microbiology research

MDPI

Article

Adenovirus-Mediated Expression of Dengue Virus 2 Envelope Ferritin Nanoparticles Induced Virus-Specific Immune Responses in BALB/c Mice

M.S.B.W.T.M. Nipuna Sudaraka Tennakoon [1], Ji-Hoon Ryu [2], Yong-Sam Jung [3], Yingjuan Qian [3] and Hyun-Jin Shin [1,2,*]

[1] Laboratory of Infectious Diseases, College of Veterinary Medicine, Chungnam National University, Daejeon 34134, Republic of Korea; nipunatennakoon55@gmail.com
[2] Research Institute of Veterinary Medicine, Chungnam National University, Daejeon 34134, Republic of Korea; jihoon0855@gmail.com
[3] MOE Joint International Research Laboratory of Animal Health and Food Safety, Jiangsu Foreign Expert Workshop, College of Veterinary Medicine, Nanjing Agricultural University, Nanjing 210095, China; ysjung@njau.edu.cn (Y.-S.J.); yqian@njau.edu.cn (Y.Q.)
* Correspondence: shin0089@cnu.ac.kr; Tel.: +82-42-821-6760

Abstract: This study provides a preliminary background for the development of a viral vector vaccine for the dengue virus using genetic material encoded by dengue envelope ferritin nanoparticles. Adenoviruses were generated for the recombinant envelope of dengue virus 2 (DENV2) and the envelope human ferritin heavy chain using a two-vector adenovirus system. The primary immunostimulatory activity of the two viruses was analyzed in mice to determine the effect of envelope ferritin nanoparticles. Transfection of a shuttle vector delivered the target gene and packaging vector carrying the packaging signal, and recombinant adenoviruses (rAds) were generated and purified using an ultracentrifugation method. Transduction efficiencies of the generated adenoviruses were confirmed in A549 cells. Purified adenoviruses (8×10^6 PFU/mL) were immunized intramuscularly into 6 weeks old BALB/c mice. Subsequently, the DENV2-specific IgG titer was evaluated 1 and 4 weeks after immunization. Envelope ferritin-immunized mice showed a significant IgG response compared to envelope-only immunized mice at 1 and 4 weeks after immunization, revealing the persistence of the dengue virus-specific IgG response. This method demonstrated the capability of the viral vector vaccine to be used as a carrier for ferritin nanoparticles, instead of direct immunization with ferritin nanoparticles.

Keywords: adenovirus; dengue virus; ferritin nanoparticles; immune responses

Citation: Tennakoon, M.N.S.; Ryu, J.-H.; Jung, Y.-S.; Qian, Y.; Shin, H.-J. Adenovirus-Mediated Expression of Dengue Virus 2 Envelope Ferritin Nanoparticles Induced Virus-Specific Immune Responses in BALB/c Mice. *Microbiol. Res.* **2024**, *15*, 1913–1923. https://doi.org/10.3390/microbiolres15030128

Academic Editor: Juan Ayala

Received: 29 July 2024
Revised: 27 August 2024
Accepted: 12 September 2024
Published: 15 September 2024

1. Introduction

Dengue is a highly contagious flavivirus that is transmitted through the bites of a few species of mosquitoes, and viral infections cause dengue fever. Four serotypes of dengue virus were identified as DENV-1, DENV-2, DENV-3, and DENV-4, according to the diverse synergy between virus types and human serum. According to reports from the WHO, it has been shown that the number of cases of dengue infection has increased over the last five years. Therefore, the establishment of virus control and prevention measures and the development of vaccines and antiviral drugs are essential for curtailing viral infection and disease severity. Although few vaccines have been reported to be used against the dengue virus, further development of vaccines with enhanced immune responses and safety is required to address the prevalence of the disease.

The genome of the dengue virus encodes three dominant structural proteins: the capsid, premembrane (prM), envelope, and seven non-structural proteins. Structural proteins are important for viral entry process of viruses into host cells [1]. In the fusion machinery

of flaviviruses, envelope proteins are the major structural elements exposed to the exterior and interact with the host cell membrane [2]. Specifically, the capsid, prM, and envelope proteins are strongly targeted antigenic elements by antibodies produced in infected patients [3]. Consequently, envelope proteins have been utilized in the development of different vaccine strategies and have demonstrated significant immune responses and virus-neutralization activity [4].

Several vaccine strategies for the dengue virus have been reported, such as live attenuated vaccines [5], inactivated virus vaccines [6], recombinant subunit vaccines [7], viral vector vaccines [8], and DNA vaccines [9]. In most vaccine candidates, the DENV envelope protein is used as the primary antigen [10]. As viral vector-based vaccines, adenovirus, alphavirus, and vaccinia virus vectors have been used in previous studies for the cloning of antigenic molecules and delivery into host cells. There have been reported benefits of using these recombinant viruses to deliver antigenic molecules, including durable immune responses, vaccine stability, large-scale manufacturing, eliciting robust antibody responses, and the induction of cellular immune responses [11]. Importantly, the ferritin nanoparticle vaccine and drug delivery systems are advanced bioengineering technologies that can be used to efficiently deliver drugs and vaccine antigens to target cells [12–15]. Although some vaccine strategies have been used with ferritin nanoparticles, with the emergence of SARS-CoV-2, a plethora of vaccine strategies have been developed using ferritin nanoparticles [16–18]. Furthermore, protein nanocages, such as ferritin, and synthetic nanocages have been used to display single antigen molecules in multiple numbers to enhance the immune response and mimic virus morphology with better protein stability [19–23]. Because of the similar morphological structure of the ferritin cage to the viral structure, it has been shown that potent CD4+ and CD8+ T cell immune responses are induced in mouse models [24]. Therefore, a ferritin nanoparticle-based vaccine delivery system is a well-established technique that provides significant immunostimulatory activity against emerging pathogens. In our study, we delivered the dengue virus envelope conjugated with the human ferritin heavy chain through the adenovirus vector system and subsequently demonstrated an enhanced IgG response from envelope ferritin nanoparticles compared to the envelope alone, revealing the formation of ferritin nanoparticles.

2. Materials and Methods

2.1. Cells and Viruses

Vero cells were maintained in Minimum Essential Medium (MEM) (WELGENE, Korea) supplemented with 10% fetal bovine serum (FBS) (Biowest, Lakewood Ranch, FL, USA), 1% antibiotic-antimycotic (AA) (Biowest, Lakewood Ranch, FL, USA), and 1% HEPES (Gibco, USA). HEK 293T and 293A cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Biowest, Lakewood Ranch, FL, USA) containing 10% FBS, 1% AA, and 1% HEPES. A549 cells were maintained in RPMI 1640 medium (WELGENE, Korea) supplemented with 10% FBS, 1% AA. The DENV-2/KBPV-VR-29 strain was used in this study.

2.2. DENV2 Virus Propagation

DENV2 was inoculated into Vero cells in serum-free MEM in the presence of 2% FBS and 1% AA and incubated for 3–5 days in a CO2 incubator at 37 °C. The virus culture was maintained for 4–5 passages in the same manner, increasing the scale. Finally, the virus was harvested and centrifuged at 12,000× *g* for 10 min at 4 °C to recover the supernatant. The cell pellet was suspended in virus stock solution, lysed with three freeze–thaw cycles, frozen in liquid nitrogen (LN2), and thawed in a 37 °C water bath to release intracellular virus particles. The cells were then centrifuged at 12,000× *g* for 10 min to pellet cell debris, and the virus-containing supernatant was mixed with the virus supernatant. The viruses were purified and concentrated via centrifugation using a sucrose gradient. Briefly, 25 mL of virus supernatant was carefully decanted into 10 mL of 20% sucrose dissolved in phosphate-buffered saline (PBS) and ultra-centrifuged in a Hitachi CP100NX ultracentrifuge at 72,000× *g* for 5 h at 4 °C. The supernatant was aspirated and 200 µL of PBS containing

1% FBS was added to the pellet, followed by incubation at 4 °C overnight. The next day, the virus pellet suspended in the solution was aliquoted into microcentrifuge tubes and stored at −80 °C.

2.3. Viral RNA Isolation, cDNA Preparation, and qPCR

Viral RNA was isolated from 300 µL of virus supernatant using Ribospin™ vRD (GeneAll, Seoul, Republic of Korea) and cDNA was prepared using TOPscript™ RT DryMIX (dN18/dN6) (Enzynomics, Daejeon, Republic of Korea). To confirm the cultured and purified viruses, 1 µL of cDNA was used for qPCR analysis with 1 µL of each previously reported primer; Primer 1, Primer 2 (Table 1) [25], and 10 µL of 2X Real-Time PCR Master Mix (BioFACT ™, Daejeon Republic of Korea) in a total reaction mixture (20 µL). RT-qPCR was performed using a QuantGene 9600 Real Time Thermal Cycler (Bioer, Binjiang, China), with a standard two-step thermal cycling profile: initial denaturation at 95 °C for 5 min, denaturation at 95 °C for 10 s, and annealing and extension at 60 °C for 20 s, with 40 cycles of repeated denaturation and annealing. Relative expression levels were calculated using cycle threshold (Ct) values.

Table 1. Primers used for the qPCR and plasmid construction.

	Primer Sequence (5′ to 3′)
Primer 1	CAAACAGCAGGACCTTGG
Primer 2	ATCCATCCTCACCTCTGT
Primer 3	GGTACCCTCGAGATGGACTACAAAGACGATGACGACAAGGCAGCAATCCTGGCATAC
Primer 4	GGAATTCCAGATCCTCTTCTGAGATGAGTTTTTGTTCACGCGTTCCTTTCTTGAACCAGTT
Primer 5	GGAATTCAGCAGCGGCACGACCGCGTCCACCTCGCA
Primer 6	CGTCTAGATTAGCTTTCATTATCACTGTCTC

2.4. Plasmids Construction

To generate rAds for DENV2 virus envelope (E) and envelope ferritin heavy chain genes (E-H), the RAPAd® CMV Adenoviral expression system (Cell Biolabs, San Diego, CA, USA) was used, which includes two vectors: pacAd5 CMV K-N pA (shuttle vector) and pacAd5 9.2–100 (packaging vector). Each E and E-H gene was cloned into the multi-cloning sites of the shuttle vector. Initially, 93 nucleotides (31 amino acids) from the C-terminus of the prM gene and 1185 nucleotides (395 amino acids) from the N-terminus of the envelope gene were amplified from the prepared cDNA using Primer 3 and Primer 4 (Table 1) from the prepared cDNA. An amplified fragment was inserted in between enzyme sites; *Xho*I and *Eco*RI in pacAd5 CMV K-N pA vector and the generated plasmids were named pAd E. To construct of envelope ferritin plasmid, the ferritin heavy chain gene which was amplified from cDNA isolated from 293T cells using Primer 5 protruding a linker (SSG) at the 5′ end, and Primer 6 (Table 1) was cloned in between *Eco*RI and *Xba*I enzyme sites downstream of prM 31 envelope gene and obtained the pAd E-H plasmid.

2.5. Transfection of Recombinant Plasmids

The day before transfection HEK 293T cells (1×10^6 cells/well) were cultured in six-well cell culture plates. When the cells achieved 80–90% confluence, 200 µL of transfection mixture containing 3 µg of plasmid DNA and 9 µL of Polyethylenimine (PEI) was transfected into the cells in serum-free media, after which the medium was replaced with cell culture media 4 h post-transfection. The cells were incubated at 37 °C in a CO_2 incubator for 48 h and harvested for expression analysis.

2.6. Adenovirus Production, Amplification and Purification

For the generation of adenoviruses, 2.4 µg of shuttle vector containing the genes of interest, and 1.2 µg of Ad backbone vector were digested with PacI enzyme for vector

linearization. One day before transfection, 2×10^6 cells were seeded in a 60 mm-cell culture dish without antibiotics. When the cells achieved 70–80% confluence, the shuttle vector and backbone vector were co-transfected with Lipofectamine® 3000 transfection reagent (ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The next day, the medium was aspirated and 4 mL of fresh culture medium (DMEM supplemented with 10% FBS and 1% ampicillin) was added. Following incubation in a CO_2 incubator at 37 °C for 7–14 days, plaques were checked intermittently. The rAds generated from pAd E and pAd E-H were named rAd E and rAd E-H, respectively.

When appropriate cytopathic effect (CPE)/plaques appeared (50% of the cells were lifted), the cells and viral supernatant were harvested and centrifuged at 2000 rpm for 10 min. The cell pellet was suspended in the virus stock solution, and three freeze–thaw cycles were performed to release the intracellular virus, followed by centrifuging at 12,000 rpm for 10 min. Then, the supernatant was mixed with the previous supernatant, inoculated into a new 10 cm dish, and incubated in a CO_2 incubator at 37 °C for 1–2 h. Thereafter, the medium was replaced with 10 mL DMEM containing 5% FBS and 1% ampicillin and subsequently kept in an incubator for 2–3 days until all cells were detached. After repeating this inoculation procedure, 100 mL of the virus supernatant was obtained.

The virus supernatant was mixed with 50 mL of 20% PEG in a 2.5 mM NaCl solution. It was then incubated overnight in an inverter at 4 °C to precipitate the virus. The precipitated virus pellet was harvested after centrifugation at 7500 rpm for 30 min, and the supernatant was discarded. The pellet was resuspended in 2 mL 20 mM Tris-HCl (pH 8.0) and centrifuged at 12,000 rpm for 10 min to remove debris and recover the adenovirus-containing supernatant. For density gradient ultracentrifugation, CsCl solutions with densities of 1.3 g/mL and 1.4 g/mL were prepared in 20 mM Tris-HCL (pH8). Sequentially, 1.5 mL of 1.4 g/mL solution, 1 mL of 1.3 g/mL solution, and 2 mL of the recovered adenovirus supernatant were added to the ultracentrifuge tube without disturbing each layer. Finally, the mixture was centrifuged at 100,000× *g* for 20 h at 4 °C. The obtained ultra-viral band was retrieved and inserted into a dialysis bag (molecular weight cutoff of 10,000) and dialyzed against 10 mM Tris-HCl (pH 8.0) overnight. Viruses were filtered through a 0.2 µM filter, aliquoted, and stored at −80 °C. To determine the titer of rAds, a TCID50 assay was performed in 293A cells following a previously published method [26], and the TCID50 values were converted into PFU/mL.

2.7. Virus Transduction and Expression Check

One day before transduction, A549 cells (1.5×10^6 cells/well) were seeded in six-well cell culture plates, followed by transduction of purified viruses into cells at different MOIs (2.5, 25, and 250) and incubated in a CO_2 incubator at 37 °C. Cells were harvested 48 h post-transduction and protein expression was assessed by western blotting.

2.8. Western Blotting

Sample preparation and western blotting were performed according to previously reported protocol [12]. Cells were lysed using Ottimolyse I lysis buffer (Jubiotech, Daejeon, Republic of Korea), and similar amounts of protein samples prepared in the sample buffer were boiled and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were transferred to a PVDF membrane (MERCK, Darmstadt, Germany), blocked with 5% skimmed milk, and treated with anti-Myc antibody (CSB-PA000085, Cusabio, Wuhan, China) or ferritin heavy chain antibody (GT1149, GeneTex, Hsinchu, Taiwan) and an anti-rabbit secondary antibody (PA489724 Cusabio, Wuhan, China). The antibody-treated membrane was developed using a western blot enhancer and the images were analyzed using a luminograph.

2.9. Mouse Immunization

Six-week-old Female BALB/c mice were maintained at the animal facility of Chungnam National University (CNU), Republic of Korea, in accordance with the guidelines of

the Institutional Animal Care and Use Committee. Mice were divided into three groups, with five mice in each group. rAds (8×10^6 PFU/mL) were prepared in PBS without adjuvant. Mice in each group were immunized intramuscularly with 100 µL of rAd E, rAd E-H, or PBS. Mice were immunized twice at 2-week intervals followed by blood being collected by retro-orbital bleeding at 1 and 4 weeks after the second booster.

2.10. DENV2 Virus-Specific IgG ELISA

A hundred-fold diluted (10^{-1}) dengue virus in PBS was added to each well of the immunoplates (100 µL/well) and incubated at 4 °C overnight. The next day, the plate was washed with PBS two times and blocked with 3% skimmed milk in PBS (blocking buffer) for 1 h. After additional washing with PBS, the plates were incubated with diluted serum samples (100 µL/well) in the blocking buffer for 2 h. A washing buffer was prepared using 1% Triton X-100 in PBS and the plates were washed three times. The plates were then incubated with HRP-conjugated anti-mouse IgG (CSB-PA644737, Cusabio, Wuhan, China) diluted (1:10,000) in blocking buffer for 1 h. After washing three times with PBS-T, 100 µL of TMB substrate and stop solution were added sequentially. The absorbance values were recorded at 450 nm using a plate reader (PerkinElmer, Hopkinton, MA, USA).

3. Results

3.1. Plasmid Construction and Expression Confirmation

A vector map of the packaging vector used to generate the adenoviruses is shown in Figure 1a. Vector maps of the adenovirus shuttle vectors with representative gene fragments are shown in Figure 1b. The 31 amino acids from prM and the envelope gene with an N-terminal Myc tag were cloned into multiple cloning sites (MCS) of the shuttle vector, following which the pAd E plasmid was obtained (Figure 1b left). The same gene arrangement with the C-terminal linker and ferritin heavy chain gene was cloned into the MCS of the shuttle vector to obtain the pAd E-H plasmid (Figure 1b right). The expression of the respective envelope and envelope ferritin genes was confirmed in HEK 293T cells. The respective protein size band (49 kDa) for the recombinant envelope protein was detected with the Myc antibody in the western blot results, but the expression level of recombinant envelope ferritin was not so significant (Figure 1c). The expected size band (68 kDa) for the recombinant envelope ferritin gene was identified with the ferritin heavy chain antibody with significant expression (Figure 1d).

3.2. rAds Generation, Purification, and Detection

Before transfection of adenovirus-specific plasmids into 293A cells, plasmid DNA digested with the PacI enzyme was confirmed by running an agarose gel with non-digested DNA. A specific cytopathic effect of rAds was observed after 12 days of transfection with both pAd E and pAd E-H (Figure 2a). The virus replication rate was low at the first passage stage, and from the third passage, it showed significant CPE within 3 days. To confirm the presence of recombinant genes inside the virus, viral DNA was isolated, and PCR amplification was performed using the respective primers, followed by the respective DNA bands for the recombinant envelope. Envelope ferritin genes were confirmed using agarose gel electrophoresis and there were no bands in the negative control (Figure 2b). After the virus was separated from the culture supernatant, clear adenoviral bands were obtained for rAd E and rAd E-H, and unpackaged viruses were observed over the clear packaged virus band (Figure 2c). Optimal buffer conditions were provided for adenoviruses, using dialysis and filtration to eliminate contamination. A clear CPE was observed after viral titration in 293A cells (Figure 2d). The titers for rAd E and rAd E-H were calculated and they were 4.64×10^{10} TCID50/mL and 3.16×10^8 TCID50/mL respectively. After converting these TCID50 values into PFU, it was obtained 2.60×10^{10} PFU/mL and 1.77×10^8 PFU/mL respectively.

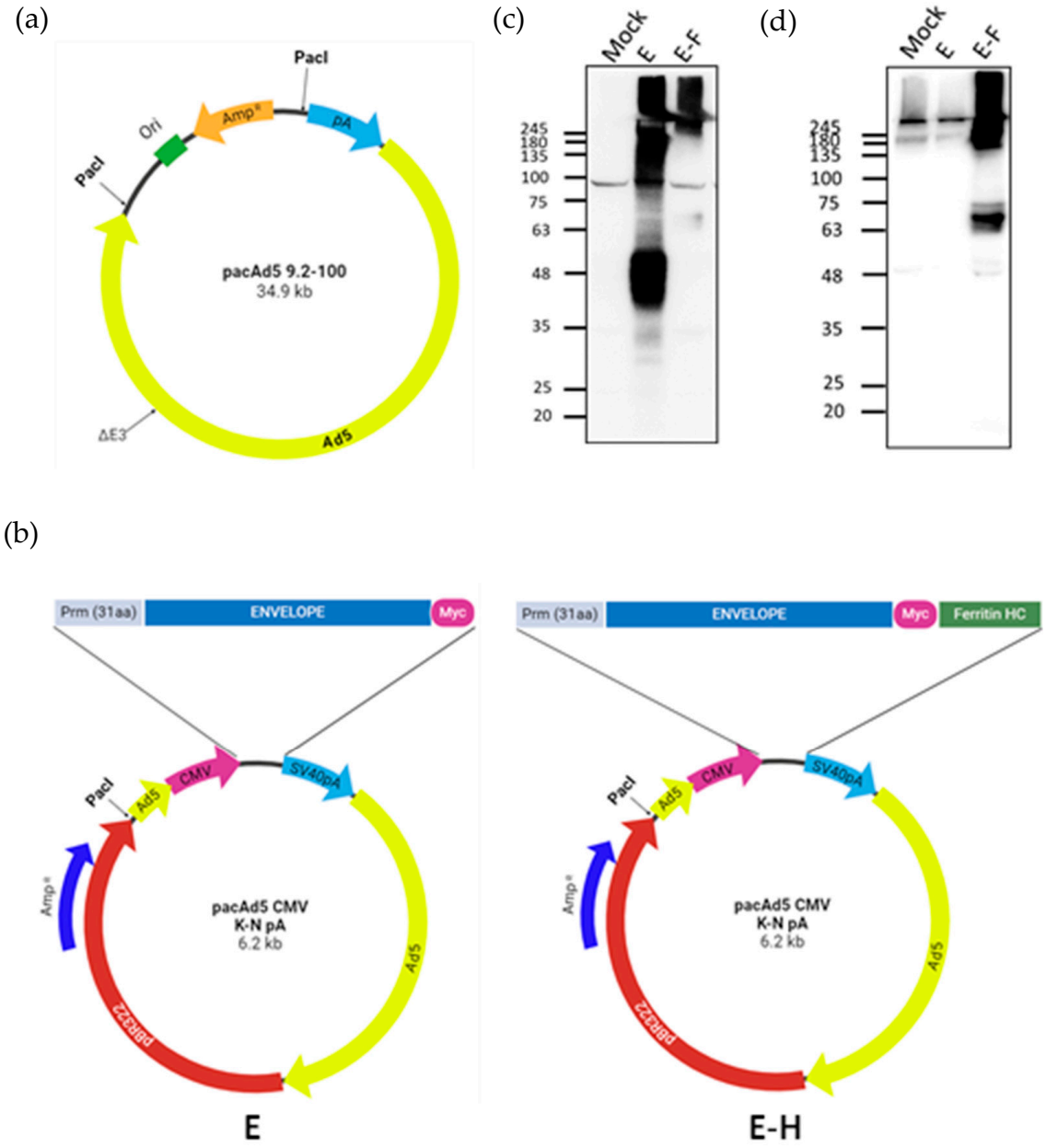

Figure 1. Plasmid construction and recombinant protein expression. (**a**) Representative vector sequence of the packaging vector. (**b**) Envelope (E) and envelope ferritin (E-H) genes were cloned into the MCS of the shuttle vector. (**c**) Envelope and envelope ferritin proteins were detected using Myc antibody. (**d**) Envelope-ferritin protein was detected using a human ferritin heavy chain antibody.

3.3. rAds Transduction Efficiency

The in vitro generation of recombinant envelope and envelope proteins was evaluated by transduction of adenovirus into A549 cells. Respectively to the transduced MOI values the expression levels of proteins were also significantly differentiated and the pattern of protein expression was also the same as the transfection expression (Figure 3). Thus, the recombinant envelope was detected at the correct level, whereas the envelope ferritin was detected very slightly using the anti-Myc antibody (Figure 3a). The expected size band of recombinant envelope ferritin was detected using the ferritin heavy chain antibody, similar to the transfection expression data (Figure 3b). Neither envelope nor envelope ferritin proteins were detected with low MOI values (2.5 and 25, respectively), whereas 250 MOI transduction induced significant expression of the two proteins (Figure 3).

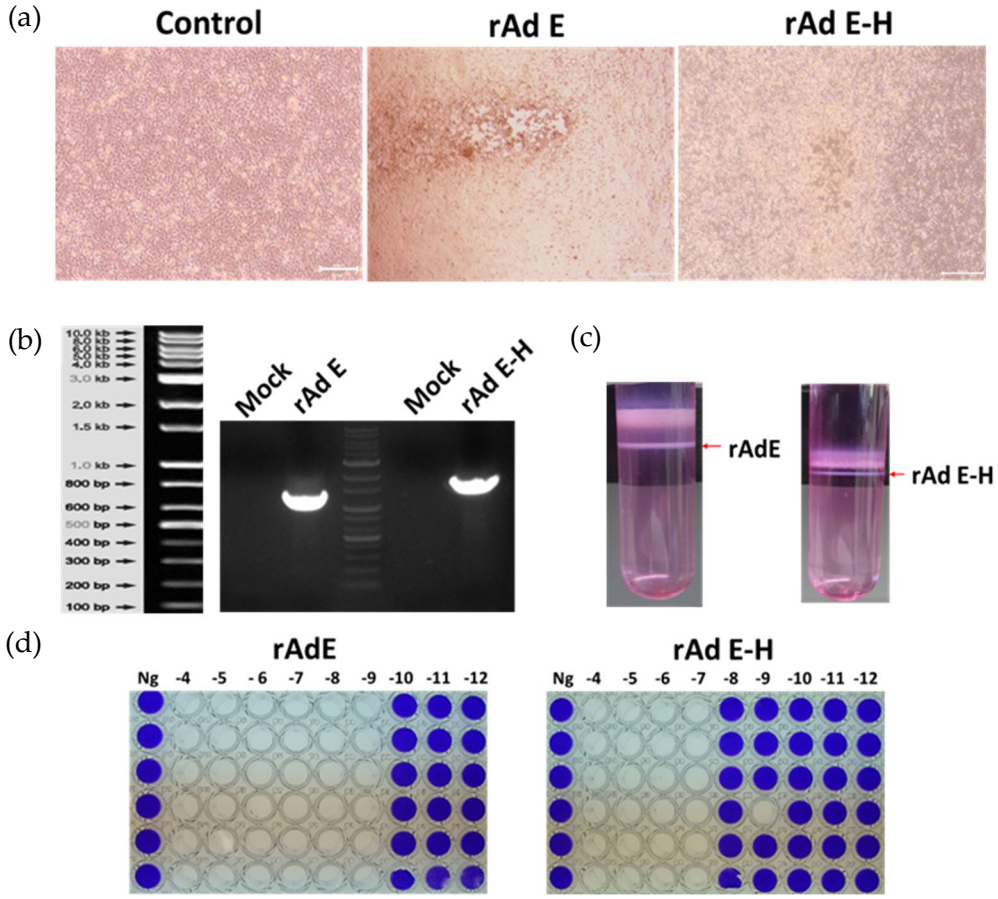

Figure 2. Generation, purification, and titration of rAds. (**a**) Adenovirus-specific CPEs were observed in 293A cells after several days of transfection compared to the vector-only transfection control. (**b**) Isolated DNA from vector only (mock), rAd E and rAd E-H culture supernatants were amplified with gene-specific primers, and envelope and envelope ferritin-specific genes were detected. (**c**) Amplified rAds were purified using the ultracentrifugation method, and clear virus bands were obtained for both viruses. (**d**) To analyze the titer of rAds, a TCID50 assay was performed in 96 well plates. Ten-fold serial dilutions were used (10^{-4} to 10^{-12}).

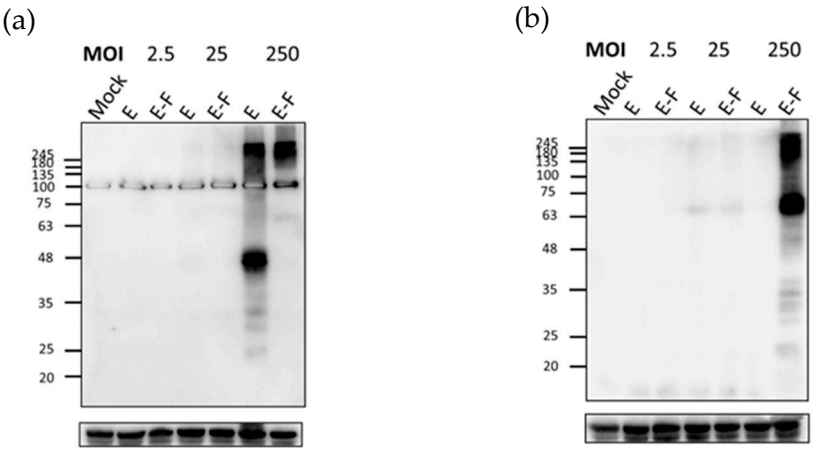

Figure 3. Confirmation of transduction efficiency in A549 cells. rAds were transduced into A549 cells at different MOI, and their respective expression was analyzed using western blotting. (**a**) Expression of rAd E was detected with Myc antibody. (**b**) Expressed rAd E-H was detected with ferritin heavy chain antibody. The lower panels in both A and B indicate actin detection as a loading control.

3.4. Dengue Virus-Specific IgG Response

Mouse immunization and serum collection schedules are shown in Figure 4a. The cultured and purified DENV2 virus strain was confirmed by qPCR analysis of the serially diluted virus samples and this virus was used as a coating antigen for virus-specific ELISA. When analyzing the results of IgG ELISA, rAd E-H induced a significant virus-specific IgG titer ($p = 0.0009$) compared to rAd E in BALB/c mice at 1 week after the final immunization (Figure 4b). Similarly, even 4 weeks after immunization, rAd E-H showed a significant IgG response ($p = 0.0003$) compared to the rAd E (Figure 4b). Furthermore, immune response of E-H immunized groups were compared at 1 week and 4 weeks after immunization. Even though the IgG titer was higher at 4 weeks post immunization, it was not significant compared to the IgG tier of 1 week post immunization (Figure 4c).

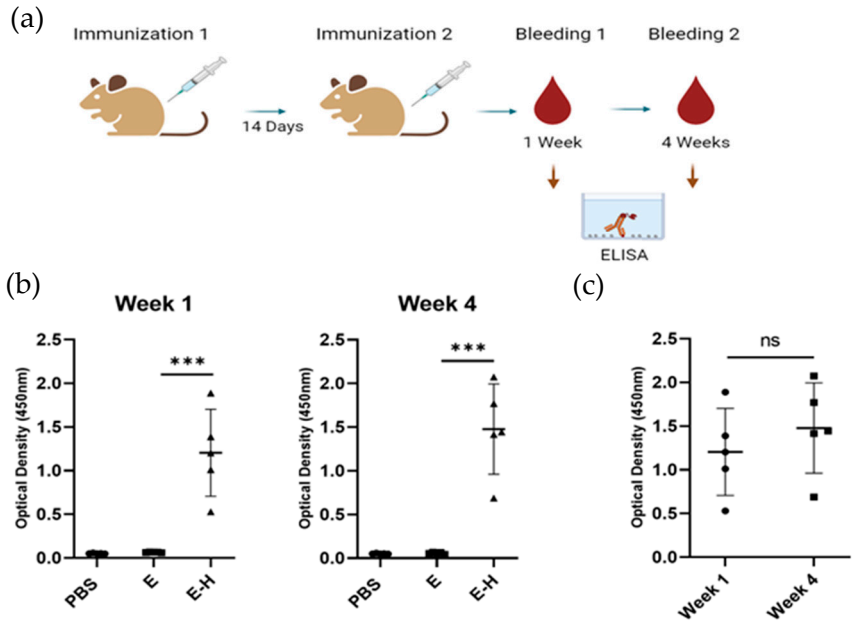

Figure 4. Mouse immunization and IgG ELISA. (**a**) Mice were immunized with 8×10^6 PFU/mL of rAds through the intramuscular route and bled as mentioned intervals (1 week and 4 weeks) in figure followed by ELISA was performed using the serum isolated from the blood. (**b**) For the DENV2 specific ELISA, DENV2 virus was coated in the plate and specific IgG response was analyzed using the mice serum obtained at 1-week and 2-week intervals (**c**) Immune responses from the E-H group at both time points were compared. ($p < 0.001$ determined by a Student's *t*-test and it was indicated as ***, $p > 0.05$ = ns).

4. Discussion

Ferritin nanoparticle vaccines are promising candidates for the enrichment of vaccine efficacy compared to single-antigen subunit vaccines. The preliminary procedure for ferritin-based vaccine development is the genetic fusion of antigenic molecules with ferritin and immunization of purified proteins. Furthermore, some studies demonstrated that ferritin nanoparticle vaccines can be generated through the delivery of mRNA encoding the antigen and ferritin [27]. However, strategies to develop ferritin vaccines using viral vector systems have not yet been established, although one study revealed that ferritin is overexpressed in an adenovirus system [28]. Our study provides preliminary data for the development of future vaccine strategies using ferritin and viral vectors.

To obtain better expression of the recombinant protein and enhance the immune response, careful selection of the antigenic molecules is important. When we cloned only the envelope protein into the adenovirus vector, the expression of the envelope protein was significantly reduced. It has been reported that a combination of 31 amino acids in the C-terminal region of the prM with the N-terminal region of the envelope protein can induce

robust expression of the dengue envelope protein in mammalian cells [29]. Furthermore, it has been reported that C-terminal truncation of envelope proteins can induce robust immunogenicity compared with full-envelope protein immunization [30]. Therefore, our envelope-only construct was groomed using the aforementioned modifications, and robust expression was demonstrated in the adenovirus system (Figure 1c). Studies have reported that peptides or molecules can be fused with human ferritin heavy chains both n-terminally and c-terminally; however, N-terminal conjugation is effective considering the stability of the ferritin cage [31]. Therefore, we linked the modified envelope protein to the N-terminal region of the human ferritin heavy chain using an immunogenic linker (SSG).

Both HEK 293T and A549 cells were used to demonstrate the transduction efficiency of the adenoviruses. Based on the medium titer of rAds, the highest MOI (250) should be transduced to induce efficient in vitro protein expression, suggesting the use of a maximum titer for immunization. To immunize rAds, we used the maximum possible viral titer that could be used for both rAds (8×10^6 PFU/mL); thus, we observed a robust immune response from rAd E-H immunization compared with rAd E immunization. We speculated that rAd E could also induce a significant immune response compared to the PBS control group if it was immunized with an enhanced virus titer. Moreover, adenovirus vaccines are better candidates for the development of long-lasting immunity [32,33]. Thus, our results also elucidated that the longevity of the rAd E-H immune response was at least one month (Figure 4).

This preliminary study elucidates the combination of two vaccine strategies (adenovirus and ferritin nanoparticles) to obtain a robust immune response. We used preliminary purification techniques for adenoviruses; however, viral titers were speculated to be enhanced using high-throughput purification strategies. Furthermore, these data can be used to develop an adenovirus-mediated ferritin tetravalent vaccine that fuses the highly immunogenic antigenic domains of the envelope proteins of all dengue virus serotypes.

Author Contributions: M.N.S.T.: conceptualization, investigation, methodology, project administration, writing—original draft preparation. J.-H.R.: conceptualization, methodology, supervision. Y.-S.J. and Y.Q. provides reagents and methodology. H.-J.S.: project administration, funding acquisition, resources, supervision, writing—review and editing. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Korea Institute of Planning and Evaluation for Technology in Food, Agriculture and Forestry (IPET) through the Animal Disease Management Technology Development Program, funded by the Ministry of Agriculture, Food and Rural Affairs (MAFRA).

Institutional Review Board Statement: The animal study protocol was approved by the Institutional animal care and use committee of Chungnam National University (202303-CNU-045).

Informed Consent Statement: Not applicable.

Data Availability Statement: The database is available upon reasonable request.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Perera, R.; Kuhn, R.J. Structural proteomics of dengue virus. *Curr. Opin. Microbiol.* **2008**, *11*, 369–377. [CrossRef] [PubMed]
2. Modis, Y.; Ogata, S.; Clements, D.; Harrison, S.C. Structure of the dengue virus envelope protein after membrane fusion. *Nature* **2004**, *427*, 313–319. [CrossRef] [PubMed]
3. Valdés, K.; Alvarez, M.; Pupo, M.; Vázquez, S.; Rodríguez, R.; Guzmán, M.G. Human Dengue Antibodies against Structural and Nonstructural Proteins. *Clin. Diagn. Lab. Immunol.* **2000**, *7*, 856–857. [CrossRef] [PubMed]
4. Alves, A.M.B.; Costa, S.M.; Pinto, P.B.A. Dengue Virus and Vaccines: How Can DNA Immunization Contribute to This Challenge? *Front. Med. Technol.* **2021**, *3*, 640964. [CrossRef]
5. Bauer, K.; Esquilin, I.O.; Cornier, A.S.; Thomas, S.J.; Del Rio, A.I.Q.; Bertran-Pasarell, J.; Ramirez, J.O.M.; Diaz, C.; Carlo, S.; Eckels, K.H.; et al. A Phase II, Randomized, Safety and Immunogenicity Trial of a Re-Derived, Live-Attenuated Dengue Virus Vaccine in Healthy Children and Adults Living in Puerto Rico. *Am. Soc. Trop. Med. Hyg.* **2015**, *93*, 441–453. [CrossRef]

6. Diaz, C.; Lin, L.; Martinez, L.J.; Eckels, K.H.; Campos, M.; Jarman, R.G.; De La Barrera, R.; Lepine, E.; Toussaint, J.F.; Febo, I.; et al. Phase I Randomized Study of a Tetravalent Dengue Purified Inactivated Vaccine in Healthy Adults from Puerto Rico. *Am. J. Trop. Med. Hyg.* **2018**, *98*, 1435–1443. [CrossRef]
7. Pintado Silva, J.; Fernandez-Sesma, A. Challenges on the development of a dengue vaccine: A comprehensive review of the state of the art. *J. Gen. Virol.* **2023**, *104*, 001831. [CrossRef]
8. Raviprakash, K.; Wang, D.; Ewing, D.; Holman, D.H.; Block, K.; Woraratanadharm, J.; Chen, L.; Hayes, C.; Dong, J.Y.; Porter, K. A Tetravalent Dengue Vaccine Based on a Complex Adenovirus Vector Provides Significant Protection in Rhesus Monkeys against All Four Serotypes of Dengue Virus. *J. Virol.* **2008**, *82*, 6927–6934. [CrossRef]
9. Sankaradoss, A.; Jagtap, S.; Nazir, J.; Moula, S.E.; Modak, A.; Fialho, J.; Iyer, M.; Shastri, J.S.; Dias, M.; Gadepalli, R.; et al. Immune profile and responses of a novel dengue DNA vaccine encoding an EDIII-NS1 consensus design based on Indo-African sequences. *Mol. Ther.* **2022**, *30*, 2058–2077. [CrossRef]
10. Akter, R.; Tasneem, F.; Das, S.; Soma, M.A.; Georgakopoulos-Soares, I.; Juthi, R.T.; Sazed, S.A. Approaches of dengue control: Vaccine strategies and future aspects. *Front. Immunol.* **2024**, *15*, 1362780. [CrossRef]
11. Travieso, T.; Li, J.; Mahesh, S.; Mello, J.D.F.R.E.; Blasi, M. The use of viral vectors in vaccine development. *NPJ Vaccines* **2022**, *7*, 75. [CrossRef] [PubMed]
12. Tu, Z.; Timashev, P.; Chen, J.; Liang, X. Ferritin-based drug delivery system for tumor therapy. *BMEMat* **2023**, *1*, e12022. [CrossRef]
13. Palombarini, F.; Masciarelli, S.; Incocciati, A.; Liccardo, F.; Di Fabio, E.; Iazzetti, A.; Fabrizi, G.; Fazi, F.; Macone, A.; Bonamore, A.; et al. Self-assembling ferritin-dendrimer nanoparticles for targeted delivery of nucleic acids to myeloid leukemia cells. *J. Nanobiotechnol.* **2021**, *19*, 172. [CrossRef] [PubMed]
14. Tang, P.; Cui, E.; Cheng, J.; Li, B.; Tao, J.; Shi, Y.; Jiao, J.; Du, E.; Wang, J.; Liu, H. A ferritin nanoparticle vaccine based on the hemagglutinin extracellular domain of swine influenza A (H1N1) virus elicits protective immune responses in mice and pigs. *Front. Immunol.* **2024**, *15*, 1361323. [CrossRef]
15. Sliepen, K.; Ozorowski, G.; Burger, J.A.; van Montfort, T.; Stunnenberg, M.; LaBranche, C.; Montefiori, D.C.; Moore, J.P.; Ward, A.B.; Sanders, R.W.; et al. Presenting native-like HIV-1 envelope trimers on ferritin nanoparticles improves their immunogenicity. *Retrovirology* **2015**, *12*, 82. [CrossRef] [PubMed]
16. Joyce, M.G.; Chen, W.H.; Sankhala, R.S.; Hajduczki, A.; Thomas, P.V.; Choe, M.; Martinez, E.J.; Chang, W.C.; Peterson, C.E.; Morrison, E.B.; et al. SARS-CoV-2 ferritin nanoparticle vaccines elicit broad SARS coronavirus immunogenicity. *Cell Rep.* **2021**, *37*, 110143. [CrossRef]
17. Sun, W.; He, L.; Zhang, H.; Tian, X.; Bai, Z.; Sun, L.; Yang, L.; Jia, X.; Bi, Y.; Luo, T.; et al. The self-assembled nanoparticle-based trimeric RBD mRNA vaccine elicits robust and durable protective immunity against SARS-CoV-2 in mice. *Signal Transduct. Target. Ther.* **2021**, *6*, 340. [CrossRef]
18. Weidenbacher, P.A.B.; Sanyal, M.; Friedland, N.; Tang, S.; Arunachalam, P.S.; Hu, M.; Kumru, O.S.; Morris, M.K.; Fontenot, J.; Shirreff, L.; et al. A ferritin-based COVID-19 nanoparticle vaccine that elicits robust, durable, broad-spectrum neutralizing antisera in non-human primates. *Nat. Commun.* **2023**, *14*, 2149. [CrossRef]
19. Hills, R.A.; Tan, T.K.; Cohen, A.A.; Keeffe, J.R.; Keeble, A.H.; Gnanapragasam, P.N.P.; Storm, K.N.; Rorick, A.V.; West, A.P.; Hill, M.L.; et al. Proactive vaccination using multiviral Quartet Nanocages to elicit broad anti-coronavirus responses. *Nat. Nanotechnol.* **2024**, *19*, 1216–1223. [CrossRef]
20. Kim, S.A.; Lee, Y.; Ko, Y.; Kim, S.; Kim, G.B.; Lee, N.K.; Ahn, W.; Kim, N.; Nam, G.H.; Lee, E.J.; et al. Protein-based nanocages for vaccine development. *J. Control. Release* **2023**, *353*, 767–791. [CrossRef]
21. López-Sagaseta, J.; Malito, E.; Rappuoli, R.; Bottomley, M.J. Self-assembling protein nanoparticles in the design of vaccines. *Comput. Struct. Biotechnol. J.* **2016**, *14*, 58–68. [CrossRef] [PubMed]
22. Vu, M.N.; Pilkington, E.H.; Lee, W.S.; Tan, H.; Davis, T.P.; Truong, N.P.; Kent, S.J.; Wheatley, A.K. Engineered Ferritin Nanoparticle Vaccines Enable Rapid Screening of Antibody Functionalization to Boost Immune Responses. *Adv. Healthc. Mater.* **2023**, *12*, 2202595. [CrossRef]
23. Rodrigues, M.Q.; Alves, P.M.; Roldão, A. Functionalizing Ferritin Nanoparticles for Vaccine Development. *Pharmaceutics* **2021**, *13*, 1621. [CrossRef]
24. Qu, Y.; Zhang, B.; Wang, Y.; Yin, S.; Sun, Y.; Middelberg, A.; Bi, J. Immunogenicity and Vaccine Efficacy Boosted by Engineering Human Heavy Chain Ferritin and Chimeric Hepatitis B Virus Core Nanoparticles. *ACS Appl. Bio Mater.* **2021**, *4*, 7147–7156. [CrossRef]
25. Neeraja, M.; Lakshmi, V.; Lavanya, V.; Priyanka, E.; Parida, M.; Dash, P.; Sharma, S.; Rao, P.L.; Reddy, G. Rapid detection and differentiation of dengue virus serotypes by NS1 specific reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay in patients presenting to a tertiary care hospital in Hyderabad, India. *J. Virol. Methods* **2015**, *211*, 22–31. [CrossRef] [PubMed]
26. Ramakrishnan, M.A. Determination of 50% endpoint titer using a simple formula. *World J. Virol.* **2016**, *5*, 85–86. [CrossRef]
27. Mu, Z.; Wiehe, K.; Saunders, K.O.; Henderson, R.; Cain, D.W.; Parks, R.; Martik, D.; Mansouri, K.; Edwards, R.J.; Newman, A.; et al. mRNA-encoded HIV-1 Env trimer ferritin nanoparticles induce monoclonal antibodies that neutralize heterologous HIV-1 isolates in mice. *Cell Rep.* **2022**, *38*, 110514. [CrossRef] [PubMed]
28. Dai, H.-Y.; He, R.; Zhang, Y.; Wu, R.-H.; Xiao, Y.-Y. Adenoviral vector mediated ferritin over-expression in mesenchymal stem cells detected by 7T MRI in vitro. *PLoS ONE* **2017**, *12*, e0185260. [CrossRef]

29. Jaiswal, S.; Khanna, N.; Swaminathan, S. Replication-defective adenoviral vaccine vector for the induction of immune responses to dengue virus type 2. *J. Virol.* **2003**, *77*, 12907–12913. [CrossRef]
30. Men, R.H.; Bray, M.; Lai, C.J. Carboxy-terminally truncated dengue virus envelope glycoproteins expressed on the cell surface and secreted extracellularly exhibit increased immunogenicity in mice. *J. Virol.* **1991**, *65*, 1400–1407. [CrossRef]
31. Qu, Y.; Wang, L.; Yin, S.; Zhang, B.; Jiao, Y.; Sun, Y.; Middelberg, A.; Bi, J. Stability of Engineered Ferritin Nanovaccines Investigated by Combined Molecular Simulation and Experiments. *J. Phys. Chem. B* **2021**, *125*, 3830–3842. [CrossRef] [PubMed]
32. Sayedahmed, E.E.; Kumari, R.; Shukla, S.; Hassan, A.O.; Mohammed, S.I.; York, I.A.; Gangappa, S.; Sambhara, S.; Mittal, S.K. Longevity of adenovirus vector immunity in mice and its implications for vaccine efficacy. *Vaccine* **2018**, *36*, 6744–6751. [CrossRef] [PubMed]
33. Shoushtari, M.; Roohvand, F.; Salehi-Vaziri, M.; Arashkia, A.; Bakhshi, H.; Azadmanesh, K. Adenovirus vector-based vaccines as forefront approaches in fighting the battle against flaviviruses. *Hum. Vaccines Immunother.* **2022**, *18*, 2079323. [CrossRef] [PubMed]