Vaccination with a codon-optimized A27L-containing plasmid decreases virus replication and dissemination after vaccinia virus challenge

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Abstract

Smallpox is a disease caused by Variola virus (VARV). Although eradicated by WHO in 1980, the threat of using VARV on a bioterror attack has increased. The current smallpox vaccine ACAM2000, which consists of live vaccinia virus (VACV), causes complications in individuals with a compromised immune system or with previously reported skin diseases. Thus, a safer and efficacious vaccine needs to be developed. Previously, we reported that our virus-free DNA vaccine formulation, a pVAX1 plasmid encoding codon-optimized VACV A27L gene (pA27LOPT) with and without Imiquimod adjuvant, stimulates A27L-specific production of IFN-\(\gamma\) and increases humoral immunity 7 days post-vaccination. Here, we investigated the immune response of our novel vaccine by measuring the frequency of splenocytes producing IFN-\(\gamma\) by ELISPOT, the TH1 and TH2 cytokine profiles, and humoral immune responses two weeks post-vaccination, when animals were challenged with VACV. In all assays, the A27-based DNA vaccine conferred protective immune responses. Specifically, two weeks after vaccination, mice were challenged intranasally with vaccinia virus, and viral titers in mouse lungs and ovaries were significantly lower in groups immunized with pA27LOPT and pA27LOPT + Imiquimod. These results demonstrate that our vaccine formulation decreases viral replication and dissemination in a virus-free DNA vaccine platform, and provides an alternative towards a safer an efficacious vaccine.

Keywords

DNA Vaccine; Adjuvant; Smallpox; Vaccinia; Virus-Free

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1. Introduction

Variola virus (VARV), a member of the Poxviridae family and causative agent of smallpox, is considered one of the most devastating infectious agents of all times. This virus is categorized as a Tier 1 select agent by the Federal Select Agent Program (CDC, USDA). Routine smallpox vaccination was discontinued in the 1970s (1). Consequently, the general population is currently unprotected or vulnerable. The possible adverse events from this vaccine rises a common concern, if a vaccination campaign had to be resumed in case of a bioterror attack using this agent.

ACAM2000 is the current smallpox vaccine (2), consisting of live vaccinia virus (VACV). ACAM2000 causes serious complications such as eczema vaccinatum and progressive vaccinia in people with previously reported skin diseases (3). Moreover, encephalitis (3–6), severe skin infections (3, 7), and even death (3, 7) were observed after administering this vaccine. According to the CDC, in the event of a smallpox emergency, the U.S. government has enough vaccine available to immunize every person in the United States (8). However, the use of this vaccine is contraindicated (9) in individuals with a compromised immune system (10), patients receiving immune-suppressive therapy, and transplant recipients (11, 12), in addition to those with a history of skin diseases. For this vast number of people, being vaccinated with the available vaccine represents life-threatening outcomes that exceed the benefits of vaccination.

DNA vaccination is a safe alternative to induce protection, and to stimulate high immune responses without the adverse effects of a live virus vaccine immunization. This type of vaccine has proven to be very effective in stimulating protective immune responses in several organisms (13–15). DNA vaccines are safe, inexpensive, easy to transport, manage and store; criteria that a good vaccine must meet (9, 16–18). Another advantage of DNA vaccines is that they penetrate the interior of the cell to emulate a viral infection so that the antigens are presented and recognized by the immune system like a virus (15, 19). There are several studies in which these types of vaccines have shown to be effective, without adverse effects on the recipient organism (20–22).

Previously, we reported a potential DNA-based vaccine against smallpox consisting of the codon-optimized A27L gene (pA27LOPT) from the VACV Western Reserve strain (23). This vaccine induces significant humoral and cellular immune responses with and without the chemical adjuvant Imiquimod (23). This codon-optimization approach has been demonstrated to enhance the immune response in many cases by an apparent increase in protein production or expression (24, 25). Consequently, the greater production of protein leads to a better stimulus for the immune system.

Since the immune response to our novel vaccine formulation is favorable, in the present study, we investigated if the elicited immune response was maintained over time, and if vaccination could protect against infection with a lethal dose of VACV. Our results show that our virus-free DNA vaccine induces memory immune responses, and significantly decreases viral replication and dissemination. Our data demonstrate that virus-free DNA vaccines represent a safe alternative to limit viral infections.
2. Materials and Methods

2.1. Mice
Female BALB/c mice between 4- and 6-week-old purchased from Charles River (Wilmington, MA, USA) were housed at the University of Puerto Rico Medical Sciences Campus (Memory Immune Response experiment) or the University of Illinois at Urbana-Champaign (VACV intranasal infection). The animal care was performed under National Institute of Health guidelines (Bethesda, MD, USA). All animal experiments and procedures were approved by the Institutional Animal Care and Use Committee at both institutions. A total of 4 mice per group were used in the immune response experiments. For the challenge experiments and the intranasal inoculation experiments, the control groups consisted of 3 mice, while the experimental groups consisted of 6 mice. Each animal was anesthetized with a mixture of Ketamine and Xylazine by an intraperitoneal injection, and euthanized via cervical dislocation to perform the experiments. Each experiment was repeated at least three times.

2.2. Vaccine design and plasmid purification
The pA27L and pA27LOPT constructs containing an IgE leader sequence, and a hemagglutinin tag sequence were synthesized and cloned into the pVAX1 (Invitrogen, Grand Island, NY, USA) as previously described [23]. The resulting plasmids were transformed individually in the E. coli Top10 strain (Invitrogen, Grand Island, NY, USA). After large scale production, plasmids were purified using the Qiagen Plasmid Giga kit (Germantown, MD, USA) following the manufacturer’s instructions.

2.3. Study design
Female BALB/c mice were either not immunized (naïve) or immunized intramuscularly three times, at two week intervals with either 100 µg pVAX1 (vector), 100 µg pVAX1 + 50 nmoles of Imiquimod, 100 µg pA27LOPT, or 100 µg pA27LOPT + 50 nmoles of Imiquimod. Also, 100 µg of the non-codon optimized pA27L were included in the viral challenge experiments. The vaccine was formulated at a DNA concentration of 1.0 mg/mL in a 0.15 M sodium citrate buffer and 0.25% bupivacaine [23].

2.4. Splenocytes preparation
Spleens from mice were collected and macerated using the back of a syringe plunger. Cells were filtered, centrifuged, and incubated for 1 min with 1 mL of lysing buffer (Sigma Aldrich, St. Louis MO, USA). Then, 15mL of RPMI media was added to stop the red blood cells lysis. If lysis is incomplete, incubation with the buffer may be repeated. Finally, cells were centrifuged and diluted for counting and analysis.

2.5. IFN-γ ELISPOT assay
Two weeks after the third immunization, the T cell-mediated immune response was measured by ELISPOT assay using the capture anti-mouse IFN-γ antibody (R&D Systems, Minneapolis, MN, USA) and High-Protein Binding IP 96-well Multiscreen TM plates (Millipore, Bedford, MA, USA) as previously described [23]. Spots-forming cells (SFCs)
were quantified using an automated ELISPOT reader system from CTL Analyzers (Cleveland OH, USA) with the ImmunoSpot software. The data were expressed as mean ± standard error of the mean.

2.6. Measurement of total IgG, IgG1 and IgG2a antibody response by ELISA

The humoral immune response was analyzed two weeks after the third vaccination by Enzyme-Linked Immunosorbent Assay (ELISA), as previously described [23]. Specifically, the IgG (115-035-008), or IgG1 (115-035-205), or IgG2a (115-035-206) HRP-conjugated goat anti-mouse from Jackson Immunoresearch (West Groce, PA, USA) were used to detect the A27-specific antibody production.

2.7. Measurement of cytokine production

Two weeks after the third immunization, splenocytes from immunized mice were stimulated for five days at 37°C, in 5% CO₂ with a pool of 25 overlapping peptides from VACV A27 protein (JPT Peptide Technologies, Berlin, Germany) diluted to a concentration of 0.5 mg/mL in culture medium. Then, the supernatants were used to detect secreted IFN-γ or IL-4 cytokines, as previously described (23), using the commercially available Quantikine Mouse IFN-γ or IL-4 immunoassays from R&D Systems (Minneapolis, MN, USA).

2.8. Intranasal Challenge and virus titers in lung and ovaries

Two weeks after the third immunization, mice were anesthetized with isoflurane (5% in O₂) inhalation using a vaporizer unit (Vetland, Louisville, KY) and inoculated with 2.5x10⁶ PFU of sucrose density gradient purified VACV in 20 µl (10 µl per nostril). The virus was kindly provided by Dr. Bernard Moss (NIAID, NIH), and propagated in Vero cells using DMEM supplemented with 2% FBS. Mice organs were harvested one week after inoculation. The left lung from each individual mouse was homogenized using the TissueLyser LT from Qiagen (Valencia, CA, USA) with two cycles of 3 minutes at 501/S oscillation. The left ovary was homogenized using the 1.5 Kimble™ BioMasherII™ tissue homogenizer from Fisher Scientific (Cayey, PR, USA).

2.9. Viral Plaque assay

Infected cell lysate from lung and ovaries harvested from different groups of mice were used to detect the viral load. Serial dilutions ranging from 10⁻¹ to 10⁻⁶ were added to confluent monolayers of Vero cells in 6-well plates for 1 h. Cell lysates were removed, and each well was covered with 2 ml of freshly prepared MEM supplemented with 2% FBS. The cells were incubated for 2 days at 37 °C with 5% CO₂. Plates were stained with crystal violet 1%. Plaques were counted and viral titers calculated.

2.10. Statistical Analyses

The data from experiments shown here were repeated at least three times and are presented as the mean ± standard error of the mean (SEM). The one-way ANOVA test was used to determine the statistical significance of differences among groups, using the GraphPad Prism 6 (La Jolla, CA, USA). Data were evaluated for equality of variance with the Brown-Forsythe tests and were transformed with X=log(x) to normalize when necessary.
3. Results

3.1. VVWR A27-Specific IFN-γ Stimulation

The capacity of pA27LOPT, either alone or in the presence of Imiquimod, to induce protective immune responses was assessed by immunizing BALB/c mice three times, two weeks apart. As controls, other mice were either not immunized (naïve), or immunized with pVAX1 in the presence or absence of Imiquimod (I).

Two weeks after the last immunization (Fig. 1), splenocytes from each group of mice were pooled and stimulated, using a mix of overlapping peptides corresponding to the complete sequence of the VACV A27 protein. A27-specific T cell responses were measured by using ELISPOT analysis (Fig. 2).

The mean frequency of the spot-forming cells (SFC) per million splenocytes are shown in Fig. 2. As expected, there were very low amounts of A27-specific T cells in naïve mice. Vaccination with pVAX1, either alone or with Imiquimod, increased the number of stimulated splenocytes, possibly due to a non-specific DNA effect on T cell responses. However, the response was further increased in mice vaccinated with pA27LOPT or pA27LOPT + I, (Fig. 2). Our data shows a statistically significant increase in T cell responses when A27L DNA was present versus absent. In summary, these data indicate that the A27L-based DNA vaccine possesses beneficial immunomodulatory properties because it significantly increased the frequency of cells that secrete IFN-γ in response to the A27 antigen.

3.2. Measuring the Cytokine Profile

Previously, we reported that our vaccine formulation triggered a significant increase in the levels of IFN-γ [23] but not IL-4, suggesting that A27-based DNA vaccines skewed a TH1 response after vaccination. We asked if this profile was stable at longer time points post-vaccination (Fig. 3). In agreement with earlier times post-vaccination there was a significant increase in IFN-γ production in stimulated splenocytes from mice immunized with pA27LOPT or pA27LOPT + I, as compared to control groups. In contrast, IL-4 secretion was not detected in any group. Thus, our vaccine formulation induced a TH1-biased immune response longitudinally.

3.3. Humoral Immune Response Analysis

We studied the A27 antigen-specific humoral memory immune response, after vaccination with a codon-optimized pA27L formulated with or without Imiquimod (Fig. 4). In this ELISA, we used the VACV A27 protein as an antigen, and an IgG capture antibody to quantify A27-specific antibody production induced by our DNA vaccine, two weeks after the third vaccination. Figure 4A shows that the production of total IgG was higher in animals immunized with pA27LOPT or pA27LOPT+I, compared to control groups.

3.4. IgG1 and IgG2a Isotypes Antibody Detection

The two antibodies of greatest interest to us were IgG2a (opsonizing antibodies that support a TH1 type response) and IgG1 (neutralizing IgG1 antibodies that support a TH2 type
response). When examining IgG2a and IgG1 in vaccinated mice, the amount of both antibodies were significantly higher in the experimental groups as compared to the control groups (Fig. 4B). Moreover, in these experiments IgG2a was the outstanding produced antibody. These data suggest that our DNA vaccination cocktail induces the generation of opsonizing antibodies, supporting a TH1 biased response.

3.5. Measuring Viral Load after Intranasal Inoculation with VACV

Our A27L-based DNA vaccine induced both T- and B-cell mediated immunity at the time of infection. One question is if this immunity would be beneficial against a poxvirus infection. To address this issue, we examined VACV replication in vaccinated mice. Animals were infected by the intranasal route with a lethal dose of VACV, two weeks after the last immunization (Fig. 5A). Six days post-infection, mice organs were harvested, and viral titers were determined in the initial site of infection (lungs) and in distal organs (ovaries). As expected, VACV replicated to high titers in the lungs of naïve mice. Whereas animals vaccinated with pVAX1 or pVAX1 + I showed a slight decrease in viral titers as compared to naïve mice. Mice immunized with a plasmid encoding a non-codon optimized A27L showed no difference in virus titers as compared to naïve mice, demonstrating that A27L without codon-optimization provides no protection against VACV replication. In contrast, mice immunized with pA27LOPT or pA27LOPT + I showed a 10-fold reduction in viral titers in lungs as compared to naïve mice (Fig. 5B).

Similar trends were observed when viral replication was assessed in the ovaries (Fig. 5C). In naïve mice, VACV disseminated from the lungs and replicated in the ovaries, as previously shown (26). Similar virus titers were observed in the ovaries of mice that were immunized with pVAX1, pVAX1 + I or the non-codon optimized A27L-based plasmid (pA27L). However, mice immunized with pA27LOPT or pA27LOPT + I showed a 10,000-fold or 1,000-fold reduction in virus titers, respectively, as compared to naïve mice. Interestingly, in addition to a decreased replication, a limited spread of VACV was observed in ovaries of mice immunized with pA27LOPT or pA27LOPT + I. While all lungs possessed detectable VACV (Fig. 5B), VACV was detected in the ovaries of only four or three mice immunized with pA27LOPT or pA27LOPT + I, respectively (Fig. 5C). These data indicate that our vaccine formulation was able to limit both, replication and spread in experimental groups, as compared to control groups.

4. Discussion

Fundamental to the success in the development of an immune therapy against viral diseases, is to generate a vaccine that induces a robust immune response able to control virus replication. ACAM2000, the current smallpox vaccine, is a live virus vaccine, and is not safe for immunocompromised individuals or patients with a history of skin diseases (27). With the intent to develop a virus-free vaccine, which is expected to be safe for all humans, we previously reported a DNA vaccine coding for the VACV A27L gene that induces strong T- and B-cell immune responses in mice soon after vaccination (23). Here, we present evidence that this same vaccine formulation also produces an adaptive immune response, which substantially decreased viral replication and spread.
In our previous study (23) the response was measured one week after the last immunization, to describe the immune effects soon after vaccination. However, our objective in this study was to capture and correlate memory responses (28) to infection. For that reason, the immune response was measured two weeks after the last immunization, coinciding with the time of the viral challenge. Opposed to our initial concern that the immune response might have faded two weeks after immunization, these data confirm not only the presence of antigen-specific immune responses, but also protection against a viral challenge at that time point (28).

The A27L gene is an attractive basis for a DNA vaccine, as its gene product is known to be immunogenic (29, 30). In this regard, several approaches have been implemented to develop an A27-coding DNA-based vaccine against smallpox. On this matter, a DNA-based vaccine consisting of A27L in addition to DNA coding for A33R, L1R, and B5R demonstrate to protect against a monkeypox challenge (31). More recently, a recombinant adenovirus-based vaccine encoding A27L, administered as a single intramuscular injection, protected mice against a lethal intranasal poxvirus challenge (32).

In our study, we demonstrate that a codon-optimized A27L gene is sufficient to mount protective immune responses, without the hazard of using a live vector. Among the modifications made to the sequence includes: (1) codon usage optimization [33, 34], (2) elimination of certain DNA motif [35], (3) adjustment guanosine-cytosine (G-C) base pair [36], (4) replacement of the sequence repetitions [37, 38], and (5) decrease possible RNA secondary structures [39]. All these modifications are intended to improve gene stability and protein expression. Consequently, a greater stimulation of the immune system leading to more T cells and antibodies [40] to recognize the antigen, and successfully induce protection in a viral challenge is obtained.

Both humoral and cellular immune responses are essential for the protection against smallpox (26). The T cell-mediated immune response is necessary for the delay in the illness progression, and the humoral-mediated response is critical for virus clearance (41, 42). In this regard, our data shows pA27LOPT or pA27LOPT + I to significantly increase the frequency of cells producing IFN-γ over time, compared to controls.

Also, pA27LOPT- or pA27LOPT + I-immunized animals generated a higher A27L-specific humoral response compared to controls. In addition, the polarization of antibodies produced after an immunization is critical in the outcome of a humoral response [43, 44]. In this regard, we found higher IgG2a and IgG1 in pA27LOPT and pA27LOPT immunized groups compared to controls. Moreover, IgG2a was the prominent antibody under those conditions.

IFN-γ is the most common cytokine in a TH1-type response and is powerful for viral removal. This cytokine promotes IgG2a class switching, which is beneficial against most viral infections (43, 44). Although we observe lower frequencies of IFN-γ producing cells in this study compared to our previous publication, as determined by ELISPOT, our ELISA analysis shows this decrease not to affect the amount of cytokine released. Conversely, the release of IFN-γ increased for the experimental groups compared to the previous study (23). This information confirms the potential of our single antigen DNA vaccine enhancing a...
TH1-biased humoral response, which is known to be essential against viral infections (43, 44).

We expected that the differences in the timing of sampling to have an impact in the results. Specifically, no significant differences in the immune response were observed between pA27LOPT and pA27LOPT + I immunized groups, as opposed to our previous report (23). This suggests that Imiquimod did not enhance the induction of immune responses in these set of experiments. We speculate that the intense immune response produced after vaccination with the optimized plasmid, at some point triggers a regulatory effect when combined with the adjuvant as a result of excessive epitope levels (45–48). We interpret this effect to induce either a higher rate of signal decay or to establish a top ceiling in the increase of the signals obtained from the adjuvanted group compared to non-adjuvanted mice. Also, adjuvant degradation could explain this effect. However, having no information regarding the rate of change for these immune responses, additional studies beyond the scope of this work would be necessary to explain for such a behavior.

Also, we observe high levels of non-specific cell-mediated immune responses in animals immunized with backbone vector. As stated before, the intense immune response observed with the optimized plasmid at some point could trigger a regulatory effect that induces a stronger rate of decay in antigen-specific immune responses, compared to non-specific responses. Also, a decline in the immune response simply due to the passage of time after vaccination is an expected effect that could account for the decrease in the signals obtained two weeks after immunization. These effects would allow non-specific immune responses in the backbone-immunized animals to stand out. Again, additional studies beyond the scope of this work are needed to explain the variations in the rates of these immune responses. It is important to highlight that even with an increase in non-specific responses, signals from vaccine-immunized mice are significantly higher compared to those obtained from the backbone controls, and these non-specific responses do not correlate with a significant decrease in the viral load assays.

Our major interest is to produce a vaccine against smallpox suitable for worldwide distribution, including developing countries. Studies show other investigators to successfully design DNA vaccines, using single antigens like D8L or a combination of A27L, A33R, L1R and B5R vaccinia genes, able to induce protection against a vaccinia virus challenge (31, 49). In other study, vaccination with both L1R and A33R gene of vaccinia virus using the gene gun technique, induce protection against lethal challenge (50). Also, using a novel skin electroporation device, a DNA vaccine containing four different antigens of vaccinia was reported to induce protection after intranasal poxvirus challenge (51).

We are aware that several investigators are testing a multi-epitope approach in their vaccine design in order to broad the spectrum of recognized antigenic determinants. With this approach, they expect to induce a higher diversity of antibodies and CTLs to target Variola. However, as the A27L gene is highly conserved among poxviruses (29), not only our group, but also other investigators design single-antigen vaccines against smallpox, expecting to induce a protective cross-reaction between VACV and VARV. Moreover, a formulation consisting of a single-antigen vaccine would accomplish the worldwide public health
requirement of inexpensive production and simple distribution among several countries. In this regard, Pulford et. al analyzed ten different genes of Vaccinia virus individually and found B5R, A33R, and A27L to induce a survival of or greater than 66% in a challenge, after four vaccine doses (52). In another study, A27 administered as a single protein was used to induce protection (29). Other investigators delivered B5R as a DNA vaccine, inducing not only a strong interferon gamma (IFN-γ) response in BALB/c mice, but also 100% protection (52). In an additional report, the investigators used the A33R clone as the antigen, and they also showed 100% protection after intranasal challenge in mice (53).

Moreover, single-antigen vaccination has not been designed only for vaccines against smallpox, nor is limited for experimental vaccines. In this regard, two Hepatitis B virus (HBV) vaccine known as Recombivax-HB\textsuperscript{®} (54) and Engerix-B (55) are licensed are in use in the USA. Also, a prophylactic Human Papilloma Virus (HPV) vaccine known as Cervarix is currently licensed (56). Therefore, vaccines based on a single-antigen are not only in development, but also currently used in several licensed vaccines.

Although DNA vaccines have been shown to be safe in animals and humans [57,58], a future study must be conducted with our vaccine candidate to address possible safety concerns [57,59]. Furthermore, we also want to assess the capacity of our DNA vaccine of inducing protection against a monkeypox challenge in nonhuman primates.

Our previous [23] and current works show a codon-optimized vaccine to induce a significant enhancement of the immune response, compared to the non-adjuvanted formulation. Moreover, our results were obtained using three doses of a single antigen vaccine without the aid of additional live-vectors, delivery systems, and novel equipment such as gene guns, electroporation, or skin tattooing machines. These devices could be a disadvantage if vaccines are intended to be administered to a large group of people, and distributed to other countries. Based on that information, we expect the significant enhancement of our codon-optimized plasmid to overcome the need of using a multi-epitope approach.

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Figure 1.
Schematic of immunization for the analysis of long-term immune responses. Three of these groups serve as controls: Naïve, used as a negative control, and pVAX1 and pVAX1 + Imiquimod (pVAX + I) used as backbone controls. The remaining two groups were the experimental groups: pA27LOPT and pA27LOPT + Imiquimod (and pA27LOPT + I).
Figure 2.
Frequencies of IFN-γ producing cell specific for A27L. Female BALB/c mice (n=4) were immunized as described in Figure 1. Two weeks after the last immunization, splenocytes from each group of mice were pooled and stimulated with VACV A27 peptides. The number of cells were determined by ELISPOT analysis. Data are expressed as mean ± standard error of the mean. Statistical significance was determined by one-way ANOVA. A p value of less than 0.05, indicated by ** = p < 0.01 and **** = p < 0.0001, was considered significant.
Figure 3.
Cytokine profile specific for the recombinant VVWR A27 by ELISA. Female BALB/c mice (n=4) were immunized as described in Figure 1. Two weeks after the last immunization, cultured splenocytes were stimulated with VACV A27 peptides. The production of both IFN-γ and IL-4 from cells supernatant were detected by ELISA and analyzed by absorbance at an OD of 450 nm. Data are expressed as the mean ± standard error of the mean (SEM) from three independent experiments. A p value of less than 0.05 was considered significant, indicated by * = p < 0.05 and **= p < 0.01.
Fig. 4.
A27L-specific humoral immune response by ELISA. (A) Generation of total IgG antibody. (B) Generation of IgG1 and IgG2a antibodies. Sera from each group (n = 4) was collected two weeks after the last DNA immunization. Serum samples from each group of mice were diluted at 1:400 and incubated in a 96-well plate previously coated with recombinant VVWR A27. Data are presented as the mean ± standard error of the mean (SEM) from three independent experiments. A p value of less than 0.05 was considered significant, indicated by * = p < 0.05, *** = p < 0.001 and **** = p < 0.0001.
Figure 5.
Effects of vaccination on replication and dissemination of VACV. (A) Female BALB/c mice were immunized as described in Figure 5A. Two weeks after the last immunization, mice were infected intranasally with vaccinia virus. Six days post-infection, mice were humanely euthanized, and organs were harvested. (B) Lungs and (C) ovaries were homogenized, and virus titers were determined by plaque assay on Vero cellular monolayers. Titers are expressed as PFUs per organ. Each symbol represents the virus titer from an individual animal, and the mean titer is indicated by a line. Statistical significance was determined and
indicated by ** = \( p < 0.01 \), and **** = \( p < 0.0001 \). The dashed line represents the limit of detection.