A replicating RNA vaccine confers protection against Crimean-Congo hemorrhagic fever in cynomolgus macaques

David W. Hawman,[a,b,*] Shanna Leventhal,[a] Kimberly Meade-White,[a] William Graham,[c] Karen Gaffney,[b] Amit Khandhar,[b] Justin Murray,[a] Jessy Prado-Smith,[d] Carl Shaia,[d] Greg Saturday,[d] Henry Buda,[c] Lenny Moise,[c] Jesse Erasmus,[b] and Heinz Feldmann[a,**]

[a]Laboratory of Virology, Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rocky Mountain Laboratories, Hamilton, MT, 59840, USA
[b]HDT Bio, Seattle, WA, 98109, USA
[c]Seromyx Systems, Woburn, MA, 01801, USA
[d]Rocky Mountain Veterinary Branch, Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rocky Mountain Laboratories, Hamilton, MT, 59840, USA

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Summary

Background Crimean-Congo hemorrhagic fever is a tick-borne febrile illness with wide geographic distribution. In recent years the geographic range of CCHFV and its tick vector have increased, placing an increasing number of people at risk of CCHFV infection. Currently there are no widely available vaccines and although the World Health Organization recommends ribavirin for treatment, its efficacy is unclear. Vaccines are critically needed for CCHFV.

Methods Here we evaluated a promising replicating RNA vaccine for CCHFV in a Cynomolgus macaque model of disease.

Findings In primed and boosted macaques, we found that our replicating RNA vaccine expressing the CCHFV nucleoprotein (repNP) was highly immunogenic, eliciting a robust non-neutralizing antibody response that conferred significant protection against CCHFV challenge. Macaques receiving a single repNP vaccination were partially protected against CCHFV challenge.

Interpretation Our data demonstrate that our repNP vaccine and NP-specific antibody can protect against CCHFV in non-human primates.

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Keywords: Crimean-Congo; Antibody; Vaccine; RNA vaccine; Non-human primates

Introduction

Crimean-Congo hemorrhagic fever (CCHF), caused by the CCHF virus (CCHFV) is a tick-borne virus that can cause a severe hemorrhagic disease in infected humans. Similar to other viral hemorrhagic fevers, CCHF begins as non-specific fever, myalgia, nausea, diarrhoea and general malaise.[1–4] In some individuals, this disease can rapidly progress to hemorrhagic manifestations and case fatality rates can be as high as 30–40% in some regions.[1,2] Currently, the only widely used therapy is ribavirin but evidence of efficacy in both humans and animal models is conflicting and suggestive of poor efficacy when treatment is started later in infection.[2,5] Besides an inactivated preparation of CCHFV grown in mouse brains licenced only in Bulgaria,[6] there are no approved vaccines for CCHFV, and prevention is limited to control of exposure to infected ticks and livestock.

We have previously evaluated a replicating RNA (repRNA) vaccine for CCHFV in mouse and rhesus macaque models.[3,4,7] This vaccine is based on an alphavirus replicon system[8] in which the structural proteins of Venezuelan Equine Encephalitis Virus are replaced with a gene-of-interest. This results in an RNA capable

*Corresponding author. HDT Bio, Seattle, WA, 98109, USA.
**Corresponding author.
E-mail addresses: David.hawman@hdt.bio (D.W. Hawman), feldmannh@niaid.nih.gov (H. Feldmann).

Research in context

Evidence before this study

Multiple vaccine platforms for CCHFV have reached various stages of pre-clinical testing and several have reached human clinical trials. However, no widely approved vaccine is yet available and the correlates of protection for vaccine-mediated protection against CCHFV is unclear.

Added value of this study

Here we show that a replicating RNA vaccine expressing just the CCHFV NP can confer protection in an established cynomolgus macaque model. This study adds to a building body of evidence that immune responses directed against the CCHFV NP can confer robust protection against CCHFV. Our data also support continued pre-clinical and clinical development of this vaccine for CCHFV.

Implications of all the available evidence

The CCHFV NP is a promising vaccine antigen for designing vaccines against CCHFV. However, vaccine platform and animal models can impact apparent vaccine efficacy.

of self-amplification, leading to dose sparing and mimicking an authentic viral infection, while being unable to spread from the initially transfected cell conferring a significant safety margin. Delivery of the repRNA is accomplished by complexing the RNA with a cationic nanocarrier, called LION™.[9] In our mouse and rhesus macaque studies we evaluated repRNA expressing either the CCHFV nucleoprotein (repNP) or the full length CCHFV glycoprotein precursor (repGPC). Protection in mice and rhesus macaques was associated with strong non-neutralizing antibody responses against the NP[3,4,7] while protection in mice was enhanced by Gc-specific cellular immunity.[3,4] In addition to CCHFV, this repRNA platform has been evaluated in pre-clinical models for SARS-CoV-2, Zika and tuberculosis[9–12] and is the basis for a vaccine that has received emergency use authorization for SARS-CoV-2 in India.[13]

To continue evaluation of this promising vaccine platform for CCHFV, we report here evaluation of our vaccine in the cynomolgus macaque model of disease that we have previously used to evaluate a DNA-based vaccine and the antiviral favipiravir.[14–17] We evaluated repNP either alone or in combination with a vaccine expressing just the glycoprotein Gc (repGc). Prime-boost vaccination with repNP alone conferred significant protection against CCHFV challenge while our repGc appeared to be largely non-immunogenic. Partial protection and significant reduction of virus in the blood and liver was observed after a single immunization with repNP alone. Together our data demonstrate significant protection afforded by the repNP vaccine in non-human primates.

Methods

Animals, biosafety and ethics

All infectious work with CCHFV and sample inactivation was performed in the maximum containment laboratory in accordance with standard operating procedures approved by the Rocky Mountain Laboratories Institutional Biosafety Committee, Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health (Hamilton, MT, USA). All animal work was performed in strict accordance with the recommendations described in the Guide for the Care and Use of Laboratory Animals of the Office of Animal Welfare, National Institutes of Health and the Animal Welfare Act of the US Department of Agriculture, in an AAALACi-accredited facility according to Rocky Mountain Laboratories Animal Care and Use Committee approved protocols (#2022-54). Animals were housed in adjoining individual primate cages that enabled social interaction, under controlled conditions of humidity, temperature and light (12-h light/12-h dark cycles). Water was available ad libitum. Animals were monitored at least twice daily (pre- and post-infection) and fed commercial monkey chow, treats and fruit twice a day by trained personnel. Environmental enrichment consisted of manipulanda, visual enrichment and audio enrichment. All procedures on nonhuman primates were performed by board-certified clinical veterinarians who also provided veterinary oversight of the study. Upon infection animals were comprehensively evaluated for disease signs using a score sheet. All necropsies were performed by board-certified veterinary pathologists. Blood chemistry and haematology was assessed using a Vetscan2 with Preventive Care profile disks (Abaxis, USA) and Procyte DX (IDEXX Labs, USA). Animals were randomly assigned to study group and the origin, age, sex and complete blood chemistry and haematology data for the animals is provided in supplemental data. Confounders such as order of treatments, measurements or animal and cage location was not controlled as this was not possible in the ABSL-4 environment.

Vaccine and vaccinations

The repNP was as previously reported[3] but the V5-epitope tag was removed. The repGc expressed the full length Gc as previously described.[4] Complexing of the repRNA with the cationic nanocarrier was done as previously described.[12] Vaccination was performed by a single intramuscular injection consisting of 25 μg of each RNA. This resulted in repNP + repGc animals receiving 50 μg of RNA while sham and repNP-only animals received 25 μg of RNA. Vaccination appeared

well tolerated with no adverse events observed following vaccinations.

Virus challenge

Animals were challenged with 1×10^5 $TCID_{50}$ of CCHFV strain Hoti divided between subcutaneous injections to the cranial dorsum and intravenously through the saphenous vein as previously described.[14] The same stock of challenge virus was used in both our prime-boost and prime-only studies and is the same stock previously described[14,18] which was obtained from the European Virus Archive (007v-EVA70) and propagated at Rocky Mountain Laboratories.

ELISA

Antibody to gamma-irradiated whole-virus antigen from CCHFV Hoti infected cells was performed as previously described[14] with serial dilutions of sera starting at the 1:100 dilution. Endpoint titres were determined as the reciprocal of the serum dilution to have an absorbance of 3 standard deviations above background. A sigmoidal curve was fit to each serial dilution series constrained to the background absorbance value and endpoint serum dilution interpolated from this curve. Samples unable to have a curve fit were considered to have an endpoint of 100. Recombinant antigen ELISA was performed at a single serum dilution using specified antigens (Native Antigen Company REC31639, REC31730, REC31615) coated on Maxisorp plates (Nunc) at 100 ng/well in PBS.

IFNγ ELISpot

Cryopreserved peripheral blood mononuclear cells (PBMCs) were evaluated for IFNγ production in response to overlapping peptides spanning the entire CCHFV NP or GPC using a 384-well human IFNγ ELISpot kit (Immunospot hIFNg-1M) as previously described[17] except that due to limited cell numbers recovered from CM, single measurements were performed. The number of spots in cells stimulated with DMSO vehicle were subtracted from cells stimulated with CCHFV peptides or PMA/Ionomycin and counts normalized to 1×10^6 cells.

Antigen-specific antibody isotyping/subclassing

The antibody isotyping assay uses fluorescently coded microspheres (Luminex) to capture up to 500 antigen specificities simultaneously and profile the isotype/subclass distribution in an antigen-specific manner. CCHFV nucleoprotein (Native Antigen Company), as well as a positive control antigen (influenza HA) and a negative control antigen (Feline Immunodeficiency Virus), was covalently coupled to beads (Luminex MC100XX-01) via primary amine conjugation. Coupled beads were diluted to a concentration of 100 microspheres/μL and incubated with cynomolgus macaque serum diluted in PBS at room temperature for 2 h on an orbital mixer, shaking at 800 rpm. For IgG1, IgG2, IgG3, IgG4, and IgA measurements, a 1:8000 dilution of serum was used. Each sample was run in duplicate. The bound antigen-specific antibodies were subsequently probed with antibodies detecting baboon-IgG1, IgG2, IgG3, IgG4 (Absolute Antibody: Ab01619-3.0, Ab01620-23.0, Ab01621-1.1, Ab01622-3.0); and monkey-IgA (Fisher Scientific PIMA516729). Fluorescence was analysed using a Stratedigm S1000EON. The data was reported as the median fluorescence intensity of phycoerythrin (PE) for a specific bead channel. Mean values of the duplicates are reported.

Antigen-specific antibody Fc receptor binding

The Fc receptor array uses fluorescently coded microspheres (Luminex) to capture up to 500 antigen specificities simultaneously and profile the effector capacity of each antigen specificity by determining the ability of these antigen-specific antibodies to interact with Fc receptors. CCHFV nucleoprotein (Native Antigen Company), as well as a positive control antigen (influenza HA, Immune Technologies IT-003-SW12p) and a negative control antigen (Feline Immunodeficiency Virus, Native Antigen REC31795), was covalently coupled to beads via primary amine conjugation. Coupled beads were diluted to a concentration of 100 microspheres/μL and incubated with cynomolgus macaque serum diluted in PBS and incubated at room temperature for 2 h on an orbital mixer, shaking at 800 rpm. For FcγR2A, FcγR2B, FcγR3, FcRn at pH 6.0, and FcRn at pH 7.4 measurements, a 1:8000 dilution of serum was used. For C1q a 1:2000 dilution was used. For TRIM21 a 1:400 dilution was used. Each sample was run in duplicate. The bound antigen-specific antibodies were subsequently stained with PE-labelled tetramerized recombinant Fc receptors rhesus-FcγR2A (Duke Protein Production Facility, DPPF), rhesus FcγR3 (DPPF), human-FcγR2B (DPPF), human-FcRn (DPPF), human-C1q (Sigma C1740) and human-TRIM21 (Sino Biological 18010-H07B) for 1 h at room temperature, shaking at 800 rpm. Fluorescence was analysed using a Stratedigm S1000EON. The data are reported as the median fluorescence intensity of PE for a specific bead channel. Mean values of the duplicates are reported.

qRT-PCR

Viral RNA in blood and tissues was quantified in RNA extracted using RNeasy and Qiamp Viral RNA kits (Qiagen). qRT-PCR and quantification by standard curve was as previously described.[19] The limit of quantitation (LoQ) was defined as the copy # of the last standard to amplify while the limit of detection (LoD) was defined as the value given by a Ct value of 40.

Histology and IHC

Histology and immunohistochemistry for the CCHFV NP antigen was performed on formalin fixed tissue sections as previously described.[19] Tissue sections were

evaluated by certified pathologists who were blinded to study groups.

Reagent validation

Our CCHFV virus stock is deep sequenced to confirm identity and exclude contamination (Genbank accession MH483984 - MH483986). Cell lines and antibodies were otherwise obtained from commercial sources with provided certificates of analysis and not validated.

Statistics

Group sizes were determined with a Fisher Exact Test based on expected differences in viral loads and previous experience with variability in the CCHF macaque model. Animals were randomly assigned to study groups, researchers were not blinded to study groups and no animals were excluded. Indicated statistical tests were performed using Prism 9 or 10 (GraphPad). Fig. 1: Statistics calculated with two-way (vaccine and time) repeated measure (RM) ANOVA with Geisser-Greenhouse correction[20] and Tukey's multiple comparisons test (b) or two-way (vaccine and isotype or Fc-binding) ANOVA Dunnett's multiple comparisons test (e and f). Fig. 2: Statistics calculated with two-way (vaccine and time) RM ANOVA with Geisser-Greenhouse correction with Dunnett's (a–d) or Sidak's multiple comparisons test (e). Lines represent mean and SD (a–d, f,g). (e) Data presented as geometric mean and SD. Fig. 3: P values calculated with a Kruskal–Wallis test. Fig. 4: Statistical tests performed using two-way (antigen and time) RM ANOVA with Sidak's multiple comparisons test (a) or two-way (vaccine and isotype or Fc-binding) Dunnett's multiple comparisons test (b). Fig. 5: Statistical tests performed using a non-parametric Kruskal–Wallis test[21] (b) Ordinary one-way ANOVA with Tukey's multiple comparisons test (c), unpaired t-test (i,j), mixed effects analysis with Geisser-Greenhouse correction and Sidak's multiple comparisons test (d–f), or two-way (vaccine and tissue) ANOVA with Sidak's multiple comparisons test (h). For each RM ANOVA we visually assessed Q–Q plots and residual plots to assess normality and homogeneity of variance respectively.

Role of funders

Funders had no role in study design, data collection, data interpretation or decision to publish.

Results

An optimized repRNA vaccine is immunogenic in cynomolgus macaques

We have previously evaluated repNP and repGPC in mice and rhesus macaques[3,7] and found significant protection. However, in these previous studies we found that repGPC elicited only cellular immunity in mice[3] while in macaques, repGPC appeared largely non-immunogenic.[7] We hypothesized that simplifying the repGPC to a repRNA encoding just the protective epitopes in Gc (repGc) would improve immunogenicity in non-human primates. In mice this resulted in similar stimulation of cellular immunity as the full length repGPC.[4] We also removed the V5-epitope tag from the NP which resulted in similar or possibly greater efficacy in mice as the epitope tagged repNP.[4] We then wanted to evaluate repNP with or without repGc in the established cynomolgus macaque model of CCHF.[14,15,22] Groups of 6 Philippine-origin, male cynomolgus macaques between the ages of 2.7 and 4 years of age were vaccinated with 25 μg of repNP or 25 μg each of repNP and repGc in a prime-boost regimen (Fig. 1a). As a control, a cohort (n = 6) received a sham vaccination consisting of a repRNA encoding an irrelevant antigen (Marburg virus GP). Vaccination appeared well tolerated in all animals and the ages of the animals in each cohort are provided in Supplemental Table S1. As expected, serum from sham vaccinated animals had little-to-no reactivity to irradiated whole CCHFV virus (Fig. 1b). Compared to sham-vaccinated animals, both repNP and repNP + repGc vaccinated animals rapidly developed CCHFV-specific IgG within two weeks after prime (Fig. 1c). In repNP-only vaccinated animals, boosting led to a further increase in titres until time of challenge (Fig. 1B). Interestingly, although boosting led to an increase in CCHFV-specific antibody two-weeks after vaccination in repNP + repGc animals, titres declined 3 and 4 weeks after boosting. At time of challenge on day 0, repNP-only vaccinated animals had 4-fold higher CCHFV-specific IgG than repNP + repGc vaccinated animals (Fig. 1b).

We also evaluated the antigen specificity of the measured response using recombinant antigens. At time of challenge, compared to sham vaccinated animals, only animals vaccinated with repNP had increased titres against recombinant NP (rNP) (Fig. 1c). These data are consistent with the decline in titres against whole virus antigen in the repNP + repGc group by day 0 (Fig. 1b) and suggest that the CCHFV-specific antibody measured by our whole-virion ELISA is largely against NP. However, overall signal in this assay was low, in contrast to the robust signal we observed against whole-virus antigen. Thus, it is likely that the absence of NP-specific signal in repNP + repGc vaccinated animals was due to the lower sensitivity of our recombinant protein assay.

We next evaluated cellular immunity elicited by the vaccine measured by IFNγ ELISpot two-weeks after boosting. Animals vaccinated with either repNP or both repNP + repGc had little specific cellular immunity against either antigen (Fig. 1d). Responses against NP were measured in some animals but little-to-no response against pools spanning the repGc antigen (pools 10–14) was measured in any animal (Fig. 1d). Cumulatively, these data indicate that vaccination of non-human primates with repNP or repNP + repGc

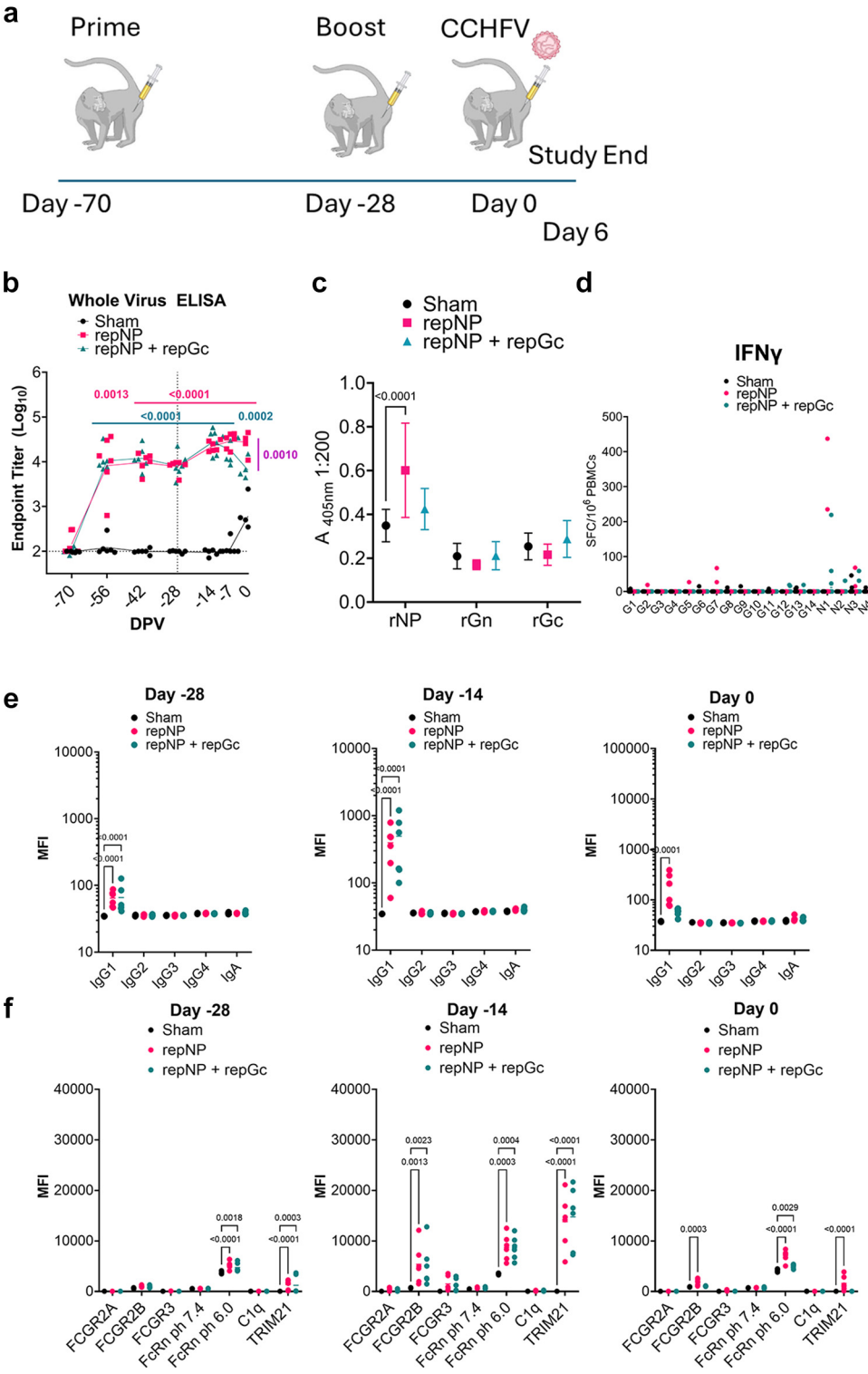

***Fig. 1:* repNP is immunogenic in cynomolgus macaques.** Groups of cynomolgus macaques were sham-vaccinated or vaccinated with repNP-alone or repNP + repGc (a). CCHFV-specific IgG to whole-virus antigen (b) or recombinant antigen (c) was quantified by ELISA. CCHFV-specific IFNγ responses at day –14 to overlapping peptide pools spanning the CCHFV GPC (G1-14) or NP (N1-5) was measured by ELISpot (d). The isotype of CCHFV NP-specific antibody was measured (e) and the ability of NP-specific antibody to bind Fc-receptors, TRIM21 or complement component C1q measured (f). Line connects means (b,d,e,f). (c) Data presented as mean and SD.

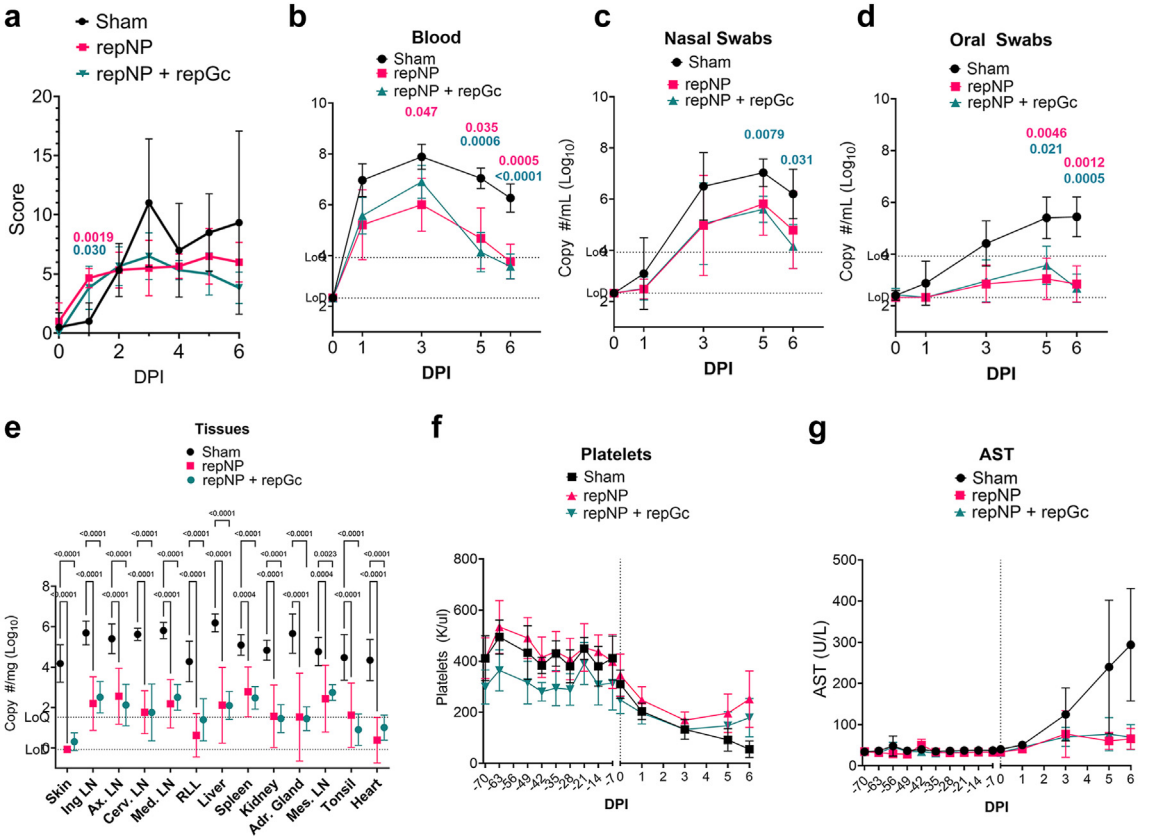

***Fig. 2:* repNP protects against CCHFV challenge in CM.** Animals were comprehensively scored daily after challenge for signs of clinical disease (a). Viral RNA in the blood (b), nasal swabs (c), oral swabs (d) and tissues (e) was quantified by qRT-PCR. Platelet count (f) and AST levels (g) are shown. (f). Lines represent mean and SD (a–d, f,g). (e) Data presented as mean and SD.

primarily leads to CCHFV-specific humoural immunity against the CCHFV NP.

We have consistently found that NP-specific antibody correlates with protection in mouse and NHP models[3,4,7] and shown that these antibodies can protect through the cytoplasmic Fc-receptor tripartite motif-containing protein 21 (TRIM21).[23,24] To more fully characterize the vaccine-elicited CCHFV-specific antibody response, we quantified the isotype of NP-specific antibody and its ability to bind Fc gamma receptor 2A (FCGR2A), FCGR2B, FCGR3, the neonatal Fc-receptor (FcRn) at pH 6.0 and 7.4, C1q and TRIM21. RepRNA vaccination primarily elicited IgG1 NP-specific antibodies (Fig. 1e). Further, we detected binding of NP-specific antibody to FCGR2B, FcRn at pH 6.0 and TRIM21 (Fig. 1f). Together, these data indicate that repNP vaccination elicits primarily IgG1 antibodies with ability to engage Fc-receptors FCGR2B and TRIM21.

repNP + repGc protects against CCHFV challenge

Vaccinated animals were challenged with 100,000 TCID50 of human clinical isolate CCHFV strain Hoti via the combined subcutaneous (SQ) and intravenous (IV) routes.[14] Exams were conducted on days 0, 1, 3, 5 and 6 PI and on day 6 PI, a scheduled necropsy was performed to evaluate viral loads and pathology in a variety of tissues. Consistent with our previous studies,[15–17] disease in most animals was mild with clinical scores <10 (Fig. 2a) although some sham-vaccinated animals developed moderate disease with clinical scores of >15 (Fig. 2a). No animals reached euthanasia criteria before scheduled necropsy. Compared to sham vaccinated animals, repNP and repNP + repGc vaccinated animals had reduced viraemia from day 3 PI onwards (Fig. 2b). We also measured reduced viral RNA in the nasal and oral swabs (Fig. 2c–d). Consistent with control of viraemia and viral shedding, compared to sham vaccinated animals, both repNP and repNP + repGc animals had reduced viral RNA in all tissues evaluated including key tissues for CCHFV such as the liver and spleen (Fig. 2e). We measured a decline in platelets in all groups after CCHFV challenge although repNP and repNP + repGc vaccinated groups began to recover on day 5 and 6 PI

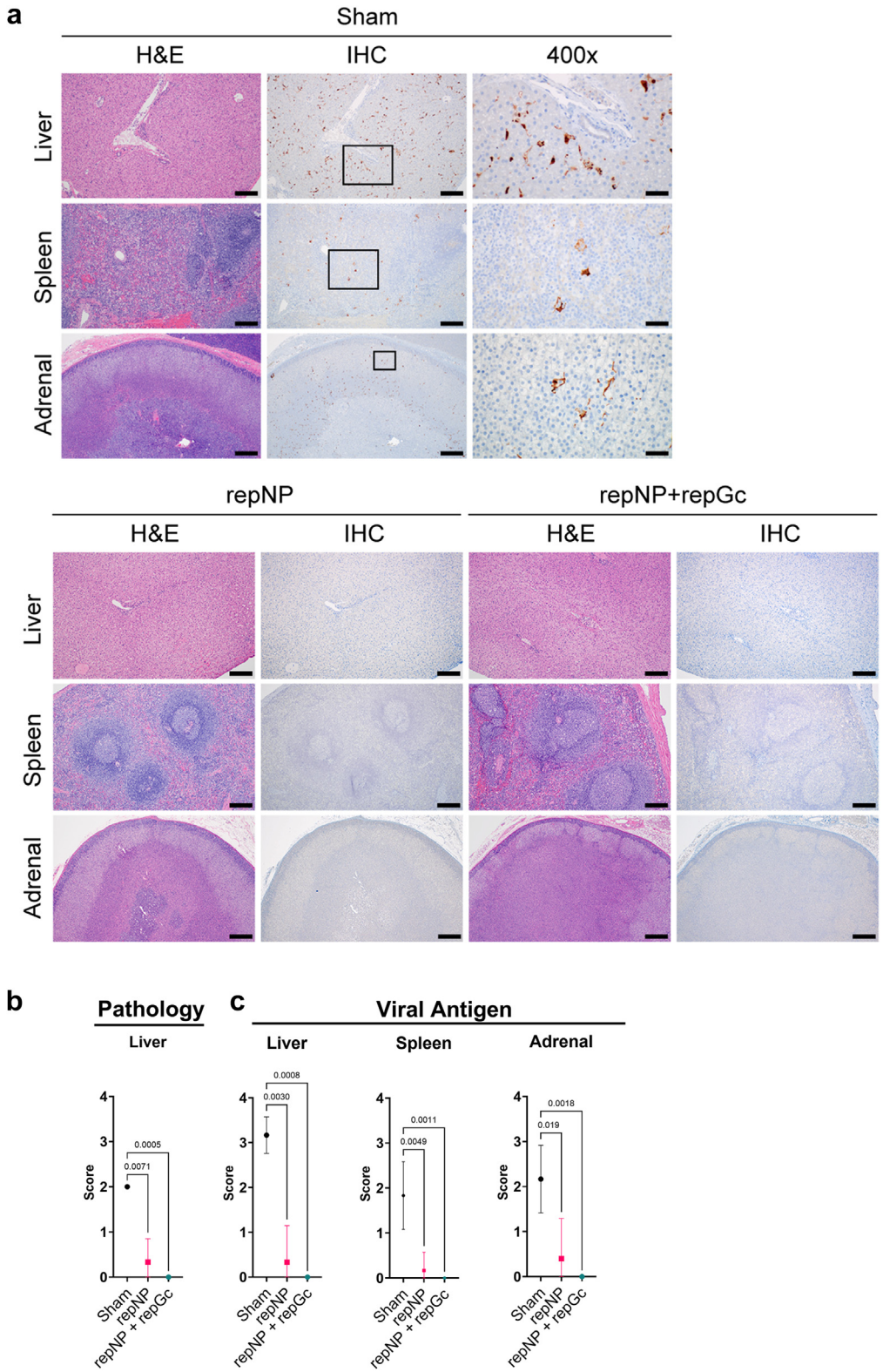

***Fig. 3:* Histological examination of vaccinated animals.** (a) Representative images for H&E and IHC for CCHFV NP antigen in the liver and spleen of sham, repNP and repNP + repGc animals is shown. (b) H&E stained sections were scored by pathologists blinded to study groups and

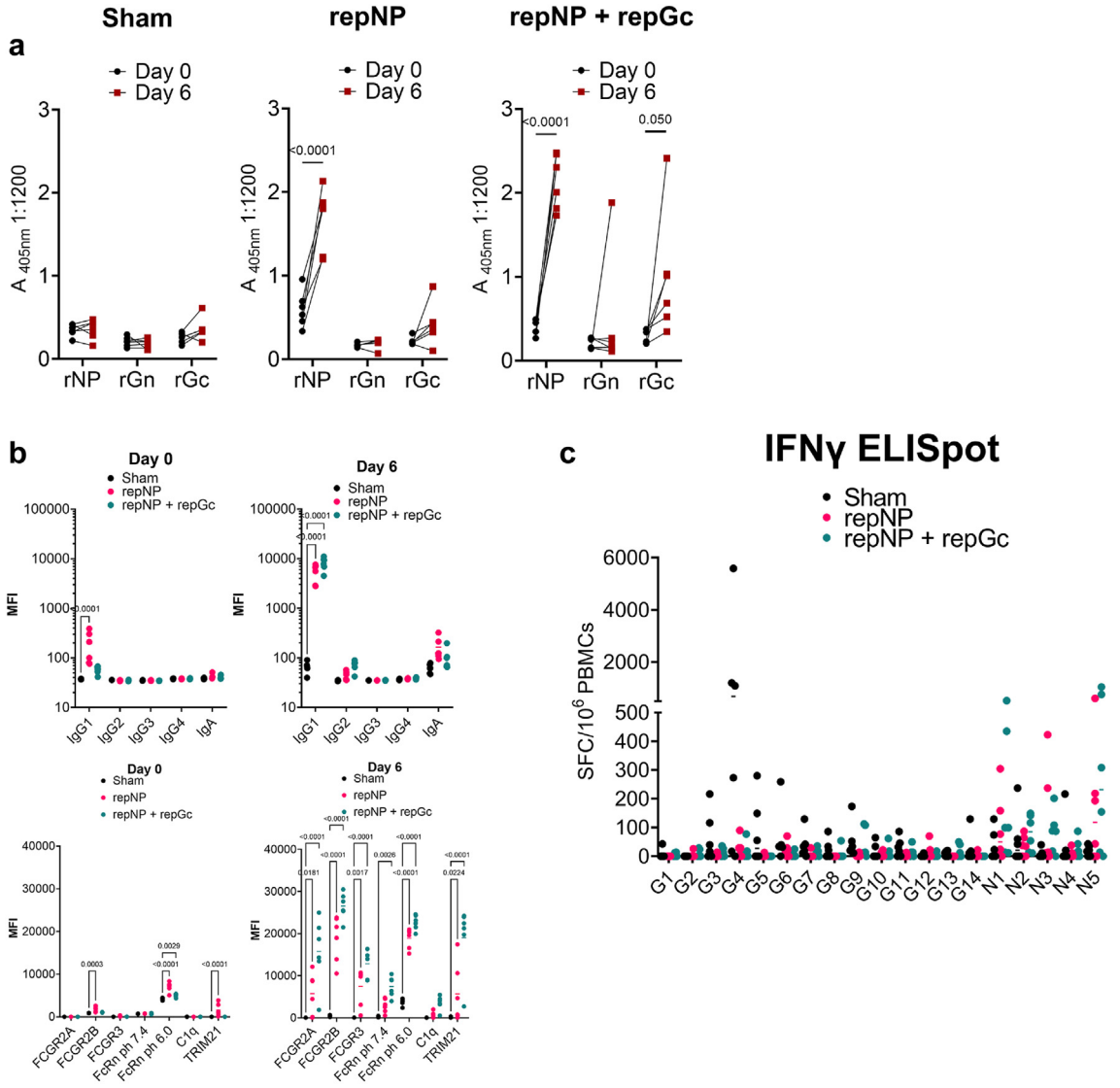

Fig. 4: **Vaccinated animals develop anamnestic antibody responses to the NP and Gc.** (a) IgG to recombinant NP, Gn and Gc was measured by ELISA at indicated timepoints relative to CCHFV challenge. (b) NP specific antibody was evaluated for its isotype and ability to bind Fc-receptors, C1q or TRIM21. Day 0 data is duplicated from Fig. 1 for comparison. (c) An IFNγ ELISpot was used to quantify anamnestic IFNγ responses to CCHFV peptides in PBMCs collected from animals at day 6 PI. (b,c) Line indicates mean.

(Fig. 1f). We also measured an increase in aspartate aminotransferase (AST) in sham but not repNP or repNP + repGc vaccinated animals (Fig. 1g). The complete blood chemistry and haematology profiles are provided in Supplemental Table S1.

We also evaluated formalin fixed sections of several tissues collected at time of necropsy but only the liver, spleen and adrenal gland had significant findings. In sham-vaccinated animals, gross lesions noted at necropsy consisted of enlarged mediastinal lymph nodes in 3 of 6 and an enlarged and/or pale liver in 4 of 6 macaques. All sham-vaccinated animals had histologic lesions of the liver consisting of minimal to mild lesions including single Kupffer cell and hepatocellular necrosis with sinusoid associated inflammation (Fig. 3a and b). Other lesions included single cell necrosis of adrenal

scored as 0 = no lesions, 1 = minimal, 2 = mild, 3 = moderate, 4 = marked and 5 = severe. (c) Sections were stained for CCHFV NP antigen and scored by pathologists blinded to study groups and presence of antigen scored as 0 = none, 1 = rare, 2 = scattered, 3 = moderate, 4 = numerous, 5 = diffuse. (b and c) Data shown as mean and SD.

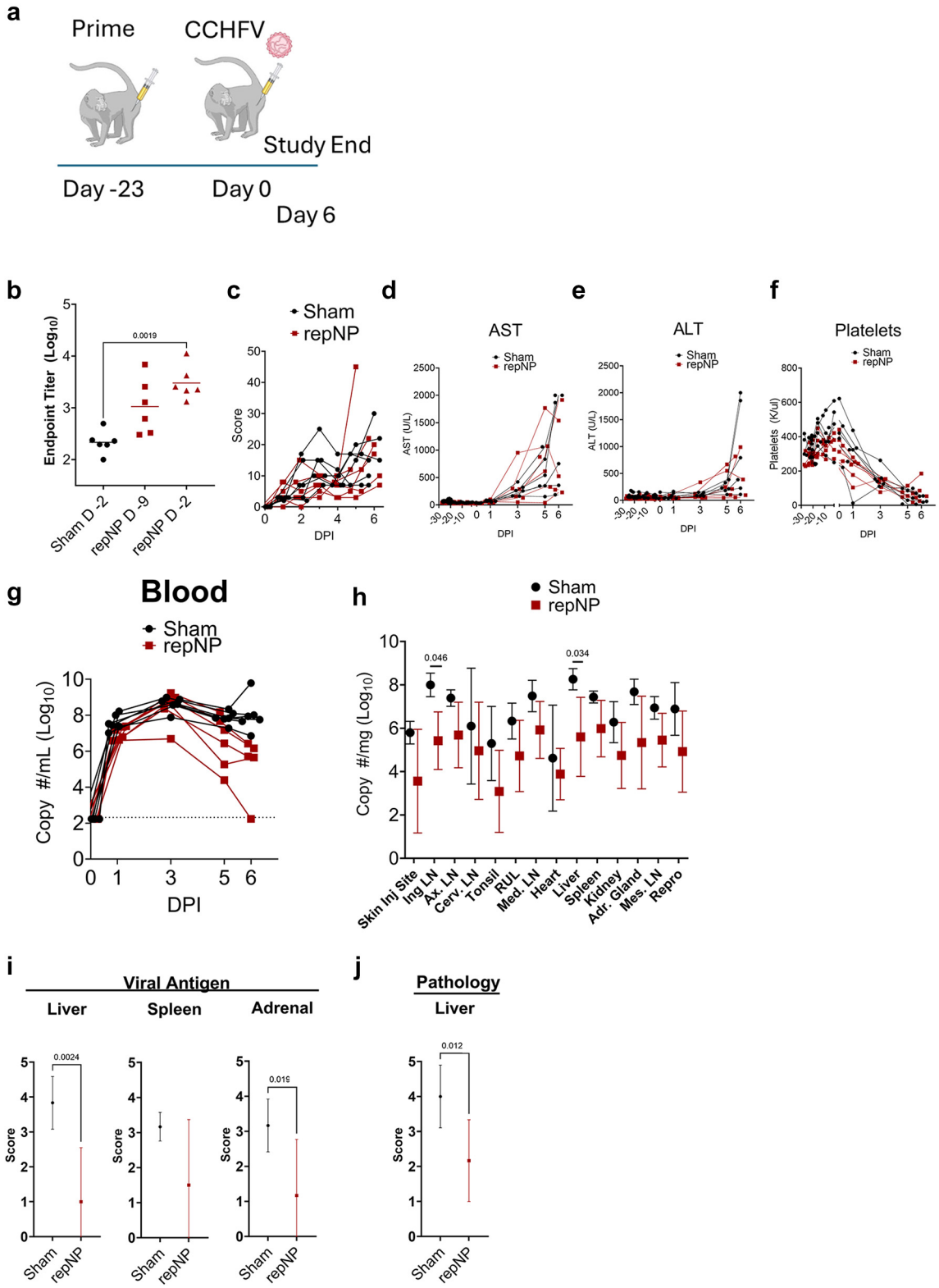

***Fig. 5:* Single immunization with repNP is partially protective against CCHFV challenge.** Groups of cynomolgus macaques were sham-vaccinated or vaccinated with repNP-alone (a). (b) CCHFV-specific IgG to whole-virus antigen was quantified by ELISA at indicated

gland cortical cells (Fig. 3a). Anti-CCHFV immunoreactivity ranged from moderate to marked in Kupffer cells and hepatocytes of the liver; minimal to moderate in red pulp monocytes within the spleen; and minimal to moderate in the adrenal gland in all sham-vaccinated animals (Fig. 3A and C). In nearly all animals (10 of 12) receiving repNP alone or repNP + repGc, no histologic lesions nor viral antigen was observed (Fig. 3A - C). Two animals in the repNP-only group had minimal pathology in the liver and in one animal, this was associated with detectable antigen in the liver, spleen and adrenal gland (Fig. 3A and B). These findings are consistent with the viral loads measured in this animal as this animal had the highest viral loads among vaccinated animals (Fig. 2E). The complete table of histological findings are shown in Supplemental Table S2. Together these data demonstrate that repNP or repNP + repGc vaccination confers protection against CCHFV challenge and signs of disease in these animals.

Anamnestic antibody and T-cell responses

Cumulatively, our data suggests that humoural immunity against NP is the primary correlate of protection, consistent with our studies in mice.[3] However, we could not exclude the possibility that repGc primed CCHFV-specific responses that were rapidly boosted upon CCHFV infection. We therefore evaluated anamnestic humoural immunity to recombinant antigens at time of necropsy on 6 PI. Anamnestic responses to the NP antigen were measured in vaccinated animals (Fig. 4A) while anamnestic responses to the Gc antigen were measured only in animals also vaccinated with repGc (Fig. 4a). Together, these data demonstrate that repRNA vaccinated animals are primed to rapidly produce CCHFV-specific antibody upon CCHFV challenge and that inclusion of repGc led to priming of low-levels of Gc-specific antibody that was rapidly boosted upon challenge.

We also evaluated serum collected on day 6 PI for NP-specific Ig isotype and Fc-receptor binding. After infection, we continued to only detect NP-specific IgG1 (Fig. 4b) although there was a slight increase in NP-specific IgA (Fig. 4b). We measured a broadening of the ability of NP-specific antibody to engage Fc-receptors after infection (Fig. 4b) likely due to an increase in the magnitude of the NP-specific response. We also measured anamnestic cellular immunity in PBMCs collected at day 6 PI. In sham-vaccinated animals, CCHFV-specific cellular immunity was measured against multiple pools in the GPC and NP (Fig. 4c) with four animals having >250 SFCs/1E6 PBMCs against pool G4 suggesting rapid development of CCHFV-specific cellular immunity in naïve animals. In repNP-only vaccinated animals, some animals had reactivity against NP peptide pools (Fig. 4c) but little-to-no activity against any GPC pools. Similarly, repNP + repGc vaccinated animals had reactivity to some NP pools but notably, little to no activity against GPC pools 9–14, comprising the Gc antigen included in repGc (Fig. 4c). These data suggest that repNP may prime NP-specific T-cells that can rapidly respond to CCHFV challenge, however, de novo responses in sham-vaccinated animals were also rapid.

Partial protection against CCHFV challenge in animals receiving a single vaccination

An optimal vaccine for CCHFV would elicit durable, protective immunity with minimal vaccinations. Since we measured CCHFV-specific immunity within two weeks of the prime vaccination (Fig. 1b) and have shown that mice are completely protected within two-weeks of a single vaccination,[24] we hypothesized that cynomolgus macaques would similarly be protected from CCHFV challenge after a single immunization with repNP. Groups of 6 Indonesian-origin cynomolgus macaques between 8.5 and 12.9 years of age were either sham vaccinated or vaccinated with just repNP as before. The ages are provided in Supplemental Table S3. We did not evaluate repGc due to the lack of a Gc-specific immune response even after boosting and prior to CCHFV-challenge in the previous study. Beginning at 14 days post-vaccination (day –9 relative to CCHFV challenge) we measured low-levels of CCHFV-specific IgG that further increased three weeks post-vaccination (day –2 relative to CCHFV challenge) (Fig. 5a). Although titres after this prime-vaccination remained lower than titres measured after boosting in our first study (Fig. 1b), we hypothesized these low titres along with the previously measured rapid anamnestic responses would be sufficient for protection. Therefore, on day 23 post-prime vaccination, animals were challenged with CCHFV as before.

Upon CCHFV challenge, prime-only repNP vaccination had no impact to clinical scores, AST or alanine aminotransferase (ALT) or platelet counts compared to sham-vaccinated animals (Fig. 5b–e) and one repNP-vaccinated animal reached euthanasia criteria on day 5 PI. However, we measured a trend towards decreased viral RNA in the blood of repNP-vaccinated animals and one repNP vaccinated animal had no detectable viral RNA in the blood at day 6 PI (Fig. 5g) suggesting repNP vaccination led to earlier control of the infection. We

timepoint. On day 23 after vaccination, animals were challenged with CCHFV and animals were comprehensively scored daily for signs of clinical disease (c) and AST (d), ALT (e) and platelet levels in the blood enumerated (f). Viral loads in the blood (g) or various tissues collected on day 5 or 6 PI (h) were quantified by qRT-PCR. Formalin fixed tissue sections were stained for CCHFV NP antigen (i) or with H&E (j) and scored by pathologists blinded to study group for presence of viral antigen or lesions. (h–j) Data shown as mean and SD. (b) Line indicates mean.

evaluated viral RNA in several tissues at time of scheduled euthanasia, day 6 PI or day 5 for the animal that reached endpoint. We measured an overall trend of reduced viral RNA in repNP-vaccinated animals with significantly reduced viral RNA in the liver and inguinal lymph nodes (Fig. 5g) compared to sham-vaccinated animals. We also stained liver, spleen and adrenal gland sections with H&E and for viral antigen. Interestingly, we observed significantly reduced presence of viral antigen in the liver and adrenal gland of repNP-vaccinated animals compared to sham-vaccinated animals (Fig. 5i). Similarly, we observed significantly less pathology in the livers of repNP-vaccinated animals (Fig. 5j) while minimal pathology was observed in the spleen or adrenal gland sections of any animal (Supplemental Table S4). The complete pathology and IHC findings are provided in Supplemental Table S4. These data indicate that a single repNP vaccination was sufficient to confer protection against viraemia and liver pathology but ultimately protection was incomplete with similar clinical disease in CCHFV-vaccinated animals compared to sham-vaccinated.

Discussion

Here we show that repNP-only vaccination of cynomolgus macaques can confer protection against CCHFV replication and disease. To date, only a DNA-based vaccine and our repRNA vaccine have been evaluated in NHPs for CCHFV.[15,17] Similar to our results for the repNP vaccine, protection was conferred by prime-boost-boost vaccination of cynomolgus macaques with just plasmid expressed NP.[17] Together, these findings demonstrate that immunity directed against the CCHFV NP is sufficient to confer robust protection against CCHFV in non-human primate models, supporting extensive evidence from mouse models across several platforms.[3,4,25,26] We have shown in mice that repNP and NP-specific antibodies require the cytoplasmic Fc-receptor TRIM21 for protection[24] and show here that vaccine-elicited NP-specific antibodies in cynomolgus macaques can bind TRIM21. We have also shown that this same serum can inhibit CCHFV replication in a TRIM21-dependent manner and coordinate close molecular interaction between NP and TRIM21 *in vitro*.[24] Prophylactic passive transfer of NP-specific antibody or an NP-specific monoclonal antibody were sufficient to confer protection against lethal CCHFV challenge[24,27] demonstrating protection in absence of any CCHFV-specific cellular immunity. Our work also supports a body of evidence that antibodies against the viral glycoproteins are neither necessary nor sufficient for protection against CCHFV.[28–31]

Despite our work in mice demonstrating that Fc effectors such as complement or activating Fc receptors are neither necessary nor sufficient for repNP-mediated protection in mice,[24] our data here indicate that prior to challenge, NP-specific antibodies mainly engaged the inhibitory Fc-receptor FCGR2B.[32] Whether NP-specific antibody engagement of inhibitory Fc-receptors has any consequence will require further study. Further, by day 6 PI, NP-specific antibodies could engage multiple Fc-receptors including activating receptors suggesting evolution of the NP-specific antibody response upon viral challenge.

The role of GPC or Gc-specific immunity in protection against CCHFV is less clear and our repGPC[7] and repGc vaccines appear to be largely non-immunogenic in non-human primates prior to challenge. However, animals vaccinated with repGc here or previously with repGPC[7] developed significant humoural immunity to Gc shortly after infection suggesting repGc or repGPC may prime low levels of Gc-specific B-cells that can rapidly respond to the infection and may contribute to protection. Our data from rhesus macaques[7] and here from cynomolgus macaques highlight the importance of non-human primate models in pre-clinical testing of candidate vaccines for CCHFV. We have consistently found repGPC or repGc to elicit potent cellular immunity in mice[3,4] while in macaques these same vaccines are poorly immunogenic. Here we also found that inclusion of repGc may have also interfered with NP-specific immunity in macaques. This was not observed in our mouse studies[3] and it is unclear why this may have occurred. Nevertheless, together these observations suggest there are species specific determinants of CCHFV antigen immunogenicity and likely efficacy. The in-bred mouse models utilized extensively to evaluate vaccine candidates for CCHFV[33] may further confound conclusions. We have found that CCHFV infection of naïve C57BL6/J mice resulted in >75% of T-cells responding to a single peptide in the N-terminus of Gc.[34] The majority of cellular immunity elicited by repGPC or repGc in mice was directed against the peptide pool containing this peptide[3,4] suggesting vaccination results in similar targeting of limited epitopes in mice. Beyond our vaccine, a DNA-vaccine elicited responses against the same region in C57BL6/J mice[28,29] while vaccination of mice on the 129 S background with a modified vaccinia vaccine expressing the GPC (MVA-GPC) elicited responses mainly against NSm and the N-terminus of Gc[35,36] suggesting similar immunodominance across multiple vaccine platforms in in-bred mice. This level of immunodominance is not seen in humans infected with CCHFV[37] nor in cynomolgus macaques vaccinated with our DNA-based vaccine.[15] These data suggest outbred populations may differ substantially from in-bred mouse models in their responses to CCHFV antigens. The MVA-GPC and a chimpanzee adenovirus vaccine expressing the GPC[38] have started human clinical trials[39,40] without apparent non-human primate testing. As data from human clinical trials of vaccines against CCHFV becomes publicly available, it will be important to compare human immunogenicity data to data from pre-clinical animal

models. The varying immunogenicity and efficacy of vaccines against CCHFV seen across multiple studies, platforms and animal models suggest that both the vaccine platform and model utilized are a critical determinant in host responses and protective capacity of vaccine expressed CCHFV antigens.

Lastly, our report adds to the variability of CCHF severity measured in NHP models. Clinical observations, liver enzymes, platelet counts, and histopathological findings suggest that disease was of greater severity in sham vaccinated animals of our second cohort than in our first cohort (Figs. 2, 3 and 5). Since the first description of the model in which 50% of animals reached euthanasia criteria,[14] severe disease has been inconsistently observed by our group and others.[15–17,22,41] It is unclear why disease was of greater severity in our second cohort, but aliquots of an identical virus stock was utilized for both cohorts arguing against differences in challenge virus. Instead, differences may be due to the differing origins (Philippine vs Indonesian), ages (<4 years vs >8.5 years) or the outbred nature of these animals. However, comparison of Chinese vs Mauritian and younger (<8 years) vs aged (>18 years) cynomolgus macaques did not significantly alter disease[22] suggesting susceptibility to severe disease upon CCHFV infection may be due to multiple host factors.

Our study has important limitations. First, the partial protection seen after a single immunization suggests further optimizations of the vaccine regimen such as through increasing the vaccine dose or including additional protective antigens such as the GP38[29] may be needed to achieve single-shot protection. Vaccination of non-human primates with up to 250 μg of a repRNA vaccine against SARS-CoV-2, 10-times the dose used here, appeared well tolerated.[11,12] The LION formulation is less reactogenic than traditional lipid nanoparticles (LNPs) suggesting higher tolerable doses may be achievable than are possible for traditional LNP-delivered mRNA vaccines.[42] Secondly, our viral challenge and vaccine antigen are both based on CCHFV strain Hoti and an ideal vaccine for CCHFV will confer broad protection against genetically diverse CCHFV. However, the CCHFV NP is well conserved across genetically distinct CCHFV isolates[43] and we have shown in mice that repNP can protect against a highly divergent strain of CCHFV.[3,4] Lastly, CCHFV is endemic in regions with limited health care resources and vaccine-mediated protection should be durable to minimize the need for boosters. We did not evaluate the durability of protection in this study, and it is unclear how long protection afforded by repNP will last. We have demonstrated significant protection against CCHFV in mice for at least one-year after vaccination,[44] and an ongoing study is evaluating the durability of the response in cynomolgus macaques.

In summary, our repRNA platform was immunogenic and conferred significant protection against CCHFV challenge in cynomolgus macaques. Our data adds to an increasing understanding of the correlates of protection for vaccines against CCHFV and demonstrates that vaccination with only the CCHFV NP is sufficient to confer significant protection in an NHP model of disease. However, our data also highlight that differences in preclinical animal models may contribute significantly to vaccine immunogenicity and efficacy against CCHFV. Therefore, as several CCHFV NP and GPC-based vaccine candidates enter human trials and data becomes available, identification of the animal models that most accurately predict immunogenicity, and likely efficacy, in humans will be paramount to support continued development of countermeasures against CCHFV.

Contributors

DWH and HF designed the studies. DWH, SL, KMW, WG, KG, JM, JPS, CS, GS, HB, LM performed the studies. DWH, AK, WG, HB, LM performed data analyses. DWH, JE and HF provided study supervision. JE and HF obtained funding. DWH wrote the manuscript and all authors have read and approved the final version. DWH and HF have verified the underlying data.

Data sharing statement

All data underlying the figures in this manuscript are available at 10.6084/m9.figshare.28673456.

Declaration of interests

J.E. and A.K. have equity interest in HDT Bio. J.E. and A.K. are co-inventors on U.S. patent application no. 62/993,307 "Compositions and methods for delivery of RNA" pertaining to formulations for RNA delivery. DWH, JE and HF are inventors on U.S. patent application number 63/365,015 "Replicating RNA vaccine for Crimean-Congo hemorrhagic fever virus" regarding the repRNA for use against CCHFV. WG, HB, LM have equity interest in Seromyx systems.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ebiom.2025.105698.

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