1   **Live attenuated influenza vaccine provides superior protection from**

2   **heterologous infection in pigs with maternal antibodies without**

3   **inducing vaccine associated enhanced respiratory disease**

4

5   **Running title: Vaccine efficacy in pigs with maternal immunity**

6

7   Amy L. Vincent,[1,*] Wenjun Ma,[1,2,#] Kelly M. Lager,[1] Jürgen A. Richt,[1,#] Bruce H. Janke,[2] Matthew R.

8   Sandbulte,[3] Philip Gauger,[1,2] Crystal L. Loving,[1] Richard J. Webby,[4] and Adolfo García-Sastre[5,6,7]

9

10   [1]Virus and Prion Diseases of Livestock Research Unit, National Animal Disease Center, USDA,

11   Agricultural Research Service, Ames, Iowa 50010; [2]Department of Veterinary Diagnostic and

12   Production Animal Medicine and [3]Department of Veterinary Microbiology and Preventive

13   Medicine, Iowa State University, Ames, Iowa 50011; [4]Division of Virology, St. Jude Children's

14   Research Hospital, Memphis, Tennessee 38105; [5]Department of Microbiology, [6]Department of

15   Medicine, Division of Infectious Diseases, and [7]Global Health and Emerging Pathogens Institute,

16   Mount Sinai School of Medicine, New York, New York 10029

17

18   *Corresponding author. Mailing address: National Animal Disease Center, PO Box 70, Ames, IA

19   50010, USA. Phone: (515) 663-7371. Fax: (515) 663-7458. E-mail: amy.vincent@ars.usda.gov

20   [#]Current address: Department of Diagnostic Medicine/Pathobiology, Kansas State University,

21   Manhattan, KS, USA

24   **Abstract**

25   Control of swine influenza A virus (IAV) in the US is hindered since inactivated vaccines do not

26   provide robust cross-protection against the multiple antigenic variants co-circulating in the field.

27   Vaccine efficacy can be further limited when administered to young pigs that possess

28   maternally derived immunity. We previously demonstrated a recombinant A/sw/Texas/4199-

29   2/1998 (TX98) (H3N2) expressing a truncated NS1 protein is attenuated in swine and has

30   potential for use as an intranasal live attenuated influenza virus (LAIV) vaccine. In the present

31   study, we compared 1 dose of intranasal LAIV with 2 intramuscular doses of TX98 whole

32   inactivated virus (WIV) with adjuvant in weanling pigs with and without TX98-specific

33   maternally-derived antibodies (MDA). Pigs were subsequently challenged with wild type

34   homologous TX98 H3N2 virus or with an antigenic variant A/sw/Colorado/23619/1999 (CO99)

35   (H3N2). In the absence of MDA, both vaccines protected against homologous TX98 and

36   heterologous CO99 shedding, although the LAIV elicited lower hemagglutination inhibiting (HI)

37   antibody titers in serum. The efficacy of both vaccines was reduced by the presence of MDA;

38   however, WIV vaccination of MDA-positive pigs led to dramatically enhanced pneumonia

39   following heterologous challenge, a phenomenon known as vaccine-associated enhanced

40   respiratory disease (VAERD). A single-dose of LAIV to MDA-positive pigs still provided partial

41   protection from CO99 and may be a safer vaccine for young pigs in field conditions where dams

42   are routinely vaccinated and diverse IAV strains are in circulation. These results have

43   implications not only to pigs but to other influenza virus host species.

Introduction

44

45 The speed and complexity of swine influenza A virus (IAV) evolution has sharply increased since

46 1998, when a new reassortant lineage with the "triple reassortant internal gene" (TRIG)

47 constellation of internal genes began to circulate and eventually predominate in the North

48 American pig population (29). As a result, many antigenic variants continue to emerge and

49 diminish the field efficacy of IAV vaccines (11, 16, 27). Fully licensed influenza vaccines for use

50 in swine in North America and Europe consist of whole inactivated virus, which may not be an

51 optimal form of antigen for inducing cross-reactive cellular and mucosal immunity against

52 antigenic variants (12). Live attenuated influenza virus (LAIV) vaccines represent an approach

53 that could potentially prime pigs for broader cross-protective immunity. Rational design of

54 attenuated IAV vaccine strains by molecular engineering has been explored in recent studies

55 (14, 18, 23). One method is truncation of the NS1 gene, which encodes an immune modulating

56 interferon antagonist (23, 24). It was previously shown that an H3N2 IAV with a truncated NS1

57 (NS1Δ126 TX98) replicated poorly in pigs after intranasal inoculation, but elicited neutralizing

58 serum antibodies as well as mucosal antibodies and provided robust protection against

59 homologous challenge in naïve pigs with a single intranasal application (26). There was a

60 comparable level of cross-protection against a serologically distinct H3N2 strain in NS1Δ126

61 TX98-vaccinated pigs, which was likely mediated in part by cross-reactive mucosal IgA. The

62 vaccine offered less but still substantial protection against challenge with an H1N1 virus, to

63 which the antibodies failed to cross-react. T-cell priming was not analyzed, but may have

64 contributed to heterologous and heterosubtypic protection. We hypothesize that replicating

65 attenuated virus delivered intranasally (IN), such as NS1Δ126 TX98, primes a more robust

4

66   cellular and mucosal immunity than an inactivated virus vaccine delivered intramuscularly (IM),

67   therefore providing greater cross-protection against variant strains.

68   A concern with inactivated adjuvanted IAV vaccines is the phenomenon of vaccine-

69   associated enhanced respiratory disease (VAERD) (4, 5, 8, 25). This phenomenon is associated

70   with the use of vaccines containing a virus of the same HA subtype as the subsequent challenge

71   strain, but with substantial antigenic drift. Our group recently described VAERD in association

72   with the use of a vaccine containing a human-like delta cluster H1N2 antigen followed by

73   challenge with 2009 pandemic H1N1 (5). A consistent predisposing factor for VAERD is the

74   presence of IgG antibodies that cross-react to the heterologous virus but lack the ability to

75   neutralize infectivity. Distinguishing pathologic features of VAERD include severe

76   bronchointerstitial pneumonia with necrotizing bronchiolitis, interlobular and alveolar edema

77   and hemorrhage (4). These pulmonary changes were accompanied by significant elevation of

78   proinflammatory cytokines.

79   Another obstacle for efficacious vaccination of pigs against IAV is interference from

80   maternally-derived immunity (MDI), particularly maternally-derived antibodies (MDA), acquired

81   through colostrum. Provided there are still sufficient antibody titers in the serum when pigs are

82   infected, MDA can reduce clinical disease (21), but the passive antibodies are less effective in

83   blocking viral shedding from the upper respiratory tract (2, 10), probably because the

84   predominant antibody isotype received in colostrum is IgG. Pigs with significant IAV-specific

85   MDA titers typically have suppressed adaptive antibody responses to homologous infection or

86   vaccination (21). This interference affects IgM, IgG, and hemagglutination inhibition (HI)

87   antibody titers in serum, as well as nasal IgA (10). T-cell proliferation assays have indicated that

5

88   the cellular immune response to IAV is less susceptible to MDA inhibition (8, 10). Analyses of

89   pigs' immune responses to pseudorabies virus, an alpha herpesvirus, have shown a similar

90   pattern, where MDA blocks the humoral but not the cellular immune response following piglet

91   vaccination or infection (19, 28). One perceived advantage of vaccination with LAIV is that

92   circulating MDA (mainly IgG) is less likely to interfere with intranasally-delivered antigen than

93   with inactivated antigen delivered by a parenteral route. In the present study we tested the

94   immunogenicity and protective efficacy of intranasal NS1Δ126 TX98 vaccine versus an

95   inactivated, adjuvanted TX98 administered intramuscularly. These vaccine strategies were

96   tested in naïve and MDA-positive weanling pigs subsequently challenged with homologous or

97   heterologous strains of H3N2 IAV.

98   **Methods**

99   *Viruses and vaccine preparation*

100   Antigen for the whole inactivated virus (WIV) vaccine was A/sw/Texas/4199-2/1998 (wt TX98),

101   grown in Madin-Darby canine kidney (MDCK) cells. Clarified virus from infected culture was

102   inactivated by UV irradiation, using the sterilize setting in an ultraviolet cross-linking chamber

103   (GS Gene Linker, Bio-Rad, Hercules, CA). Inactivation of the virus was confirmed by failure to

104   replicate in 2 serial passages on MDCK cells. A commercial adjuvant was added at a 1:5 ratio

105   (Emulsigen D, MVP Laboratories, Inc., Ralston, NE). Each dose of WIV contained approximately

106   128 HA units of virus. Attenuated virus for the LAIV was generated via reverse genetics as

107   previously described (24). The attenuated vaccine virus contained an NS1 gene with a 3'

108   premature termination plus the insertion of four stop codons in the three frames after,

109   producing a protein 126 amino acids in length with a carboxy-terminal truncation (TX98-

6

110   NS1Δ126). The remaining seven gene segments were from wild type TX98. The challenge

111   viruses included wild-type TX98 H3N2 and a heterologous A/SW/CO/23619/99 H3N2 (CO99).

112   The TX98 and CO99 were shown previously to have limited serologic cross-reactivity (22).

113   Vaccine and challenge viruses were grown in MDCK cells.

114   *Experimental design*

115   Eight sows obtained from a high-health herd free of IAV and porcine reproductive and

116   respiratory syndrome virus (PRRSV) were vaccinated with the TX98 WIV. Each vaccinated sow

117   received 3 doses at 2 week intervals beginning in mid-gestation. Six sows from the same high-

118   health source were not vaccinated for IAV. All sows delivered their pigs without surgical

119   intervention and pigs suckled their own dams. Pigs were bled for evaluation of transfer of MDA

120   at 1 week of age and were weaned at 2 weeks of age. They were treated with ceftiofur

121   crystalline free acid (Pfizer, New York, NY) at weaning to reduce respiratory bacterial

122   contaminants. Pigs were demonstrated to be free of influenza virus by nasal swab sampling,

123   and those born to non-vaccinated sows were shown by serum HI assay to be free of anti-

124   influenza antibody prior to piglet vaccination.

125   To evaluate both vaccines when given in the presence or absence of H3N2 IAV-specific

126   MDA, 51 pigs with MDA were divided into 7 groups and 52 pigs without MDA were divided into

127   7 groups (Table 1). Pigs in the LAIV groups were vaccinated with 2 mL of TX98-NS1Δ126 at 1 X

128   10^6 50% tissue culture infective doses ($TCID_{50}$) per ml by slowly dripping vaccine in the nose.

129   LAIV was administered once at weaning, approximately 14 days of age. Pigs in the WIV groups

130   were vaccinated intramuscularly with 1 ml of the formulation described above, at

131   approximately 14 and 28 days of age. At 8 weeks of age non-vaccinated pigs with MDA were

7

132   determined to have HI titers below 1:40, indicating waning of MDA prior to challenge. Pigs in

133   each challenge group were inoculated with 2 ml (1 X 10^5 TCID$_{50}$/ml) of the indicated virus.

134   Challenge viruses were given intratracheally while the pigs were anesthetized following an

135   intramuscular injection of a cocktail of ketamine (8 mg/kg), xylazine (4 mg/kg), and Telazol (6

136   mg/kg, Fort Dodge Animal Health, Fort Dodge, IA). Challenge groups were housed in individual

137   isolation rooms and cared for in compliance with the Institutional Animal Care and Use

138   Committee of the National Animal Disease Center.

139   ***Clinical observation and sampling***

140   To compare the efficacies of WIV and LAIV against infection with homologous and heterologous

141   viruses, infected pigs were observed daily for clinical signs. Nasal swabs were taken on 0, 3, and

142   5 days post-infection (dpi), placed in 2 ml minimal essential media (MEM) and frozen at -80°C

143   until study completion. All animals were humanely euthanized 5 dpi with a lethal dose of

144   pentobarbital (Sleepaway, Fort Dodge Animal Health, Fort Dodge, IA). After euthanasia, each

145   lung was lavaged with 50 ml MEM to obtain bronchoalveolar lavage fluid (BALF). Nasal swab

146   specimens were filtered (0.45 mm), and a 200 µl aliquot of each was plated onto confluent

147   phosphate buffered saline (PBS) washed MDCK cells in 24-well plates. After 1 hour incubation

148   at 37°C, 200 µl serum-free MEM supplemented with 1 µg/ml TPCK trypsin and antibiotics was

149   added per well. All wells were evaluated for cytopathic effect (CPE) between 48-72 hours. Ten-

150   fold serial dilutions in serum-free MEM supplemented with TPCK trypsin and antibiotics were

151   made with each BALF sample and virus isolation positive nasal swab filtrate sample. Each

152   dilution was plated in triplicate in 100 µl volumes onto PBS-washed confluent MDCK cells in 96-

153   well plates. Plates were evaluated for CPE between 48-72 hours post infection. At 72 hours,

8

154   plates were fixed with 4% phosphate-buffered formalin and stained using

155   immunocytochemistry with an anti-influenza A nucleoprotein monoclonal antibody as

156   previously described (8). A $TCID_{50}$ titer was calculated for each sample using the standard

157   method (20).

158   ***Pathologic examination of lungs***

159   At necropsy, lungs were removed and evaluated for the percentage of the lung affected with

160   purple-red consolidation typical of IAV infection. The percentage of the surface affected with

161   pneumonia was visually estimated for each lung lobe, and a total percentage for the entire lung

162   was calculated based on weighted proportions of each lobe to the total lung volume (6). Tissue

163   samples from the trachea and right middle or affected lung lobe were fixed in 10% buffered

164   formalin for histopathologic examination. Tissues were processed by routine histopathologic

165   procedures and slides stained with hematoxylin and eosin. Microscopic lesions were evaluated

166   by a veterinary pathologist blinded to treatment groups. Individual scores were assigned to

167   each of three parameters: percent of intrapulmonary airways demonstrating epithelial necrosis

168   or proliferation, percent of bronchi and bronchioles demonstrating peribronchiolar lymphocytic

169   cuffing (PBLC) and magnitude of neutrophil exudation in bronchioles and alveoli. The

170   intrapulmonary airway epithelium was scored according to the following criteria: (0.0) no

171   significant lesions; (1.0) a few airways affected with bronchiolar epithelial damage; (1.5) more

172   than a few airways affected (up to 25%); (2.0) 50% airways affected often with interstitial

173   pneumonia; (2.5) approximately 75% airways affected, usually with significant interstitial

174   pneumonia; (3.0) greater than 75% airways affected, usually with significant interstitial

175   pneumonia. Peribronchiolar lymphocytic cuffing was scored according to the following criteria:

9

176   (0.0) no significant lesions; (1.0) a few airways with light PBLC; (1.5) more than a few airways

177   with PBLC (up to 25%); (2.0) 50% airways with PBLC; (2.5) approximately 75% airways with

178   PBLC; (3.0) greater than 75% airways with PBLC. Neutrophil (PMN) exudation in bronchioles and

179   alveoli were scored according to the following criteria: (0.0) none to minimal presence of

180   neutrophils; (1.0) small clusters of PMNs present in occasional airways; (2.0) Prominent small to

181   large aggregates of PMNs in bronchiolar lumens, minimally in alveoli. A composite score was

182   computed using the sum of the three individual scores. The average group composite score

183   was used for statistical analysis.

184   The trachea was evaluated with a single score based on the magnitude of epithelial

185   attenuation or necrosis. Trachea scores were based on the following criteria: (0.0) normal

186   epithelium the entire circumference; (1.0) focal epithelial attenuation; (2.0) Extensive epithelial

187   attenuation or necrosis.

188   ***Serologic and mucosal antibody assays***

189   Serum samples were collected by anterior vena cava or jugular venipuncture at the following

190   points: pre-weaning (-7 dpv), primary vaccination (0 dpv), WIV secondary vaccination (14 dpv),

191   2 weeks post-secondary vaccination (28 dpv), challenge inoculation (49 dpv / 0 dpi), and

192   necropsy (5 dpi). For use in the HI assay, sera were heat inactivated at 56°C and treated to

193   remove non-specific agglutinators with a 20% suspension of Kaolin (Sigma Aldrich, St. Louis,

194   MO) followed by adsorption with 0.5% turkey red blood cells (RBC). HI assays were done using

195   TX98 and CO99 viral antigens and turkey RBC using standard techniques and with a maximum

196   titer of 1:640 (17).

197   Enzyme-linked immunosorbent assays (ELISA) to detect total IgG and IgA antibodies

198   against whole virus preparations of TX98 and CO99 present in serum and BALF were performed

199   as previously described (Gauger et al., 2011) with the following modifications. Serum samples

200   were diluted to 1:2000 for the IgG ELISA. BALF samples were diluted to 1:4 for IgG and IgA

201   ELISAs. Samples were diluted in bovine serum albumen (Fraction V BSA, Life Technologies) and

202   PBS with a final concentration of 5% BSA to block non-specific antibodies. Independent assays

203   were conducted using 50 μL of whole virus TX98 or CO99 at 100 HA units per well as ELISA

204   antigen and coated plates were blocked with 150 μL of a commercial blocking buffer (Starting

205   Block, Thermo Fisher). Anti-swine IgG (Kirkegaard and Perry) and anti-swine IgA (Bethyl

206   Laboratories) were used at a 1:1500 dilutions in blocking buffer. Each sample was analyzed in

207   duplicate. The optical density (O.D.) was measured at 405 nm wavelength with an automated

208   ELISA reader. Antibody levels were reported as the mean O.D. for each sample and the means

209   for each treatment group were compared.

210   ***Statistical analysis***

211   Macroscopic and microscopic pneumonia scores, $\log_{10}$ transformed BALF and nasal swab virus

212   titers, $\log_2$ transformations of HI reciprocal titers, and mean O.D. for ELISA assays were

213   analyzed using analysis of variance (ANOVA) with a p-value ≤ 0.05 considered significant

214   (GraphPad Prism software, La Jolla, CA). Data from treatment groups infected with different

215   virus strains (TX98 versus CO99) were analyzed separately. Response variables shown to have a

216   significant effect by treatment group were subjected to pair-wise mean comparisons using the

217   Tukey-Kramer test.

218   **Results**

11

219 ***Serology***

220 Serum antibody responses to the vaccines displayed different profiles compared between

221 MDA-negative and MDA-positive pigs. In MDA-negative pigs, WIV induced high HI responses in

222 sera against TX98 with a geometric mean reciprocal titer of 556 at 49 dpv, whereas LAIV

223 induced TX98-specific HI titers only marginally above the limit of detection (Fig. 1A). Similarly,

224 WIV induced greater levels of TX98-specific serum IgG in MDA-negative pigs (Fig. 1C). WIV

225 vaccination induced a modest level of cross-reacting HI titers in sera against CO99 in MDA-

226 negative pigs, with a geometric mean reciprocal titer of 61 at 0 dpi (Fig. 1B), and there was a

227 corresponding increase in CO99 serum IgG (Fig. 1D). In contrast, LAIV induced no detectable HI

228 or total IgG cross-reactivity against CO99, even in MDA-negative pigs (Fig. 1B and 1D,

229 respectively).

230 Pigs that suckled immunized dams acquired MDA, as measured by serum HI titers

231 against the vaccine strain TX98 at seven days before vaccination (-7 dpv) with a geometric

232 mean reciprocal titer of 312 and a range between 40 and ≥640; however, pigs in the MDA-

233 positive groups did not respond to LAIV or WIV vaccination with increases in HI antibody titers

234 to TX98 (Fig. 1A). In these MDA-positive pigs, weakly cross-reactive HI titers against CO99 were

235 detectable at -7 dpv (Fig. 1B). Homologous HI titers in the MDA-positive pigs declined by the

236 day of challenge (49 dpv) to levels below or near the lower limit of detection (≤40). However,

237 prior to challenge maternally-derived serum IgG specific to TX98 was still detectable by ELISA in

238 non-vaccinated controls (Fig. 1C) but not against the heterologous CO99 (Fig. 1D). Although IgG

239 levels in the WIV-vaccinated MDA-positive group were significantly higher than in the non-

240 vaccinated MDA-positive group, WIV vaccination resulted in significantly higher IgG levels in the

12

241 MDA-negative pigs against both the TX98 and CO99 viruses (Fig. 1C and 1D, respectively). LAIV

242 given to MDA positive pigs failed to induce an increase in the pre-existing total IgG in sera

243 against TX98 or CO99 (Fig. 1C and 1D), similar to the HI response. Serum IgG responses after

244 experimental challenge with TX98 or CO99 (5 dpi) displayed a similar pattern to the pre-

245 challenge results (Fig. 1E and 1F), with the notable exception of a boost in IgG antibodies

246 binding to CO99 in the pigs given LAIV in the absence of MDA and challenged with CO99 (Fig.

247 1F).

248 ***Mucosal Antibody Responses***

249 After TX98 challenge (5 dpi), there were significant levels of TX98-specific IgG in lungs of WIV

250 vaccinates, whether vaccinated in the presence or absence of MDA (Fig. 2A). LAIV vaccinates

251 had significant levels of TX98-specific IgG in lungs only if the vaccine was administered in the

252 absence of MDA (Fig. 2A). This was consistent in pigs challenged with CO99 as well (Fig. 2B). In

253 sharp contrast, statistically significant IgA levels were only detected in the lungs of pigs

254 vaccinated with LAIV in the absence of MDA. Results were similar in pigs challenged with either

255 virus and in both the TX98 and CO99 ELISA assays (Fig. 2C and 2D, respectively). Thus, similar to

256 the serum antibody profiles, mucosal antibody responses also differed between vaccine types

257 and were impacted by the presence of MDA at the time of vaccination.

258 ***Replication of challenge viruses***

259 Distinct differences were detected in replication of challenge virus based on MDA and vaccine

260 status. Nasal replication of TX98 and CO99 challenge viruses was monitored by virus isolation

261 and titration of virus in nasal swabs. As expected, no virus was detected in any of the pigs on

262 the day of challenge (data not shown). In MDA-negative, non-vaccinated (NV) animals the two

13

263   viruses reached similar nasal titers at 3 and 5 dpi, between 10^4 and 10^5 TCID$_{50}$/ml (Fig 3). Non-

264   vaccinated pigs that received TX98-specific MDA shed 10- to 100-fold less TX98 virus 3 and 5 dpi

265   (Fig 3), even though their passive serum HI titers were near the lower limit of detection at the

266   time of challenge (Fig 1A). In contrast, nasal shedding of CO99 was not significantly inhibited by

267   the presence of residual TX98-specific MDA (Fig 3B). In MDA-negative pigs vaccinated with WIV

268   or LAIV, both vaccines provided effective protection against the nasal shedding of TX98 and

269   CO99, as almost no virus was detected 3 or 5 dpi in these treatment groups. Pigs that had been

270   vaccinated with WIV in the face of MDA demonstrated reduced TX98 virus shedding at both

271   samplings, but not complete prevention as in the MDA-negative, WIV-vaccinated pigs. The

272   single dose of LAIV vaccine in the face of MDA also did not prevent shedding of TX98 at 3 dpi,

273   but it led to a statistically significant reduction in nasal titers by 5 dpi, when compared with NV

274   pigs with MDA. Finally, when administered in the face of MDA, both vaccines failed to protect

275   against nasal replication of heterologous CO99, although statistically significant reductions in

276   group mean titers were detected at both time points in the LAIV-vaccinated MDA pigs.

277   Virus titers were also measured in BALF specimens collected at necropsy, 5 dpi (Fig 4A).

278   BALF collected from NV pigs had mean titers of 10^5-10^6 TCID$_{50}$/ml. Residual MDA appeared to

279   provide a very limited amount of protection against TX98 replication in the lung –

280   approximately a 10-fold reduction in titer – but not against CO99 (Fig 4A). These results are

281   similar to what was observed for nasal titers at 5 dpi, though reductions due to MDA were

282   greater in the nose compared to the lung. BALF samples from MDA-negative WIV and LAIV

283   vaccinees contained no detectable TX98 or CO99, which closely followed the prevention of

284   nasal shedding in these vaccinated groups. Even in the face of MDA, both vaccines significantly

14

285   reduced the TX98 virus loads in BALF (Fig. 3A). However, CO99 BALF viral loads were not

286   reduced in WIV vaccinates when the vaccine was administered in the face of MDA. MDA also

287   interfered with the efficacy of LAIV against CO99 although less dramatically than for WIV, as

288   LAIV provided for a significant reduction in BALF CO99 titers.

289   ***Lung pathology***

290   Challenge with either H3N2 strain caused mild lung pathology in pigs lacking maternal or

291   vaccine-induced immunity, consistent with previous reports (22, 26). MDA-positive pigs

292   vaccinated with WIV subsequently developed enhanced macroscopic pneumonia when

293   challenged with either homologous TX98 or heterologous CO99 (Fig 4B and 5C) as compared to

294   their respective MDA-negative counterparts. This WIV-associated enhancement was

295   particularly evident after CO99 challenge, with a group mean of 35% of the lung area affected

296   with pneumonia. Importantly, no enhancement in macroscopic pneumonia was seen with LAIV

297   under either MDA condition with either challenge virus and there was a general trend for

298   reduction in pneumonia. Non-vaccinated and LAIV groups challenged with CO99 had average

299   pneumonia percentages of 4.2% and 2.2%, respectively. Likewise the LAIV vaccine significantly

300   reduced the percentage of macroscopic lung pathology in MDA-negative animals challenged

301   with TX98. In pigs lacking MDA, both of the vaccines were associated with significantly reduced

302   macroscopic lung pathology following heterologous CO99 challenge. Although TX98-specific

303   serum HI titers from MDA had largely waned in non-vaccinated pigs by the time of challenge,

304   macroscopic pneumonia was less extensive in non-vaccinated pigs that had received MDA than

305   in those without MDA (Fig 3B). This difference in severity of macroscopic lesions corresponded

306   with a similar trend in BALF viral titer 5 dpi (Fig. 4A).

15

307   When administered to MDA-negative animals, both vaccines showed protective effects

308   against both challenge viruses with respect to microscopic lung lesion scores (Fig 4C). The

309   protective effects of LAIV against both challenge viruses, which did not reach statistical

310   significance, appeared to be maintained when the vaccine was given to MDA-positive animals.

311   However, as with macroscopic pneumonia, there were distinctly different outcomes when

312   MDA-positive, WIV-vaccinated pigs were challenged with homologous TX98 versus

313   heterologous CO99. Those vaccinated with WIV in the face of MDA, then challenged with TX98,

314   had microscopic lesion scores not different from the non-vaccinated MDA-positive group. In

315   contrast, WIV vaccination of MDA-positive pigs not only failed to provide protection against

316   heterologous CO99, but a significant enhancement in lesion severity was demonstrated

317   microscopically, paralleling the dramatic difference that was seen macroscopically. Importantly,

318   LAIV did not contribute to enhanced microscopic lesion severity with either challenge virus.

319   ***Tracheal pathology***

320   Regardless of MDA status, TX98 tended to induce more tracheal lesions than CO99 (Fig. 4D). In

321   MDA negative pigs, both vaccines provided statistically significant reduction of these TX98

322   lesions, and in MDA positive pigs, LAIV still significantly reduced TX98-induced tracheal damage.

323   However, tracheal damage was sharply higher in pigs that received WIV vaccine in the face of

324   MDA and then were challenged with CO99. Thus, the overall enhanced respiratory disease from

325   heterologous infection of the MDA-positive WIV vaccinates was clearly evident in the trachea as

326   well as in the lung.

Discussion

327

328 The antigenic diversity of contemporary and emerging IAV strains is a major obstacle to

329 effective and reliable vaccines for swine (16). IAV vaccines currently licensed around the world

330 contain inactivated viral antigens representing H1N1, H3N2, H1N2, and 2009 pandemic H1N1

331 strains (12). Inactivated IAV vaccines elicit systemic neutralizing antibodies and protection

332 against homologous challenge, but their efficacy against antigenically distinct strains is often

333 diminished (1, 9). Intranasal vaccination with an attenuated virus is considered likely to elicit

334 more cross-reactive T cells and mucosal antibodies against antigenically variant strains (12).

335 Several attenuated viral constructs made by targeted genetic mutations have been tested in

336 recent years (13, 18, 24). We previously reported the immunogenicity and protective efficacy of

337 TX98 virus attenuated by truncation of the NS1 gene, which encodes a type I interferon

338 antagonist protein (23, 26). This virus (identical to LAIV in the present report) was shown to

339 have attenuated replication in the upper respiratory tract. When administered as an intranasal

340 vaccine to young seronegative pigs, the TX98 LAIV elicited a mucosal IgA response, modest

341 titers of serum HI antibodies, and antigen-specific T cells, while conferring protection against

342 homologous challenge and a degree of cross-protection against variant strains with a single or

343 two-dose regimen (7, 26). Our previous work indicated that two intranasal applications of the

344 LAIV did not confer any benefit against homologous challenge compared to one dose (26) and

345 one dose would be highly preferred for use in the swine population. Here we show that a single

346 dose was highly efficacious against the homologous TX98 and heterologous CO99 in MDA-

347 negative pigs. Although future studies are necessary to investigate whether two doses would

348 improve efficacy in the presence of MDA, the impact of the findings of this study come from the

349 differences between WIV and LAIV in the face of MDA with heterologous challenge.

350   One aim of the present study was to compare the efficacy of intranasal LAIV versus

351 intramuscular WIV vaccination in seronegative pigs. WIV vaccination induced high serum HI

352 titers to the homologous antigen, while HI responses following LAIV vaccination were weak or

353 below detection limits. Even before the WIV vaccinees were boosted with a second dose, they

354 achieved higher HI titers than LAIV vaccinees (Fig. 1A). Cross-reactive HI antibody titers against

355 heterologous CO99 tended to be 4- to 16-fold lower than homologous titers, and these were

356 only detectable in WIV vaccinees. Despite the marked differences in serological responses, both

357 vaccines supplied significant protection in the absence of MDA against the replication of not

358 only homologous TX98 but also heterologous CO99. Based on these data, protective immunity

359 induced by LAIV vaccination was likely mediated by T cells and/or mucosal antibodies, and here

360 we demonstrate a robust IgA response in the lower respiratory tract when the LAIV was

361 administered in the absence of MDA. Higher levels of IgG in the serum were also detected at 5

362 dpi, indicating a cross-reacting boost of antibody to the CO99 virus exposure. It is not clear if

363 the higher levels of serum (and mucosal) antibodies to CO99 at 5 dpi in the MDA-negative pigs

364 were specific only to the CO99 challenge virus or if the CO99 challenge boosted the TX98

365 primed response, particularly against epitopes that are shared between TX98 and CO99. While

366 there was no evidence of virus replication, the immune system had likely formed prior

367 immunity against common epitopes contained in the LAIV vaccine virus that may have been

368 boosted upon exposure to the CO99 virus. The protection provided by WIV vaccine against

369 CO99 in the non-MDA pigs was surprising, since previous studies reported limited HI cross-

18

370   reactivity between TX98 and CO99 (22) and the geometric mean cross-reactive HI titer at the

371   time of challenge in this study was 61, about ten-fold lower than the geometric mean

372   homologous HI titer to TX98. The cross-reacting HI antibodies and mucosal IgG antibodies

373   detected at 5 dpi are likely to have played a role in the heterologous protection, perhaps

374   enabled by the magnitude of the systemic antibody response to WIV. Cross-reactive T-cells

375   primed by the WIV may have also contributed to the protective effect against the heterologous

376   CO99 infection. Consistent with this, a similarly formulated H1N2 WIV vaccine was shown to

377   prime T cells in antibody-negative young pigs (7).

378   IAV vaccination of sows is a widespread practice in North American swine herds (3). This

379   presents a second practical problem concerning IAV vaccines in the swine industry: antibodies

380   transferred in colostrum from sows to their litters can interfere with subsequent vaccination of

381   the piglets (8) and are often poorly matched to viruses circulating on the sow farm or in down-

382   stream production stages. Colostrum-borne maternal antibodies, which are predominantly IgG,

383   are not expected to infiltrate the nasal mucosa of the upper respiratory tract, so we

384   hypothesized that the LAIV vaccine would be less sensitive to inhibition by MDA. Our serological

385   results demonstrated that MDA indeed prevented pigs from mounting active HI antibody

386   responses to WIV vaccine (Fig. 1A). Despite this, WIV vaccine administered in the face of MDA

387   still provided significant protection against homologous TX98 challenge, including decreased

388   nasal shedding (Fig. 3) and replication in the lung (Fig. 4A), but did not prevent damage to the

389   lower respiratory tract (Fig. 4B-D). This pointed again to the possibility that the adjuvanted WIV

390   in the face of MDA primed a cellular immune response that contributed to protection against

19

391   homologous virus. Moderate T-cell priming was demonstrated in pigs vaccinated with a similar

392   formulation of inactivated H1N2 IAV (7).

393   Critically, though, there was a pronounced failure of WIV vaccination in MDA-positive

394   pigs that were challenged with heterologous CO99. In this group, the vaccine failed to reduce

395   viral replication in nasal passages and the viral load in BALF (Fig. 3 and 4A). Strikingly,

396   macroscopic lung lesions were exacerbated (Fig. 4B and 5C) in a manner similar to another

397   VAERD model (4, 5, 25). Composite microscopic lung and tracheal lesions (Fig. 4 C-D) were

398   enhanced and similar in character to VAERD lesions previously reported (4). In sharp contrast,

399   there was no enhancement of pathologic lung changes in the MDA-positive group vaccinated

400   with LAIV; in fact, LAIV was partially protective against CO99 in terms of reducing lung damage

401   and viral load on 5 dpi (Fig. 4 and Fig. 5). Thus, although WIV and LAIV had similar efficacy in

402   naïve pigs, the presence of MDA titers at the time of vaccination followed with heterologous

403   viral challenge produced sharply different outcomes between the two vaccines. The abrogation

404   of the LAIV-induced IgA response in the lower respiratory tract (Fig. 2D) may explain the

405   reduction in efficacy in the MDA-positive pigs compared to LAIV given to MDA-negative pigs.

406   Evaluation of the antibody profile in the upper respiratory tract (nasal mucosa) was not

407   conducted in this study but should be considered in future studies to understand how MDA

408   interferes with the LAIV-induced mucosal antibody response and if the inhibition is limited to

409   the lower respiratory tract. We speculate that the cellular immune response to LAIV in MDA-

410   positive pigs was a key factor in cross-protection, since no lung IgA or cross-reacting serum HI

411   antibodies were evident above the limits of detection. It is also unclear if there was a role for

412   the presumably non-neutralizing IgG in the lungs of MDA-positive WIV vaccinates with VAERD

20

413   following heterologous challenge with CO99 in comparison to the absence of IgG in the lungs of

414   MDA-positive LAIV vaccinates without VAERD.

415   In a previous study, MDA-positive pigs that received a bivalent IAV vaccine containing

416   inactivated classical H1N1 were primed for enhanced pneumonia upon heterologous H1N1

417   challenge, whereas vaccination of MDA-negative pigs provided cross-protection (8). This

418   detrimental interaction between passive immunity and WIV vaccination parallels what we

419   observed in the present study with H3N2 viruses. Although the earlier study did not include an

420   LAIV treatment group for comparison, it did show evidence that intramuscular vaccination with

421   an inactivated virus administered in the face of MDA was ineffective at priming protective T-cell

422   memory. The mechanism(s) responsible for the enhancement of respiratory disease in our

423   model is not completely clear. It can be hypothesized that MDA's bind to vaccine antigen and

424   the method of antigen processing and presentation is different than when vaccine antigen is

425   not bound to antibody (seronegative pig). This change in antigen uptake and subsequent

426   presentation may alter the adaptive immune response (both humoral and cell-mediated) in the

427   piglet, possibly directing it away from neutralizing epitopes to conserved, albeit non-

428   neutralizing epitopes shared between the vaccine virus and challenge virus. After 2 doses of

429   WIV, HI antibodies against neither TX98 nor CO99 were detected in MDA-positive pigs, whereas

430   HI antibodies against both were detected in MDA-negative pigs. Although there was no HI

431   antibody response in MDA-positive pigs receiving WIV, there appeared to be a modest increase

432   in total IgG specific to TX98 in the serum prior to challenge (Fig. 1C). Total IgG was also present

433   in the lung at 5 dpi (Fig. 2 A and B) when there was still no detectable HI response in the serum

434   in this group (data not shown). This indicates that MDA interfered with the induction of

21

435   neutralizing HI antibodies by WIV. Upon challenge with the heterologous virus, immune

436   complexes may form between non-neutralizing antibodies and challenge virus that trigger

437   inflammatory responses such as those implicated in vaccine-enhanced respiratory syncytial

438   virus (RSV) infection of infants (3). The involvement of antibody in generating immune

439   complexes associated with severe respiratory disease and pulmonary damage has also been

440   described for 2009 pandemic H1N1 influenza disease (15). Roles for specific IgG subclasses have

441   not been clearly defined in the pig; thus, this type of analysis is not available for further

442   interrogation of within type differences in antibody responses induced by WIV vaccination in

443   the face of MDA. However, functional or qualitative differences in the antibodies produced in

444   response to WIV and LAIV in the presence or absence of MDA appear likely to have a critical

445   role in the clinical outcome after infection.

446   Another hypothesis, though not mutually exclusive, is that differences in antigen

447   processing and presentation of WIV vaccine antigen in MDA-positive pigs may alter the kinetics

448   of the response or may prime a qualitatively different T cell response and these T cells play a

449   role in immunopathology. A different subset of memory T cells may develop in MDA-positive,

450   WIV vaccinated pigs that upon heterologous challenge, when cross-neutralizing antibody is not

451   present, are activated and contribute to pathology, possibly through granzyme release and

452   killing of infected cells. Additional studies are needed to further characterize differences in the

453   antibody and T cell responses that develop following WIV vaccination of MDA-positive and

454   MDA-negative pigs and elucidate the immunopathogenic mechanism of enhanced disease

455   following heterologous challenge.

456   Collectively, the results of this experiment demonstrate very distinct outcomes of IAV

457   vaccination and infection with heterologous virus, with pivotal factors including the format and

458   route of administration of vaccines, the presence or absence of MDA, and the antigenic

459   similarity of challenge virus to the vaccine strain. The differences go beyond protection versus

460   non-protection, and point to realistic scenarios in the field where vaccinating sows and their

461   piglets could potentiate more severe respiratory disease. This underscores the need to re-

462   evaluate the way in which efficacy studies are designed for swine influenza vaccine licensure for

463   use in pigs in the United States. Methods of IAV vaccine evaluation that focus simply on

464   protection against homologous challenge in seronegative pigs would be unlikely to identify this

465   problem. Determining the immune correlates of protection versus disease exacerbation would

466   significantly aid the improvement of vaccine safety and efficacy under field conditions.

467   **Acknowledgements**

References

476

477   1. **Bikour, M. H., E. Cornaglia, and Y. Elazhary.** 1996. Evaluation of a protective immunity

478   induced by an inactivated influenza H3N2 vaccine after an intratracheal challenge of pigs.

479   Can J Vet Res **60:**312-314.

480   2. **Choi, Y. K., S. M. Goyal, and H. S. Joo.** 2004. Evaluation of transmission of swine influenza

481   type A subtype H1N2 virus in seropositive pigs. Am J Vet Res **65:**303-306.

482   3. **Delgado, M. F., S. Coviello, A. C. Monsalvo, G. A. Melendi, J. Z. Hernandez, J. P. Batalle, L.**

483   **Diaz, A. Trento, H. Y. Chang, W. Mitzner, J. Ravetch, J. A. Melero, P. M. Irusta, and F. P.**

484   **Polack.** 2009. Lack of antibody affinity maturation due to poor Toll-like receptor stimulation

485   leads to enhanced respiratory syncytial virus disease. Nat Med **15:**34-41.

486   4. **Gauger, P. C., A. L. Vincent, C. L. Loving, J. N. Henningson, K. M. Lager, B. H. Janke, M. E.**

487   **Kehrli, Jr., and J. A. Roth.** 2012. Kinetics of Lung Lesion Development and Pro-Inflammatory

488   Cytokine Response in Pigs With Vaccine-Associated Enhanced Respiratory Disease Induced

489   by Challenge With Pandemic (2009) A/H1N1 Influenza Virus. Vet Pathol.

490   5. **Gauger, P. C., A. L. Vincent, C. L. Loving, K. M. Lager, B. H. Janke, M. E. Kehrli, Jr., and J. A.**

491   **Roth.** 2011. Enhanced pneumonia and disease in pigs vaccinated with an inactivated

492   human-like (delta-cluster) H1N2 vaccine and challenged with pandemic 2009 H1N1

493   influenza virus. Vaccine **29:**2712-2719.

494   6. **Halbur, P. G., P. S. Paul, M. L. Frey, J. Landgraf, K. Eernisse, X. J. Meng, M. A. Lum, J. J.**

495   **Andrews, and J. A. Rathje.** 1995. Comparison of the pathogenicity of two US porcine

496   reproductive and respiratory syndrome virus isolates with that of the Lelystad virus. Vet

497   Pathol **32:**648-660.

24

498   7. **Kappes, M. A., M. R. Sandbulte, R. Platt, C. Wang, K. M. Lager, J. N. Henningson, A.**

499   **Lorusso, A. L. Vincent, C. L. Loving, J. A. Roth, and M. E. Kehrli, Jr.** 2011. Vaccination with

500   NS1-truncated H3N2 swine influenza virus primes T cells and confers cross-protection

501   against an H1N1 heterosubtypic challenge in pigs. Vaccine.

502   8. **Kitikoon, P., D. Nilubol, B. J. Erickson, B. H. Janke, T. C. Hoover, S. A. Sornsen, and E. L.**

503   **Thacker.** 2006. The immune response and maternal antibody interference to a

504   heterologous H1N1 swine influenza virus infection following vaccination. Vet Immunol

505   Immunopathol **112:**117-128.

506   9. **Lee, J. H., M. R. Gramer, and H. S. Joo.** 2007. Efficacy of swine influenza A virus vaccines

507   against an H3N2 virus variant. Can J Vet Res **71:**207-212.

508   10. **Loeffen, W. L., P. P. Heinen, A. T. Bianchi, W. A. Hunneman, and J. H. Verheijden.** 2003.

509   Effect of maternally derived antibodies on the clinical signs and immune response in pigs

510   after primary and secondary infection with an influenza H1N1 virus. Vet Immunol

511   Immunopathol **92:**23-35.

512   11. **Lorusso, A., A. L. Vincent, M. L. Harland, D. Alt, D. O. Bayles, S. L. Swenson, M. R. Gramer,**

513   **C. A. Russell, D. J. Smith, K. M. Lager, and N. S. Lewis.** 2010. Genetic and antigenic

514   characterization of H1 influenza viruses from United States swine from 2008. J Gen Virol.

515   12. **Ma, W., and J. A. Richt.** 2010. Swine influenza vaccines: current status and future

516   perspectives. Anim Health Res Rev **11:**81-96.

517   13. **Masic, A., J. S. Booth, G. K. Mutwiri, L. A. Babiuk, and Y. Zhou.** 2009. Elastase-dependent

518   live attenuated swine influenza A viruses are immunogenic and confer protection against

519   swine influenza A virus infection in pigs. J Virol **83:**10198-10210.

520   14. **Masic, A., X. Lu, J. Li, G. K. Mutwiri, L. A. Babiuk, E. G. Brown, and Y. Zhou.** 2010.

521   Immunogenicity and protective efficacy of an elastase-dependent live attenuated swine

522   influenza virus vaccine administered intranasally in pigs. Vaccine **28:**7098-7108.

523   15. **Monsalvo, A. C., J. P. Batalle, M. F. Lopez, J. C. Krause, J. Klemenc, J. Z. Hernandez, B.**

524   **Maskin, J. Bugna, C. Rubinstein, L. Aguilar, L. Dalurzo, R. Libster, V. Savy, E. Baumeister, G.**

525   **Cabral, J. Font, L. Solari, K. P. Weller, J. Johnson, M. Echavarria, K. M. Edwards, J. D.**

526   **Chappell, J. E. Crowe, Jr., J. V. Williams, G. A. Melendi, and F. P. Polack.** 2011. Severe

527   pandemic 2009 H1N1 influenza disease due to pathogenic immune complexes. Nat Med

528   **17:**195-199.

529   16. **Olsen, C. W.** 2002. The emergence of novel swine influenza viruses in North America. Virus

530   Res **85:**199-210.

531   17. **Palmer, D. F., M. T. Coleman, W. D. Dowdle, and G. O. Schild.** 1975. Advanced laboratory

532   techniques for influenza diagnosis. Immunology series no. 6. U.S. Dept. of Health, Education,

533   and Welfare, Washington, D.C.

534   18. **Pena, L., A. L. Vincent, J. Ye, J. R. Ciacci-Zanella, M. Angel, A. Lorusso, P. C. Gauger, B. H.**

535   **Janke, C. L. Loving, and D. R. Perez.** 2011. Modifications in the polymerase genes of a

536   swine-like triple-reassortant influenza virus to generate live attenuated vaccines against

537   2009 pandemic H1N1 viruses. J Virol **85:**456-469.

538   19. **Pomorska-Mol, M., I. Markowska-Daniel, and Z. Pejsak.** 2010. Evaluation of humoral and

539   antigen-specific T-cell responses after vaccination of pigs against pseudorabies in the

540   presence of maternal antibodies. Vet Microbiol **144:**450-454.

541 20. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty percent end points. .

542   American Journal of Hygiene **27:**5.

543 21.**Renshaw, H. W.** 1975. Influence of antibody-mediated immune suppression on clinical, viral,

544   and immune responses to swine influenza infection. Am J Vet Res **36:**5-13.

545 22. **Richt, J. A., K. M. Lager, B. H. Janke, R. D. Woods, R. G. Webster, and R. J. Webby.** 2003.

546   Pathogenic and antigenic properties of phylogenetically distinct reassortant H3N2 swine

547   influenza viruses cocirculating in the United States. J Clin Microbiol **41:**3198-3205.

548 23. **Richt, J. A., P. Lekcharoensuk, K. M. Lager, A. L. Vincent, C. M. Loiacono, B. H. Janke, W. H.**

549   **Wu, K. J. Yoon, R. J. Webby, A. Solorzano, and A. Garcia-Sastre.** 2006. Vaccination of pigs

550   against swine influenza viruses by using an NS1-truncated modified live-virus vaccine. J Virol

551   **80:**11009-11018.

552 24. **Solorzano, A., R. J. Webby, K. M. Lager, B. H. Janke, A. Garcia-Sastre, and J. A. Richt.** 2005.

553   Mutations in the NS1 protein of swine influenza virus impair anti-interferon activity and

554   confer attenuation in pigs. J Virol **79:**7535-7543.

555 25. **Vincent, A. L., K. M. Lager, B. H. Janke, M. R. Gramer, and J. A. Richt.** 2008. Failure of

556   protection and enhanced pneumonia with a US H1N2 swine influenza virus in pigs

557   vaccinated with an inactivated classical swine H1N1 vaccine. Vet Microbiol **126:**310-323.

558 26. **Vincent, A. L., W. Ma, K. M. Lager, B. H. Janke, R. J. Webby, A. Garcia-Sastre, and J. A.**

559   **Richt.** 2007. Efficacy of intranasal administration of a truncated NS1 modified live influenza

560   virus vaccine in swine. Vaccine **25:**7999-8009.

561 27. **Webby, R. J., K. Rossow, G. Erickson, Y. Sims, and R. Webster.** 2004. Multiple lineages of

562   antigenically and genetically diverse influenza A virus co-circulate in the United States swine

563   population. Virus Res **103:**67-73.

564 28. **Wittmann, G., and V. Ohlinger.** 1987. Aujeszky's disease vaccination and infection of pigs

565   with maternal immunity: effects on cell- and antibody-mediated immunity. Arch Virol **92:**87-

566   101.

567 29. **Zhou, N. N., D. A. Senne, J. S. Landgraf, S. L. Swenson, G. Erickson, K. Rossow, L. Liu, K.**

568   **Yoon, S. Krauss, and R. G. Webster.** 1999. Genetic reassortment of avian, swine, and

569   human influenza A viruses in American pigs. J Virol **73:**8851-8856.

570 **Table 1.** Study design for comparison of LAIV and WIV vaccines

Treatment Group	MDA Status[1]	Piglet Vaccine	Piglet Challenge	N
MDA / NV / NC	+	None	Sham	4
MDA / NV / TX	+	None	wt TX98	8
MDA / NV / CO	+	None	wt CO99	8
MDA / WIV / TX	+	TX98 WIV	wt TX98	8
MDA / WIV / CO	+	TX98 WIV	wt CO99	8
MDA / LAIV / TX	+	TX98 LAIV	wt TX98	8
MDA / LAIV / CO	+	TX98 LAIV	wt CO99	7
no MDA / NV / NC	-	None	Sham	4
no MDA / NV / TX	-	None	wt TX98	8
no MDA / NV / CO	-	None	wt CO99	8
no MDA / WIV / TX	-	TX98 WIV	wt TX98	8
no MDA / WIV / CO	-	TX98 WIV	wt CO99	8
no MDA / LAIV / TX	-	TX98 LAIV	wt TX98	8
no MDA / LAIV / CO	-	TX98 LAIV	wt CO99	8

571 [1] MDA(+) pigs suckled sows which were previously vaccinated with three doses of TX98 WIV.

572 MDA(-) pigs suckled sows which were not vaccinated against IAV.

29

573 **Figure 1**. Serum antibody levels due to maternal derived antibody and/or response to vaccine.

574 Reciprocal geometric mean hemagglutination inhibition (HI) titers at multiple time points prior

575 to challenge against TX98 H3N2 antigen (A) and against CO99 antigen (B). MDA designates

576 groups with maternally-derived antibody induced by immunizing dams with TX98 vaccine.

577 Treatment groups were non-vaccinated (NV), vaccinated at 0 days post-vaccination (dpv) and

578 14 dpv with TX98 whole inactivated virus (WIV), or vaccinated intranasally with TX98 live-

579 attenuated influenza virus (LAIV) at 0 dpv only. Mean optical density (O.D.) of serum IgG in

580 whole virus ELISA assays against TX98 antigen (C) and against CO99 antigen (D) at 49 dpv (0

581 days post infection). Mean optical density (O.D.) of IgG in whole virus ELISA assays at 5 days

582 post challenge against TX98 antigen for groups challenged with TX98 (E) and against CO99

583 antigen for groups challenged with CO99 (F). Open bars designate groups without MDA and

584 solid bars designate groups with MDA. Statistically significant differences between MDA

585 statuses within a vaccine group are marked with asterisks and differences between vaccine

586 treatment groups with matched MDA status are identified by connecting lines ($P < 0.05$).

587 **Figure 2.** Antibody levels in broncho-alveolar lavage fluid at five days post infection. Mean

588 optical density (O.D.) of IgG in whole virus ELISA assays against TX98 antigen (A) and against

589 CO99 antigen (B) and of IgA against TX98 antigen (C) and against CO99 antigen (D). Groups

590 challenged with TX98 are represented in panels A and C whereas groups challenged with CO99

591 are represented in panels B and D. Open bars designate groups without MDA and solid bars

592 designate groups with MDA. Statistically significant differences between MDA statuses within a

593 vaccine group are marked with asterisks and differences between vaccine treatment groups

30

594   with matched MDA status and challenge virus strains are identified by connecting lines ($P <$
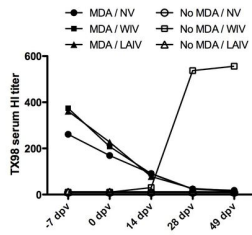
595   0.05).

596   **Figure 3.** A. Nasal shedding of challenge virus at 3 (A) and 5 (B) dpi in nasal swabs (NS). Piglets

597   were vaccinated in the presence or absence of circulating MDA against TX98. At vaccination,

598   piglets received no vaccine (NV), two intramuscular doses of TX98 WIV, or one intranasal dose

599   of TX98 LAIV. Forty-nine days after the initial vaccine dose, piglets were challenged by intra-

600   tracheal inoculation with TX98 or CO99. Nasal swab specimens were collected from 3 and 5

601   days post-infection (dpi), and titrated by $TCID_{50}$ assay on MDCK cells. Statistically significant

602   differences between MDA statuses within a vaccine group are marked with asterisks and

603   differences between vaccine treatment groups with matched MDA status and challenge virus

604   strains are indentified by connecting lines ($P < 0.05$).

605   **Figure 4**. Adjuvanted TX98 WIV administered to MDA-positive piglets enhances the severity of

606   subsequent infection with heterologous H3N2 strain CO99, whereas TX98 LAIV vaccine partially

607   cross-protects. MDA-positive pigs suckled colostrum from TX98-vaccinated sows and MDA-

608   negative pigs suckled from naïve sows. WIV was delivered intramuscularly at 2 and 4 weeks of

609   age, while LAIV was delivered intranasally only at 2 weeks of age. At 8 weeks of age (49 dpv)

610   pigs were challenged intratracheally with TX98 or CO99. At 5 days post-infection (dpi), pigs

611   were euthanized, BALF samples were collected, and necropsy was conducted. BALF samples

612   were titrated by $TCID_{50}$ assay on MDCK cells (A). Macroscopic lesions were scored as the

613   percentage of total lung surface area involved (B). Microscopic pneumonia (C) and tracheal

614   damage (D) were scored as described in Materials and Methods. Statistically significant

615   differences between MDA statuses within a vaccine group are marked with asterisks and
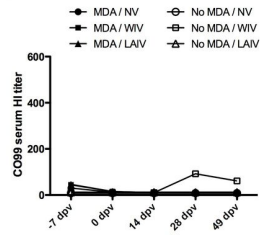
31

616   differences between vaccine treatment groups with matched MDA status and challenge virus

617   strains are identified by connecting lines ($P < 0.05$).

618   **Figure 5.** Photographs of macroscopic lung pathology in pigs positive for MDA at the time of

619   vaccination, shown 5 days after heterologous challenge with CO99. Photographs of ventral

620   surfaces of lungs are representative of three vaccine treatment groups: non-vaccinated

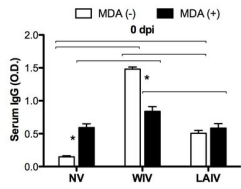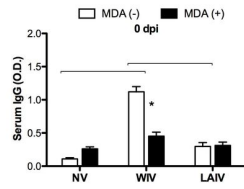621   challenge controls (A); TX98 LAIV-vaccinated pigs (B); and TX98 WIV-vaccinated pigs (C).
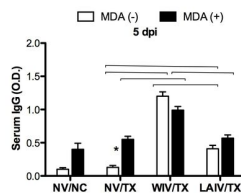
A. TX98

B. CO99

C. TX98

D. CO99

E. TX98

F. CO99

A. TX98

B. CO99

C. TX98

D. CO99

A.

MDA (-) MDA (+)

3 dpi

Nasal swab $\log_{10}$ titer

NV/NC NV/TX WIV/TX LAIV/TX NV/CO WIV/CO LAIV/CO

B.

MDA (-) MDA (+)

5 dpi

Nasal swab $\log_{10}$ titer

NV/NC NV/TX WIV/TX LAIV/TX NV/CO WIV/CO LAIV/CO