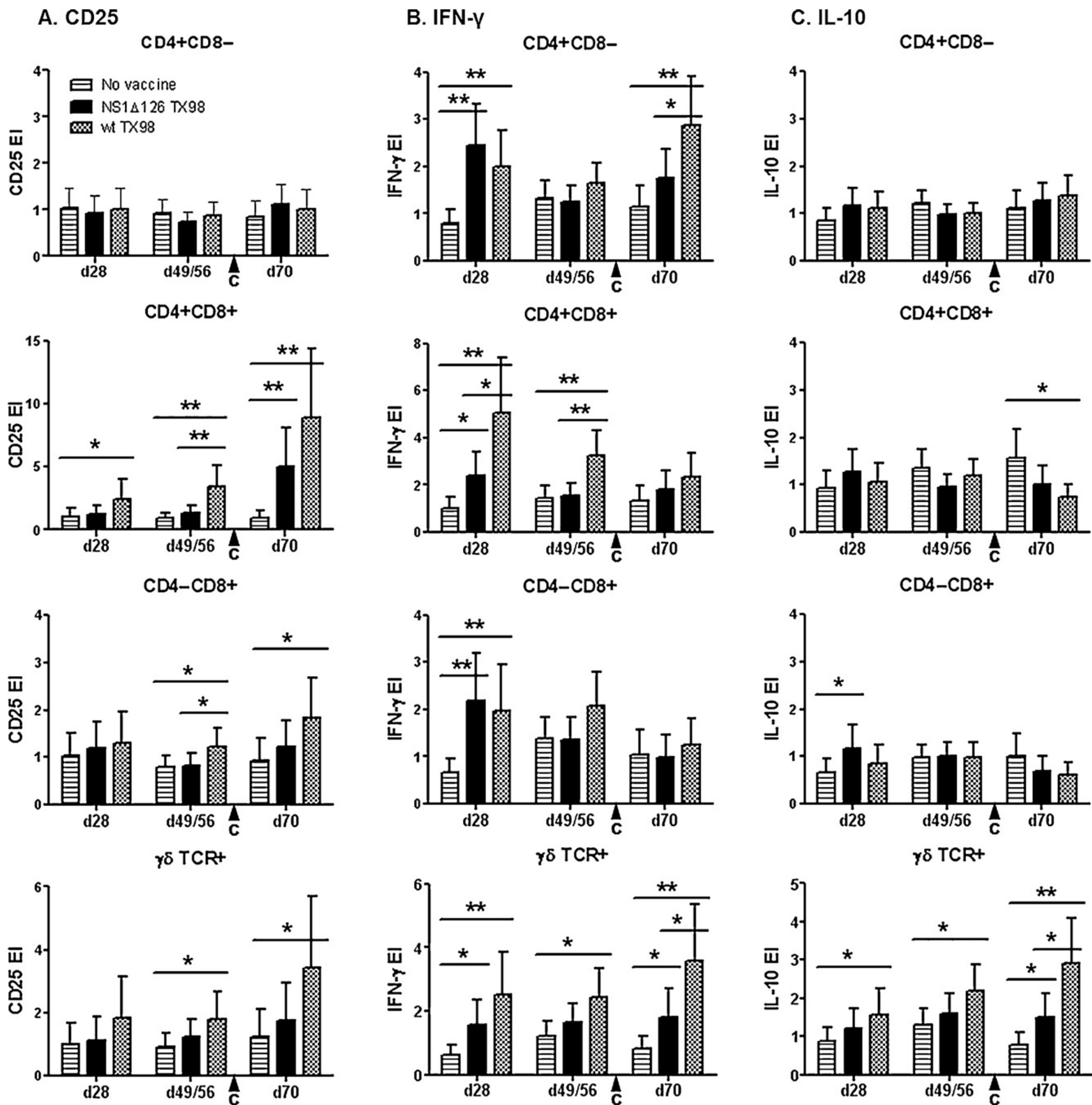

Fig. 3. Flow cytometry-based analyses of T-cell priming. Pigs were immunized on day 0, with either wild type TX98 (H3N2), NS1Δ126 TX98, or a sham inoculum. PBMC were isolated early after immunization (day 28), twice in the period immediately before challenge (day 49 and day 56), and 5 days after heterologous H1N1 challenge with IA04 (day 70). PBMC were dispensed into tissue culture plates with live TX98 (H3N2) virus or media alone, and incubated for 4 days, concluding with 4 h of Brefeldin A treatment. T cells were fluorescently labeled with antibodies specific to subset markers CD4, CD8, and γδ TCR; activation marker CD25; and intracellular cytokines IFN-γ and IL-10. Expression indices were calculated for CD25 (A), IFN-γ (B), and IL-10 (C), as described in Section 2. Horizontal bars marked with one or two asterisks denote statistically significant differences between groups ($^{*}p < 0.05$; $^{**}p < 0.001$). Values are reported as group mean ± SE.

IFN-γ recall response ($CD4^+CD8^+$ and γδ T-cell subsets). Wild type TX98 inoculation primed for a modest increase in intracellular IL-10 levels in γδ TCR^+ cells, but NS1Δ126 TX98 vaccination did not (Fig. 3C).

3.3. Heterosubtypic challenge

Pigs in all three treatment groups were challenged with the H1N1 isolate IA04, differing in subtype of both major glycoproteins as compared to TX98. Thus, the challenge virus was heterosubtypic to the vaccine strain in regard to surface glycoproteins, but internal genes of the two strains had higher similarity. The predicted amino acid sequence identities between TRIG segments of these two viruses were >90%, whereas HA and NA identities were considerably lower (40–50%) (Supplemental Table 1). Shedding of the heterosubtypic challenge virus was monitored in nasal swabs collected 3 and 5 days post-infection. At 3 days post-infection (dpi), nasal swab virus titers were approximately 10^3 $TCID_{50}$/ml, regardless of the vaccine treatment group (Fig. 4A). The lack of early protection against replication in vaccinated animals was expected, given the difference in subtype between vaccine and challenge strains. By 5 dpi, however, there were significant differences in virus titers among vaccine treatment groups (Fig. 4B). In pigs given the sham inoculation with no SIV antigen, nasal swab titers

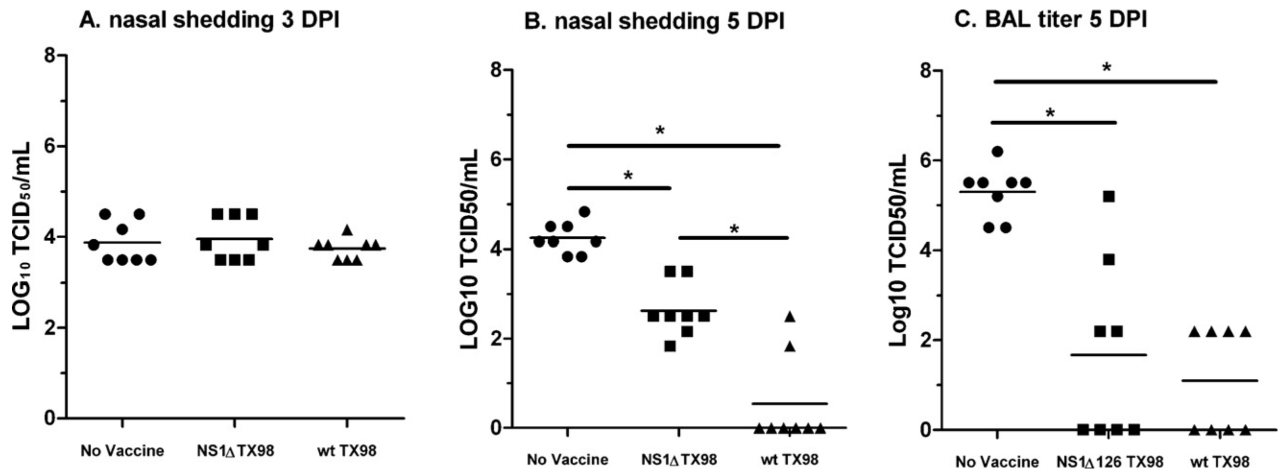

Fig. 4. Viral shedding after heterologous H1N1 virus challenge. Sixty-five days after primary immunization, all treatment groups were inoculated intranasally with IA04. Nasal swabs were collected at 3 and 5 days post infection. BAL fluid was collected from lungs at necropsy (day 5). Each specimen was titrated by infectivity on MDCK cell monolayers. Titers were computed from results of replicate dilutions by the Reed–Muench method. Log_{10}-transformed data were analyzed by one-way ANOVA and the Tukey HSD test for pairwise comparisons. Horizontal bars linking two treatment groups denote statistically significant differences in mean titer between groups ($p < 0.05$).

remained approximately 10^4 $TCID_{50}$/ml. In contrast, very little virus was detectable in pigs that had been primed by wild type H3N2 virus inoculation. Virus shedding by NS1Δ126 TX98 vaccinated pigs at 5 dpi was intermediate; almost 100 fold lower than in the non-vaccinated controls. Virus was also titrated in BALF samples collected upon necropsy at 5 dpi (Fig. 4C). By this parameter there was an even greater disparity between non-vaccinated animals and those that had been immunologically primed with attenuated or wild type TX98. The mean BALF titer in the naïve group exceeded 10^5 $TCID_{50}$/ml, significantly different from the mean titers in both groups with prior heterosubtypic immunity, which were between 10^1 and 10^2 $TCID_{50}$/ml. In contrast to nasal swab titers, 5 dpi BALF titers did not show a difference in protection between groups primed with wild type TX98 versus attenuated NS1Δ126 TX98.

Groups immunized with NS1Δ126 TX98 or wild type TX98 did not have reduced lung lesions after heterologous IA04 challenge (5 dpi), compared with the non-vaccinated group. Mean macroscopic lesion scores (percentage of surface area ± standard error) of the non-vaccinated, NS1Δ126 TX98, and wild type TX98 groups after challenge were 5.6 ± 0.7, 9.5 ± 0.8, and 12.1 ± 2.6, respectively. The difference between non-vaccinated and wild type TX98 groups was statistically significant ($p = 0.021$), but the NS1Δ126 TX98 group's mean score was intermediate between the two, and not significantly different from either of them. Lung sections examined by histology showed no statistically significant difference between the microscopic lung lesion scores of non-vaccinated (1.59 ± 0.24), NS1Δ126 TX98 (1.72 ± 0.31), and wild type TX98 (1.63 ± 0.46) groups (Supplemental Fig. 1).

3.4. BALF cytokine levels after challenge

T cell-associated cytokines were analyzed in BALF samples collected 5 days following heterosubtypic challenge (Fig. 5). The levels of interleukin-2 (IL-2) and TNF-α were moderately but significantly higher in the group primed with wild type TX98 than in the non-vaccinated or NS1Δ126 TX98 groups. The difference was more pronounced in terms of IFN-γ, as pigs primed with wild type TX98 had 4–5 times higher levels than pigs in the other groups. There were no significant differences in IL-10 protein concentrations among groups in BALF post-challenge.

3.5. Antigen-specific T cell responses following heterosubtypic challenge infection

We collected PBMC from pigs a final time at 5 dpi (70 dpv) and assayed T cells for *in vitro* activation and cytokine secretion

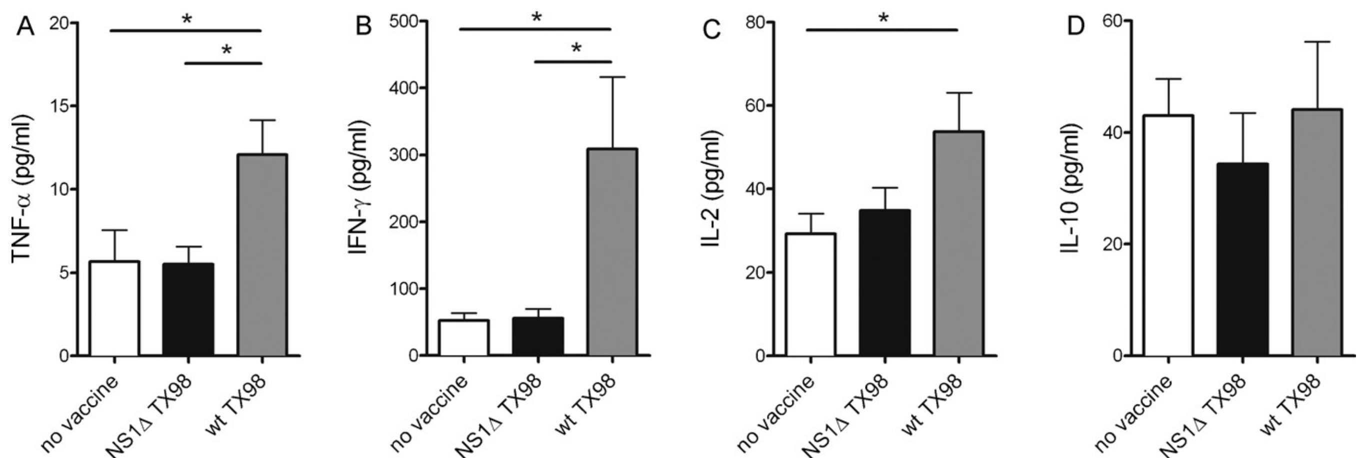

Fig. 5. Cytokine detection in bronchoalveolar lavage fluid samples of pigs 5 days post IA04 (H1N1) challenge infection. Cytokines were assayed by multiplex ELISA. The average of duplicate samples for each pig were used for statistical analysis. Statistical analysis was by one-way ANOVA and the Tukey test for pairwise comparisons. Horizontal bars linking two treatment groups denote statistically significant differences between groups ($p < 0.05$).

in response to wild type TX98 stimulation. The IFN-γ data from Day 70 suggested a modest anamnestic response by $CD4^+CD8^-$ and $\gamma\delta$ T-cells of pigs initially exposed to wild type TX98 (Fig. 3B). Heterologous challenge did not lead to a measurable rise in the IFN-γ response of the $CD4^+CD8^+$ T cells. However, the most notable change in *in vitro* recall responses of T cells after challenge was in the CD25 expression index of $CD4^+CD8^+$ cells. Prior to heterosubtypic challenge, this response was only statistically significant in the wild type TX98-primed treatment group. After challenge, $CD4^+CD8^+$ T cells from the NS1Δ126 TX98 group showed an abrupt anamnestic increase in virus-stimulated CD25 expression compared with corresponding T cells from the non-vaccinated group (Fig. 3A). This indicates the NS1Δ126 TX98 vaccine induced a $CD4^+CD8^+$ T cell memory response, despite the absence of detectable levels in pre-challenge peripheral blood samples.

4. Discussion

Multiple molecular strategies have been developed in recent years for the design of live-attenuated influenza A vaccine candidates. NS1 deletion or truncation attenuates an influenza virus by reducing its ability to antagonize the type I IFN response [28]. It was hypothesized that this class of mutant viruses would elicit robust adaptive immune responses, despite attenuated replication, because restraints on the early innate response would be relaxed. Indeed, viruses with deleted or truncated NS1 have been shown to protect against virulent challenge in mice and pigs [20,29]. A recent study investigated adaptive immune responses in mice given intranasal inoculation with H1N1 strain A/Puerto Rico/8/1934 (PR8) mutated by deletion or truncations of NS1 [30]. In that study, even the most attenuated (NS1 deletion mutant, which did not replicate to a detectable level) and intermediately attenuated (126 amino acids, equal in length to our TX98 NS1Δ126) mutant viruses elicited long-term systemic T- and B-cell responses, including CD8 T cells that expanded after secondary challenge. There appear to be parallels between those observations in mice and our observations in pigs immunized with a similar NS1Δ126 TX98 construct. In both models, only very low levels of vaccine virus replication were detectable in the days following intranasal inoculation, and serum HI antibody responses were lower than those induced by wild type virus. Nonetheless, in both models the vaccines primed antigen-specific T cells, whose numbers were sharply elevated upon subsequent infection of the host. Pigs receiving intranasal NS1Δ126 TX98 also had influenza-specific IgA and IgG in BALF; these mucosal antibody levels were indistinguishable from levels in pigs primed with wild type TX98.

In general, we observed greater T-cell priming in pigs immunized with wild type TX98 infection than in pigs given the attenuated NS1Δ126 TX98 vaccine, especially in terms of CD25 and IFN-γ expression. Cytokine analysis of BALF samples after H1N1 challenge also suggests that more T cells producing Th1-type cytokines were present in lungs of pigs primed with wild type TX98. Consistent with these immunological data, the nasal shedding of heterosubtypic H1N1 challenge virus was controlled more rapidly in wild type TX98-primed animals, although the two groups had similar low viral loads in the BALF. Although the wild type TX98 group had the greatest heterologous protection in terms of viral shedding, possibly due to T-cell memory, it also had the highest macroscopic lung lesion scores at necropsy. Conversely, pigs given the sham vaccine had much higher lung viral titers than other groups, but significantly lower macroscopic lung lesion scores. It has been recognized that cellular immune mediators in lungs can cause collateral tissue damage in the process of clearing viral infections (reviewed by Bruder et al. [31]). In this study, significantly elevated levels of TNF-α, IL-2 and IFN-γ were measured in the BALF of the wild type TX98 treatment group as compared to the non-vaccinated controls. The elevated cytokine data and increased lung pathology resulting from the more reactive immune response primed by wild type TX98 infection may indicate a trade-off between protection and pathology in the influenza infected hosts.

In our study, immunization with live or attenuated virus primed both the $CD4^+CD8^-$ and $CD4^+CD8^+$ T-cell populations for early IFN-γ recall responses. In subsequent assays, prior to challenge, these IFN-γ responses leveled off or declined. After heterologous challenge the characteristic recall responses of these two $CD4^+$ populations became noticeably different, as $CD4^+CD8^+$ cells shifted to a profile of strong CD25 upregulation and low IFN-γ secretion. This is consistent with the established properties of porcine $CD4^+CD8^+$ T cells, which are unique compared to other mammalian hosts in their high frequency outside the thymus [32]. The porcine $CD4^+CD8^+$ population contains memory T helper cells that recognize antigens in an MHC class II-restricted manner [33]. The $CD4^+CD8^+$ are multifunctional, as they express perforin and mediate antigen-specific cytolytic activity against virus-infected target cells [34,35]. It is not clear why the $CD4^+CD8^+$ CD25 EI responses of animals vaccinated with NS1Δ126 TX98 remained low prior to challenge. One possibility is that the wild type TX98 recall antigen suppressed *in vitro* activation of quiescent memory cells. However, we also tested recall responses *in vitro* with UV-inactivated TX98 and NS1Δ126 TX98 viruses, and observed very similar CD25 and intracellular cytokine profiles (data not shown). A more plausible explanation is that these double-positive cells localized preferentially to lymphoid tissues in non-infected animals and were not detectable in the periphery until after challenge.

The recall response of $CD4^+CD8^+$ cells after challenge included an upregulation in CD25 expression, indicative of cell activation, but not an increase in the number of IFN-γ or IL-10 producing cells. This represented a change from $CD4^+CD8^+$ cells at Day 28, when the NS1Δ126 TX98 and wild type TX98 groups had much lower CD25 responses to re-stimulation, but statistically significant IFN-γ responses. These results suggest that the long-term memory cells were uncommitted to either a Th1 or Th2 biased phenotype. Such cells may be comparable to the “central memory” T lymphocyte population, described in mice, which traffic preferentially in lymphoid tissues and are not committed to a Th1, Th2, or Th17 phenotype [36,37]. This would help explain their low frequency in peripheral blood after pigs were immunized, followed by the sharp rise after challenge.

Distinct lymphocyte trafficking patterns might be partially responsible for another surprising result, the minimal detection of antigen-primed $CD4^-CD8^+$ T cells following immunization and challenge. Porcine $CD4^-CD8^+$ T cells have been characterized as classical cytotoxic T lymphocytes (CTL). One goal of developing replicating influenza vaccine viruses, such as NS1Δ126 TX98, is to elicit CTLs through MHC class I-restricted antigen presentation. Future studies will test whether TX98 NS1Δ126 primes $CD4^-CD8^+$ T cells that reside in secondary lymphoid organs or lungs. However, given the very low level of NS1Δ126 TX98 virus detected in pigs' nasal swabs after intranasal inoculation, it is also possible that the vaccine did not supply enough endogenous antigen to prime a large population of $CD4^-CD8^+$ T cells. Viruses engineered with even shorter NS1 proteins than that of NS1Δ126 TX98 are paradoxically less attenuated, in both pigs and mice [17,30]. It is reasonable to predict that mutants such as those would elicit greater $CD4^-CD8^+$ T cell responses.

T-cell response data indicate that both NS1Δ126 TX98 and wild type TX98 induced antigen-specific $\gamma\delta$ T cells. This included intracellular IFN-γ and IL-10 recall responses, before and after heterosubtypic challenge. Porcine $\gamma\delta$ T cells have not been extensively characterized, but they can outnumber $\alpha\beta$ T cells in the circulation

of young pigs [38]. Antigen-specific memory γδ T-cell responses were previously reported in pigs inoculated with porcine reproductive and respiratory syndrome virus [39]. The potential of γδ T cells to protect against influenza infection is not known. In our study, the treatment group with the greatest protection against heterosubtypic challenge, pigs primed with wild type TX98, also had the most robust γδ T-cell responses, suggesting these cells may have had a protective role.

Previous work showed that intranasal NS1Δ126 TX98 elicited significant IgG and IgA antibody titers in lungs, and the vaccine protected pigs against challenge with TX98 or a similar H3N2 strain [21]. In that study, NS1Δ126 TX98 vaccination conferred partial protection against the heterosubtypic IA04 challenge. Our present data provide insight into the cross-reactivity of T-cell epitopes in the TX98 vaccine virus and IA04 challenge virus. The two viruses both contain internal proteins encoded by the TRIG cassette, while HA and NA subtypes are mismatched (H3N2 versus H1N1). Challenge with IA04 appears to have triggered anamnestic responses from T cells that had been primed by NS1Δ126 TX98 or wild type TX98 immunization (Fig. 3A). This is consistent with the concept that many influenza T-cell epitopes occur in internal proteins and are conserved between heterosubtypic viruses [40]. Our analyses of T-cell priming after vaccination point to $CD4^+$ T cell populations as likely factors in NS1Δ126 TX98 vaccine-induced cross-protection, in addition to mucosal antibodies. The results show that highly attenuated SIV vaccines derived by truncating NS1 are capable of inducing broadly reactive cellular immunity and providing protection against strains with dissimilar surface antigens.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2011.10.098.

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