

**A DNA vaccine for Crimean-Congo hemorrhagic fever protects against disease and death
in two lethal mouse models**

Aura R. Garrison^{1¶*}, Charles J. Shoemaker^{1¶}, Collin J. Fitzpatrick¹, John J. Suschak¹, Michelle J. Richards¹, Catherine V. Badger¹, Carolyn M. Six¹, Jacqueline D. Martin¹, Drew Hannaman⁴, Marko Zivcec⁵, Eric Bergeron⁵, Jeffrey W. Koehler², Joseph W. Golden¹, and Connie S. Schmaljohn^{3*}

Running title: Crimean-Congo hemorrhagic fever DNA vaccine

¹Virology Division, ²Diagnostics Systems Division, ³Headquarters Division, United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland, USA;

⁴Ichor Medical Systems, Inc., San Diego, California 92121-3209

⁵Viral Special Pathogens Branch, National Center for Emerging and Zoonotic Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia.

*Corresponding authors

E-mail: connie.s.schmaljohn.civ@mail.mil

E-mail: aura.r.garrison.civ@mail.mil

¶Authors contributed equally

Abstract

Crimean-Congo hemorrhagic fever virus (CCHFV) is a tick-borne virus capable of causing a severe hemorrhagic fever disease in humans. There are currently no licensed vaccines to prevent CCHFV infection. We developed a DNA vaccine expressing the M-segment glycoprotein genes of CCHFV and assessed its' immunogenicity and protective efficacy in two lethal mouse models of disease: type I interferon receptor knockout (IFNAR^{-/-}) mice; and a novel transiently immune suppressed (IS) mouse model. Vaccination of mice by muscle electroporation of the M-segment DNA vaccine elicited strong antigen-specific humoral immune responses with neutralizing titers after three vaccinations in both IFNAR^{-/-} and IS mouse models. To compare the protective efficacy of the vaccine in the two models, groups of vaccinated mice (7-10 per group) were intraperitoneally (IP) challenged with a lethal dose of CCHFV strain IbAr 10200. Weight loss was markedly reduced in CCHFV DNA-vaccinated mice as compared to controls. Furthermore, whereas all vector-control vaccinated mice succumbed to disease by day 5, the DNA vaccine protected >60% of the animals from lethal disease. Mice from both models developed comparable levels of antibodies, but the IS mice had a more balanced Th1/Th2 response to vaccination. There were no statistical differences in the protective efficacies of the vaccine in the two models. Our results provide the first comparison of these two mouse models for assessing a vaccine against CCHFV and offer supportive data indicating that a DNA vaccine expressing the glycoprotein genes of CCHFV elicits protective immunity against CCHFV.

Author summary

Crimean-Congo Hemorrhagic Fever Virus (CCHFV) is a tick-borne virus capable of causing lethal human disease against which there are currently no approved vaccines. In this study, we

compared the immunogenicity and protective efficacy of a candidate DNA vaccine expressing the glycoprotein precursor gene of CCHFV in two mouse models. In addition to the recently established IFNAR^{-/-} mouse pathogenesis model, we also tested the vaccine in a novel murine system in which the interferon (IFN) α/β signaling response of immunocompetent mice is transiently suppressed. We found that the DNA vaccine elicited high humoral immune responses and provided significant protection against challenge with CCHFV in both mouse models. These findings further our understanding of the requirements for a CCHFV vaccine and provide a new mouse model for the development of CCHFV vaccines.

Introduction

Crimean-Congo hemorrhagic fever virus (CCHFV) is a tick-borne virus with a wide geographical distribution, including Africa, the Balkans, the Middle East, Russia and western Asia (1). CCHFV, a member of the *Nairoviridae* family in the *Bunyavirales* order, has a tripartite, negative-sense RNA genome comprising small (S), medium (M) and large (L) segments. The small segment encodes the nucleocapsid protein (N), the M segment encodes the glycoprotein open reading frame (ORF) that is cleaved into two structural glycoproteins (G_N and G_C) and non-structural proteins, and the L segment encodes the RNA-dependent RNA polymerase (reviewed in (2)). CCHFV infection can cause Crimean-Congo hemorrhagic fever (CCHF), a severe, often fatal, human disease characterized by hyperthermia and hemorrhage. Humans appear to be uniquely affected by CCHFV as infection in other animals, including agricultural animals, does not cause significant disease and the virus is generally cleared after a brief period of viremia (3, reviewed in (4)). Human infection can result from the bite of infected ticks, as well as from exposure to infected agricultural animals during butchering (5).

Nosocomial CCHFV infections primarily impacting medical staff have also been reported (6, 7). Between 1953 and 2010, around 6000 cases of CCHF were reported worldwide with mortality rates ranging from 5-67%, and from 2002 to 2016 more than 9700 CCHF patients were reported in Turkey alone (5, 8, 9). There is also some evidence that the range of CCHFV is expanding, as CCHFV infected ticks were found in Spain in 2010 and the first reported human infections in Southwestern Europe occurred in Spain in 2016 (10, 11). As of 2017, CCHFV has been designated as one of ten priority emerging infectious diseases by the World Health Organization. This has led to an increased awareness of the need for medical countermeasures aimed at preventing this disease.

To date, the only CCHFV vaccine tested in humans is a formalin inactivated, suckling mouse brain-derived, virus preparation formulated with an aluminum hydroxide adjuvant, which was developed in Bulgaria (12). Evaluation of this vaccine in healthy human volunteers showed that four vaccinations elicited high levels of total IgG but only low levels of neutralizing antibodies (13). Individuals vaccinated four times were also found to have T-cell responses to N that were approximately ten-fold higher than those individuals receiving a single vaccination. The historical absence of a lethal animal model of CCHF has precluded laboratory evaluation of the efficacy of this vaccine, and controlled human studies have not been reported.

Although CCHFV is apathogenic in wild-type mice, two lethal mouse models, a STAT-1 knockout mouse model and an interferon α/β receptor 1 (IFN- α/β) knockout (IFNAR^{-/-}) mouse model, have been developed that recapitulate the clinical features of CCHF in humans, including severe hepatic injury (14-16). Both of these mouse systems have been used to evaluate CCHFV vaccines. A study in the STAT-1 mouse model showed that a CCHFV subunit vaccine could elicit strong neutralizing antibodies; however, the mice were not protected from lethal disease,

indicating that the STAT-1 model, which has defects in both type I and II interferon signaling systems, is perhaps too sensitive to CCHFV for vaccine evaluation (17, 18). In contrast, experimental CCHFV vaccines have recently been reported to show protective efficacy in the IFNAR^{-/-} (A129) model (19, 20). This includes a formalin inactivated CCHFV (cell culture derived Turkey-Kelkit06) vaccine that demonstrated protective efficacy in IFNAR^{-/-} (A129) against a lethal infection with the homologous strain of CCHFV. Additionally, a modified vaccinia Ankara (MVA)-vectored vaccine expressing the CCHFV M- segment ORF (MVA-GP), from the IbAr 10200 strain, provided complete protection from lethal infection with the homologous strain of CCHFV (19, 21). Investigation of vaccine-induced immune responses with the MVA-GP vaccine suggested that both the cellular and humoral arms were critical for protective efficacy. A CCHFV DNA vaccine comprised of three separate plasmids encoding G_N, G_C, and N, tethered to a ubiquitin coding sequence, was also shown to elicit protective immunity in IFNAR^{-/-} A129 mice (22).

We previously developed a CCHFV DNA vaccine encoding the CCHFV M-segment ORF (23). We showed that this vaccine induced neutralizing antibodies in mice when delivered by gene gun, but that the response was inconsistent. Efficacy testing was not possible at the time due to the lack of a lethal animal model for CCHFV. Here, we report the improvement of this DNA vaccine by gene optimization of the full length M segment. We evaluated the immunogenicity and protective efficacy of this optimized vaccine when delivered by intramuscular electroporation (IM-EP) in two lethal CCHFV models, IFNAR^{-/-} mice and a novel transiently immune-suppressed (IS) C57BL/6 mouse CCHFV model (Golden , J.W., unpublished data). The IS mouse model exploits a monoclonal antibody (MAb-5A3) that blocks signaling via the IFNAR-1 subunit of the murine IFN α/β receptor. This transient IFN blockade has been used

in several other viral studies to examine the role of type I IFN in disease (24-26). The advantage of the transient IFN- α/β blockade model is that vaccines can be evaluated in mice with intact IFN- α/β signaling, and then during challenge IFN- α/β can be blocked to test protective efficacy. To our knowledge, this is the first direct comparison of the IFNAR^{-/-} and IS mouse model for assessing the immunogenicity and efficacy of a CCHFV vaccine.

Materials and methods

Ethics Statement

This work was supported by an approved by the USAMRIID IACUC animal research protocol. Research was conducted under an IACUC approved protocol in compliance with the Animal Welfare Act, PHS Policy, and other Federal statutes and regulations relating to animals and experiments involving animals. The facility where this research was conducted is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 2011 (27). This research was conducted at a facility that is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). Humane endpoints were used during these studies, and mice that were moribund, according to an endpoint score sheet, were humanely euthanized. Mice were euthanized by CO₂ exposure using compressed CO₂ gas followed by cervical dislocation. However, even with multiple observations per day, some animals died as a direct result of the infection.

Cells and virus

HepG2 cells were propagated in Modified Eagles Medium with Earle's Salts (MEM) (Corning) supplemented with 10% fetal bovine serum (FBS) (Gibco), and 1X Glutamax (Gibco). BHK-21 cells were cultured in Dulbecco's modified eagles medium (DMEM) (Corning) supplemented with: 10% heat-inactivated FBS, 1% Penicillin/Streptomycin (Gibco), 1% Sodium Pyruvate (Sigma), and 1% L-Glutamine (HyClone). SW13 cells were cultured in DMEM supplemented with 10% FBS, 1% Penicillin/Streptomycin, 1% HEPES (Sigma), 1% non-essential amino acids (Gibco), and 1% L-Glutamine. COS-7 cells were propagated in MEM supplemented with 10% heat-inactivated FBS, 1% Penicillin/Streptomycin, and 1% L-Glutamine. All cells were maintained at 37°C/5% CO₂. CCHFV strain IbAr10200 (USAMRIID collection) was used for all experiments. This virus was previously passaged nine times in suckling mouse brain and then propagated three times in HepG2 cells. The virus was collected from clarified cell culture supernatants and stored at -80°C. All CCHFV work was handled in BSL-4 containment.

DNA vaccine construction

The M-segment ORF of strain IbAr10200 (Accession # AAA86616) was optimized by GeneArt for human codon usage and deletion of known motifs that are detrimental to mRNA stability or expression. The optimized gene was *de novo* synthesized and cloned into pCAGGS (a generous gift from Robert Doms, University of Pennsylvania). The codon-optimized M-segment was subcloned into the mammalian expression vector pWRG7077 at the NotI sites to create the CCHFV-M DNA vaccine (23, 28). Nucleotide sequences were determined prior to vaccination.

Flow cytometry

COS-7 cells were propagated in 12-well tissue culture plates (Corning) to 70-90% confluency in MEM. The medium in these plates was replaced with an Opti-MEM (Gibco) solution containing 2% FBS and 0.1% Gentamicin (Sigma) (cOpti-MEM). The DNA plasmids were transfected into

COS-7 cells in a dilution series of 500, 250, 100, 50, or 0 ng in triplicate using Eugene 6 (Promega) according to manufacturer's directions. Transfected cells were incubated for 44 h, washed once with PBS and then detached by adding 100 μ L of trypsin-EDTA (Gibco) per well and incubating at ambient temperature. The cells were washed three times in FACS buffer solution, PBS with 2% heat-inactivated FBS and 0.1% sodium azide (Sigma), fixed in Cytofix buffer (BD Biosciences) for 30 minutes at 4°C, and then washed once with FACS buffer as above. To detect the intracellular CCHFV glycoprotein, cells were permeabilized with Perm/Wash buffer (BD Biosciences) at ambient temperature for 15 minutes, and then centrifuged for five minutes at 980 x g, at 4°C. The permeabilized and non-permeabilized cells were incubated with 20 μ g/ml of anti-CCHFV G_C mouse monoclonal antibody 11E7 (USAMRIID) in Perm/Wash buffer or PBS with 2% FBS respectively, and incubated for 30 minutes at 4°C. Cells were washed three times in Perm/Wash buffer and centrifuged for five minutes at 980 x g, at 4°C between each wash. Alexa Fluor 488-conjugated goat anti-rabbit IgG (Life Technologies) was diluted 1:200 and incubated with the cells for 20 minutes at 4°C, and then washed three times with FACS buffer. Cell pellets were re-suspended in 500 μ L FACS buffer and analyzed on a FACSCalibur flow cytometer (BD Biosciences). Cells staining positive for intracellular glycoprotein were shown as a percentage of total cells per 10,000 events. Histograms and dot plots were generated using FlowJo flow cytometry analysis software (Tree Star Inc).

Western blot

Following the aforementioned 44 h incubation post-transfection, COS-7 cells were lysed with 1X Protein Loading Buffer (LI-COR). The cell lysates were probe sonicated for 15-20 seconds each, mixed 9:1 with 2-mercaptoethanol (Sigma), and heated at 70°C for 10 min. Proteins were separated by SDS-PAGE in 10% Bis-Tris gels (Nu-PAGE) and transferred to polyvinylidene

192 difluoride membranes (Invitrogen). The membranes were blocked with Odyssey Blocking Buffer
193 in Tris-buffered Saline (TBS, LI-COR) and probed for either G_C with 4.1 µg/ml of monoclonal
194 antibody 11E7 (USAMRIID) or G_N with 1:500 rabbit polyclonal anti-CCHFV G_N sera (a
195 generous gift from Robert Doms, University of Pennsylvania) (29) prepared in TBS (Sigma)
196 supplemented with 0.2% Tween-20 (TBST, Sigma) and incubated at 4°C overnight. The
197 membranes were washed 3 times with TBST and incubated with IR680-conjugated anti-rabbit or
198 IR800-conjugated anti-mouse secondary antibodies (LI-COR) diluted in TBST at ambient
199 temperature for 1 hour. The membranes were washed an additional 3 times with TBST and
200 imaged using an Odyssey CLx imaging system (LI-COR).

201 **DNA vaccination**

202 Groups of 10 IFNAR^{-/-} or C57BL/6 mice were vaccinated in the anterior tibialis muscle with 25
203 µg of either the CCHFV-M co-DNA vaccine (IbAr10200) or the pWRG7077 empty vector using
204 the Ichor TriGrid® IM-EP system (30), under isoflurane anesthesia. All mice were vaccinated
205 three times at three weeks intervals. Blood was obtained via submandibular bleeds prior to each
206 vaccination.

207 **CCHF VLP production**

208 Production of IbAr10200 strain of CCHF VLPs (CCHF_{VLP}) was performed with slight
209 modification of methods reported previously (31). Briefly, BHK-21 cells were propagated to 70-
210 80% confluency in 10 cm² round tissue culture plates and then transfected with 10 µg pC-M Opt
211 (IbAr10200), 4 µg PC-N, 2 µg L-Opt, 4 µg T7-Opt, and 1 µg Nano-luciferase encoding mini-
212 genome plasmid using the Transit LT-1 (Mirus Bio) transfection reagent according to
213 manufacturer's instructions. Three days post-transfection, supernatants were harvested, cleared
214 of debris, and VLPs were pelleted through a cushion of 20% sucrose in virus resuspension buffer

(VRB; 130 mM NaCl, 20 mM HEPES, pH 7.4) by centrifugation for 2 h at 106,750 x g in an SW32 rotor at 4°C. VLPs were resuspended overnight in 1/200 volume VRB at 4°C, and then frozen at -80°C in single-use aliquots. Individual lots of CCHF_{VLP} were standardized by Western Blot analysis based on incorporation of N relative to a parallel gradient of recombinant N loaded on the same SDS-PAGE reducing gel. CCHF_{VLP} were also quantified using a TCID₅₀ assay on SW13 cells in 96-well, black-walled, clear-bottom plates (Corning). Plates were incubated with tenfold dilutions of the CCHF_{VLP} overnight and were then processed for Nano Luciferase (Promega) expression. Wells that displayed a Nano Luciferase signal 3 standard deviations or greater above background levels were considered positive for VLP signal. VLP stock concentrations (TCID₅₀ per mL) were calculated using the Reed and Muench formula (32).

CCHF VLP neutralization assay

One day prior to the assay, 50,000 SW13 cells were seeded into a 96 well black-walled, clear bottom tissue culture plate. All serum samples were heat inactivated at 56°C for 30 m. Half-log serial dilutions were made in duplicate from 1:25 to 1:25,368 and then an equal volume of medium with IbAr10200 VLPs containing 237 TCID₅₀ units was added and incubated at 37°C/5% CO₂ for 1 h. Final effective dilutions of analyte sera ranged from 1:50 to 1:50,736. Half of this reaction mixture (50 µl) was then added to the previously aspirated target cell plate. Cells were incubated for 24 h before being lysed using NanoGlo Lysis buffer mixed with 1/50 dilution of NanoGlo substrate (Promega). Samples were mixed and incubated for 5 min at ambient temperature prior to the luminescent signal being measured on a Modulus Microplate Reader (Turner Biosystems) with an integration time of 5 s per well. To measure the effect of complement on neutralization, Low-Tox Guinea Pig Complement (Cederlane Labs) was reconstituted in DMEM, filtered, and added to the VLP/sera mixture at a final concentration of

5% and the assay was carried out as above. Data were analyzed as previously reported using GraphPad Prism software (GraphPad Software) (33).

CCHF_{VLP} ELISA

High Bind ELISA plates (Corning) were coated overnight at 4°C with approximately 1 ng N equivalent of CCHF_{VLP} diluted in PBS per 96-well plate. The following day, plates were washed and then blocked with 3% goat serum/3% skim milk for 1 h at 37°C. All washes were done with PBS containing 0.2% Tween-20 (PBST, Sigma). Plates were washed again, prior to being loaded with two-fold serial dilutions of mouse sera in duplicate (dilution range 1:200 to 1:25,600). Serum dilutions were carried out in blocking buffer. Plates were incubated at ambient temperature for 1 h prior to being washed, and then incubated with a 1:1000 dilution of horse radish peroxidase (HRP) conjugated goat anti-mouse (SeraCare Inc.) in PBST for 1 h at ambient temperature. Plates were washed again and then developed with TMB substrate (SeraCare Inc.). Absorbance at the 450 nm wavelength was detected with a Tecan M1000 microplate reader. Pooled naïve sera collected prior to vaccination was used as an internal control on each assay group. A plate cutoff value was determined based on the average absorbance of the naïve control starting dilution plus 3 standard deviations. Only sample dilutions whose average was above this cut-off were registered as positive signal. Additional analysis was carried out using GraphPad Prism 6.

Antibody isotype analysis

Plates were coated with CCHF_{VLP} as previously described. The following day, plates were washed and blocked, and two-fold serial dilutions of mouse sera starting at 1:100 were added to the wells of replicate plates. After 1 h incubation at ambient temperature, the plates were washed, and then incubated for 1 h with a 1:10,000 dilution of either anti-mouse IgG1 HRP conjugated

antibody (Bethyl) or anti-mouse IgG2c HRP conjugated antibody (Bethyl). Plates were then washed, and TMB substrate was added and absorbance at 450 nm absorbance was recorded.

Antibody avidity

Plates were coated with CCHF_{VLP}. The following day, plates were washed, blocked and loaded with 1:200 dilutions of experimental sera for 1 h at ambient temperature. Plates were then washed before being exposed to concentrations of Sodium Thiocyanate (Sigma-Aldrich) ranging from 0-5.0 M. Samples were incubated for 15 m at ambient temperature before being washed. Secondary antibody incubation and development with TMB substrate was then performed as previously stated.

Virus challenge and MAb-5A3 treatment

C57BL/6 mice were treated by the intraperitoneal (IP) route with MAb-5A3 (Leinco Technologies Inc) 24 h prior to (2.0 mg) and 24 h after (0.5mg) CCHFV challenge. IFNAR^{-/-} and IS C57BL/6 mice were challenged with 100 pfu of CCHFV strain IbAr 10200 by the IP route four weeks following the final vaccination. The mice were monitored daily for weight change, clinical score, and survival. Twenty-eight days following challenge the surviving mice were euthanized by exsanguination under deep anesthesia, and sera were collected for post-challenge analysis.

N ELISA

N antibodies in challenged mice were detected by ELISA. Recombinant N was produced as previously reported (34) with minor modifications. Briefly, CCHFV N (strain IbAr10200) was amplified and cloned into the vector pQE-30 (Qiagen) to have an N-terminal histidine tag. This insert was transferred into the plasmid pFastbac-1 (Invitrogen) in order to generate recombinant

283 baculovirus according to manufacturer's instructions using the Bac-to-Bac Baculovirus
284 Expression System (Invitrogen). *Spodoptera frugiperda* (Sf9) insect cells were infected with the
285 recombinant baculovirus and incubated at 28°C for two days at 150 rpm. Cells were lysed (50
286 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, 1% Tween20, pH 8.0), clarified, filtered
287 through a 0.45 µm filter, and loaded by syringe onto a nickel column (HisTrap FF, GE
288 Healthcare). The column was washed (50 mM NaH₂PO₄, 300 mM NaCl, 100 mM imidazole, pH
289 8.0), and the recombinant protein eluted (50 mM NaH₂PO₄, 300 mM NaCl, 150 mM imidazole,
290 pH 8.0). The elution buffer was exchanged with PBS through three successive concentrations
291 (10k YM centrifugal device, Millipore). Antigen concentration was determined using the
292 QuantiPro BCA Kit (Sigma-Aldrich).

293 For the ELISA, clear 96-well EIA (Corning) plates were coated overnight at 4°C with
294 100 µg of the purified N per well. The plates were then blocked in PBST with 3% nonfat dry
295 milk (BD Biosciences) and 3% goat serum (Corning) for 2 h at 37°C, and washed with PBST.
296 Twenty-eight day post-challenge, the sera were heat treated for 30 min at 56°C to inactivate virus.
297 Sera were diluted in half-log dilutions in blocking buffer, starting at 1:50. The diluted sera were
298 added to the wells and the plates were incubated at 37°C for 1 h. The plates were then washed
299 with PBST, and probed for 1 h at 37°C with a 1:1000 dilution HRP-conjugated goat anti-mouse
300 antibody (Abcam), and then washed in PBST. The HRP was detected with TMB (SeraCare Inc.)
301 and the plates were read at 450 nm absorbance.

302 **Statistics**

303 Weight loss significance was determined using two-way ANOVA with the Bonferroni
304 correction. Survival statistics utilized the log-rank test. Statistical significance of CCHF_{VLP}
305 neutralization titers and ELISA data were assessed using the unpaired, two-tailed Student's *t* test

with log transformed titers. Significance levels were set at a p value less than 0.05. All analyzes were performed using GraphPad Prism v.6.

Results

Expression of a gene-optimized CCHFV-M DNA vaccine construct

In earlier studies we found that a DNA vaccine expressing the M-segment ORF of CCHFV did not consistently elicit neutralizing antibodies in vaccinated mice (23). In studies with a DNA vaccine for Venezuelan equine encephalitis virus, we found that gene optimization could lead to a dramatic improvement in expression and immunogenicity (30). Consequently, we generated a new construct in which the CCHFV M-segment gene was optimized to reflect the codon bias of humans and to remove known elements that impact mRNA stability and expression. Using flow cytometry, we showed that a monoclonal antibody to CCHFV G_C detected viral protein both on the surface of transfected non-permeabilized COS-7 cells and within permeabilized cells (Fig 1A). Expression levels were observed to be dose dependent (data not shown). Furthermore, we confirmed expression of both CCHFV glycoprotein genes by Western blot using a rabbit polyclonal antibody to detect G_N and a mouse monoclonal antibody to detect G_C (Fig 1B).

Fig 1. *In vitro* expression of the glycoprotein genes from the CCHFV-M DNA vaccine

plasmid. A. The total (permeabilized cells) and surface presence (non-permeabilized cells) of G_C was examined 44 h after transfection of COS-7 cells with CCHFV-M or empty vector, 250 ng of each plasmid. B. *In vitro* expression by Western blot of G_N (37 kDa) and G_C (75 kDa) in COS-7 cells 44 h after transfection of CCHFV-M or empty vector, 250 ng of each plasmid.

328

329 **Vaccination and challenge of IFNAR^{-/-}, and IS mice**

330 To compare the protective efficacy of the optimized CCHFV-M DNA vaccine in two lethal
331 mouse models, we vaccinated groups of 10 IFNAR^{-/-} (C57/BL6 background) mice or
332 immunocompetent C57/BL6 mice three times at 3-week intervals by IM-EP with 25 µg of either
333 the CCHFV-M vaccine or empty pWRG7077 DNA plasmid vector. The mice were bled prior to
334 each vaccination to measure immune responses. Four weeks after the third vaccination, mice
335 were challenged by IP injection with 100 pfu of CCHFV. We chose the IP route for challenge, as
336 IP challenge of IFNAR^{-/-} mice was previously found to result in a more rapid onset of disease
337 than challenge by subcutaneous, intranasal, or intramuscular routes (15); thus, the IP route
338 should provide a stringent test of the vaccine's efficacy. For the IS model, the vaccinated
339 C57/BL6 were immunosuppressed by treatment with an antibody to the IFN-α/β receptor 1 day
340 before and 1 day after challenge as described in Methods.

341 Following challenge, group weights (Fig 2A) and disease scores (not shown) were
342 obtained daily. All of the mice in both empty vector control groups displayed dramatic weight
343 loss and died or were euthanized between days 3 and 5 post-infection (Fig 2A, B). Both the
344 CCHFV-M vaccinated IFNAR^{-/-} and IS mice lost between 5%-10% of their group weights by
345 day 6, but survivors returned to their starting weights by day 7 (Fig 2B) and had no visible signs
346 of illness (lethargy, ruffling). Three mice in the CCHFV-M DNA vaccinated IFNAR^{-/-} group
347 died during manipulations three weeks following the final vaccination and prior to challenge.
348 Two out of seven (29%) CCHFV-M DNA-vaccinated IFNAR^{-/-} mice died between days 4 and 5
349 post-infection, and four out of 10 (40%) CCHFV-M vaccinated C57/BL6 mice died or were
350 euthanized on day 5 post-infection. There was no significant difference between the survival

rates of CCHFV-M-vaccinated mice in the two mouse models, although there was a significant difference in both models as compared to mice that were vaccinated with empty vector (Fig. 2B).

Fig 2. The CCHFV-M DNA vaccination provided protection in both mouse models. Weight (A) and survival (B) following CCHFV challenge with 100 pfu by the IP route, vaccinated C57BL/6 mice were transiently immunosuppressed prior to challenge (IS-B6). CCHFV-M vaccinated group in each mouse strain compared to empty vector vaccinated group in the same strain. Confidence intervals were set to 95%, *p=0.0002, **p<0.0001.

Comparison of antibody responses of IFNAR^{-/-} and immunocompetent C57BL/6 mice

Total CCHFV glycoprotein-specific antibodies were measured after each vaccination by ELISA using a CCHF_{VLP} antigen. All of the CCHFV-M vaccinated mice developed CCHFV-specific antibody responses following three vaccinations. The kinetics of the antibody responses were the same for the immune competent C57/BL6 mice and the IFNAR^{-/-} mice; i.e., both mouse strains displayed detectable antibody responses after the first vaccination, large increases after the second vaccination, and a smaller increase after the third vaccination (Fig 3A). Although significantly higher total antibody titers were measured for individual IFNAR^{-/-} mice vaccinated with CCHFV-M as compared to C57/BL6 CCHFV-M vaccinated mice, there was no correlation with ELISA titer and survival after challenge for either mouse strain (Fig 3B).

Fig 3. CCHFV specific IgG ELISA titers increase following each vaccination. The CCHFV specific ELISA titers following three vaccinations are similar in IFNAR^{-/-} and WT C57BL/6 mice before challenge. A) Mouse sera were pooled in each vaccination group and the CCHFV specific IgG antibodies were measured by CCHF_{VLP} ELISA following each vaccination with the optimized CCHFV-M vaccine. Vaccinations were performed at weeks 0, 3, and 6. B). The CCHFV IgG ELISA titers for individual mice, mice that died after CCHFV challenge are shown in red. *Confidence intervals were set to 95%.

As an indirect measure of the Th1 vs Th2 response to the CCHFV-M DNA vaccine, we performed IgG2c vs IgG1-specific ELISAs on samples collected 2 weeks after the final vaccination. Both strains of mice had higher IgG2c than IgG1 responses (Fig 4A) indicating a predominant Th1 response, which is consistent with previous trends seen in mice vaccinated by IM-EP or needle delivery (30). All of the CCHFV-M vaccinated mice developed measureable IgG2c responses and there was no significant difference between titers observed in the two mouse strains. There was a significant difference in the IgG1 response between the IFNAR^{-/-} group and the C57/BL6 group, with 42.8% (3 out of 7) of the IFNAR^{-/-} mice having detectable IgG1, and 80% (8 out of 10) of the WT C57/BL6 mice having detectable IgG1. The ratio of IgG2c to IgG1 was greater in the IFNAR^{-/-} mice than in the WT C57/BL6 (data not shown), indicating that overall the immunocompetent mice may have a more balanced response than the IFNAR^{-/-} mice. We also confirmed the ability of our CCHFV-M DNA vaccine to induce affinity matured B cell responses by avidity ELISA (Fig 4C). Estimated avidity of the CCHFV specific antibodies in the CCHFV-M vaccinated IFNAR^{-/-} group was significantly higher than the WT C57BL/6 group, however, this did not correlate to a higher survival in the IFNAR^{-/-} mice.

Fig 4. The majority of the CCHFV-M vaccinated mice developed both IgG1 and IgG2c responses following three vaccinations. Prior to challenge, CCHFV-specific antibody isotypes and the antibody avidity were examined by ELISA against the CCHF_{VLP}. A) The CCHFV specific IgG1 and IgG2c response in individual mice following three vaccinations. Pooled sera from IFNAR^{-/-} and WT C57BL/6 mice vaccinated with empty vector were tested concurrently and had no detectable signal (data not shown). B) The avidity of the CCHFV specific antibody response in vaccinated mice following three vaccinations were measured. A) and B) Mice that died after CCHFV challenge are shown in red. *Confidence intervals were set to 95%.

To assess the neutralizing antibody responses to the CCHFV-M DNA vaccine we used a CCHF_{VLP} neutralization assay as described in the Methods. All of the CCHFV-M DNA-vaccinated IFNAR^{-/-} mice and 90% of the C57/BL6 mice developed neutralizing antibodies at >1:50 dilution. Although there was no significant difference in the group titers of the two mouse strains, the IFNAR^{-/-} mice all had consistent antibody responses as compared to one another whereas there was a wide range of responses among the C57/BL6 mice. There was no significant difference in the CCHFV-specific neutralizing response between the survivors and the non-survivors in either mouse model. For both mouse strains, the addition of complement significantly increased the neutralizing antibody titers (Fig 5).

Fig 5. CCHFV-M vaccinated mice developed CCHFV-specific neutralizing antibody following three vaccinations, and the response was enhanced with the addition of complement. Prior to challenge, neutralization titers were measured against the CCHF_{VLP}, with and without complement. Mice that died following challenge are highlighted in red. *Confidence intervals were set to 95%.

Our CCHFV-M DNA vaccine was immunogenic in both mouse models, as all of the vaccinated mice developed antibody responses to CCHFV. There was no clear correlation between the humoral response to the vaccine and survival after CCHFV challenge in either IFNAR^{-/-} or WT C57/BL6 mice. Mice that developed a higher CCHFV-specific antibody response, a higher IgG1 response, higher neutralizing antibody titers, or higher antibody avidity did not have an increased chance of survival.

Seroconversion of vaccinated mice following challenge

To determine if mice that survived CCHFV challenge had overcome viral replication we assessed antibodies to CCHFV N in sera collected from mice four weeks after challenge. CCHFV N was not encoded in our vaccine construct, so the presence of anti-N antibodies would suggest viral replication. All of the IFNAR^{-/-} mice and all but one mouse of the IS C57BL/6 group had detectable antibodies to CCHFV N, indicating that the vaccine did not provide sterile immunity (Fig 6). There was no difference in the N antibody response between the IFNAR^{-/-} and the IS mice.

Fig 6. N-specific antibodies in post-challenge sera of CCHFV-M vaccinated mice. Sera from vaccinated mice that survived CCHFV challenge (28 days post-challenge) were examined by ELISA for antibodies against N.

Discussion

We generated a DNA vaccine construct encoding the M-segment ORF of CCHFV, which was optimized for expression in mammalian cells. We compared and quantified both the humoral response and protective efficacy of our optimized DNA vaccine in two murine challenge models (IFNAR^{-/-} and IS) with the same genetic background (C57BL/6). We found that the optimized CCHFV-M DNA vaccine delivered by IM-EP was highly immunogenic, with 100% of IFNAR^{-/-} vaccinated mice and 90% of C57BL/6 mice developing CCHFV-specific immune responses, including neutralizing antibodies. The single mouse in the IS model that failed to produce neutralizing antibodies did develop G_N/G_C-specific antibodies, albeit at low levels. The immunocompetent C57BL/6 mice developed a more balanced IgG2c/IgG1 response than the IFNAR^{-/-} mice, which may be due to cytokine signaling differences in the immunocompetent mice. In both mouse models, the CCHFV-specific IgG ELISA titers of vaccinated mice significantly increased between the second and third boosting vaccinations. Because we did not test additional vaccinations, we do not know if we reached the maximum response possible.

As we were preparing this manuscript, another study reported DNA vaccination of IFNAR^{-/-} mice with an A129 background using separate plasmids expressing CCHFV G_N, G_C, or N genes, each tethered to a ubiquitin coding sequence (22). In general, our findings of the humoral immune response to DNA vaccination are in agreement with those in the other DNA

vaccine report; however, numerous differences between the two studies make it difficult to directly compare results. For example, our vaccine expresses the complete M-segment ORF of CCHFV, whereas in the other study a mixture of plasmids was used, which included an N construct along with two constructs encoding the individual glycoprotein genes, from which the mucin-like domain and GP38 coding regions were deleted. Also, our vaccine does not express either the N gene or the ubiquitin sequence. The ubiquitin was intended to broaden the cell-mediated immune response, but it is difficult to determine if it did due to the small number of mice used and the limited sample volumes that precluded a comprehensive assessment of cell-mediated immunity. In addition to differences in the DNA vaccine constructs and the differing genetic backgrounds of the IFN^{-/-} mice, the studies differed in that we used a lower dose of DNA and a different delivery method (IM-EP vs intradermal EP) and compared the IFNAR^{-/-} mouse model responses to antibody responses of immunocompetent mice

In our studies, we could not identify a correlate of protective immunity in either mouse model. While we were able to elicit specific anti-CCHFV antibody responses in all vaccinated mice, post-challenge seroconversion ELISA results revealed that the vaccine was not able to prevent viral replication in the majority of the CCHFV-M DNA-vaccinated mice. These results are similar to those reported previously with a MVA-GP vaccine, and in the recent CCHFV DNA vaccine study (19, 22). Also consistent with the MVA-GP and the DNA vaccine studies, we found no direct correlation between the humoral response(s) and survival in CCHFV-M DNA-vaccinated mice (19, 21) suggesting that anti-CCHFV glycoprotein antibodies alone elicited by these vaccines are not sufficient for protection to viral challenge. This is in agreement with results of an earlier study demonstrating that the passive transfer of serum antibodies from MVA-GP vaccinated mice into a naïve host did not confer protection to CCHFV challenge (21).

MVA-GP vaccine studies further suggested, through adoptive transfer of T-cells and passive sera transfer studies, that both the cellular and humoral responses to the MVA-based CCHFV vaccine were necessary to provide protection, as determined by a statistically significant delay in time to death. Contrary to this, earlier passive transfer studies with monoclonal antibodies directed against CCHFV G_N/G_C show that individual neutralizing and non-neutralizing antibodies alone can provide 100% protection both before and after challenge in suckling mice (35). However, monoclonal antibody studies have not been reported to confirm that this protection holds true in an adult mouse model. As we have not yet assessed cell-mediated immune responses to our DNA vaccine, we cannot eliminate the necessity for inclusion of additional immunogens, such as N, to elicit T cell responses, although the immune response elicited by the MVA-GP vaccine was fully protective, whereas MVA-N vaccine was unable to protect.

We show in this study that immune competent mice can be used to evaluate CCHFV vaccines and protective efficacy can be examined by transient inhibition of IFN-I using MAB-5A3 proximal to the time of challenge. IFN- α/β signaling is critical for the generation of potent adaptive immune responses, for example by promoting antigen presenting cell maturation, driving the T cell, and subsequent B cell, response (36, 37). IFN- α/β also amplifies B cell receptor sensitivity, boosting the ability of naïve B cells to produce antibodies upon antigen recognition (38). Furthermore, IFN- α/β signaling promotes the generation of memory T and B cell pools. Although we did not observe significant differences either in antibody responses or protective immunity in the IFNAR^{-/-} vs the IS models with our CCHFV M DNA vaccine, the ability to vaccinate immune intact mice might be advantageous for other DNA vaccine approaches or for other types of CCHFV vaccines. For example, in the same study where the mixed CCHFV DNA vaccine plasmids were tested, a transcriptionally-competent CCHF VLP

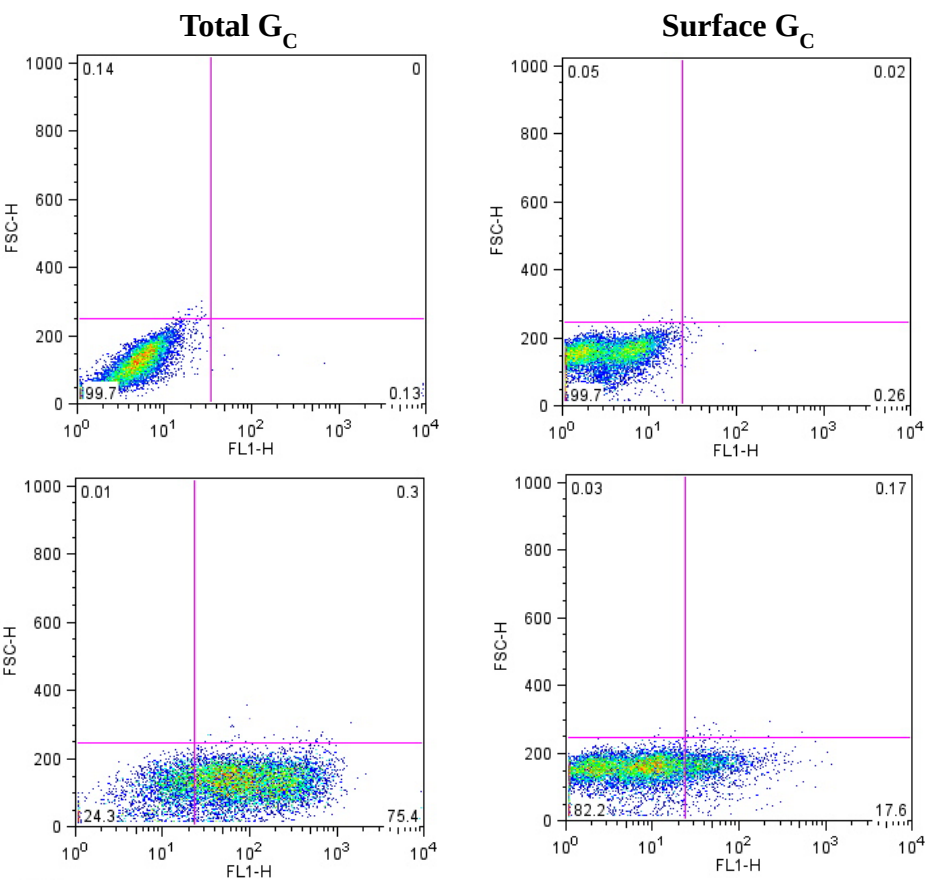
(tcVLP) vaccine was given alone or in a prime-boost regimen with the DNA. The IFNAR^{-/-} mice vaccinated with the tcVLP or the prime-boost were less protected than the DNA vaccine alone (22). The authors concluded that a type I IFN responses may be required for the development of a protective immune response against the tcVLP vaccine. Therefore, the ability to study CCHFV vaccines in immune intact mice and then testing protective efficacy by disrupting IFN signaling only at the time of challenge might have important advantages over the IFNAR^{-/-} CCHFV vaccination model, particularly when T cell responses are critical for protection (39, 40).

In summary, here we show that a novel CCHFV M-segment DNA vaccine can elicit protective immune responses to CCHFV challenge in two lethal mouse models of CCHF. The exact mechanism of protection remains unclear, but it is evident that a DNA vaccine encoding the CCHFV M-segment ORF can generate protective immunity. It remains to be seen if this vaccine can provide cross-protective immunity to more genetically distant CCHFV strains. Overall, our results provide further insight into the protective capabilities of a CCHFV DNA vaccine and will help in the development of a more rationally tailored CCHFV vaccine.

A.

Empty vector

CCHFV-M

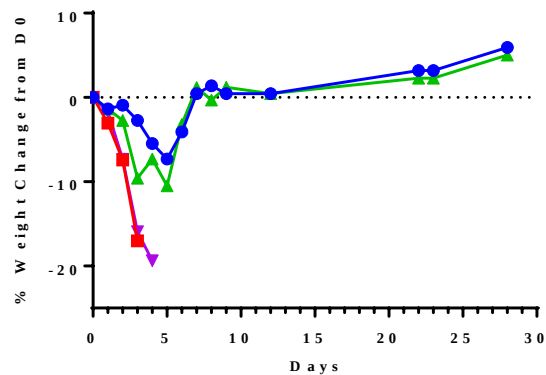


B.



Fig 1. *In vitro* expression of the glycoprotein genes from the CCHFV-M DNA vaccine plasmid. A. The total (permeabilized cells) and surface presence (non-permeabilized cells) of G_C was examined 44 h after transfection of COS-7 cells with CCHFV-M or empty vector, 250 ng of each plasmid. B. *In vitro* expression by Western blot of G_N (37 kDa) and G_C (75 kDa) in COS-7 cells 44 h after transfection of CCHFV-M or empty vector, 250 ng of each plasmid.

A.



B.

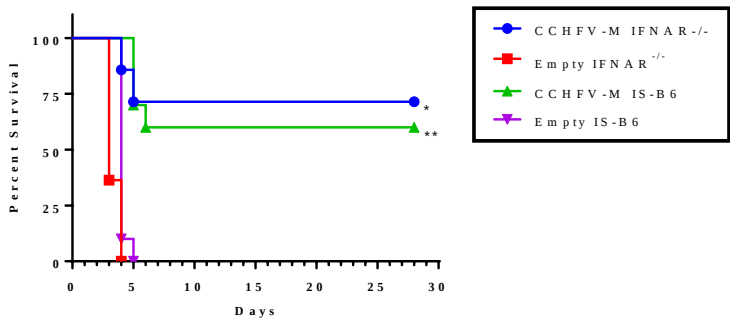
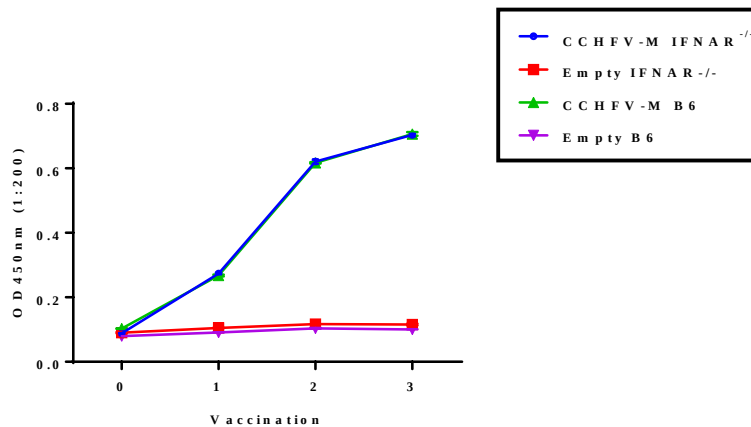


Fig 2. The CCHFV-M DNA vaccination provided protection in both mouse models. Weight (A) and survival (B) following CCHFV challenge with 100 pfu by the IP route, vaccinated C57BL/6 mice were transiently immunosuppressed prior to challenge (IS-B6). CCHFV-M vaccinated group in each mouse strain compared to empty vector vaccinated group in the same strain. Confidence intervals were set to 95%, *p=0.0002, **p<0.0001.

A.



B.

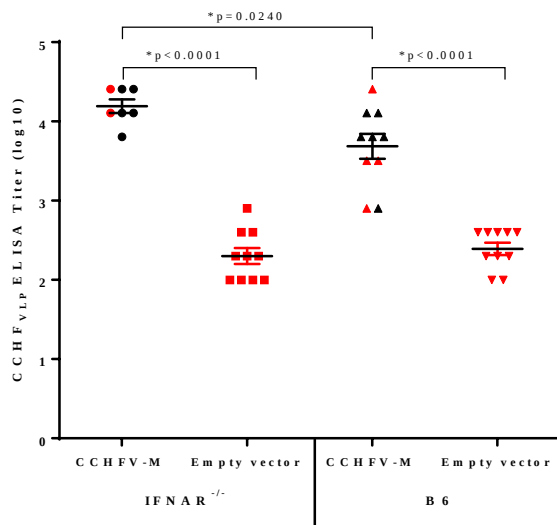
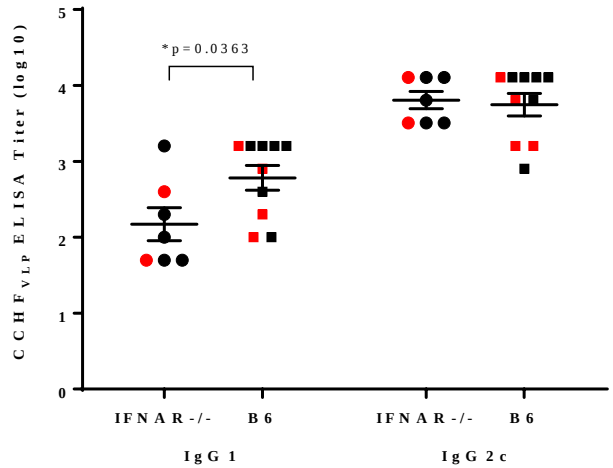


Fig 3. CCHFV specific IgG ELISA titers increase following each vaccination. The CCHFV specific ELISA titers following three vaccinations are similar in IFNAR^{-/-} and WT C57BL/6 mice before challenge. A) Mouse sera were pooled in each vaccination group and the CCHFV specific IgG antibodies were measured by CCHFV_{VLP} ELISA following each vaccination with the optimized CCHFV-M vaccine. Vaccinations were performed at weeks 0, 3, and 6. B). The CCHFV IgG ELISA titers for individual mice, mice that died after CCHFV challenge are shown in red. *Confidence intervals were set to 95%.

A.



B.

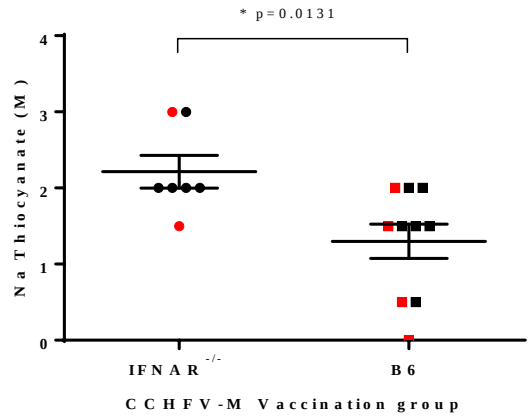


Fig 4. The majority of the CCHFV-M vaccinated mice developed both IgG1 and IgG2c responses following three vaccinations. Prior to challenge, CCHFV-specific antibody isotypes and the antibody avidity were examined by ELISA against the CCHF_{VLP}. A) The CCHFV specific IgG1 and IgG2c response in individual mice following three vaccinations. Pooled sera from IFNAR^{-/-} and WT C57BL/6 mice vaccinated with empty vector were tested concurrently and had no detectable signal (data not shown). B) The avidity of the CCHFV specific antibody response in vaccinated mice following three vaccinations were measured. A) and B) Mice that died after CCHFV challenge are shown in red. *Confidence intervals were set to 95%.

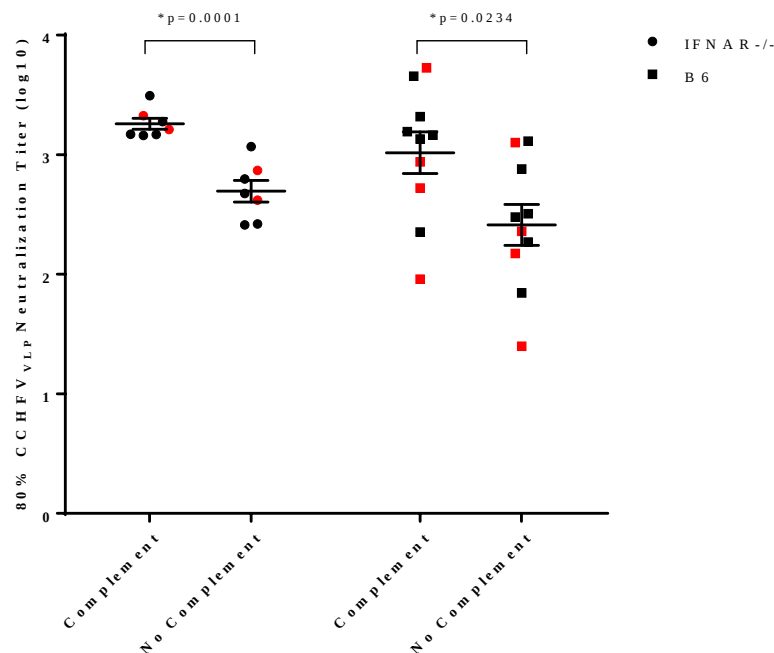
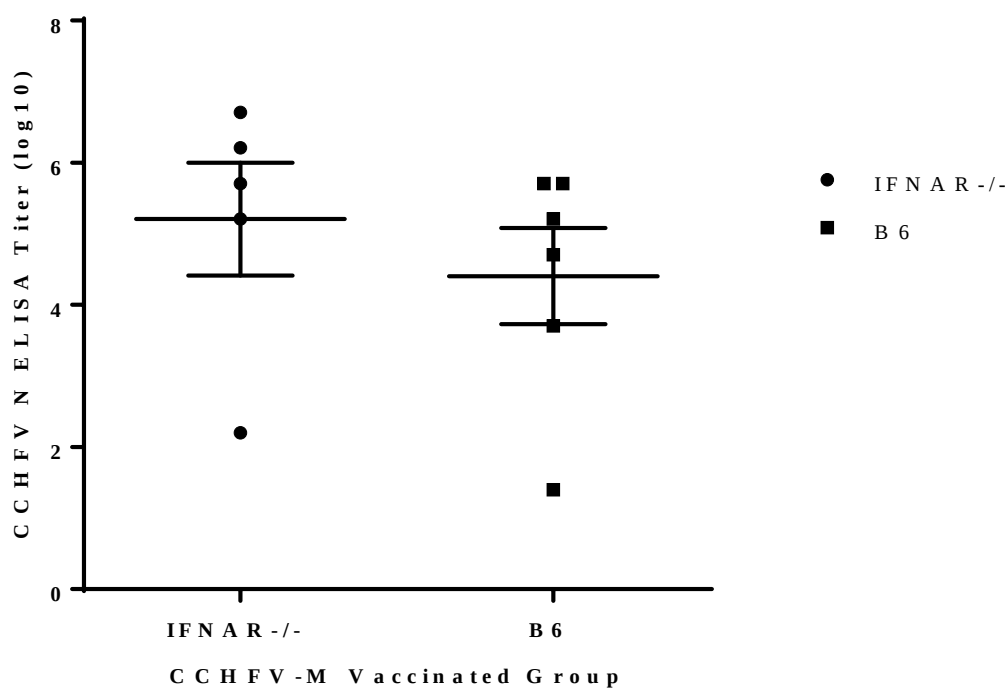


Fig 5. CCHFV-M vaccinated mice developed CCHFV-specific neutralizing antibody following three vaccinations, and the response was enhanced with the addition of complement. Prior to challenge, neutralization titers were measured against the CCHF_{VLP}, with and without complement. Mice that died following challenge are highlighted in red. *Confidence intervals were set to 95%.

582



583

584

Fig 6. N-specific antibodies in post-challenge sera of CCHFV-M vaccinated mice. Sera from

585

vaccinated mice that survived CCHFV challenge (28 days post-challenge) were examined by

586

ELISA for antibodies against N.

587

Funding: This work was funded by the Military Infectious Diseases Research Program.

Conflict of interest: All authors report no conflicts of interest.

Disclaimer: The content of this publication does not necessarily reflect the views or policies of the US Department of the Army, the US Department of Defense or the US Department of Health and Human Services or of the institutions and companies affiliated with the authors.

Acknowledgements: The authors would like to thank Dr. Steve KWilas for his advice on the design and analysis of the CCHF_{VLP} neutralization assay. We are grateful to Dr. Robert Doms for providing the codon-optimized pCAGGS CCHFV-M, and Ms. Kristin Ostrowski for technical assistance in cloning. We would also like to thank Ms. Sarah M. Johnson for her assistance in producing some of the CCHF_{VLP} preps used.

References

1. Flick R, Whitehouse CA. Crimean-Congo hemorrhagic fever virus. Current molecular medicine. 2005;5(8):753-60.
2. Schmaljohn CS, Nichol ST. Bunyaviridae. In: PM KDaH, editor. Fields Virology. 5th edn. Philadelphia: Lippincott Williams & Wilkins; 2007. p. 1741-89.
3. Shepherd AJ, Leman PA, Swanepoel R. Viremia and antibody response of small African and laboratory animals to Crimean-Congo hemorrhagic fever virus infection. The American journal of tropical medicine and hygiene. 1989;40(5):541-7.

- 609 4. Spengler JR, Bergeron E, Rollin PE. Seroepidemiological Studies of Crimean-Congo
610 Hemorrhagic Fever Virus in Domestic and Wild Animals. PLoS neglected tropical diseases.
611 2016;10(1):e0004210.
- 612 5. Mertens M, Schmidt K, Ozkul A, Groschup MH. The impact of Crimean-Congo
613 hemorrhagic fever virus on public health. Antiviral research. 2013;98(2):248-60.
- 614 6. Conger NG, Paolino KM, Osborn EC, Rusnak JM, Gunther S, Pool J, et al. Health care
615 response to CCHF in US soldier and nosocomial transmission to health care providers,
616 Germany, 2009. Emerging infectious diseases. 2015;21(1):23-31.
- 617 7. Smego RA, Jr., Sarwari AR, Siddiqui AR. Crimean-Congo hemorrhagic fever: prevention
618 and control limitations in a resource-poor country. Clin Infect Dis. 2004;38(12):1731-5.
- 619 8. Leblebicioglu H, Ozaras R, Irmak H, Sencan I. Crimean-Congo hemorrhagic fever in
620 Turkey: Current status and future challenges. Antiviral research. 2016;126:21-34.
- 621 9. Ince Y, Yasa C, Metin M, Sonmez M, Meram E, Benkli B, et al. Crimean-Congo
622 hemorrhagic fever infections reported by ProMED. International journal of infectious
623 diseases : IJID : official publication of the International Society for Infectious Diseases.
624 2014;26:44-6.
- 625 10. Estrada-Pena A, Palomar AM, Santibanez P, Sanchez N, Habela MA, Portillo A, et al.
626 Crimean-Congo hemorrhagic fever virus in ticks, Southwestern Europe, 2010. Emerging
627 infectious diseases. 2012;18(1):179-80.
- 628 11. Garcia Rada A. First outbreak of Crimean-Congo haemorrhagic fever in western Europe
629 kills one man in Spain. Bmj. 2016;354:i4891.
- 630 12. Papa A, Papadimitriou E, Christova I. The Bulgarian vaccine Crimean-Congo haemorrhagic
631 fever virus strain. Scand J Infect Dis. 2010;43(3):225-9.

- 632 13. Mousavi-Jazi M, Karlberg H, Papa A, Christova I, Mirazimi A. Healthy individuals'
633 immune response to the Bulgarian Crimean-Congo hemorrhagic fever virus vaccine.
634 Vaccine. 2012;30(44):6225-9.
- 635 14. Bente DA, Alimonti JB, Shieh WJ, Camus G, Stroher U, Zaki S, et al. Pathogenesis and
636 immune response of Crimean-Congo hemorrhagic fever virus in a STAT-1 knockout mouse
637 model. J Virol. 2010;84(21):11089-100.
- 638 15. Zivcec M, Safronetz D, Scott D, Robertson S, Ebihara H, Feldmann H. Lethal Crimean-
639 Congo hemorrhagic fever virus infection in interferon alpha/beta receptor knockout mice is
640 associated with high viral loads, proinflammatory responses, and coagulopathy. The Journal
641 of infectious diseases. 2013;207(12):1909-21.
- 642 16. Berezsky S, Lindegren G, Karlberg H, Akerstrom S, Klingstrom J, Mirazimi A. Crimean-
643 Congo hemorrhagic fever virus infection is lethal for adult type I interferon receptor-
644 knockout mice. J Gen Virol. 2010;91(Pt 6):1473-7.
- 645 17. Akira S. Functional roles of STAT family proteins: lessons from knockout mice. Stem cells.
646 1999;17(3):138-46.
- 647 18. Kortekaas J, Vloet RP, McAuley AJ, Shen X, Bosch BJ, de Vries L, et al. Crimean-Congo
648 Hemorrhagic Fever Virus Subunit Vaccines Induce High Levels of Neutralizing Antibodies
649 But No Protection in STAT1 Knockout Mice. Vector borne and zoonotic diseases.
650 2015;15(12):759-64.
- 651 19. Buttigieg KR, Dowall SD, Findlay-Wilson S, Miloszezewska A, Rayner E, Hewson R, et al. A
652 novel vaccine against Crimean-Congo Haemorrhagic Fever protects 100% of animals
653 against lethal challenge in a mouse model. PLoS One. 2014;9(3):e91516.

- 654 20. Canakoglu N, Berber E, Tonbak S, Ertek M, Sozdutmaz I, Aktas M, et al. Immunization of
655 knock-out alpha/beta interferon receptor mice against high lethal dose of Crimean-Congo
656 hemorrhagic fever virus with a cell culture based vaccine. PLoS neglected tropical diseases.
657 2015;9(3):e0003579.
- 658 21. Dowall SD, Graham VA, Rayner E, Hunter L, Watson R, Taylor I, et al. Protective effects
659 of a Modified Vaccinia Ankara-based vaccine candidate against Crimean-Congo
660 Haemorrhagic Fever virus require both cellular and humoral responses. PloS one.
661 2016;11(6):e0156637.
- 662 22. Hinkula J, Devignot S, Akerstrom S, Karlberg H, Wattrang E, Bereczky S, et al.
663 Immunization with DNA Plasmids Coding for Crimean-Congo Hemorrhagic Fever Virus
664 Capsid and Envelope Proteins and/or Virus-Like Particles Induces Protection and Survival
665 in Challenged Mice. J Virol. 2017.
- 666 23. Spik K, Shurtleff A, McElroy AK, Guttieri MC, Hooper JW, SchmalJohn C.
667 Immunogenicity of combination DNA vaccines for Rift Valley fever virus, tick-borne
668 encephalitis virus, Hantaan virus, and Crimean Congo hemorrhagic fever virus. Vaccine.
669 2006;24(21):4657-66.
- 670 24. Pinto AK, Daffis S, Brien JD, Gainey MD, Yokoyama WM, Sheehan KC, et al. A temporal
671 role of type I interferon signaling in CD8⁺ T cell maturation during acute West Nile virus
672 infection. PLoS pathogens. 2011;7(12):e1002407.
- 673 25. Teijaro JR, Ng C, Lee AM, Sullivan BM, Sheehan KC, Welch M, et al. Persistent LCMV
674 infection is controlled by blockade of type I interferon signaling. Science.
675 2013;340(6129):207-11.

- 676 26. Smith DR, Hollidge B, Daye S, Zeng X, Blancett C, Kuszpit K, et al. Neuropathogenesis of
677 Zika Virus in a Highly Susceptible Immunocompetent Mouse Model after Antibody
678 Blockade of Type I Interferon. PLoS neglected tropical diseases. 2017;11(1):e0005296.
- 679 27. Council NR. Guide for the Care and Use of Laboratory Animals. 8th ed. Washington, D.C.:
680 National Academies Press; 2011.
- 681 28. Hooper JW, Custer DM, Thompson E, Schmaljohn CS. DNA vaccination with the Hantaan
682 virus M gene protects Hamsters against three of four HFRS hantaviruses and elicits a high-
683 titer neutralizing antibody response in Rhesus monkeys. J Virol. 2001;75(18):8469-77.
- 684 29. Altamura LA, Bertolotti-Ciarlet A, Teigler J, Paragas J, Schmaljohn CS, Doms RW.
685 Identification of a novel C-terminal cleavage of Crimean-Congo hemorrhagic fever virus
686 PreGN that leads to generation of an NSM protein. J Virol. 2007;81(12):6632-42.
- 687 30. Dupuy LC, Richards MJ, Ellefsen B, Chau L, Luxembourg A, Hannaman D, et al. A DNA
688 vaccine for venezuelan equine encephalitis virus delivered by intramuscular electroporation
689 elicits high levels of neutralizing antibodies in multiple animal models and provides
690 protective immunity to mice and nonhuman primates. Clinical and vaccine immunology :
691 CVI. 2011;18(5):707-16.
- 692 31. Zivcec M, Metcalfe MG, Albarino CG, Guerrero LW, Pegan SD, Spiropoulou CF, et al.
693 Assessment of Inhibitors of Pathogenic Crimean-Congo Hemorrhagic Fever Virus Strains
694 Using Virus-Like Particles. PLoS neglected tropical diseases. 2015;9(12):e0004259.
- 695 32. Lindenbach BD. Measuring HCV infectivity produced in cell culture and in vivo. Methods
696 in molecular biology. 2009;510:329-36.
- 697 33. Kwilas S, Kishimori JM, Josleyn M, Jerke K, Ballantyne J, Royals M, et al. A hantavirus
698 pulmonary syndrome (HPS) DNA vaccine delivered using a spring-powered jet injector

elicits a potent neutralizing antibody response in rabbits and nonhuman primates. Current gene therapy. 2014;14(3):200-10.

34. Saijo M, Qing T, Niikura M, Maeda A, Ikegami T, Prehaud C, et al. Recombinant nucleoprotein-based enzyme-linked immunosorbent assay for detection of immunoglobulin G antibodies to Crimean-Congo hemorrhagic fever virus. J Clin Microbiol. 2002;40(5):1587-91.

35. Bertolotti-Ciarlet A, Smith J, Strecker K, Paragas J, Altamura LA, McFalls JM, et al. Cellular localization and antigenic characterization of crimean-congo hemorrhagic fever virus glycoproteins. J Virol. 2005;79(10):6152-61.

36. Luft T, Pang KC, Thomas E, Hertzog P, Hart DN, Trapani J, et al. Type I IFNs enhance the terminal differentiation of dendritic cells. J Immunol. 1998;161(4):1947-53.

37. Gallucci S, Lolkema M, Matzinger P. Natural adjuvants: endogenous activators of dendritic cells. Nat Med. 1999;5(11):1249-55.

38. Braun D, Caramalho I, Demengeot J. IFN-alpha/beta enhances BCR-dependent B cell responses. Int Immunol. 2002;14(4):411-9.

39. Ishii KJ, Kawagoe T, Koyama S, Matsui K, Kumar H, Kawai T, et al. TANK-binding kinase-1 delineates innate and adaptive immune responses to DNA vaccines. Nature. 2008;451(7179):725-9.

40. Suschak JJ, Wang S, Fitzgerald KA, Lu S. A cGAS-Independent STING/IRF7 Pathway Mediates the Immunogenicity of DNA Vaccines. J Immunol. 2016;196(1):310-6.