RESEARCH REPORT
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SWINE HEALTH

Title: Immune correlates of clinical outcomes in maternal antibody-positive piglets vaccinated with attenuated or killed SIV and challenged with an antigenic variant. **NPB #11-061.**

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INDUSTRY SUMMARY

OBJECTIVES OF THE STUDY.

Current SIV vaccines, which contain whole inactivated virus (WIV), do not provide broad cross-protection against new variants, and maternal antibodies inhibit pigs' immune response to vaccination. Intranasal vaccination of pigs with a live attenuated influenza virus (LAIV) is considered likely to induce more cross-reactive T cells and respiratory tract antibodies capable of providing broad protection. Objectives of the study were the following:

1. Compare the effects of maternally-derived antibodies on antibody and T cell responses to intranasal LAIV versus intramuscular WIV vaccines.
2. Test the efficacy of one LAIV dose versus the two-dose LAIV regimen tested previously.
3. Identify immune mechanisms that correlate with the cross-protective property of LAIV vaccine.
4. Identify immune mechanisms which correlate with vaccine-associated respiratory disease (VAERD).

RESULTS

Weaned piglets, with and without maternal antibodies, were immunized with LAIV or WIV. Neither vaccine induced high serum hemagglutination inhibition antibody titers. Pigs that received LAIV had greater T cell responses and higher levels of lung antibodies. Antibody responses were reduced in pigs with maternal antibodies, while T cell responses were not inhibited. Pigs were experimentally infected with an H3N2 virus mismatched to the vaccines. LAIV vaccine provided strong cross-protection, even in pigs that had maternal antibodies, in terms of lung lesions, virus replication, and clinical disease. Pigs that had received the WIV vaccine were not protected against challenge infection, but had enhanced clinical disease and lung lesions. The presence of maternal antibodies at vaccination did not significantly affect disease severity. Immune factors associated with protection included IgA antibodies in the respiratory tract and virus-specific T cells in lymphoid tissue. The results support LAIV as an influenza vaccine platform for the swine industry, since a single dose protected against the mismatched challenge strain even when administered to pigs with maternal antibodies.

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KEY WORDS

Swine influenza virus, vaccine, immune response, vaccine-associated enhancement of respiratory disease (VAERD), heterologous immunity

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SCIENTIFIC ABSTRACT

Background. Vaccine-associated enhanced respiratory disease (VAERD) can occur in pigs given whole-inactivated virus (WIV) influenza vaccine upon infection with an antigenically divergent strain of swine influenza virus (SIV). VAERD was first characterized with H1 viruses, and later described in pigs vaccinated with H3N2 WIV and challenged with heterologous H3N2, whereas live-attenuated virus vaccine (LAIV) was protective. An additional factor predisposing to H3N2 VAERD was maternally-derived antibodies (MDA) present at immunization. The present study was aimed at identifying immune correlates of VAERD and cross-protection.

Experimental Design. Piglets that acquired H3N2-specific MDA from immunized dams, along with seronegative controls, were vaccinated with H3N2 WIV (2 doses) or LAIV (1 or 2 doses). Humoral and cellular immune responses to vaccines were monitored at the systemic level and locally in the respiratory tract, followed by challenge infection with heterologous H3N2.

Results. SIV-binding IgG was detected in serum after WIV vaccination, but hemagglutination inhibition antibody titers remained very low. WIV induced low IgA and moderate IgG levels in lungs, but both responses were inhibited by MDA. Systemic cellular responses following WIV were detected at modest levels, with evidence of MDA inhibition. LAIV elicited cross-reactive mucosal antibodies and T cells. While the presence of MDA at LAIV vaccination inhibited SIV-specific antibody production, it did not interfere with T cell priming. Piglets given 1 or 2 LAIV doses were protected against heterologous challenge. Lesions and clinical disease were more pronounced in WIV-vaccinated than non-vaccinated groups, and in contrast to the previous study, MDA did not accentuate H3N2 VAERD.

Conclusions. High levels of mucosal antibodies were associated with protection, but LAIV was also protective in MDA-positive LAIV vaccinees that had reduced mucosal antibody responses. Since T-cell responses were one immune component not inhibited by MDA, cellular immunity may have had a significant role in LAIV-mediated cross-protection. These data support LAIV as an SIV vaccine platform for the swine industry, as a single dose protected against heterologous challenge even when administered to MDA-positive piglets.

Introduction

North American and European swine are commonly vaccinated against swine influenza virus (SIV), but multiple factors hinder the success of current vaccines. Current licensed vaccines, containing whole-inactivated viral (WIV) antigens, typically elicit functional antibodies to antigenically similar strains, but new strains with a few mutations in the viral surface proteins frequently evade neutralization by existing antibodies [1]. Therefore, it is difficult for vaccine formulations to keep pace with evolution of the various SIV subtypes and lineages that co-circulate. Immunizing sows to stimulate colostrum-mediated transfer of antibodies to their piglets is a common practice in the swine industry. Passive, circulating antibodies protect pigs against infection with similar strains until they wane below a protective threshold [2]. Piglets that are vaccinated while maternally-derived antibodies (MDA) are present in circulation often suppress immune responses to the vaccine [3]. Therefore, even in a sow herd with routine SIV vaccination it is difficult to achieve lasting protective immunity in the young animals.

Live-attenuated influenza virus (LAIV) vaccines have shown promise as a means to induce T cells and mucosal immunity that cross-react with antigenically drifted and heterosubtypic strains, both in pigs and humans [4, 5]. We previously

reported protective immunity against heterologous H3N2 and heterosubtypic H1N1 infections in young pigs immunized with an experimental LAIV engineered to express a truncated NS1 protein [6, 7]. This LAIV, administered in a two-dose regimen, provided cross-protection even when administered to piglets with high titers of MDA [8]. This was in marked contrast to a WIV vaccine that failed, and in fact exacerbated heterologous infection severity, when given to piglets with maternal immunity. H3N2 LAIV induced humoral immunity such that lung washes of pigs 5 days post heterologous challenge contained significant levels of IgA and IgG with reactivity to the challenge strain, although not in MDA-positive piglets [8]. The localization and quantity of mucosal antibodies before challenge were not explored, and T cell induction by LAIV was also not monitored. A trivalent seasonal LAIV vaccine (FluMist®, MedImmune, Mountain View, CA) is licensed for use in children and adults and shown to induce T cell and mucosal antibody responses cross-reactive to antigenically drifted strains (reviewed by Carter and Curran [9]). Determining correlates of cross-protective immunity in swine could facilitate improved regulatory evaluation of commercial LAIV vaccines and provide insights into design of effective vaccination programs. This is an important need because traditional serological parameters, such as serum hemagglutination inhibition titers, are not induced reliably by LAIV immunization in swine or humans [10].

The adverse outcome of WIV vaccination and heterologous challenge was similar to vaccine-associated enhanced respiratory disease (VAERD) phenomenon previously described in pigs that were vaccinated with an H1N2 WIV and challenged with heterologous H1N1 [11, 12], except that MDA were not involved in the H1-subtype VAERD pathogenesis. Both the H3 and H1 models of VAERD are characterized by macroscopic pneumonia, severe bronchointerstitial pneumonia with necrotizing bronchiolitis, interlobular and alveolar edema, and hemorrhage [8, 12]. Data from both models indicate a likely role in pathogenesis for SIV-specific IgG antibodies that fail to neutralize the challenge virus. High levels of such antibodies were detected in lungs of pigs affected by H3N2 VAERD, 5 days post infection. The mechanism by which non-neutralizing antibodies might promote inflammation in this model and the possible involvement of vaccine-primed B and T cells have not been resolved. Developing a more complete understanding of immunological factors in H3N2 VAERD, and the degree of viral replication in affected hosts, could facilitate improving the safety of inactivated, adjuvanted SIV vaccines.

Objectives

Objectives of the study were the following:

1. Determine the effects of maternally-derived antibodies (MDA) on young piglets' antibody and T cell responses to intranasal LAIV and intramuscular WIV vaccines both systemically and locally.
2. Test the efficacy of a single LAIV dose versus a two-dose LAIV regimen, in piglets with and without MDA.
3. Identify immune mechanisms which correlate with VAERD in pigs that are initially vaccinated with H3N2 WIV vaccine and later infected with a heterologous H3N2 virus.
4. Identify immune mechanisms which correlate with the cross-protective property of LAIV vaccine, potentially including mucosal antibodies in the respiratory tract and cross-reactive T lymphocytes.

Materials and Methods

Viruses and vaccine preparation. Antigen for the WIV vaccine was A/sw/Texas/4199-2/1998 (wild-type TX98), grown in Madin-Darby canine kidney (MDCK) cells or 9-day-old fertilized hen eggs. The WIV vaccine for sows contained MDCK-grown virus, while the WIV vaccine for piglets contained egg-grown virus. Clarified virus supernatants from infected culture were inactivated by UV irradiation, using the "sterilize" setting in a UV cross-linking chamber (GS Gene Linker; Bio-Rad, Hercules, CA). Inactivation of the virus was confirmed by failure of the virus to replicate in 2 serial passages on MDCK cells. A commercial adjuvant (Emulsigen D; MVP Laboratories, Inc., Ralston, NE) was added at a 1:5 volume ratio,

along with sterile PBS to adjust the final mixture to 128 units of hemagglutination (HA) activity. The attenuated virus for the LAIV was generated via reverse genetics as previously described [13]. The attenuated vaccine virus contained an NS1 gene with a 3' premature termination plus the insertion of four stop codons in the three frames after, producing a protein of 126 amino acids with a carboxy-terminal truncation (NS1Δ126 TX98). The remaining seven gene segments were from wild-type TX98. The challenge virus, A/sw/Colorado/23619/99 (H3N2) (CO99), was cultivated in MDCK cells. The TX98 and CO99 viruses were shown previously to have limited serologic cross-reactivity [14].

Experimental design. Sows obtained from a high-health herd free of SIV and porcine reproductive and respiratory syndrome virus (PRRSV) were vaccinated with the TX98 WIV or immunologically naive. The vaccine was administered to sows in 3 doses, once before breeding and twice during mid-late gestation. Approximately 2 weeks before farrowing, vaccinated and non-vaccinated sows were delivered to Veterinary Resources Inc. (Ames, IA), which provided animal care and housing through the remainder of the study. One week after farrowing, piglets were bled to evaluate transfer of MDA and confirm seronegative status of litters from non-vaccinated sows. At 14-19 days of age, piglets were weaned and randomly assigned to vaccine treatment groups within the appropriate MDA status (Table 1). They were treated with ceftiofur crystalline-free acid (Excede®, Zoetis Inc., New York, NY) at weaning. The initial doses of WIV or LAIV were administered at 15-20 days of age. Pigs in the LAIV groups were immunized intranasally (I.N.) with 2 ml of NS1Δ126 TX98, at 10^6 50% tissue culture infective doses ($TCID_{50}$) per ml. Pigs in the WIV groups were vaccinated intramuscularly with 2 ml of the formulation described above. LAIV-vaccinated pigs were housed in separate rooms. Booster doses were administered 14 days later to the WIV groups and designated LAIV groups. Extra groups of pigs in some treatments were moved to separate housing at NADC late in the vaccine regimen to be sacrificed for pre-challenge specimens (as denoted in Table 1). HI titers in non-vaccinated MDA-positive pigs decreased to ≤40 before challenge. At 50-55 days of age [0 days post infection (dpi)], pigs were inoculated intratracheally (2 ml) and intranasally (1 ml) with virus suspension containing $10^{3.7}$ $TCID_{50}$/ml. Non-challenged control groups were housed in separate isolation rooms from infected animals. Pig studies at VRI and NADC were conducted in compliance with their respective Institutional Animal Care and Use Committees.

Clinical observation and sampling. To compare the efficacies of WIV and LAIV against infection with heterologous virus, infected pigs were observed daily for clinical signs. Nasal swabs were taken at 0, 1, 3, and 5 days postinfection (dpi), placed in 2 ml minimal essential medium (MEM), and frozen at -80°C until testing. Daily clinical observations included scoring three parameters of morbidity (behavior, difficulty breathing, coughing) and rectal temperature measurements. All animals were euthanized humanely at 5 dpi with a lethal dose of pentobarbital (Sleepaway; Fort Dodge Animal Health, Fort Dodge, IA). At necropsy, each lung was subjected to lavage with 50 ml MEM to obtain bronchoalveolar lavage fluid (BALF). Nasal swab specimens were processed and analyzed by virus isolation as described previously [8]. Tissue culture infectious dose 50 ($TCID_{50}$) virus titers in BALF samples and each virus isolation-positive nasal swab sample were determined as described previously [8].

Pathological examination of lungs. At necropsy, lungs were removed and evaluated for the percentage of the lung affected with purple-red consolidation typical of IAV infection. The percentage of the surface affected with pneumonia was estimated visually for each lung lobe, and the total percentage for the entire lung was calculated based on the weighted proportion of each lobe relative to the total lung volume [15]. Tissue samples from the trachea and right middle or affected lung lobe were fixed in 10% buffered formalin for histopathologic examination. Tissues were processed by routine histopathologic procedures, and slides were stained with hematoxylin and eosin. Microscopic lung and tracheal lesions were evaluated by a veterinary pathologist blinded to treatment groups, using a scoring system described previously [16]. Briefly, lung scores were based on airway inflammation, airway necrosis, peribronchiolar

lymphocyte cuffing, septal inflammation, and edema. A composite score was computed using the sum of the individual scores. Microscopic tracheal lesions were scored similarly, based on two parameters: epithelial necrosis and degree inflammation.

IHC staining of lymphocytes and viral antigen. Virus was detected in lung tissues by use of a previously described immunohistochemical (IHC) method [16]. Briefly, tissue sections were deparaffinized, hydrated, quenched in 3% hydrogen peroxide, and treated with 0.05% protease for 2 min. Slides were labeled with primary monoclonal antibody HB65 (ATCC, Manassas, VA), specific for nuceloprotein (NP), peroxidase-labeled anti-mouse IgG, and the Dako LSAB2-HRP detection system (Dako, Carpinteria, CA). The slides were counterstained with Gill's hematoxylin. Antigen detection was assessed using two scores: (i) airway epithelial labeling and (ii) alveolar/interstitial labeling. For airway epithelium, the following 5-point scale was used: 0, none; 1, few cells with positive labeling; 2, mild scattered labeling; 3, moderate scattered labeling; and 4, abundant scattered labeling (>50% of the epithelium was positive in affected airways). For the interstitium/alveoli, the following 4-point scale was used: 0, none; 1, minimal focal signals; 2, mild multifocal signals; and 3, abundant signals.

Serologic and mucosal antibody assays. Serum samples were collected at the following time points: pre-weaning (-12 days post-vaccination [dpv]), first vaccination (0 dpv), second vaccination (14 dpv), 27 dpv, 34 dpv, and 40 dpv (necropsy). For use in the HI assay, sera were heat inactivated at 56°C and treated with 20% kaolin (Sigma-Aldrich, St. Louis, MO) followed by adsorption with 0.5% turkey red blood cells (RBC) to remove nonspecific agglutinants. HI assays were performed with TX98 and CO99 viral antigens and turkey RBC, using standard techniques [17]. Enzyme-linked immunosorbent assays (ELISA) to detect total IgG and IgA antibodies against whole virus preparations of TX98 and CO99 present in serum and BALF were performed as previously described [11], with the following modifications. Serum samples were diluted to 1:2,000 for the IgG ELISA. BALF samples were diluted to 1:4 for IgG and IgA ELISAs. Antibody levels were reported as the mean OD for duplicate wells of each sample.

Multi-parameter flow cytometry (MP FCM) analysis of SIV-specific T cells. Peripheral blood mononuclear cells (PBMC) were stimulated by ex vivo culture with virus and tested for response markers by MP-FCM, using a previously described method [18]. Briefly, PBMC of each pig were incubated for 4 days in the absence (unstimulated control) or presence of TX98, CO99, or supernatant of mock-infected MDCK cells. Antibodies to the CD4, CD8 and γδ TCR markers were used with fluorochrome-conjugated secondary antibodies to label T cell subsets. The activation marker CD25 and intracellular cytokines interferon-γ (IFN-γ) and interleukin-10 (IL-10) were labeled as measures of SIV-specific responses. The net increase in the percentage of a subset to express each response marker was calculated by subtracting the marker-positive cell percentage of mock-stimulated from the marker-positive cell percentage of virus-stimulated cells. If the net result was negative, it was adjusted to 0 before statistical analysis.

ELISPOT analysis of SIV-specific T cells.
Lymphocytes were purified from tracheobronchial lymph nodes (TBLN) as described previously [19]. ELISpot assay for IFN-γ secreting cells (IFN-γ SCs) was performed as previously described with slight modification [20]. Briefly, 96-well membrane plates were coated overnight at 4°C with 6 µg/ml anti-pIFN-γ (P2G10, BD Biosciences, San Jose, CA), then washed and blocked. Next, 5×10^5 PBMCs were plated per well, along with TX98 virus or CO99 virus, and plates were incubated for 18 h at 37 °C 5% CO_2. Anti-IFN-γ detection antibody (P2C11, BD Biosciences) was applied for 2 h at 37 °C to detect IFN-γ SCs. Plates were developed using ELISpot Blue Color Module (R&D Systems, Minneapolis, MN) and scanned using CTL-ImmunoSpot® S5 UV Analyzer and ImmunoSpot 5 software. The reported values were calculated as the average number of spots counted for wells receiving each virus.

Statistical analysis. Macroscopic and microscopic pneumonia scores, log10-transformed BALF and nasal swab virus titers, mean ODs for ELISAs, and T-cell results (flow cytometry and ELISpot) were analyzed using analysis of variance (ANOVA) and the Tukey test for pairwise comparisons between groups, with P values < 0.05 considered significant (Prism software; GraphPad, La Jolla, CA).

Results

Serological response to vaccines.

Immunization of sows with multiple doses of TX98 WIV vaccine elicited homologous serum HI antibody titers of 160 or greater. Non-vaccinated sows had HI titers ≤20, and were also seronegative by nucleoprotein-specific commercial ELISA (data not shown). Neonatal piglets from the vaccinated sows acquired passive HI antibody at similar high titers, which waned steadily (Fig. 1A). There was very limited HI cross-reactivity to the eventual challenge strain, CO99 (Fig. 1B), consistent with previous studies [8, 14]. Piglets were weaned from their dams and vaccinated the following day with WIV or LAIV. Secondary WIV doses were administered to the WIV treatment group and secondary LAIV doses to designated groups. Serum HI antibody responses to both vaccines were low or below detection limits prior to challenge (Fig 1A), which was unexpected in WIV vaccinees. The WIV vaccine did, however, elicit virus-binding IgG antibodies, which were detectable by ELISA, including moderate cross-reactivity with the eventual challenge strain, CO99 (Fig. 1C-D).

Mucosal antibody response to vaccines

Subgroups of pigs in several treatments were sacrificed at 0 dpi to enable analysis of respiratory tract local immunity prior to infection. BALF samples were analyzed for SIV-specific IgA and IgG antibodies by ELISA (Fig. 2). There was minimal evidence of maternally derived SIV-specific IgA in lungs of non-vaccinated (NV) piglets. In MDA(-) piglets, a single LAIV dose elicited a much greater IgA response than two doses of WIV, and these antibodies cross-reacted with CO99. The BALF IgA response to LAIV was strongly inhibited in MDA(+) piglets, to levels near the threshold of detection. With regard to SIV-specific IgG, passive maternal antibodies apparently remained in lungs of NV pigs at moderate levels, 7 weeks after piglets had received colostrum. LAIV induced the highest BALF IgG response in MDA(-) groups but, like the IgA isotype, this was clearly inhibited by MDA. WIV immunization induced moderate BALF IgG, but this response also appeared inhibited by MDA (approximately 2-fold).

SIV-specific IgA levels were also measured in nasal wash (NW) specimens collected at 0 dpi from the same piglets. Although the magnitude of IgA signal samples was lower in NW, the pattern of results approximately mirrored results from 0 dpi BALF samples. In the context of MDA-negative piglets, LAIV vaccinees had significantly more TX98-binding IgA than WIV-vaccinated counterparts (Fig. 3). There was considerable variability among NW IgA responses of individuals within the LAIV-vaccinated group, and the presence of MDA at vaccination largely blocked this response. The cross-reactivity of LAIV-induced NW IgA against CO99 appeared rather low, although results of assays on the two strains are not directly comparable. Overall, results from pre-challenge samples indicate that LAIV vaccination induces robust local antibody defenses in the respiratory tract, while WIV vaccinees developed moderate quantities of lung IgG only, but the presence of MDA at vaccination suppressed these responses.

Cellular immune response to vaccines

T cell priming by the vaccines was analyzed by testing ex vivo recall responses to homologous and heterologous viruses. Lymphocytes were isolated from peripheral blood and tracheobronchial lymph nodes (TBLN), loci expected to feature distinct lymphocyte trafficking after immunization. PBMC from two bleedings (-7 and -1 dpi) were stimulated with the TX98 and CO99 viruses and analyzed by MP-FCM for expression of CD25, IFN-γ, and IL-10 in four main cell populations:

CD4 single positive (CD4+), CD8 single positive (CD8+), CD4/CD8 double positive (CD4+CD8+), and γδ TCR positive. There was unexpected dissimilarity between results from the two time points, as most of the response readouts at -1 dpi were markedly lower than on the initial assay at -7 dpi. It is unclear if the diminished signals at -1 dpi are an anomaly of the assay or reflect rapid contraction of the number of T cells that expanded after the booster doses of vaccines 2-3 weeks earlier. Because group-to-group differences are more discernible at -7 dpi, results of that initial assay are reported here.

Antigen-driven increases in the expression of CD25 (Fig. 4) were nearly undetectable in the CD4+ T cells of all treatment groups. The CD4+CD8+ population, which have been previously characterized as memory cells in swine, had much group-to-group variation in response to homologous TX98 virus stimulation. The two treatment groups for which the CD25 marker was significantly upregulated compared to NV controls were MDA(-) WIV and MDA(+) LAIVx2. Although CD25 recall responses varied widely among the MDA(+) LAIVx2 animals, the mean was significantly higher than in either the MDA(+) LAIVx1 or MDA(-) LAIVx2 groups. In contrast, WIV vaccination only primed CD4+CD8+ cells for significant CD25 responses if the vaccine was given to MDA(-) piglets. CD25 expression on the other two T-cell populations also indicated priming by WIV and LAIV, but differences did not reach statistical significance. Similar patterns were present in CD25 data from cells stimulated with CO99 virus, including significant recall responses in the CD4+CD8+ population of MDA(+) LAIVx2 piglets.

Analysis of cytokine expression in virus-stimulated T cells showed vaccine-associated activity mainly in the CD4+CD8+ and γδ TCR+ populations. Like the CD25 marker, virus-induced IFN-γ (Fig. 5) and IL-10 (Fig. 6) were consistently detected in cells from WIV and LAIVx2 vaccinees. Also like CD25, the IFN-γ and IL-10 recall responses tended to be higher in the MDA(+) group that received LAIVx2 than in the MDA(-) group that received the same vaccine, although differences were not statistically significant. Among WIV vaccinees, there was fairly intense antigen-driven expression of IFN-γ and IL-10 in the γδ cell population.

Lymphocytes isolated from TBLN of the pig subgroups that were sacrificed pre-challenge (-1 dpi) were tested by IFN-γ ELISpot (Fig. 7). After WIV-vaccinated pigs' TBLN cells were stimulated with either TX98 or CO99, modest numbers of IFN-γ secreting cells were detected. Greater numbers of IFN-γ secreting cells were detected from TBLN of the LAIVx1 groups. (TBLN were not obtained from LAIVx2 vaccinated piglets.) The data reveal no evidence that TBLN T cells' recall responses were inhibited or enhanced in MDA(+) piglets. ELISpot results from corresponding PBMC samples confirm similar results to the MP-FCM IFN-γ assay, i.e. SIV-specific IFN-γ SCs were detected in most WIV and LAIV vaccinees, with the highest frequencies found in the MDA(+) LAIVx2 treatment group.

Heterologous challenge

Three weeks after secondary vaccination, piglets were experimentally infected with CO99 virus. The infectious dose of virus was 1-2 logs lower than administered in the previous study that established the vaccine-challenge model. Nasal swabs collected at 1 dpi, 3 dpi, and 5 dpi were analyzed by virus isolation and titration of virus in positive specimens. Shedding from the nasal cavity was rather limited even in non-vaccinated animals, as virus was only detected in 3 of 8 MDA(-) NV piglets and in 7 of 8 MDA(+) NV piglets. The frequencies of virus-positive swabs in the WIV vaccinated groups were similar to those of the corresponding NV control groups (data not shown). LAIV vaccinated groups had fewer positive swabs, including none in MDA(-) pigs. All positive swabs had infectivity titers below 10^3 $TCID_{50}$/ml. The CO99 inoculum was not shed as much from the nasal cavity as in the previous studies [6, 8], possibly because the dose of virus was lower. In lungs, the challenge virus replicated more extensively, based on 5 dpi BALF infectivity titers and viral antigen detection in lung tissue (Figure 8). BALF viral titers in the MDA(-)and MDA(+) non-vaccinated groups were only moderate at this stage (near 10^3 $TCID_{50}$/ml). None of the LAIV-vaccinated pigs had detectable virus in BALF or lung tissue. Among MDA(-) WIV piglets there was very little infectious virus in 5 dpi BALF, yet the amount of viral antigen detected in

the lung tissues was as high as in NV animals. WIV vaccination was ineffective in reducing lung viral load in MDA(+) animals.

Clinical disease was monitored daily in all pigs during challenge infection, with scores reported for coughing, lethargy, and difficulty breathing (Fig. 9A). Composite scores taken from these values indicate that clinical signs peaked about 3-4 dpi, and that disease was most severe in WIV-vaccinated groups. The NV groups both had mild clinical disease, while in the LAIV-vaccinated groups almost no clinical signs were observed. Daily rectal temperatures were also recorded throughout infection (Fig. 9B). Mean temperatures of different groups did not vary greatly, although the NV and WIV groups had at least marginally higher temperatures than LAIV groups at 3-4 dpi. At 5 dpi, the day of necropsy, temperatures of the MDA(+) NV and MDA(+) WIV unexpectedly rose further, to about 105°F, while temperatures in equivalent MDA(-) groups had decreased toward the baseline of approximately 103°F. Based on the clinical disease scores, WIV vaccination exacerbated the severity of heterologous infection, while LAIV was cross-protective.

Enhanced macroscopic pneumonia is a hallmark of VAERD in H3N2 and H1N1 heterosubtypic challenge models [8, 11]. In the present study WIV-vaccinated piglets again had higher macroscopic lung lesion scores than NV counterparts (Fig. 10A). In the prior study we observed more severe macroscopic pneumonia in MDA(+) WIV vaccinees than in the MDA(-) WIV group, but in the present study this was not reproduced, perhaps due to the lower challenge dose. The mean percentage of lung area affected with pneumonia was 27% in the MDA(-) WIV group and 17% in the MDA(+) WIV group. A statistically significant difference between macroscopic lesion scores of WIV and NV groups only occurred in the MDA(-) context. Piglets in LAIV-vaccinated groups had little evidence of macroscopic pneumonia, regardless of MDA status or the number of LAIV doses received. Tracheal and lung tissue sections were also analyzed microscopically (Figure 10B-C). Tracheal microscopic lesion scores, which reflect necrosis and inflammation, were slightly higher in WIV-vaccinated groups than NV groups, whereas LAIV-vaccinated groups had the lowest scores among CO99-challenged piglets. Lung microscopic lesion scores reflect airway inflammation, airway necrosis, peribronchiolar lymphocyte cuffing, septal inflammation, and edema/hemorrhage. Microscopic lesions of lung tissues were most pronounced in the WIV-vaccinated groups, and the group-to-group comparisons were similar to those observed in trachea. Overall, analysis of respiratory tract pathological lesions shows a similar pattern to clinical disease scores, pointing to exacerbation of heterologous infection by WIV and cross-protection by LAIV, without a great impact by the presence of MDA.

Post-challenge mucosal antibody responses

As discussed above, 0 dpi BAL fluid specimens were collected from groups of piglets immunized with the WIV and LAIV vaccines, and ELISA results showed that one dose of LAIV elicited substantial levels of lung IgA and IgG that bound to both TX98 and CO99 (Fig. 2). These antibody responses were nearly undetectable in other treatment groups, including those vaccinated with WIV and the group vaccinated with one dose of LAIV in the presence of MDA. At necropsy (5 dpi), BALF specimens were collected from lungs of infected and control animals for a post-challenge vantage point on mucosal antibody responses. BALF TX98- and CO99-binding IgG were present at higher levels compared to the corresponding treatment groups euthanized at 0 dpi (Fig. 11). These IgG levels continued to be lower in groups that had been vaccinated in the face of MDA. Similarly, SIV-specific IgA reached higher levels in 5 dpi BALF than in equivalent 0 dpi samples, although these levels were still several-fold lower in the MDA(+) animals. The MDA(-) group vaccinated with WIV had an intermediate level of IgA, which was apparently primed by vaccination and boosted during infection. MDA(-) LAIV vaccinees continued to have the highest levels of SIV-specific IgA at 5 dpi. Interestingly, there was little or no additional lung IgA in groups that had received two doses of LAIV, compared with one dose.

Discussion

H3N2 influenza viruses of several antigenically distinct lineages circulate extensively in North American swine. Because of the diversity and rapid evolution of these viruses, commercial vaccines containing WIV antigens often lack efficacy in field conditions. Sows are frequently immunized with WIV vaccines to enable colostrum-mediated protection of young piglets, although it is recognized that MDA can interfere with immunization of the young pigs. Our previous study tested immune responses and cross-protective efficacy of H3N2 WIV versus LAIV, in pigs with and without MDA [8]. Both vaccines conferred protection against homologous challenge, but in MDA(+) animals the outcomes of heterologous H3N2 challenge were markedly different. LAIV provided moderate cross-protection in terms of viral replication and respiratory tract lesions, even in MDA(+) pigs which had very limited antibody responses. WIV in MDA-positive animals failed to limit viral replication and it primed for more severe respiratory tract lesions. This VAERD outcome was associated with high levels of virus-binding IgG that lacked HI cross-reactivity, which has also been observed in an H1N1 model of VAERD [11]. The present study was aimed at identifying immune mediators that underlie the cross-protection or disease enhancement associated with heterologous infection. We hypothesized that LAIV vaccination induces cross-reactive T cells and mucosal antibodies localized to the respiratory tract, which contribute to cross-protection in the event of heterologous challenge. Regarding WIV vaccination, we hypothesized that VAERD pathogenesis involves non-HI virus-binding IgG coupled with a reduction or qualitative changes in the balance of the CMI response.

Experimental parameters of the present study introduced two possibly significant variations from the initial study. First, the WIV vaccine administered to piglets induced unusually low homologous titers of serum HI antibodies (≤80) (Fig. 1), although IgG ELISA and assays of CMI confirmed that it was immunogenic. This lot of vaccine contained virus cultured in fertilized eggs, while the WIV lot administered earlier to the sows contained virus cultured in MDCK cells. In relation to heterologous CO99 challenge virus, the lack of HI cross-reactivity in WIV-induced antibodies does not differ greatly from the initial study. Second, the CO99 challenge inoculum had about 20-fold less viral titer in the present study ($10^{3.7}$ $TCID_{50}$/ml compared with 10^5 $TCID_{50}$/ml) and was administered to non-anesthetized pigs. Virus isolation and titration of nasal swabs indicated that upper respiratory tract infection was rather limited. Titers of virus in BALF of NV pigs at necropsy were also lower in the present study. The CO99 experimental challenge in the present study was thus less severe in virological terms, but respiratory tract lesions in NV control animals had similar severity to those reported previously and there was clear clinical disease, indicating a rigorous test of vaccine efficacy.

LAIV-vaccinated groups had robust cross-protection against the CO99 challenge. Regardless of the dose regimen (1 or 2 doses) or the MDA status at vaccination, the LAIV protected pigs in terms of clinical disease, macroscopic and microscopic pneumonia, and viral load in the airways. WIV-vaccinated groups, both MDA(-) and MDA(+), displayed enhanced clinical disease signs and developed more severe respiratory tract lesions than NV controls. That is to say, WIV immunization led to VAERD, with features highly similar to what was reported previously [8], except that the presence of MDA at immunization was not a predisposing factor. In fact, the MDA(-) WIV group developed more severe lesions and clinical disease. In the previous study, MDA(+) WIV-vaccinated pigs developed VAERD, while MDA(-) WIV-vaccinated pigs were protected against heterologous challenge. The different outcomes may stem from the difference in viral loads of the two challenge infections, a greater quantity of non-HI antibodies induced by WIV alone in the present study, or other variables. The results reported here align more closely with the H1 subtype VAERD model described previously, where inactivated vaccine is sufficient to prime for enhanced disease after heterologous challenge.

Vaccine-induced mucosal immunity was analyzed by measuring SIV-specific IgA and IgG in pre-challenge NW, pre-challenge BALF, and post-challenge BALF. LAIV vaccinees possessed abundant mucosal IgA in both of these pre-challenge specimens and IgG in the BALF only. In comparison, the WIV vaccine (i.m.) elicited moderate lung IgG responses before

challenge but no mucosal IgA. For the most part, local IgA and IgG appear to have cross-reacted well with CO99 challenge virus. These findings are consistent with the concept that locally administered LAIV's elicit superior mucosal antibody responses. It is a reasonable conclusion that the cross-reactive mucosal antibodies in LAIV vaccinees were sufficient to block viral infection from being established. We had also hypothesized that LAIV induces mucosal antibody responses effectively in pigs with circulating maternal IgG, but this did not occur. In fact, MDA(+)pigs had almost complete inhibition of local IgA and IgG responses, in both the WIV and LAIV groups. Pre-challenge mucosal samples were not tested from pigs vaccinated twice with LAIV, so it is possible that MDA interference with mucosal antibody induction was less absolute in the 2-dose LAIV groups.

BALF samples were also collected from infected pigs (5 dpi) and assayed for SIV-specific antibodies, which could have entered lungs or been produced by activated local B cells during infection. Vaccinated groups had increased BALF IgA and IgG levels after infection. An increase in BALF IgA was notable in WIV vaccinees after infection, although the LAIV groups (1 and 2 dose) continued to have higher levels. MDA interference with lung antibodies was still very evident post-infection. Based on mucosal antibody levels only, one might predict MDA(+) LAIV vaccinees to be vulnerable to heterologous infection. However, this group was solidly cross-protected, suggesting that memory B cells were primed by the vaccine to make an anamnestic antibody response early in infection and/or that T cells formed a strong defense against viral replication.

Cellular immune responses were analyzed before infection of the pigs by ex vivo antigenic stimulation of T cells isolated from both the peripheral blood and TBLN, draining lymph nodes of the lower respiratory tract. T cells from both sources tended to be nearly as responsive to heterologous CO99 virus as to homologous TX98 virus. This confirms that the cellular immune response is more broadly reactive than the systemic antibody response, especially HI activity. Peripheral blood cells were assayed with MP-FCM assays that differentiate major subset markers in addition to markers of cell activation and function. The two T-cell subsets with the most significant responses to viral stimulation were the CD4+CD8+ and γδ TCR+ populations. Porcine CD4+CD8+ cells have been characterized as memory cells with T helper function (reviewed by Gerner et al [21], but also with potential cytolytic activity that might mediate clearance of virally infected cells [22]. Priming of this T cell subset was evident in both WIV and LAIV vaccinated pigs, in terms of all response markers (CD25, IFN-γ, and IL-10). However, MDA had distinct effects on CD4+CD8+ cell responses to WIV versus LAIV: a dampening effect for WIV and an enhancing effect for LAIV. This seems consistent with likely differences in antigen presentation pathways for the inactivated versus replicating antigens. The functions of porcine γδ T cells are poorly understood, particularly in relation to acute viral infection, so it is difficult to speculate on the protective or non-protective roles of these cells primed against SIV.

Between the treatment groups that received 1 or 2 doses of LAIV, higher recall responses were observed in the peripheral T cells of the 2-dose groups, and the MP-FCM assay was often not sensitive enough to detect responses in pigs with one LAIV dose. One possible explanation is that the first dose primed only a small population of cells. Another possibility is that the second dose (14 days before the assay) stimulated transient cell trafficking from lymphoid tissue into the circulation. The results of IFN-γ ELISpot with pre-challenge TBLN cells point to cell traffic as the more likely answer. IFN-γ responses were readily detected from the TBLN cells of single-dose LAIV-vaccinated pigs, higher than the responses of WIV vaccinees. (TBLN specimens were not collected from two-dose LAIV vaccinees.) Data captured from the TBLN, which drain the site of infection, are a valuable complement to data from peripheral blood cells. Both vaccines, particularly LAIV, elicited effector or memory cells that were positioned to respond to infection. Taken together, these data indicate that cellular immunity induced by the LAIV vaccine was not impeded by MDA or by the antigenic divergence between H3N2 strains. Cellular immunity may have been the primary defense against heterologous infection in the MDA(+) LAIV group, which was deficient in mucosal antibodies.

Data from this study highlight the concept that SIV vaccines can confer protection against infection without meaningful titers of systemic neutralizing antibodies. In the absence of such systemic neutralizing antibodies, other facets of the adaptive immune response become pivotal in determining if infection will be resolved quickly or exacerbated. In our model, LAIV-induced immunity supplies multiple cross-reactive factors (mucosal antibodies and T cells before infection, anamnestic systemic antibody response after infection). When one major factor is minimized (i.e. MDA interference with mucosal antibody induction), other mediators are available to supply cross-protection. On the other hand, our lab-formulated WIV primes an immune response that has detrimental effects to the host respiratory tract when antibodies cannot block viral infection. The results support further development of LAIV as an influenza vaccine platform for the swine industry.

TABLES

Table 1.

Treatment group	MDA status[a]	Piglet vaccine[b]	Challenge virus	*n*
MDA(-) NV/NC	–	None	Sham	4
MDA(+) NV/NC	+	None	Sham	4
MDA(-) NV	–	None	CO99[c]	8
MDA(+) NV	+	None	CO99	8
MDA(-) WIV	–	WIV, 2 doses	CO99	8
MDA(+) WIV	+	WIV, 2 doses	CO99	8
MDA(-) LAIVx1	–	LAIV, 1 dose	CO99	8
MDA(+) LAIVx1	+	LAIV, 1 dose	CO99	8
MDA(-) LAIVx2	–	LAIV, 2 doses	CO99	8
MDA(+) LAIVx2	+	LAIV, 2 doses	CO99	8
MDA(-) NV*	–	None	NC[d]	4
MDA(+) NV*	+	None	NC	4
MDA(-) WIV*	–	WIV, 2 doses	NC	8
MDA(+) WIV*	+	WIV, 2 doses	NC	8
MDA(-) LAIVx2*	–	LAIV, 1 dose	NC	8
MDA(+) LAIVx2*	+	LAIV, 1 dose	NC	8

[a] MDA-positive pigs were from sows that had received 3 doses of TX98 WIV vaccine. MDA-negative pigs were from non-vaccinated sows of the same source farm.

[b] Vaccines are derived from A/Texas/4199-2/1998 (H3N2)

[c] A/swine/Colorado/23619/1999 (H3N2)

[d] NC, not challenged. Subgroups were euthanized at -1 dpi for pre-challenge specimens.

FIGURE LEGENDS

Figure 1. Serum antibody levels due to maternally derived antibodies and responses to vaccination. Reciprocal geometric mean HI titers against TX98 H3N2 antigen (A) and CO99 antigen (B) are shown for time points prior to and after challenge. MDA(+) pigs were from sows immunized with the TX98 WIV vaccine. Treatment groups were non-vaccinated (NV), vaccinated I.M. at 0 dpv and 14 dpv with TX98 WIV, vaccinated I.N. with TX98 LAIV at 0 dpv only (LAIVx1), or vaccinated I.N. with LAIV at 0 dpv and 14 dpv (LAIVx2). IgG antibodies binding to TX98 (C) and CO99 (D) whole viral antigens were measured by ELISA in 34 dpv sera. Open bars designate groups without MDA, and solid bars designate groups with MDA. Statistically significant differences between MDA statuses within a vaccine group are marked with asterisks, and differences between vaccine treatment groups with matched MDA statuses are identified by connecting lines ($P < 0.05$).

Figure 2. Antibody levels in bronchoalveolar lavage fluid at 0 days post-infection (35 dpv). Mean O.D. values in whole-virus ELISAs are shown for IgA antibodies against TX98 (A) and CO99 (B) and for IgG antibodies against TX98 (C) and CO99 (D). Open bars designate groups without MDA, and solid bars designate groups with MDA. Statistically significant differences between MDA statuses within a vaccine group are marked with asterisks, and differences between vaccine treatment groups with matched MDA statuses and challenge virus strains are identified by connecting lines ($P < 0.05$).

Figure 3. IgA antibody levels in nasal washes at 0 days post-infection (35 dpv). Mean O.D. values in whole-virus ELISA are shown for IgA binding to TX98 (A) and CO99 (B). Open bars designate groups without MDA, and solid bars designate groups with MDA. Statistically significant differences between MDA statuses within a vaccine group are marked with asterisks, and differences between vaccine treatment groups with matched MDA statuses and challenge virus strains are identified by connecting lines ($P < 0.05$).

Figure 4. Vaccine priming of T cells for SIV-specific upregulation of CD25. PBMC isolated from pigs 28 dpv were cultured ex vivo for 4 days in the presence of TX98, CO99, or no virus. CD25 and T-cell subset markers were quantified by MP-FCM. Within each T-cell subset, the virus-specific gain in percentage of cells with surface expression of CD25 is plotted for each animal. Open symbols represent MDA(-) pigs, and filled symbols represent MDA(+) pigs. Group mean values and standard errors of the means are denoted with bars. Statistically significant differences between vaccine treatment groups with matched MDA status, or between MDA statuses within a vaccine treatment, are identified by connecting lines ($P < 0.05$).

Figure 5. Vaccine priming of T cells for SIV-specific upregulation of IFN-γ production. PBMC isolated from pigs 28 dpv were cultured ex vivo for 4 days in the presence of TX98, CO99, or no virus. Intracellular IFN-γ and T-cell subset surface markers were quantified by MP-FCM. Within each T-cell subset, the virus-specific gain in percentage of cells with surface expression of IFN-γ is plotted for each animal. Open symbols represent MDA(-) pigs, and filled symbols represent MDA(+) pigs. Group mean values and standard errors of the means are denoted with bars. Statistically significant differences between vaccine treatment groups with matched MDA status, or between MDA statuses within a vaccine treatment, are identified by connecting lines ($P < 0.05$).

Figure 6. Vaccine priming of T cells for SIV-specific upregulation of IL-10 production. PBMC isolated from pigs 28 dpv were cultured ex vivo for 4 days in the presence of TX98, CO99, or no virus. Intracellular IL-10 and T-cell subset surface markers were quantified by MP-FCM. Within each T-cell subset, the virus-specific gain in percentage of cells with surface expression of IL-10 is plotted for each animal. Open symbols represent MDA(-) pigs, and filled symbols represent MDA(+) pigs. Group mean values and standard errors of the means are denoted with bars. Statistically significant differences

between vaccine treatment groups with matched MDA status, or between MDA statuses within a vaccine treatment, are identified by connecting lines ($P < 0.05$).

Figure 7. Vaccine priming of SIV-specific T cells in lung-draining lymph nodes. TBLN cells were harvested from non-infected pigs that were sacrificed 35 dpv, and tested by IFN-γ ELISpot for responses to TX98 and CO99. The mean number of spot-forming cells in duplicate wells is plotted for each pig, and the treatment group means and standard errors of the means are denoted with bars. Statistically significant differences between vaccine treatment groups with matched MDA status or between MDA statuses within a vaccine treatment, are identified by connecting lines ($P < 0.05$).

Figure 8. Detection of CO99 heterologous challenge virus in lungs 5 days post-infection. Groups of pigs, with and without MDA, received no vaccine (NV), two I.M. doses of TX98 WIV, one I.N. dose of TX98 LAIV, or two I.N. doses of LAIV. Thirty-five days after the initial vaccine dose, piglets were challenged by I.T. inoculation with CO99. (A) BALF specimens were collected at necropsy (5 dpi) and titrated by $TCID_{50}$ assay on MDCK cells. (B) Lung tissue sections were stained for SIV antigen by IHC.

Figure 9. Following heterologous challenge with CO99, pigs were monitored for clinical disease signs and fever. Daily cinical scores were comprised of sub-scores for coughing, lethargy, and difficulty breathing, and daily rectal temperatures were recorded. Open symbols represent MDA(-) groups, and filled symbols represent MDA(+) groups, with error bars indicating standard errors of the means.

Figure 10. Lung lesion severity upon heterologous H3N2 SIV challenge. At 35 dpv, vaccinated and non-vaccinated treatment groups were experimentally infected with CO99 by I.T. and I.N. routes. At 5 dpi, pigs were euthanized and necropsy was conducted. (A) Macroscopic lesions were scored as the percentage of total lung surface area involved. Microscopic lesions of lung (B) and trachea (D) were scored as described in Materials and Methods. Statistically significant differences between MDA statuses within a vaccine group are marked with asterisks, and differences between vaccine treatment groups with matched MDA statuses are identified by connecting lines ($P < 0.05$).

Figure 11. Antibody levels in BALF at 5 days post-infection with heterologous CO99. Mean O.D. values in whole-virus ELISAs are shown for IgG antibodies against TX98 (A) and CO99 (B) and for IgA antibodies against TX98 (C) and CO99 (D). Open bars designate groups without MDA, and solid bars designate groups with MDA. Statistically significant differences between MDA statuses within a vaccine group are marked with asterisks, and differences between vaccine treatment groups with matched MDA statuses are identified by connecting lines ($P < 0.05$).

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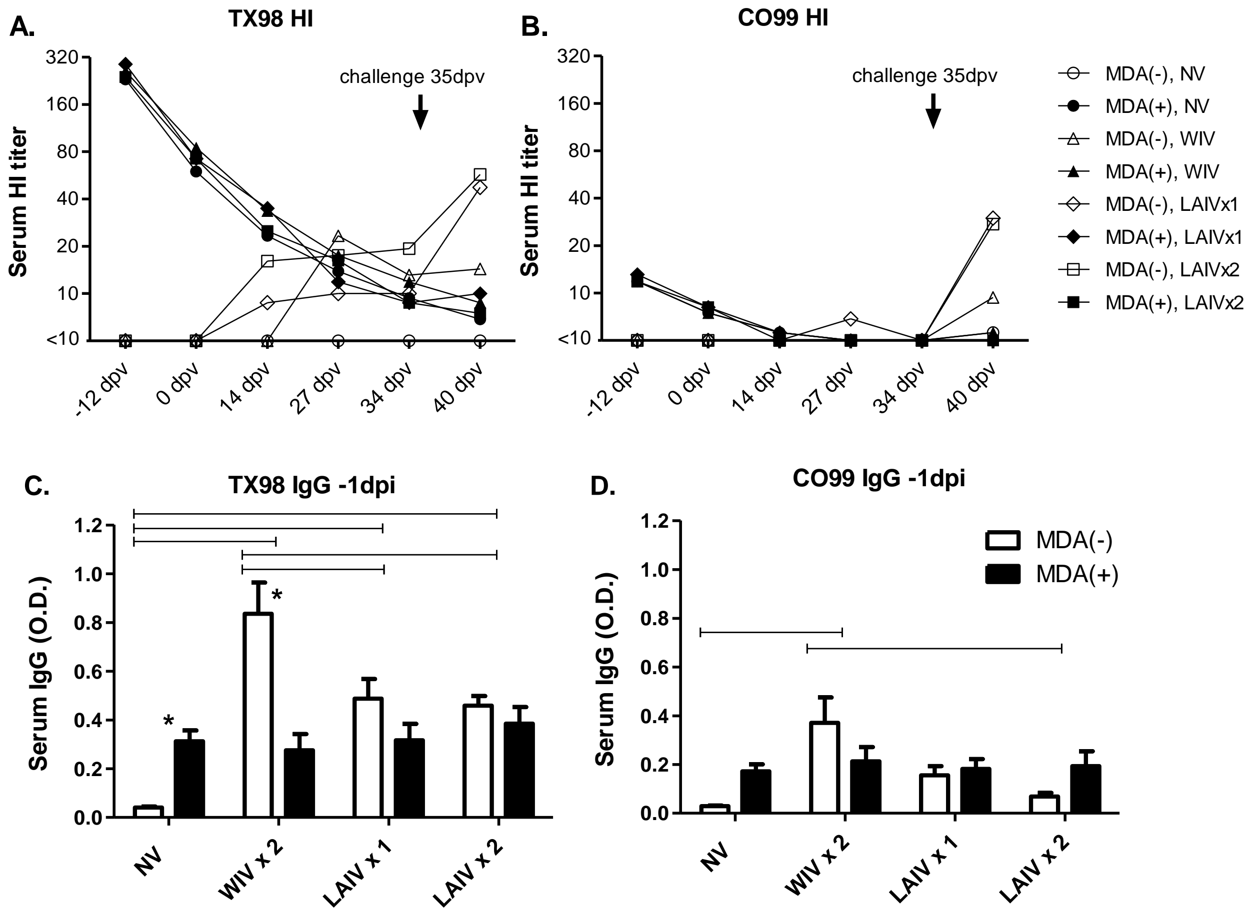
FIGURES



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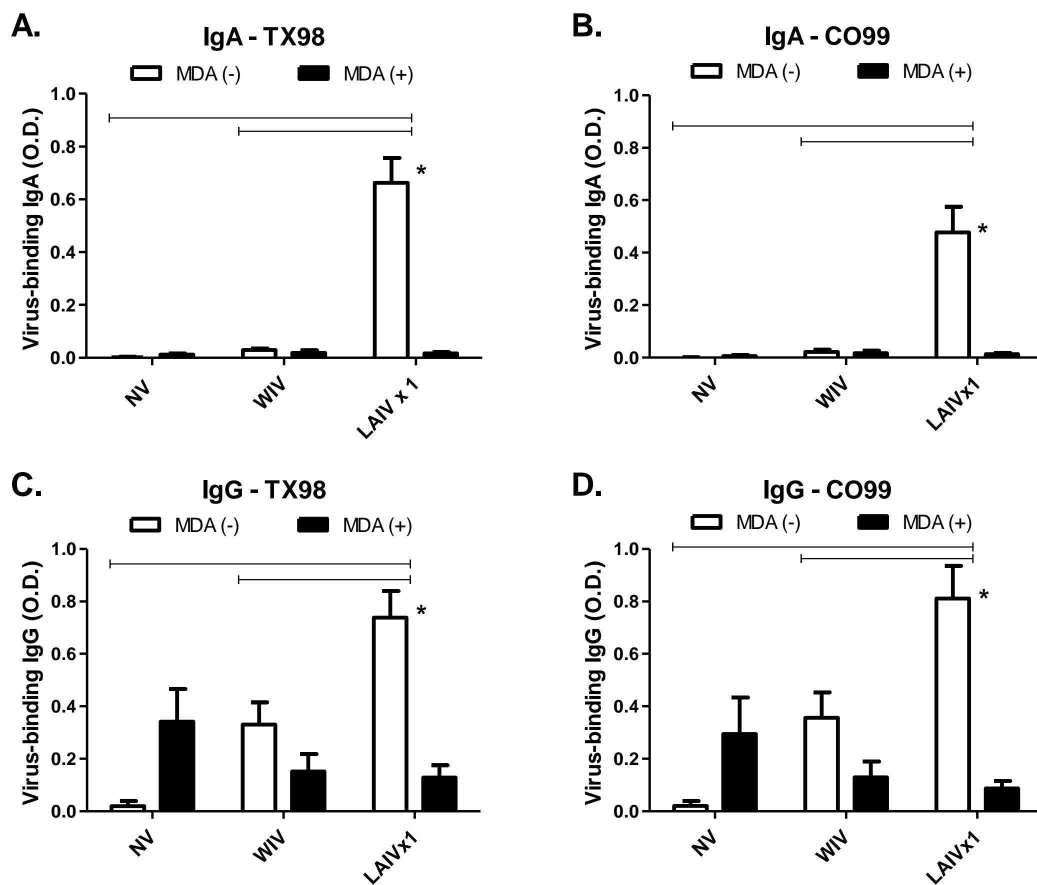

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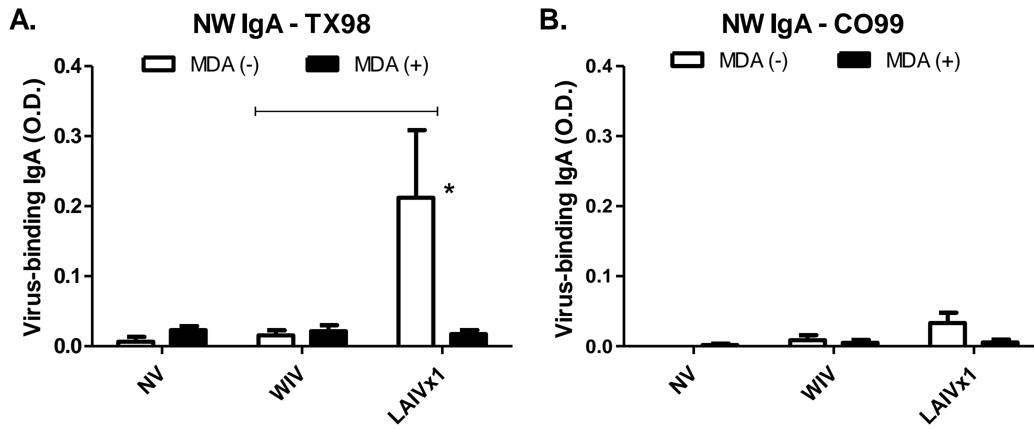
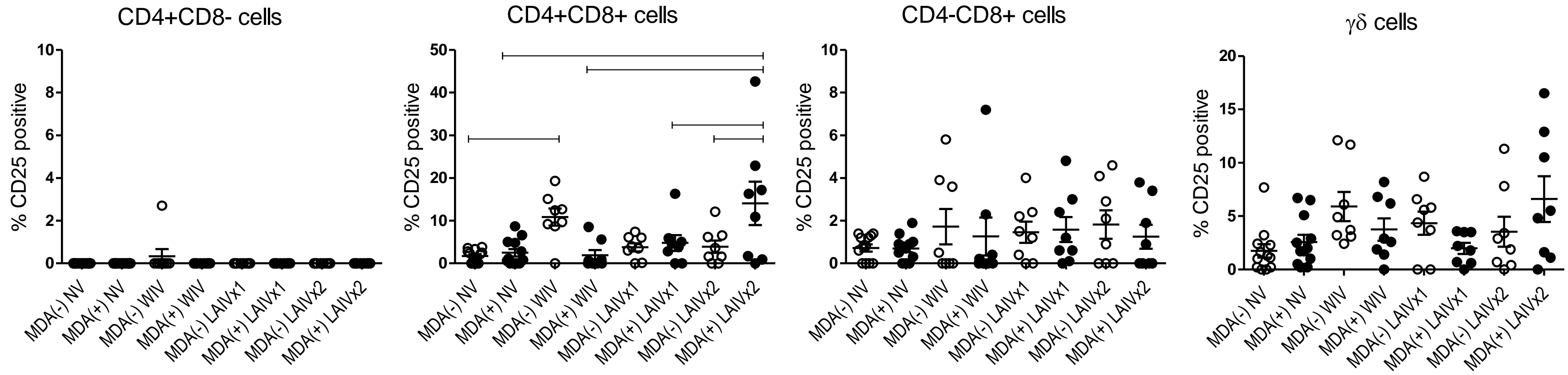


Figure 3.

CD25 responses -7 dpi

TX98 antigen



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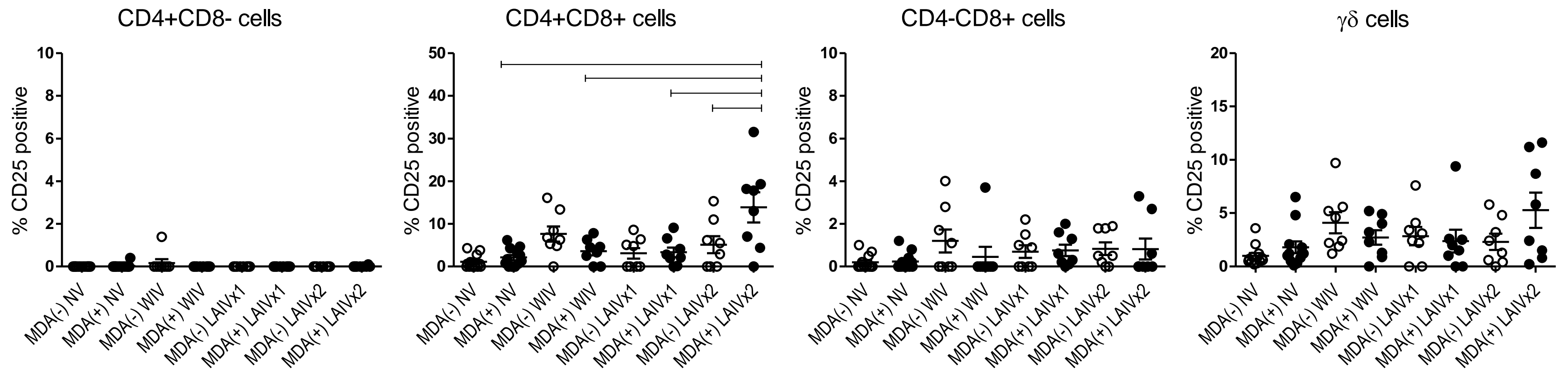

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IFN-γ responses -7 dpi

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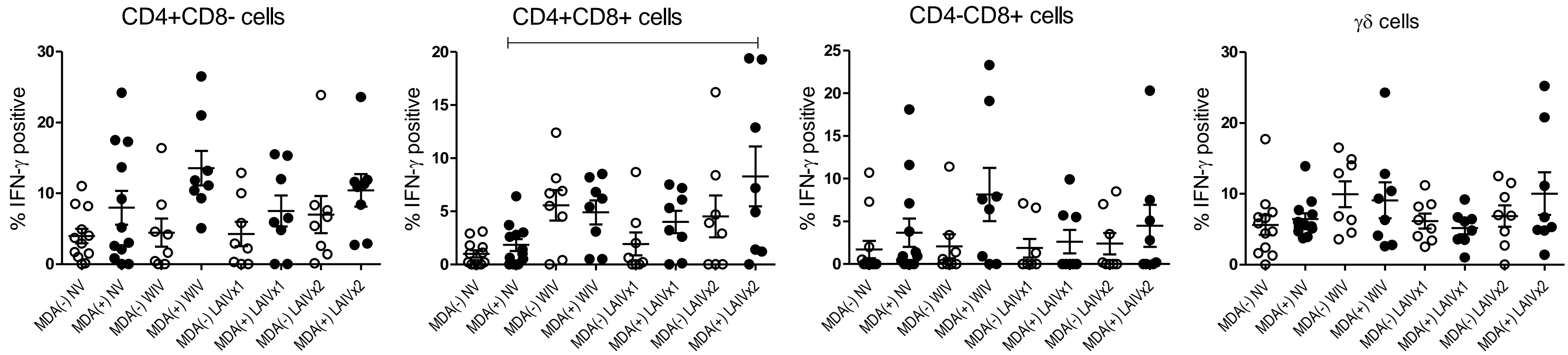

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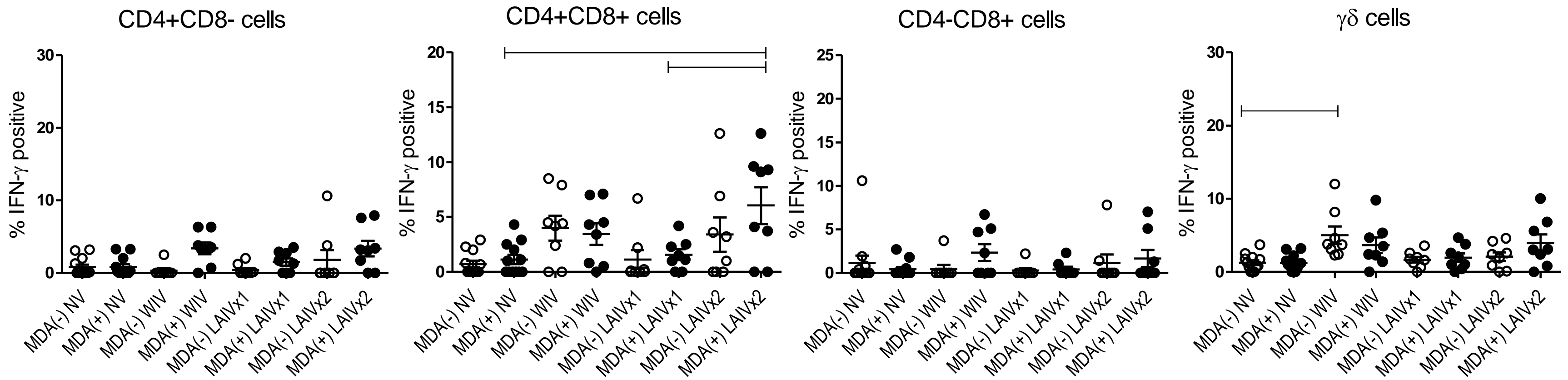

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IL-10 responses -7 dpi

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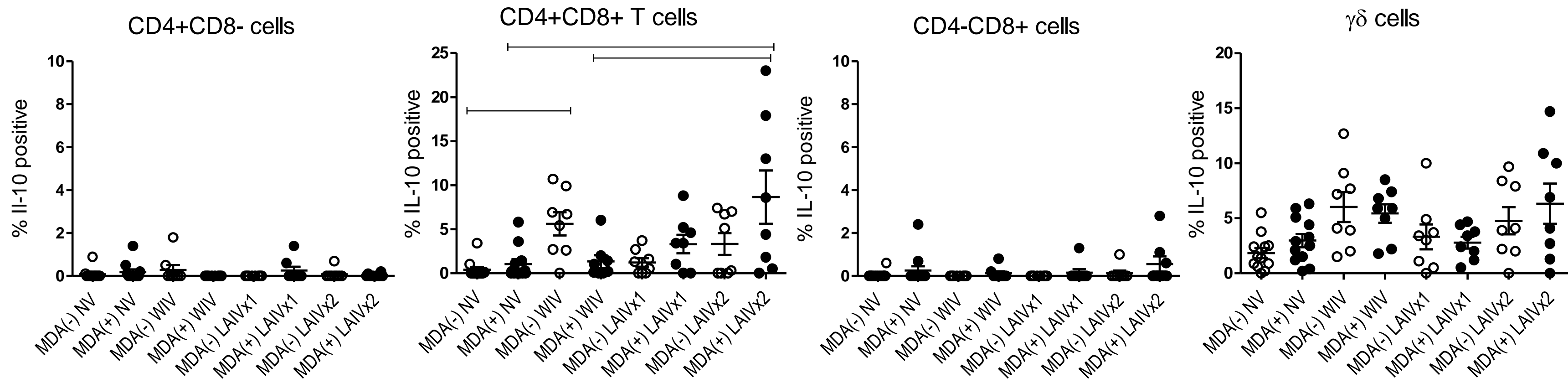

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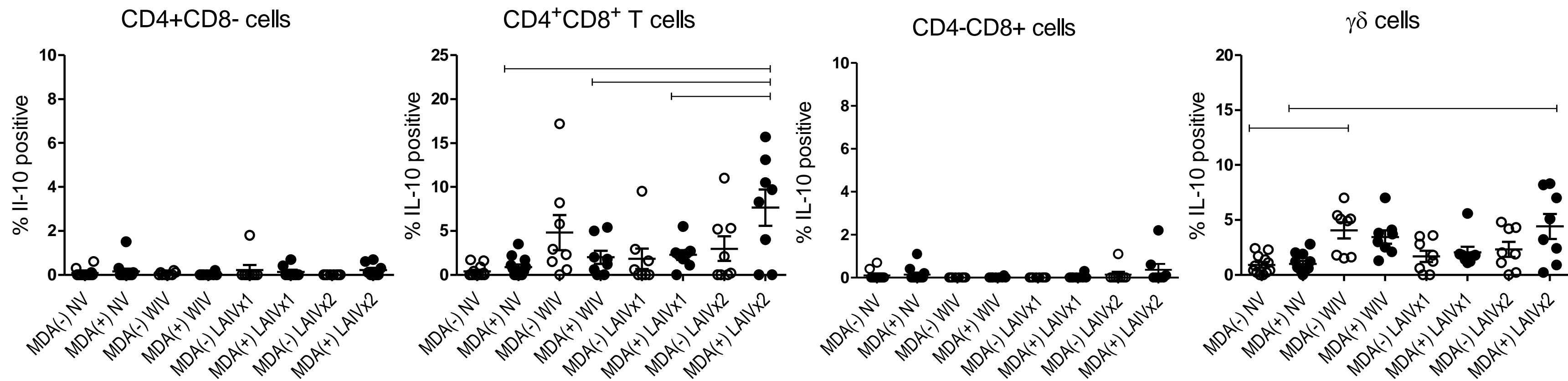

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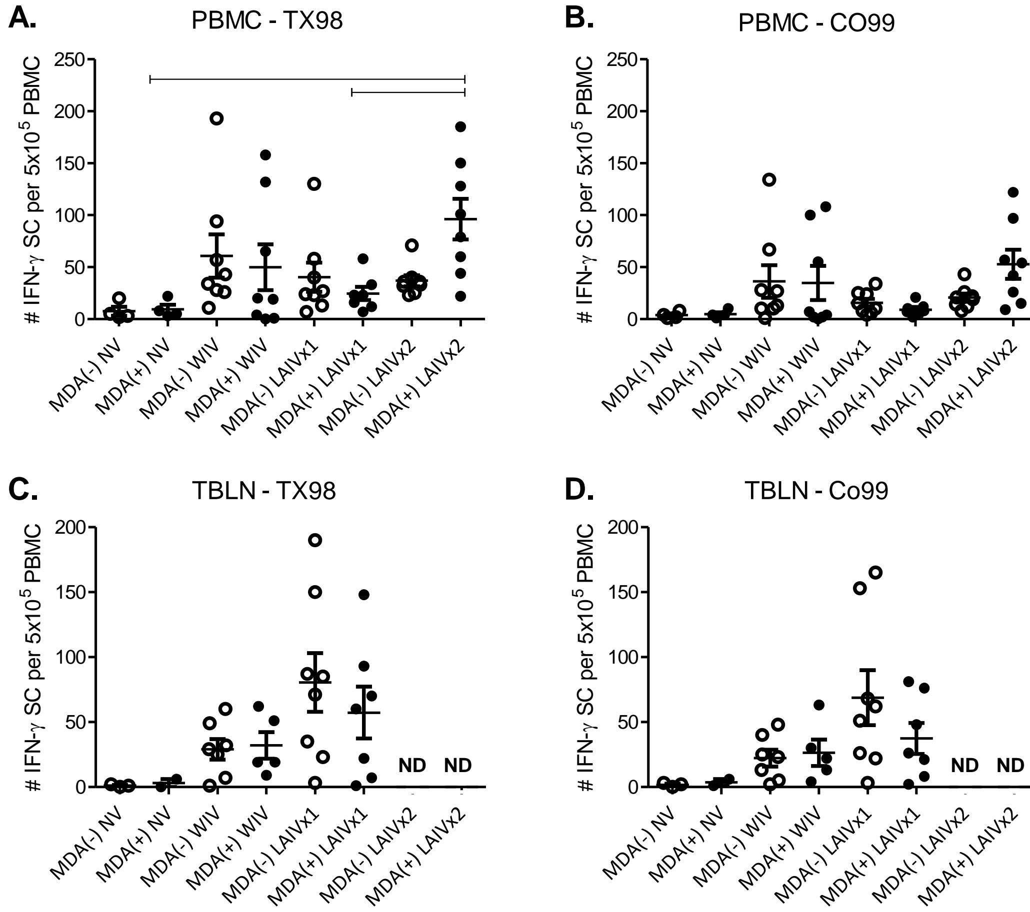

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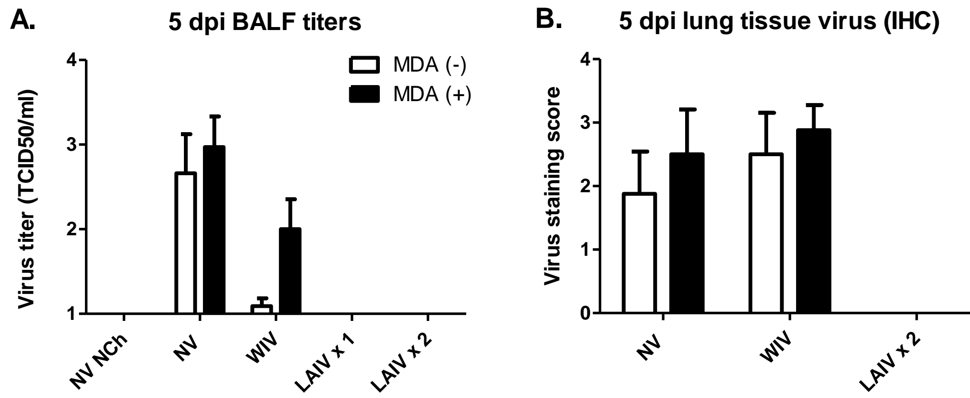

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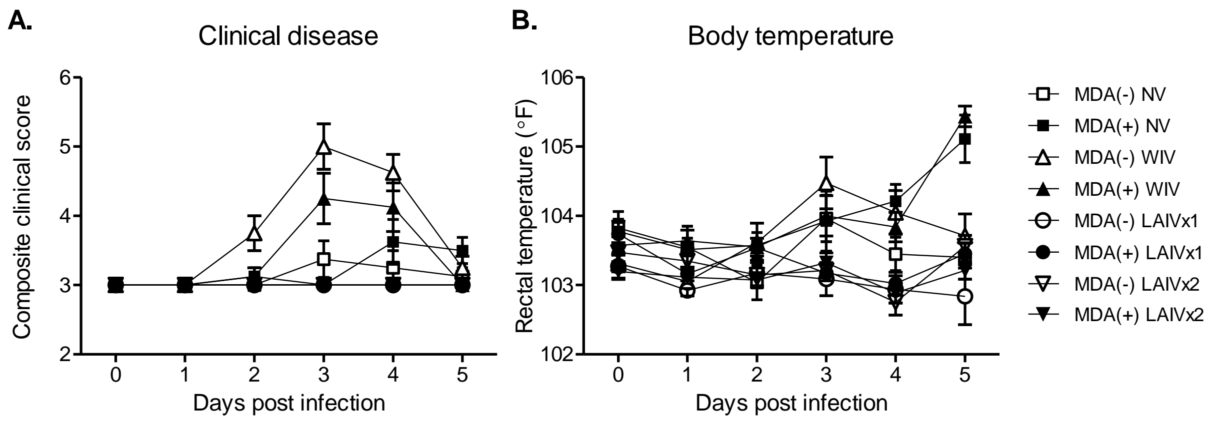

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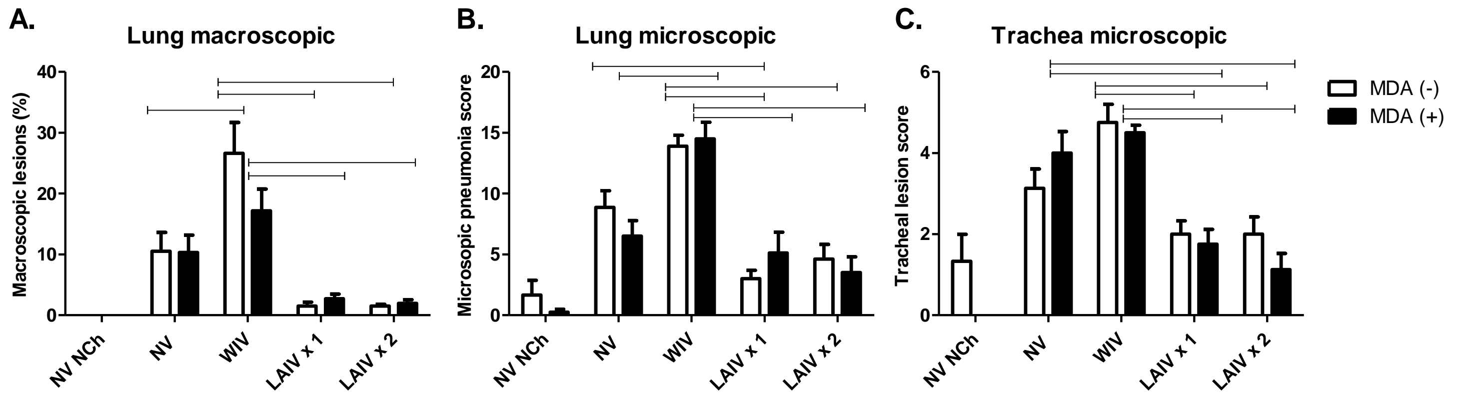

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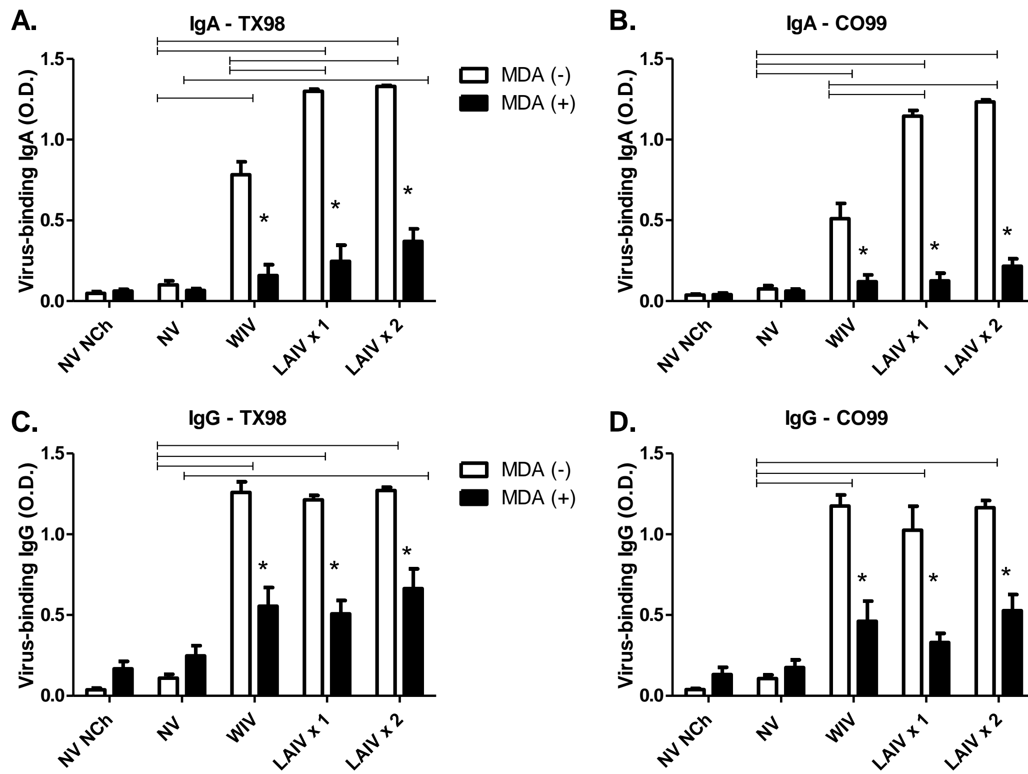

Figure 11.