Heliyon 7 (2021) e08161

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Development of a protective inactivated vaccine against Crimean–Congo hemorrhagic fever infection

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ARTICLE INFO

Keywords:
Crimean–Congo hemorrhagic fever virus
Bunyavirus
Hemorrhagic fever
Inactivated vaccine
Protective response
Long-term immunity
Humoral immune response

ABSTRACT

Crimean–Congo hemorrhagic fever (CCHF) is an emerging zoonotic infectious disease caused by Crimean–Congo hemorrhagic fever virus (CCHFV). The first clinical CCHF infection was described in 1944 in the Crimean Peninsula, exclusively in humans, with case-fatality rates exceeding 30%. The increasing number of cases, high mortality rate, and lack of effective therapy make CCHF a serious threat to public health and a potential bio-terrorism agent. The present study evaluated the development, immunogenicity, and immune response durations for cell-culture-derived inactivated vaccine (CCVax) formulations in comparison with those of mouse-brain-derived vaccine (MBVax) formulations. In this study, the Kelkit06 CCHF virus strain was propagated in both suckling mice and Vero E6 cells, and purified with a sucrose gradient. Formalin-inactivated vaccine candidates were formulated at various doses [low dose (LD), 5 μg; medium dose (MD), 10 μg; high dose (HD), 20 μg)] and mixed with an alum adjuvant. BALB/c mice received the same doses of the vaccine formulations three times at 3-week intervals. The humoral endpoint IgG responses were evaluated and compared for the MBVax and CCVax treatments. The duration of the presence of IgG and neutralizing antibody (Ab) titers was evaluated and compared until up to 1 year after immunization. The humoral IgG responses indicated that the CCVax and MBVax candidates enhanced the IgG endpoint titers in a dose-dependent manner, which were induced more strongly in all the CCVax groups than in the MBVax mice. The fold changes in neutralizing Ab levels were also found to be higher in the CCVax groups: between 2- and 7.6-fold after the second week of the last immunization. The neutralization titers peaked 4 months after immunization in all the vaccine-receiving groups, but these were still comparable at the end of the first year. The CCVax formulations induced higher IgG and neutralizing Ab titers at all the measured time points. In this study, we showed that cell-culture-purified and formalin-inactivated vaccine candidates induced strong and robust immunity in vaccinated mice dose-dependently, more so than mouse-brain-derived vaccines.

1. Introduction

Crimean–Congo hemorrhagic fever (CCHF) is considered an emerging infectious disease that is transmitted to humans by infected ticks or close contact with the body fluids of infected patients or animals [1]. Nosocomial infections have also been reported during hospitalization [2]. The causative agent of this disease is a member of the *Orthabunyavirus* genus within the Nairoviridae family, which was recently classified under the Bunyavirales order and has the largest group of viruses in the viral taxonomy [3, 4]. CCHF disease was first characterized by marked hemorrhage and increased fever in the Crimean Peninsula in 1944 and, subsequently, was first isolated in Congo in 1956. CCHF was initially reported with small outbreaks in an area called Kelkit Valley, which includes the cities of Tokat and Sivas in Turkey. At the time of writing, the CCHF virus has spread to broader geographical locations in Turkey. Outbreaks of the disease have stretched beyond the Kelkit Valley [5, 6]. The first epidemiological results in human sera showed 9.21% prevalence from west coast of Turkey; these results were published several

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https://doi.org/10.1016/j.heliyon.2021.e08161
Received 3 April 2021; Received in revised form 9 August 2021; Accepted 8 October 2021

decades before the first clinical cases were reported [7]. No reports or clinical cases that showed evidence of the virus' presence in Turkey were reported until the 2000s. The first CCHF-associated clinical cases appeared in Turkey in early 2002, and outbreaks have been reported annually since then [1, 3]. Currently, outbreaks are seasonally reported in a sporadic manner in more than 50 countries throughout the Mediterranean, Middle East, Central Asia, and southern European countries, and various case-fatality rates have been estimated, between 5 and 30%, in regions with an estimated mean annual case number of 432 [3, 8, 9, 10].

The vector ticks, mainly the *Hyalomma* species, are reservoirs of the virus, and tick-bite-related infections are very common where CCHF is endemic [11, 12]. As the CCHFV infection of animals is seemingly asymptomatic, people who have close contact with domesticated or wild animals due to occupational necessity such as farmers, slaughterhouse workers, and veterinarians may unsuspectingly become infected with CCHFV during animal care and handling [13]. Regarding the clinical stages and pathogenesis of CCHF, the progress of the disease is well described and documented [3, 14]. CCHF begins with a sudden-onset fever, headache, and muscular pain and has a relatively short incubation period that ranges from 3 to 7 days, followed by a pre-hemorrhagic stage. The disease rapidly progresses into hemorrhagic manifestations that can result in convalescence or death, depending on the host factors and immune responses [14, 15]. Fatalities occur in the hemorrhagic stage, even if there is no replicating virus present, and patients demonstrate heavy blood leakage from the blood vessels, coagulative abnormalities, and multi-organ failure and insufficiency according to autopsy findings [16].

The treatment options are limited and include supportive therapy options, such as serum and platelet transfusions. An antiviral therapy, ribavirin, has also shown success in hospitalized patients during the early stage of clinical illness [17, 18, 19]. The humoral immune response occurs 7–9 days after the onset of infection, and people die with low or unmeasurable IgG Ab responses, which are believed to play a role in protection [20, 21]. Survivors develop long-term IgG responses that can remain detectable even 3–5 years after the infection [1, 22, 23].

Preventive measurements are necessary due to the lack of available adequate medical treatment. Therefore, a safe and protective vaccine is needed to prevent disease and control the spread of the virus among the public.

A preventive vaccine against CCHFV is available from the National Center for Infectious and Parasitic Diseases (BulBio-NCIPD Ltd.) in Bulgaria, which is produced from mouse brains and inactivated using chloroform. In the past, this vaccine was mainly distributed to soldiers employed in rural districts [24, 25]. However, the inactivated mouse-brain-derived vaccine has not been experimentally shown to be protective against virus challenge in mouse studies, and there is an absence of controlled human studies and laboratory assessments of the efficacy of this vaccine. In addition, the production of vaccines from mouse brains also poses the risk of autoimmune diseases developing in humans, such as autoimmune encephalitis [26, 27]; therefore, mouse-brain-derived vaccines are not allowed in many countries. Because of these highlighted concerns and the lack of a safe vaccine for human use, safe and more effective vaccines are needed for controlling CCHF in human populations.

The aim of this study was to develop an inactivated CCVax candidate against CCHF. The efficacy of this vaccine was compared with that of MBVax in BALB/c animals. Serum IgG and virus-neutralizing Ab were compared using ELISA and permissive cell culture systems, respectively. In this study, we describe formalin-inactivated and alum-formulated CCHFV vaccines for human use to prevent the development of mortality and morbidity caused by virus infection. Various vaccine formulations (LD, 5 μg; MD, 10 μg; HD, 20 μg) were tested on 4–6-week-old BALB/c mice. The doses were delivered via the intraperitoneal (IP) route three times at 3-week intervals, and serum samples were tested for up to 1 year. In this study, we showed that the highest IgG response and robust, prolonged neutralization titers were seen in the CCVax-immunized mice compared with the MBVax treatment; in addition, the responses remained detectable for 1 year.

2. Materials and methods

This study was conducted according to guidelines of the local ethics committee of the institutional animal care board. All the animal procedures involved in this study were reviewed by the local ethics committee (HDEE/FU) (protocol number 40/07) and approved by the Institutional Board Committee of Firat University (CAR/FU protocol IP-1–13) and the Turkish Environmental Agency (TEA/Protocol 5199–3). All experiments were conducted in the BSL-3 facility located at the Virology Department of the Faculty of Veterinary Medicine, Firat University, 23119, Elazig, Turkey. The biological samples were handled according to the guidelines on biosafety for biohazard materials, and a waste management system was implemented following the protocols of *Biosafety in Microbiological and Biomedical Laboratories* (5th Edition, 2009; CDC) [27]. The present study and the inactivated vaccine platform are summarized in Figure 1.

2.1. Production of vaccine candidates

The seed virus, Turkey-Kelkit06, was obtained from a human diagnosed and hospitalized with CCHFV. The Turkey-Kelkit06 virus was registered in GenBank under the accession numbers GQ337053, GQ337054, and GQ337055 [28, 29]. To produce working and vaccine candidate antigens from the seed virus, Turkey-Kelkit06 was used to inoculated a permissive Vero E6 cell culture (ATCC, CRL-1586) at various multiplicities of infection (MOI; 1, 0.1, and 0.01). To quantify the non-cytopathic CCHF virus titer, a modified pseudo-plaque assay was performed [30]. Fully grown Vero E6 cells were inoculated in 24-well plates with serially diluted (log_{10}) virus inoculum (DMEM F-12; Sigma–Aldrich, Germany, D8900) and incubated at 37 °C for 1 h. The inoculum was replaced with 1% carboxymethyl cellulose containing DMEM F-12 (1% FBS; Sigma-Aldrich, Germany, F2442) and incubated at 37 °C for 5 days. The cells were fixed (using 10% neutral buffered formaldehyde, Sigma-Aldrich, Germany, F8775) and permeabilized (with 0.1% Triton X-100; Bio-Rad, USA, 1611047) in PBS, followed by blocking (in 5% skim milk in PBS) for 20 min each. Polyclonal mouse sera provided by Canakoğlu et al. [31] were raised against CCHFV and diluted in TBST-20 [1:1500; 1% Tween-20 (Bio-Rad, USA, 1706531)-containing Tris-buffered PBS], and the cells were incubated for 1 h at RT. The cells were incubated for another hour at RT with goat anti-mouse β-gal conjugate (1:1500 in TBST-20; Southern Biotech, USA, 1010-06) after washing the cells three times with TBST-20. The β-gal substrate reagents [50 mg/ml X-gal (Sigma-Aldrich, Germany, B4252); 83 mg/ml NBT (Sigma-Aldrich, N6876)] were prediluted in 5 mM $MgCl_2$ (Merck, Germany, S4845833 730)-containing PBS (1/300) and mixed before being added to the wells for color development. The plates were kept at 37 °C for 15–30 min. The wells were thoroughly washed with PBS after color development had been observed. The virus growth was observed under a fluorescent microscope, followed by a fluorescent focus assay, as described previously [30]. Fully grown Vero cells on 8-well chamber slides (Nunc, Lab-Tek, 154453) were inoculated with CCHFV as described above. Formalin-fixed and permeabilized cells were pre-incubated with mouse polyclonal sera [31] and treated with goat anti-mouse IgG (1:1000; Southern Biotech, USA, 1030-02) for 1 h after washing the cells. The cells were observed under a fluorescent microscope (Olympus BX50, Japan).

To produce CCVax antigens, Vero E6 cell monolayers in 175 cm^2 flasks (Corning, NY, USA, 431079) were inoculated with 0.01 MOI of Turkey-Kelkit06 CCHFV after the assessment of virus growth in MOI optimization studies. The virus was diluted in 5 ml of DMEM F-12 (w/o serum), and the cells were covered with the virus inoculum. The flasks were left at 37 °C for 60 min and hand-rotated every 15 min during

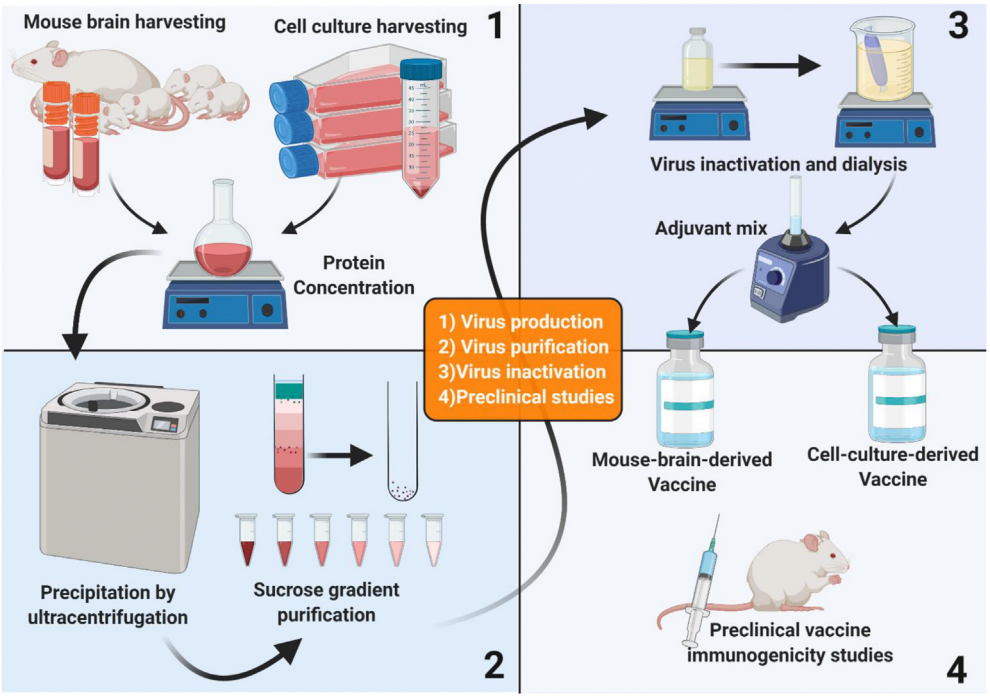

Figure 1. Study design and graphical abstract of the inactivated CCHFV vaccine platform.

incubation. The inocula were discarded, and the cells were washed twice with PBS; then, the flasks were filled with 20 ml of virus growth medium (2% FBS in DMEM F-12) containing 1% antibiotic (Pen/Strep/Amph) (Sigma-Aldrich, Germany, A5955). The flasks were left at 37 °C in a constant 5% CO_2-supplemented humidified cell-culture chamber until cells showing lysis began to detach from the bottom of the flask. To produce the MBVax, 1×10^4 PPFU of Turkey-Kelkit06 was administered to 3-day-old suckling BALB/c mice intracerebrally through the forehead. The mice were left in their home cages with their mothers. Mice were euthanized when they started to show partial limb paralysis (hind limb paralysis was seen between approx. 4 and 5 days after inoculation). The brains were harvested from the paralytic infected mice, minced into several pieces and equally (w/v) mixed with the virus growth medium. The brain samples were freeze–thawed twice, subjected to bench-top cooled centrifugation at 12,000 rpm for 30 min to remove brain debris, and stored at −80 °C in a freezer in aliquots [32, 33].

2.2. Purification of vaccine candidates

Virus suspensions were harvested from the Vero E6 cell culture by repeated freeze–thaw cycling. Crude cell contaminants were separated from the propagated viruses by cooled (4 °C) centrifugation at 2000 rpm for 30 min. Pooled supernatants were subjected to 15% PEG8000 (50% prepared from PEG800-Promega, V3011); then, 10% NaCl (23% prepared from NaCl-Germany, K92033000 546) was added to the final volume to precipitate the viruses at 4 °C overnight on a slowly agitating magnetic stirrer. Concentrated viruses were obtained through a series of centrifugation steps (the pellet was recovered after 30 min of centrifugation at 12,000 rpm; then, the supernatant was collected after 20 min of centrifugation at 5500 rpm). The resulting pellets were diluted with a Tris-EDTA-NaCl (TEN) buffer (1:10 w/v) directly after the first centrifugation, and then, the pooled supernatants were subsequently subjected to ultracentrifugation (Beckman-Coulter, Japan) at 24,000 rpm for 2 h to obtain concentrated virus pellets [34, 35]. To remove contaminating myelin-based residues from the mouse-brain-produced viruses, a protamine sulfate (Merck, Germany, K36409023 701) treatment was applied to the brain homogenates (2 mg/ml), which were then left to rest at 4 °C for 2 h. The myelin contaminants were cleared by centrifugation at 3000 rpm for 5 min. The supernatants were collected and subjected to ultracentrifugation at 24,000 rpm for 2 h, and the resulting pellets were resuspended in a TEN buffer. MBVax antigens were subjected to sucrose (Sigma-Aldrich, Germany, S7903)-gradient purification. Discontinuous sucrose-gradient separation was performed to remove cellular proteins and other contaminants to purify both the CCVax and MBVax candidates. Sucrose gradients that constituted overlays of 10%, 20%, 30%, 40%, 50%, and 60% sucrose solutions (prepared in a TEN buffer) in SW41 collection tubes were prepared 3 h before centrifugation at room temperature. The virus suspensions were loaded on top of the overlay, and high-speed centrifugation was applied at 24,000 rpm for 16 h at 4 °C. The sucrose layers were collected using a gradient collector (Frac 920, GE), starting from the bottom of the tubes, and were dispensed in 0.5 ml fractions to determine the virus density in the sucrose gradient [34]. The protein concentrations in each fraction were determined by the Lowry assay (Bio-Rad, USA, 500-0113 and 500-0114), measuring the 650 nm absorbance (Biotek, ELx 800). Virus-specific proteins were confirmed by using mouse polyclonal sera that were raised against the Turkey-Kelkit06 virus in a Western blotting assay after the separation of viral proteins with 10% SDS-PAGE [31, 34, 35]. Co-localized virus fractions were pooled and diluted with a TEN buffer (1:10 v/v). The virus antigens were concentrated by centrifugation at 24,000 rpm for 2 h, and the resulting pellets were dissolved in a TEN buffer.

2.3. Inactivation of vaccine candidates

Semi-purified viral antigens were treated with 0.05% formalin for 7 days at room temperature (RT) to inactivate the infectious viruses. The formalin was neutralized (1:400) with sodium bisulfate (3.75%; Merck, Germany, S6058956 018). Contaminating chemical residues were removed by replacing the TEN buffer with PBS during the dialysis steps. The inactivated vaccine candidates were filter sterilized using a 0.22 μm filter (Millipore, Merck, SLGVM33RS), and the virus inactivation was tested using Vero E6 cells. A pseudo-plaque assay and fluorescent microscopy analysis were performed to determine the efficiency of the virus inactivation [30].

2.4. Formulation of vaccine candidates and immunization schedules

The protein contents of the vaccine candidates were measured as described by the Lowry method [36]. The vaccine formulations were created on the basis of the protein contents of the purified and inactivated antigen bulks. Each dose of the vaccine was formulated as follows: 5 μg for LD, 10 μg for MD, and 20 μg for HD. Alum (Imject Alum, Thermo, 77161) was added (1:1 v/v) to the formulated vaccine as an adjuvant at the concentration that was suggested by the provider prior to each immunization. Four-to six-week-old BALB/c female mice were randomly assigned to vaccine and control groups, with six mice in each group. The BALB/c mice were immunized via the IP route with either cell-culture-originating vaccine formulations (CCVax-LD, CCVax-MD, and CCVax-HD) or mouse-brain-originating vaccine formulations (MBVax-LD, MBVax-MD, and MBVax-HD). The same vaccine type and formulation was used for the second and third vaccinations. Each group received the second and third doses of the same formulated vaccine at 3-week intervals. The control group received a PBS-mock vaccine that was also formulated with the alum adjuvant. Blood was collected from the submandibular veins of mice before each vaccination, and sera were obtained from the collected blood specimens for IgG and virus neutralization studies. The serum sample collection was also repeated 14, 35, and 56 days after the first immunization and continued up to 1 year at 2-month intervals after the final booster shot.

2.5. Vaccine immunogenicity and protection studies

The vaccine immunogenicity was evaluated according to the humoral immune response. The humoral IgG responses were quantified using a homemade ELISA, as described by Canakoğlu et al. [31]. Flat-bottomed 96-well plates (Nunc, M9410) were coated with 0.1 μg of the purified viral antigen in a 0.05 M carbonate-bicarbonate (Sigma-Aldrich, Germany, C3041) buffer (pH 9.6) at 4 °C overnight, and blocked with 5% skim milk in PBS for 1 h at 37 °C. Serially diluted mouse serum samples were added to the wells, and then, the plates were incubated at 37 °C for 1 h, followed by incubation with a horseradish-peroxidase-conjugated polyclonal goat anti-mouse IgG (Southern Biotech, 1010-05). The plates were washed three times between incubations with 0.05% PBST-20. The TMB chromogenic reagent (Sigma-Aldrich, Germany, T3405) substrate was reacted with the HRP-bound Abs for color development. The reaction was stopped by adding 0.2 M H_2SO_4 (Merck, Germany, 100731) to the wells after 10 min of incubation at RT in the dark. The absorbance at a wavelength of 450 nm (OD 450) was quantified using a spectrophotometer (Biotek, ELx 80). The end point of the Ab titer was determined after the normalization of the OD values to those of pre-immunized negative-control sera. To measure the vaccine-related protection, we performed a pseudo-plaque reduction neutralization by 50% (PPRNT50) assay in Vero E6 cells as described previously [37].

2.6. SDS-PAGE and immunoblotting

The viral proteins were separated on 10% resolving and 5% stacking SDS-PAGE gels in a mini-electrophoresis unit (Bio-Rad, USA) after loading 20 μg of protein into each well. For SDS-PAGE staining, the gel was directly stained with 0.05% Coomassie Brilliant Blue (Sigma-Aldrich Germany, 42660) dye at RT for 6 h, or the proteins were transferred onto a PVDF membrane (Millipore, USA, IPVH00010) in wet conditions using trans-blot apparatus (Bio-Rad, USA, 1703930) for Western immunoblotting. The stained gel was washed with a wash buffer (methanol, dH_2O, and glacial acetic acid; 4:5:1) several times, and then once more overnight, and was left in dH_2O in a glass container. The protein transferred to the membrane was blocked with 5% skimmed milk in a PBST-20 buffer for 1 h at room temperature, and the membrane was then incubated with polyclonal rabbit sera (1:3000) raised against the CCHFV antigen by the hyper-immunization method [31]. The primary-Ab-incubated membrane was immunoblotted with Ab, followed by HRP-conjugated goat anti-rabbit Ab (1:2000; Southern Biotech, 4030-05) after washing it three times with PBST-20. The immunoblotting procedure was completed at RT on a rotator shaker platform. Finally, to detect the Abs that reacted with the blotted viral antigen proteins, the membrane was treated with an ECL substrate solution (Thermo Pierce ECL, 32106) for 5 min and immediately exposed to an autoradiograph film (Sigma-Aldrich Germany, F5763), for 45 s to 5 min. The film was developed using a Kodak developer buffer (Sigma-Aldrich Germany, F5763) for 1 min, and fixation was performed using a fixing buffer (Sigma-Aldrich Germany, P7067) by soaking the film in the buffer containers. Finally, chemiluminescence-exposed film was washed several times in dH_2O and hung to dry for imaging. The entire detection procedure was carried out in a dark room without using any dim light.

2.7. Statistics

The statistical analyses were performed with the GraphPad Prism software (GraphPad Prism Version 7, San Diego, CA). Tukey's multiple-comparisons test was applied after two-way ANOVA to estimate the significance of differences in MOIs and vaccine-induced immune responses. Dunnett's multiple-comparisons test was also performed for measuring the significance of the obtained total protein yields via one-way ANOVA statistics.

3. Results

3.1. Virus purification pipeline and inactivation

The flasks of Vero E6 cells that inoculated with the virus were incubated at 37 °C for 6 days. Cellular lysis was observed at 5 days post-infection (dpi) (Figure 2A, B). The virus growth was observed by fluorescence microscope imaging after reacting the infected cultures with CCHF-virus-specific polyclonal Abs. Fluorescent focus analyses of the viruses up to 4 dpi revealed that the cellular lysis was caused by virus growth in Vero E6 cells (Figure 2C). The virus titer of Turkey-Kelkit06 was measured in a pseudo-plaque assay at 4.8×10^5 PFU/ml (Figure 2D).

The virus growth kinetics revealed that a higher virus titer was yielded by a 0.01 MOI at 48 h post-inoculation in Vero E6 cells (Figure 3A). To determine the virus harvesting time for infected cells, we measured the total protein at different time points up to 6 dpi. The highest amount of total protein was measured at 5 and 6 dpi, though there was no significant difference ($p = 0.9998$) between 5 and 6 dpi (Figure 3B). Protein analysis by SDS-PAGE and Western blotting indicated that viral products such as nucleoprotein NP (55 kDa) and glycoprotein Gn (37 kDa) were increased up to 5 dpi in Vero E6 cells (Figure 3C, D). We optimized the virus production in Vero E6 cells at 5 dpi by using a 0.01 MOI. Suckling mice started developing paralysis and neurological sign-like symptoms between 4 and 5 dpi; mice were humanely euthanized when they presented tremors and partial paralysis. The virus production and titer were confirmed with a PPFU assay.

We recorded 5–7.5 μg of purified viral antigens in each 175 cm^2 flask during virus concentration and the downstream purification steps, whereas a 50 μg protein yield from each suckling mouse brain homogenate after sucrose gradient purification was recorded (Figure 4A). There were 5- to 10-fold differences in viral antigen yield between the mouse brain and cell-culture systems. The results from this purification step also indicated that the CCVax candidates were located between fraction numbers 7 and 13 (Figure 4B, C). Unlike the cell-culture-propagated viral antigens, the MBVax CCHF viral antigens were co-localized between the 13th and 19th fractions (Figure 4B–D).

Virus inactivation in the presence of formalin was carried out at room temperature for 7 days. PPFU and FFU microscopic image analysis indicated that CCHFV was completely inactivated at 48 h of treatment (Figure 5A, B). The longevity of the inactivation was confirmed for up to 7 days to ensure complete inactivation. The viral protein integrity for both vaccine candidates was confirmed by SDS-PAGE and Western

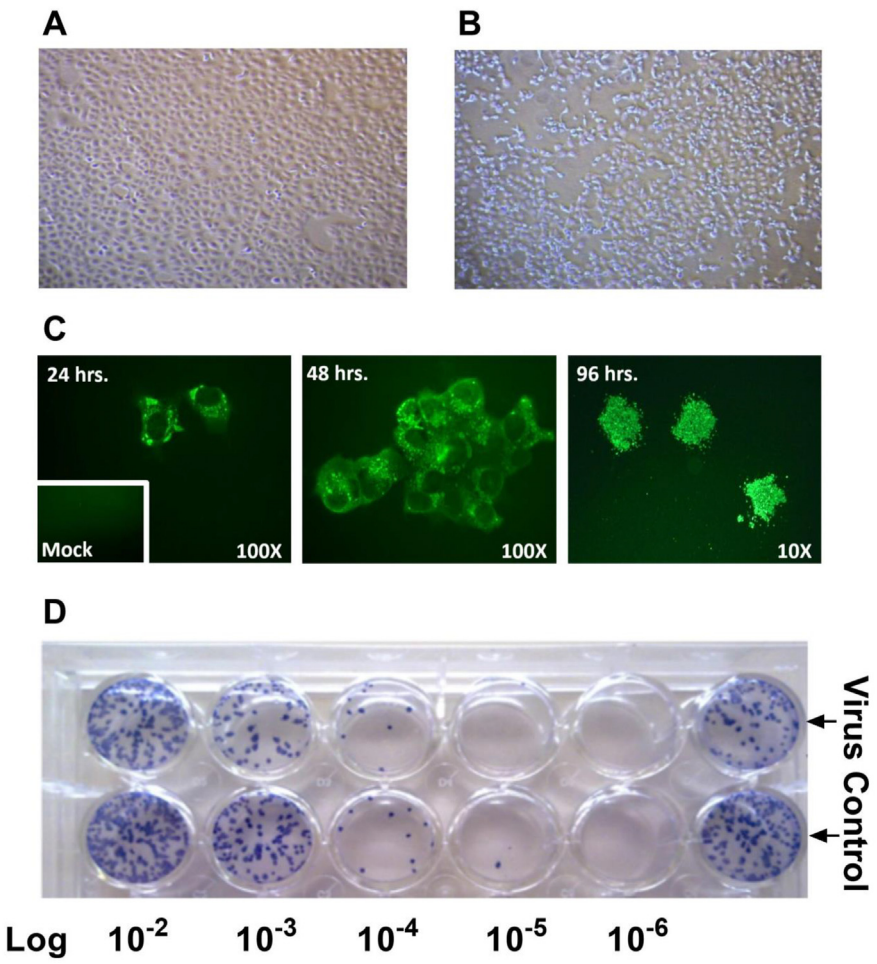

Figure 2. Characterization of Turkey-Kelkit06 virus growth in Vero E6 cells. Vero E6 cells (A) were infected with CCHFV, and the cells showed lysis and detachment from the bottom of the cell culture flasks at 5 dpi (B). Fluorescent focus analysis confirmed that virus-infected cells showed kinetic growth, and the focus sizes became larger over time (C). A 24-well plate demonstrating virus titers increasing logarithmically (Log 10) in a PPFU assay after the addition of NBT substrate (D).

blotting after inactivation and dialysis, and was compared with unpurified antigens, in consideration of the viral protein conformations (Figure 5C, D). The results showed that the inactivation processes did not alter the protein integrity or molecular weights.

3.2. Vaccine formulations and immunogenicity of vaccine candidates

To evaluate the immunogenicity of the CCVax and the MBVax vaccines, BALB/c animals received MBVax and CCVax formulations via the intraperitoneal route at three (LD, MD, and HD) concentrations, and two boosters of the same dose formulations were administered at three-week intervals (Figure 6A). The first immunization of mice with the CCVax and MBVax stimulated IgG responses but at low levels (Figure 6B, C). The levels of IgG were subsequently increased after the administration of the second and third vaccine doses in all the mice except those in the mock immunization group. The results showed that CCVax induced significantly comparable IgG responses in all the dose groups, and these were higher than those of the mice that received MBVax formulations at 2 weeks after the second immunization. We found that the IgG titer obtained from MBVax-LD (endpoint dilution: 51,200) induced a higher titer than that obtained from MBVax-HD (endpoint dilution: 40,133) at the second week after the last immunization (Day 56; Figure 6B, C). To examine the durability of the immune response to the two vaccine preparations, virus-specific IgGs were measured every 2 months for 12 months. The peak ELISA Ab titers were obtained at 4 months post-vaccination with CCVax and MBVax in the HD groups (1:102,366 and 1:49,600, respectively), and the titers were twofold higher in the CCVax group than in the MBVax group (Table 1; Figure 6D and E). The CCVax-MD and -LD groups in mice also showed the highest Ab titers at 4 months post-vaccination (1:101,400 and 1:64,200, respectively) (Table 1 and Figure 6D). The MBVax-MD and -LD groups reached the highest IgG Ab titers at 4 months post-vaccination (1:41,200 and 1:28,300, respectively) (Table 1; Figure 6E). The virus-specific Abs in the mice gradually decreased but remained detectable at the end of the first year post-vaccination in all of the vaccinated mouse groups. The results indicate that the BALB/c animals that received the CCVax candidate vaccine exhibited stronger anti-CCHFV IgG responses than those receiving the MBVax immunizations at all three concentrations after 1 year of study.

Despite the lack of a neutralization assay for CHFV, the PPRNT50 assay was used as described to measure the neutralization titer (NT). We could not detect measurable PPRNT50 titers after the first immunizations. The PPRNT50 results in the LD group did not reveal a significant difference between the MBVax and CCVax candidates after the second booster, but results were seen 2 weeks after the third immunization. Additionally, there was statistical significance when the MD and HD PPRNT50 results were compared for each vaccine candidate after the administration of the second dose (Figure 7). The highest virus titer was measured in the CCVax-HD group, and it was recorded after the third immunization. We found that the highest neutralization Ab titer was induced by the CCVax candidate ($^*p < 0.05$) compared with the MBVax treatment (Figure 7). To assess the durability of the neutralization Ab response to the two vaccine preparations, we measured the neutralizing

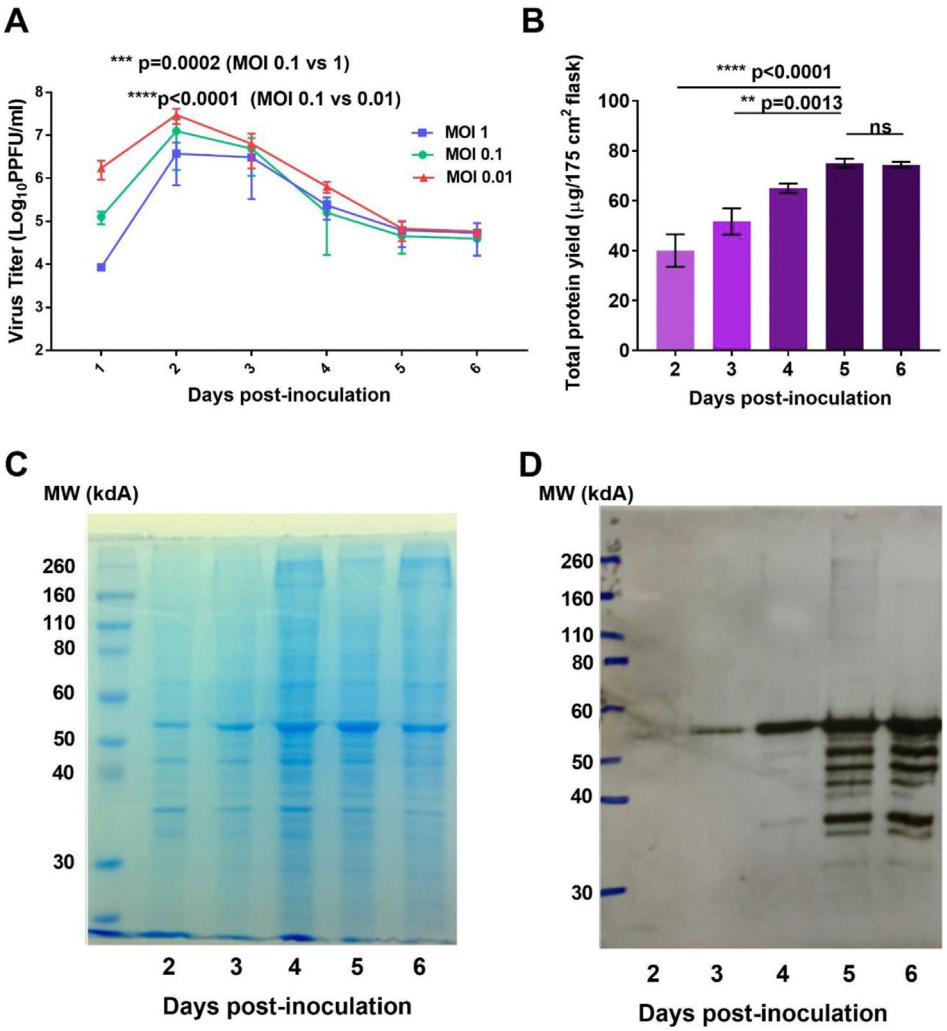

Figure 3. Optimization of Turkey-Kelkit06 viral growth and kinetics in Vero E6 cells. Cells were infected with three (1, 0.1, and 0.01) MOIs, and viral titers were measured at different time points (A). Total protein quantification by the Lowry assay, showing the daily total protein yield per infected (175 cm^2) flask (B). Viral protein analysis was performed by SDS-PAGE (C) and Western blotting (D) for CCVax candidate production in Vero E6 cells (Gn, 37 kDa; NP, 55 kDa).

Ab responses at 2-month intervals for 12 months. The neutralization Ab titers in the CCVax-LD and MBVax-LD groups (Figure 7A) reached a plateau at 4 months post-vaccination (1:80 and 1:14, respectively), similar to the IgG titers, and gradually decreased but remained detectable for up to 365 days post-vaccination (1:18 and 1:6.6, respectively). The neutralization Ab titers in the MD and HD groups in BALB/c animals also reached a plateau at 4 months post-vaccination (1:149 and 1:104, respectively) and gradually decreased but remained high for up to 365 days post-vaccination (1:45.3 and 1:24, respectively) (Table 1; Figure 7B, C). The neutralization Ab titers in the MBVax-MD dose group (Figure 7B) reached a plateau at 4 months post-vaccination (1:22), and the MBVax-HD dose group reached the highest level of neutralization Abs at 2 months post-vaccination (1:59.1) (Figure 7C). The titers gradually decreased but remained detectable at up to 365 days post-vaccination (1:16, 1:9). The longevity of the neutralization Ab response stimulated by the CCVax candidate at all the formulated doses was found to be higher ($^*p < 0.05$) than that obtained in the mice immunized with the MBVax formulations (Figure 7).

4. Discussion

In this study, the aim was to develop a safe vaccine candidate against CCHF disease due to the limited treatment options and the lack of vaccine availability for CCHF. The efficacy and immunogenicity of a novel vaccine were evaluated in BALB/c mice and compared with those of MBVax, which is produced in a similar manner to the unapproved Bulgarian CCHFV vaccine. We found that the Vero-cell-propagated and formalin-inactivated CCVax induced a robust, long-lasting humoral immune response compared to the mouse-brain-propagated and inactivated vaccine. Although an immunocompetent wild-type animal model suitable for studying virus pathogenesis is lacking, signal transducer gene-knockout mice (STAT-1^{KO}, which fail to activate interferon signaling to IFN-α/γ) and type I interferon (IFN-α/β receptor 1)-knockout mice ($IFNAR^{KO}$) have been introduced as an animal model for CCHF infection. These mouse models can present some clinical manifestations, including lethality, that are similar to those of human infections [38, 39]. Studies in STAT-1^{KO} mice that were immunized with insect-cell-based viral Gn and Gc proteins resulted in higher neutralizing Abs but failed to show protection against lethal virus challenge [40]. In a mouse-model comparison study, it was highlighted that immunocompetent mice developed more efficacious and well-balanced IgG responses than the $IFNAR^{KO}$ mouse model, suggesting that cytokine signaling might be essential for the establishment of the protective immune response [41]. Seemingly, interferon-altered transgenic mice provide an excellent model for studying CCHF infection, but the immune responses need to be investigated more widely. A recent study also suggested that vaccine development studies in immunocompromised mice may not be suitable for prognosticating vaccine efficacy in humans [42]. Accordingly, in this

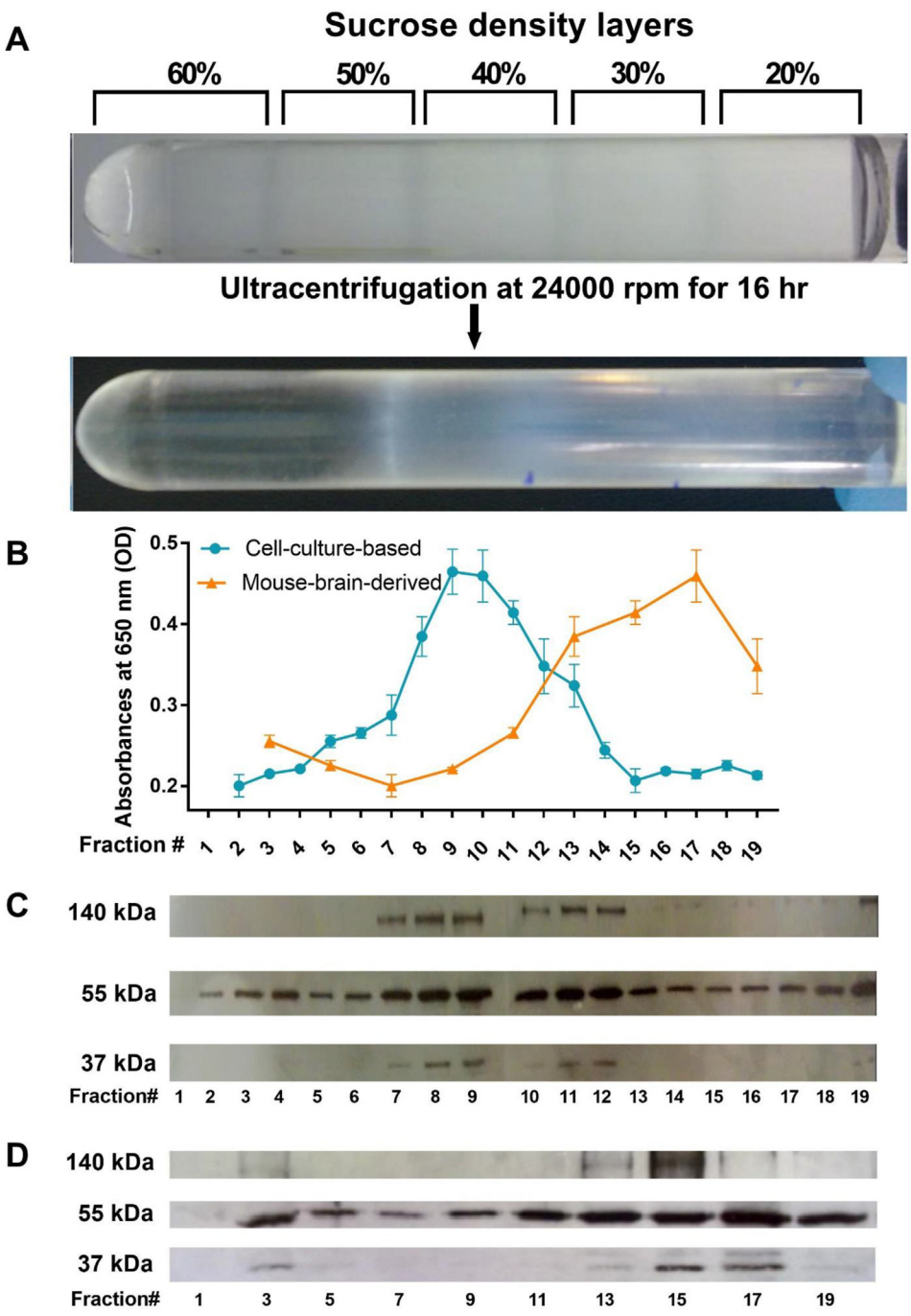

Figure 4. Separation and purification of virus particles through a discontinuous sucrose gradient ultracentrifugation. Sucrose gradient layers were overlaid on each other from 60% to 20% in SW41 ultraclear tubes, and virus suspensions were loaded on top of the gradient layers (A). Viral antigens were centrifuged through the gradient layers and fractioned to smaller volumes. Fractions were tested for protein quantification, and OD values at 650 nm absorbance were obtained (B); viral particle localizations were visualized in the cell-culture- (C) and mouse-brain-derived (D) fractions (Gn, 37kDa; NP, 55 kDa; Gc, 75 kDa; Pre-Gn, 140 kDa).

study, we aimed to investigate long-term humoral immune responses without the assessment of the protection against viral challenge in immunocompetent Balb/c animals.

A study showed that the Bulgarian mouse-brain-based CCHFV vaccine elicited a cellular and humoral response to CCHF, but the neutralizing Ab titers were low, even though the recipients received four doses of the vaccine [32]. In the present study, the CCVax resulted in a higher CCHFV-specific humoral immune response than the MBVax formulations for a 12-month period. The CCVax dose groups reached a plateau at 4 months post-vaccination, with mean neutralization Ab titers of 149.3, 104, and 80 in the HD, MD, and LD vaccine formulation groups, respectively. The mean neutralization Ab titers obtained from the MBVax formulations at 4 months post-vaccination were 42.6, 22, and 14 in the HD, MD, and LD vaccine formulation groups, respectively (Table 1). We interpreted these results as indicating that the CCVax could ensure higher protection than mouse-brain-derived vaccine formulations, and the protection was higher for both the MD and HD formulations. In terms of vaccine dose formulation, we suggest that the MD vaccine formulation is as efficient as the HD formulation of CCVax.

Several vaccine-antigen delivery systems, including transgenic plants, modified vaccinia Ankara, herpes viral vector delivery, and subunit-based vaccine candidates against CCHFV have been studied in animal models [41, 43, 44, 45, 46, 47]. It was revealed that protection against CCHFV was obtained through both the humoral and cellular immune responses in a modified vaccinia virus vaccine-based immunization study [48]. Our results showed that inactivated vaccine candidates stimulated robust IgG and neutralizing Abs in a dose-dependent manner, especially when CCVax-MD and CCVax-HD vaccines were used. However, there was

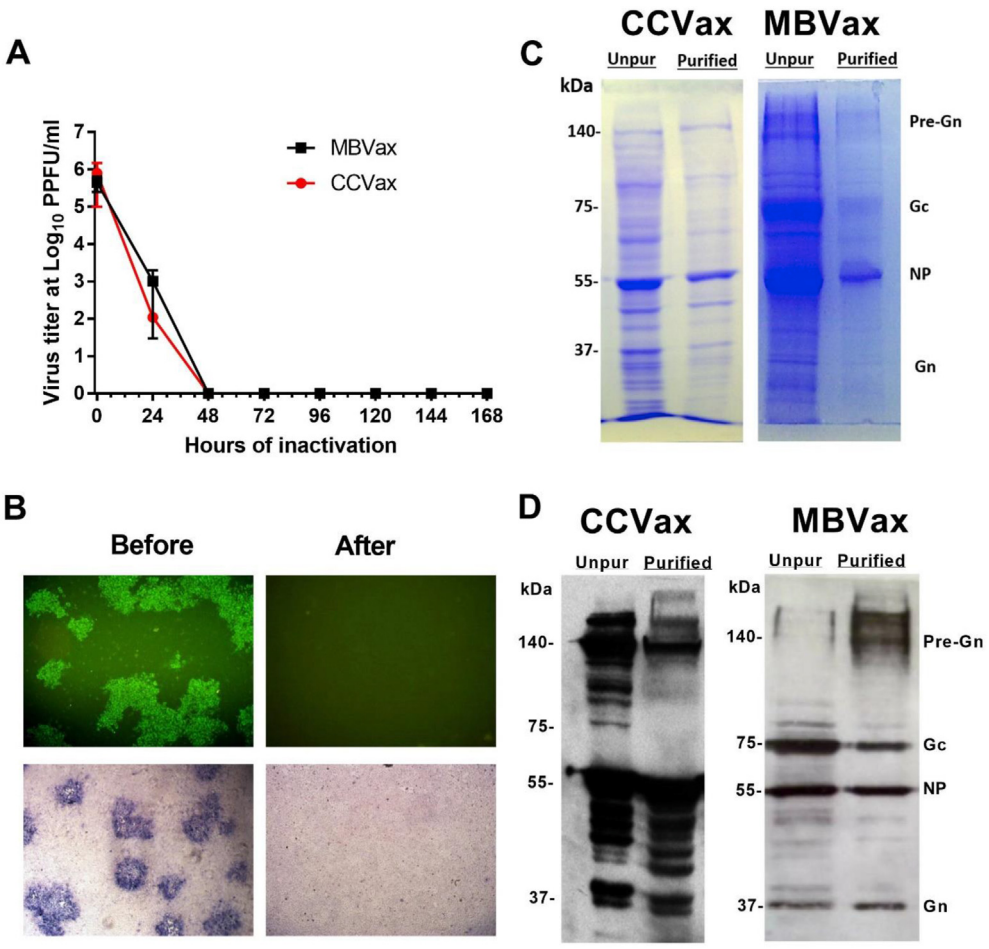

Figure 5. Inactivation of Turkey-Kelkit06 virus' infectivity by formalin (A), in Vero E6 cells, as determined by a fluorescent focus and pseudo-plaque formation assay, before and after formalin inactivation (B). SDS-PAGE (C) and Western blotting (D) analyses were performed for the CCVax and MBVax candidates to reveal protein purification and conformations (Gn, 37kDa; NP, 55 kDa; Gc, 75 kDa; Pre-Gn, 140 kDa).

no significant difference between CCVax-MD and CCVax-HD for both IgG and neutralizing Ab responses in terms of long-term durability. In a previous study, we showed that IFNAR^{KO} mice that received three doses of the inactivated vaccine showed an increased survival rate of around 80% in a viral challenge experiment compared to mock-infected mice. We suggest that the inactivated vaccine provided a higher neutralizing Ab titer, and therefore, greater protection was observed [35]. We also showed vaccine-mediated protection in immunocompromised animal models in our own group, but the cellular immune response remains unknown, and an evaluation of the vaccine candidate's long-term immune protection was lacking [35].

Within the last decade, some pre-clinical and clinical studies with other highly virulent lethal viral infections have shown that humoral Abs isolated and concentrated from survivors of natural infection were promising after administration to humans, even after the onset of the disease's symptoms [49, 50, 51, 52]. These studies imply that the role of the humoral immune responses in protection is indispensable. With respect to the importance of humoral immune responses in CCHFV cases, people receiving convalescent serum therapy upon the early onset of the illness showed increased viral clearance and lower mortality in the ICU. The success of convalescent therapy remains debatable because a placebo-controlled study is not possible for ethical reasons [53]. The extent of the relative protection against CCHF infection remains unidentified. In an experimental study in IFNAR^{KO} mice, a combination of non-neutralizing and neutralizing monoclonal Ab treatment provided modest protection, but the protection reached 60% when non-neutralizing monoclonal Ab was administered alone at a higher concentration after infection. The study results obtained by Golden et al. suggested that Abs targeting the viral glycoprotein GP38 could confer protection, regardless of whether the neutralizing Abs were induced, through unexplained mechanisms [54]. More remarkably, a study that screened broadly occurring virus-specific memory B cells taken from convalescent human survivors described complete neutralizing Abs in vitro that targeted the viral Gn/Gc complex in several CCHFV clades and virus isolates, and this broadly neutralizing bispecific Ab afforded full protection in immunocompromised mice, even when a single dose was administered. This study shows the importance of specific neutralizing Ab development in the serologic response after natural infection in humans [55]. Furthermore, a plasmid DNA vaccine encoding the CCHFV Gn, Gc, and N regions linked with ubiquitin in a state-of-the-art treatment to induce the cellular immune response produced a successful Th1-type immune response in all IFNAR^{KO} survivors. Hinkula et al. did not exclude the importance of neutralizing Abs from their study, in which a substantial quantity of neutralizing titers were also observed in surviving mice, but they suggested that the cellular immune response, especially Th1, was more likely to be involved in protection against viral challenge [56]. However, the role of the T cell immune response alone in vaccine-mediated protection is less defined and remains elusive. As a case in point, the transfer of CD3^{+} T cells in passive protection therapy failed to protect mice, but complete protection was achieved when the cells were administered with a combination of sera obtained from immunized mice using a modified vaccinia Ankara vector expressing CCHF viral glycoproteins, suggesting that both humoral and cellular immunity play unique roles in protection against CCHFV [48].

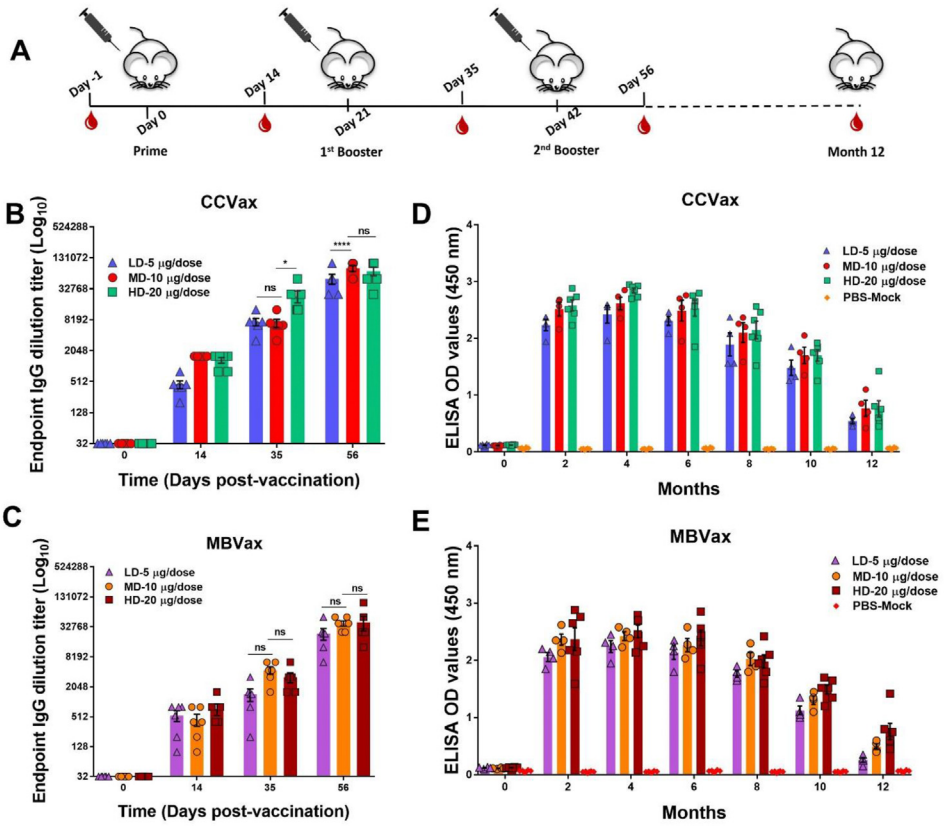

Figure 6. Vaccine schedule and vaccine-induced IgG titers in BALB/c animals. The immunization schedule was three injections at 3-week intervals, and serum samples were collected from mice before and after each immunization, and then every 2 months after the last serum samples were collected following the booster (A). Indirect ELISA measuring the Turkey-Kelkit06 virus-specific IgG responses in the sera of mice (n = 6) immunized with CCVax and MBVax formulations (B and C). Evaluation of the OD of the serum IgG titer; values were measured for 1 year for both CCVax (D) and MBVax candidate recipients (E). The error bars indicate the standard deviations. The two-way ANOVA comparison test was performed to measure the significance of each dose formulation (*p < 0.05, ****p < 0.0001, and ns: not significant).

Table 1. Evaluation of the longevity of CCVax- and MBVax-induced humoral IgG and neutralizing Ab responses in BALB/c animals.

Vaccine	Formulation	Humoral immune responses									
		4 months		6 months		8 months		10 months		12 months	
		IgG[a]	PPRNT50[b]	IgG	PPRNT50	IgG	PPRNT50	IgG	PPRNT50	IgG	PPRNT50
CCVax	HD	102.3	149.3*	53.2	96	30.8	64	15.8	58.6	7.5	45.3
	MD	101.4	104[ns]	48.2	64	25.6	44	12.8	32	6.4	24
	LD	64.2	80	32	64	19.7	48	7.5	32	3.3	18
MBVax	HD	49.6	42.6	40.3	40	22.8	29.3	12.8	20	7	16
	MD	41.2	22	38.5	14	19.5	12	7.5	10	3.2	9
	LD	28.3	14	22.4	10	12.8	9.3	6.4	8	2.1	6.6

[ns] CCVax-HD vs. CCVax-MD; not significant.
[a] The Ab titers from mice were calculated individually via ELISA, and the means are presented (n = 6).
[b] Neutralizing Ab titers (PPRNT50) were assayed individually for serum samples from mice in Vero E6 cells, and the means are presented (n = 6).
* CCVax-HD vs. CCVax-LD; p < 0.05.

Recently, a DNA vaccine candidate encoding NP along with the Gn and Gc proteins was tested in primates (*Cynomolgus macaques*) and showed improved clinical outcomes after viral challenge experiments. The results revealed that the vaccine induced efficacious virus-specific humoral IgG and virus neutralization titers, along with T cell immune responses in immunocompetent immunized primates, highlighting that DNA-vaccine-mediated protection could be conferred by antigen-specific IgG and T cell responses. Therefore, the vaccine reduced the morbidity and clinical manifestations [42]. NP-based vaccines might be efficacious, but the results can vary depending on the vaccine platform used [57, 58]. Collectively, these studies suggest that the NP, Gn, and Gc viral proteins should be intact and present in vaccine candidates to induce protective immune responses against CCHF infection both immunocompromised and immunocompetent animals or humans to induce humoral and cellular immune responses. Here, in this study, we developed a whole inactivated CCVax vaccine candidate, with a description of the vaccine production platform and the downstream purification processes, along with vaccine-induced humoral immune responses in BALB/c mice in comparison with MBVax. The vaccine regimens and schedules in the current study were similar to those of previous studies showing that repeated immunization was required to elicit IgG and neutralization titers in humans receiving the mouse-brain-derived Bulgarian CCHF vaccine [32]. Our results also suggest that at least two immunizations were required to achieve comparable peak IgG and neutralization titers in the CCvax-MD and CCvax-HD mouse groups.

There are some important limitations should be addressed and warrant further investigation. One was the lack of evaluation of the cellular immune responses, including vaccine-mediated $CD3^+$ T cells, especially the antigen-specific $CD3^+$ $CD8^+$ T cell response and the duration of memory T cell responses. We could also determine the plasma cytokine repertoire that can

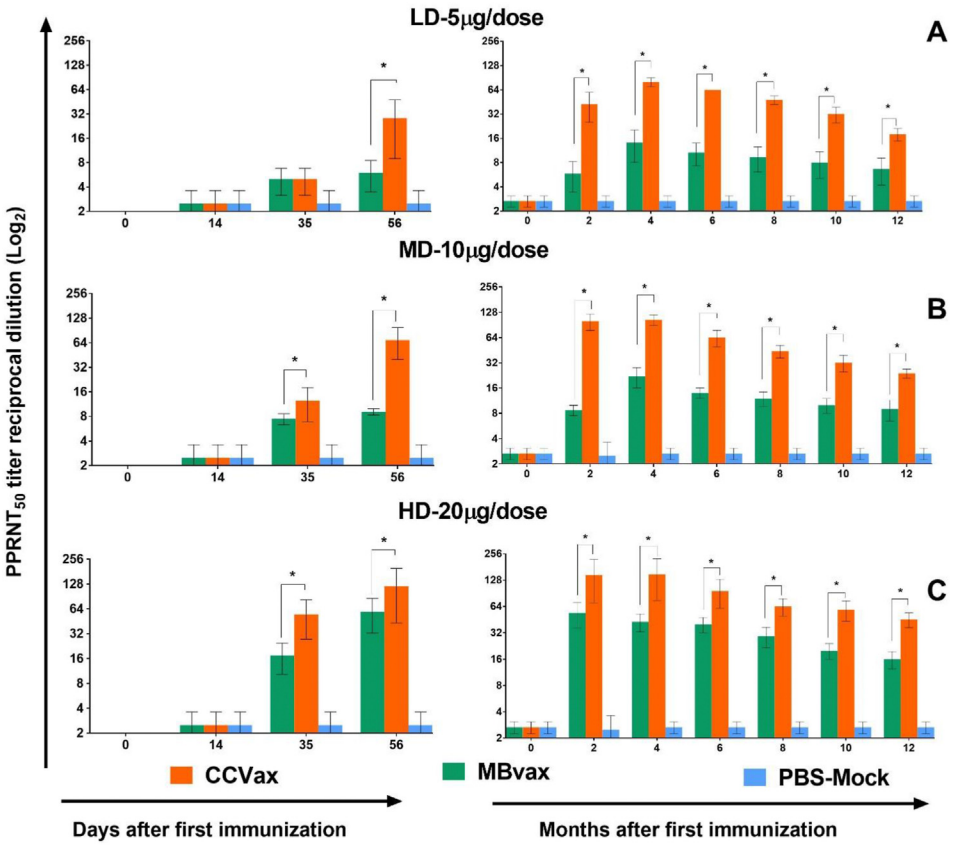

Figure 7. PPRNT50 titers were assessed in serum samples obtained after and during immunization. Vaccine formulations of CCVax and MBVax were compared, including HD (A), MD (B), and LD (C). PPRNT50 titers were calculated on the basis of the results obtained from a 50% reduction in the virus titer from before immunization in each mouse. The error bars show the standard errors of the mean. The two-way ANOVA comparison test was performed to assess the significance of each dose formulation (*$p < 0.05$).

predict the type of cellular immune response, such as Th1 or Th2. The other concerns are related to the vaccination schedule of three immunizations, which could be impractical for humans but could be achieved through booster doses after the second dose in people who are living in endemic regions. This concern may be addressed by human-phase clinical studies. A double-blind Phase I clinical study was initiated to evaluate the basic pharmacodynamics and the pharmacological and toxicological effects of the newly developed CCHF vaccine for humans, and it has been registered (NCT03020771) at Clinicaltrials.gov [59]. A patent related to CCVax production from current study, and from PhD thesis of corresponding author [34], has been licensed and protected under the European Patent Office (EPO) with the release numbers EP 2 766 038 B1 and international publication WO 2014/039021 [60]. We could also investigate whether the immunogenic characterization of the antigens produced from different platforms provides different IgG and neutralization titers, which might be related to structural and peptide differences; this needs to be explored by mass spectrophotometry or crystallography.

We have evaluated and reported CCVax's immunogenicity in the long term in comparison with that of MBVax, which is produced in a similar manner to the Bulgarian CCHFV vaccine. The results obtained in this study revealed that the formalin-inactivated CCVax against CCHF induced broad humoral immune responses both an IgG and a neutralizing titer manner in BALB/c mice. The CCVax against CCHF elicited significantly higher levels of neutralizing Ab than the mouse-brain-derived vaccine, indicating that the cell-culture-derived vaccine potentially elicits a greater protective response than mouse-brain-derived vaccine formulations. Although similar protein patterns were demonstrated for both Vero-cell-derived and mouse-brain-derived viral antigens, such differences in immune responses might be related to the various glycosylated carbohydrate-binding proteins of the viral proteins that were produced by different vaccine platforms [61]. Taking these points together, we have described a suitable inactivated CCVax CCHFV vaccine candidate that could be beneficial for use in humans.

5. Conclusions

Here, we describe an inactivated cell-culture platform for producing a vaccine candidate to protect against CCHF. The findings from this study indicate that CCVax induces a durable and prolonged humoral response in BALB/c mice, and it could be considered a safe vaccine against CCHFV for human use. The CCVax responses protective against CCHFV showed promising results, in terms of both the IgG responses and neutralization titers. Humoral immune responses could be responsible for providing protection induced by inactivated vaccines in a dose-dependent manner, but cellular immunity should also be investigated to understand vaccine-induced protection. This vaccine platform can be readily adjusted for more practical and large-scale vaccine production platforms with some modifications.

Declarations

Author contribution statement

Engin Berber: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Nurettin Çanakoğlu, Şükrü Tonbak: Performed the experiments; Analyzed and interpreted the data.

Aykut Ozdarendeli: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Funding statement

This work was supported by TUBITAK KAMAG 1007, grant number 108G126.

Data availability statement

Data included in article/supp. material/referenced in article.

Competing interest statement

The authors declare the following conflict of interests: Engin Berber, Nurettin Çanakoğlu, and Aykut Ozdarendeli; had a patent investor role in this study, and a patent was assigned to Firat University, where this study was performed. The affiliations other than Firat University did not play a role and are not involved in this patent application. The funder and affiliations had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Additional information

No additional information is available for this paper.

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