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RESEARCH

Cellular immunity to nucleoproteins (NP) of Crimean-Congo hemorrhagic fever virus (CCHFV) and Hazara Virus (HAZV)

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Abstract

Crimean-Congo Hemorrhagic Fever Virus (CCHFV) is a globally significant vector-borne pathogen with no internationally-licensed preventative and therapeutic interventions. Hazara virus (HAZV), on the other hand, a related *Orthonairovirus*, has not been reported as a human pathogen. HAZV has been proposed as a surrogate model for studying CCHFV, bisosafety level 4 (BSL-4) agent. Previously, we investigated the humoral immune responses between NPs of these viruses and in this study, we extended the scrutiny to cellular immune responses elicited by NPs of CCHFV and HAZV. Here, mice were immunized with recombinant CCHFV NP and HAZV NP to evaluate the correlates of cell-mediated immunity (CMI). Delayed-type hypersensitivity (DTH) responses were assessed by challenging immunized mice with CCHFV-rNP or HAZV-rNP on the footpad and lymphocyte proliferation assays (LPAs) were performed by stimulating splenocytes in vitro with CCHFV-rNP or HAZV-rNP to compare cellular immune responses. In all test groups, strong DTH and LPA responses were detected against homologous and heterologous challenging antigens. To assess the cytokine response, an RT-qPCR -specific for cytokine mRNAs was utilized. Interestingly, CCHFV NP stimulated groups exhibited a significantly elevated mRNA level of interleukin 17 A (IL-17) compared to HAZV NP, indicating a notable difference in immune responses. This study presents comparison between CMI elicited by NPs of CCHFV and HAZV and contributes to the understanding of a highly pathogenic virus, particularly in the context of the declaration of CCHFV by World Health Organization's (WHO) as a major viral threat to the world.

Keywords CCHFV · HAZV · Nucleocapsid protein · Cellular immune response · Antigenic similarity

Introduction

CCHFV and HAZV are RNA viruses with negative polarity, classified in the *Nairoviridae* family of the *Bunyavirales* order. The initial outbreak of Crimean-Congo hemorrhagic fever (CCHF) was observed among Russian soldiers in Crimea in the 1940s. The disease presented as an acute febrile illness with notable symptoms of bleeding and shock [1, 2]. In the late 1960s, it was noticed that the causative agent of hemorrhagic disease in Crimea was similar to the causative agent of hemorrhagic disease in the Democratic Republic of Congo (formerly known as Belgian Congo). Consequently, this pathogen was named Crimean-Congo hemorrhagic fever virus [3]. In recent years, Türkiye, the Middle East, the Caucasus region, Africa, and the majority of Asia have all been affected by the hemorrhagic fever caused by the CCHFV, which is spread by ticks of the *Ixodidae* family. Case fatality rates range according to epidemics; however, they commonly vary between 5% and 30% [4, 5]. Currently, there is no universally accepted preventive vaccine nor treatment regimen for the disease. CCHFV is also considered as a possible bioterrorism agent by international authorities and requires a high level of biosafety to be handled. Hazara virus, on the other hand, has not been reported to cause disease in human or animals [6]. The global distribution of HAZV, initially found in ticks of the *Ixodes redikorzevi* species in Kaghan Valley, Pakistan in 1964, has

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not been thoroughly examined. However, antibodies against HAZV have been found in the sera of wild rodents [2, 7, 8].

CCHFV and HAZV are genetically and serologically related [9, 10]. The virus has a genome comprising of three single stranded RNA segments, namely S, M, and L, which encode for viral nucleoprotein (NP), glycoprotein precursor (GPC), and RNA-dependent RNA polymerase (RdRp), respectively. While the CCHFV S segment encodes a nonstructural protein (NSs) through an ambisense coding strategy, it is unclear if the HAZV S segment also contains NS in the ambisense genome. No studies have been conducted to investigate this matter [11]. As with all bunyaviruses, the NPs of CCHFV and HAZV oligomerize and encapsulate the three viral RNAs in the genome to form the ribonucleoprotein (RNP) structure, which is essential for the virus's vital functions [12]. Genetic sequences of CCHFV strains from different geographical regions demonstrate 20%, 31%, and 22% differences in the S, M, and L segments, respectively. Notably, NP appears to be the most conserved protein among the isolates [13]. Studies conducted in the 1970–1980 s revealed antigenic similarities between the two viruses in hemagglutination inhibition and neutralization tests [10, 14]. Subsequent research failed to validate the initial findings. Smirnova et al. conducted cross-neutralization tests, demonstrating effective neutralization of CHF and Congo virus strains by immune sera, including convalescent CHF antisera; however, the viruses remained active when treated with anti-Hazara sera [15]. In a recent study, it was found that experimental HAZV infections do not affect sheep or cattle. Furthermore, there was slight cross-reactivity observed in immunofluorescence and immunoblot assays, although this was not detected in widely used commercial CCHFV ELISAs commonly employed in field studies [16]. Cross-neutralization studies suggest potential glycoprotein similarities, and the investigation into antigenic similarity between the two viruses was constrained to the initial unverified reports.

Nucleoproteins play crucial roles in various stages of the viral life cycle, including replication, transcription, and virion assembly, and are the most abundant protein in many RNA viruses [17]. The RNA hybridization test demonstrated the S-segment sequence similarity of the two viruses in a previous study and the reported amino acid sequence similarity between the S segments of CCHFV and HAZV JC280 is 60% [18]. Our laboratory has been examining the antigenic similarity between CCHFV and HAZV in recent years. Our previous study revealed a notable cross-reactivity in humoral immune responses against NPs in both immune serum of patients with confirmed CCHFV infection and experimental animals that were immunized with CCHFV NP [19]. The current report expands the investigation to the CMI stimulated by NPs.

Studies on the specific role of NP in CMI are considerably scarce. Epitope mapping studies on NP has shown that it possesses numerous antigenic sites [20, 21]. Various vaccines have been developed against CCHF using different platforms [22–25]. Despite the neutralizing effect observed in glycoprotein-based vaccines, their low protective efficacy necessitates the inclusion of NP in the vaccines [23, 24]. Some studies using NP as an antigen in vaccine design have shown the induction of robust cellular responses and provided 100% protection [23, 26]. Although immune responses to glycoproteins are critical, the genetic diversity among glycoproteins suggests that NP, which is genetically conserved, may play an essential role in disease protection [20, 27–29]. Similarly, in the context of COVID-19, cellular immune responses against NP have been associated with positive clinical outcomes and survival [30, 31]. Another well studied NP is of the influenza viruses, and it is more conserved than other influenza viral proteins. This prompted some to suggest that NP could be a valuable target for creating influenza vaccines capable of providing broad protection against various influenza strains [32]. It has been well established that nucleocapsid proteins of various negative stranded human RNA viruses such as measles, mumps, respiratory syncytial virus, metapneumovirus, and Hantaan virus induce robust cellular immune responses [33]. Additionally, in some viral hemorrhagic fevers (VHF) such as Lassa fever, dengue fever, Ebola, the roles of CMI have been demonstrated and the protein is suggested to be included in vaccine formulations [34–36].

Cell mediated immune responses are typically attributed to playing a significant role in recovering from the disease, rather than in preventing infection. However, in some viral infections, CMI is held responsible for the pathogenesis [37]. Therefore, identifying the role of CMI in CCHF could potentially contribute to understanding the development of pathogenic picture. Hence, a detailed comprehension and potential interventions in the pathogenesis necessitate a thorough understanding of the elements that stimulate the CMI response triggered by individual proteins of closely related CCHFV and HAZV. Therefore, in this study, we addressed the comparative features of CMI against NPs of two *Nairoviruses*, namely HAZV and CCHFV, the latter of which is listed as one of the top priority viruses to be investigated by WHO [38].

Materials and methods

Study design

Plasmid construction, protein expression and purification

DNA sequence of HAZV NP was obtained from HAZV-infected cell lysate and cloned into pET-30b(+) (Addgene,

Cambridge, MA, USA). The resulting plasmid was transformed into One Shot BL21(DE3) competent *Escherichia coli* (Thermo Fisher Scientific, Waltham, MA, USA) cells to express HAZV rNP. The cloning and protein expression process is described in detail previously [19]. Bacterial-optimized CCHFV Kelkit06 NP DNA (GenBankAccession no. GQ337053) was cloned in plasmid pUC19. CCHFV rNP was produced and purified as described in earlier [39].

Western blot of his-tagged proteins

Recombinantly expressed HAZV rNP and CCHFV rNP were separated using a 12% polyacrylamide gel electrophoresis (PAGE). Subsequently, the proteins were transferred onto a 0.45-µm nitrocellulose membrane (Amersham Protran) at 25 V for 7 min utilizing a Trans-blot SD semidry blotter (Bio-Rad). Following the transfer process, the membrane underwent blocking in a solution of 5% nonfat dry milk and 1× TBST (10 mM Tris-HCl 8pH 7.4), 0.9% NaCl, 0.02% Tween 20) for 2 h and was then incubated overnight with a secondary antibody, which was a horseradish peroxidase-conjugated anti-6×His tag antibody (HRP; Abcam, UK), at the dilution recommended by the manufacturer. After this incubation, the membrane underwent five washes and was visualized using chemiluminescence detection (WesternBright Sirius chemiluminescence detection kit; Advansta, CA, USA), following the provided manufacturer's instructions.

Animal experiments

Male Balb/C mice, aged between six to eight weeks, were employed for both rNP, as positive control BSA and as negative control PBS immunizations. Each group had consisted of five mice. Blood samples were collected from the facial vein two weeks following each immunization to assess the efficacy of the immunization process. After three days of DTH to perform LPA, mice were humanely euthanized through cervical dislocation under isoflurane anaesthesia. Blood was then directly drawn from the heart using a 5 ml syringe (22G needle). Following blood collection, the samples were incubated at room temperature for 30 min to facilitate clotting, and sera were separated via centrifugation at 1,000xg for 10 min. The aliquots of separated serum samples were stored at -20 °C until further use.

Immunizations

Balb/C mice, aged six to eight weeks, were obtained from Bezmialem Vakif University Research Animals Laboratory. Mice were allocated randomly into six groups and housed individually for each group in cages under pathogen-free conditions. The mice had ad libitum access to food and water and were kept in an air-conditioned room throughout the study. They were subcutaneously inoculated in the shoulder blade or flanks with 100 µl of 0.5 mg/ml CCHFV rNP or HAZV rNP emulsified in Freund's Complete Adjuvant (CFA) (Sigma-Aldrich, Taufkirchen, Germany), resulting in a final vaccination dose of 50 µg rNP. At two-week intervals, mice received three boosts with the same antigen dose emulsified in Incomplete Freund's Adjuvant (IFA) (Sigma-Aldrich, Taufkirchen, Germany) via the same route. Positive control groups were administered bovine serum albumin (BSA) (Sigma-Aldrich, Taufkirchen, Germany) emulsified in relevant adjuvants, with a final vaccination dose of 50 µg BSA. In contrast, mice in the negative control groups were mock immunized with PBS emulsified in relevant adjuvants. Immunizations were conducted following the described schedule. Each experimental group comprised five mice, and detailed information on the immunization and stimulation combinations is provided in Table 1.

Delayed-type hypersensitivity (DTH) response

After last immunization, humoral immune responses were tested by EIA with using relevant antigens and immunized mice sera. One week after last immunization, mice received 15 µg of either HAZV rNP or CCHFV rNP in 50 µl of sterile saline in the right hind footpad and the same volume of PBS into their left hind footpad. As the control antigen, Bovine Serum Albumin (Sigma-Aldrich) in PBS were used for the control groups. Mice received equivalent amount of BSA in PBS or PBS injected into the right hind footpad and the same volume of PBS into their left hind footpad. The thickness formed in the right hind footpad after antigen stimulation, subtracted by the thickness formed in the left hind footpad after PBS stimulation, highlights the swelling response indicative of delayed-type hypersensitivity (DTH) within 24 h after antigen stimulation. Footpad swelling was assessed using an External Electronic Caliper Gauge (Swiss Precision Instruments, Melville, NY 11747, USA) at baseline and at 24-hour intervals for three consecutive days.

Isolation of murine splenocytes and lymphocytes

Three days after DTH assay, all mice were terminated humanely via cervical dislocation under isoflurane anesthesia. For LPA, the spleen and lymph nodes were aseptically excised and homogenized using a sterile slide to obtain a single-cell culture. The cells were washed twice in Dulbecco's phosphate-buffered saline (DPBS) (Gibco, Thermo Fisher Scientific, Waltham, USA) and centrifuged at 400×g for five minutes. Spleen cells were homogenized and suspended in Ammonium-Chloride-Potassium (ACK) Red

Table 1 The mice utilized in the study were grouped as outlined in the table. Each group, including both positive and negative controls, consisted of five mice. *CICS* CCHFV NP immunized CCHFV NP stimulated, *HIHS* HAZV NP immunized HAZV NP stimulated, *CIHS* CCHFV NP immunized HAZV NP stimulated, *HICS* HAZV NP immunized CCHFV NP stimulated, *BSA* Bovine serum albumin, *NP* nucleoprotein, *PBS* Phosphate buffered saline

		Stimulation			
		CCHFV	HAZV	PBS	BSA
Immunization	CCHFV	CICS	CIHS	-	-
	HAZV	HICS	HIHS	-	-
	PBS	-	-	PBS	-
	BSA	-	-	-	BSA

Cell Lysing Buffer (pH 7.4) containing 150 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM Na_2EDTA. The suspension was incubated twice for two minutes at room temperature to lyse red blood cells. Then, 10 ml of DPBS was added and spun at 400×g for 5 min. The supernatants were discarded, and the splenocytes and lymphocytes were resuspended in RPMI 1640 containing 10% (v/v) FBS and 1% (v/v) PES. The cells were pooled after determining the cell number by trypan blue prior to conducting LPA.

Lymphocyte proliferation assay (LPA)Lymphocyte proliferation test was achieved by using commercial 5-Bromo-2'-deoxyuridine (BrdU) colorimetric kit (Roche Diagnostics, Indianapolis, USA). Briefly, 0.5×10^5 splenocytes/well and 10^5 lymphocytes/well were combined and seeded in a 96-well flat-bottom tissue culture plate (TPP, Sigma-Aldrich, Taufkirchen, Germany). Plates were incubated at time zero with different concentrations (1 and 0.5 µg/ml in 100 µl) and different combinations of rNPs as indicated in Table 1. Concanavalin A (ConA) was used at final concentration 1 µg/ml as positive control stimulant. Experiments were performed in triplicate with RPMI 1640 supplemented with 10% (v/v) FBS and 1% (v/v) PES as culture medium. After two days of incubation, cells were labeled with 10 µM BrdU labeling solution for 24 h. At the end of the experiment, the cell proliferation was determined by performing the BrdU-based ELISA test according to the manufacturer's instructions. The rate of BrdU incorporation is measured and the results are shown as percent response for antigen. Experiments were carried out in triplicate. The percent response was calculated as [(mean OD of the antigen-stimulated group–mean OD of the negative control group)/mean OD of the negative control group] x100.

In-house IgG EIA

An in-house IgG EIA was employed to assess the antibody response to HAZV rNP or CCHFV rNP. Sera from immunized mice were diluted in PBST (pH 7.2) containing 5% non-fat dairy milk and 0.2% Tween 20. The diluted sera were then utilized against HAZV rNP or CCHFV rNP at a 1:100 dilution. Each well of polystyrene 96-well microtiter plates (Immulon1B; Dutscher Scientific) was coated with 1 µg of the respective antigen at 4 °C overnight, using a coating buffer consisting of 35 mM $NaHCO_3$ and 15 mM Na_2CO_3 in 500 mL distilled water with a pH of 9.6. After coating, plates were blocked with 200 µL of 5% non-fat dairy milk in PBST at room temperature for 2 h. Following aspiration, 100 µL of a 100-fold dilution of polyclonal anti-HAZV rNP or anti-CCHFV rNP mice sera in 5% non-fat dairy milk in PBST was added to the wells and incubated at 37 °C for 1 h. Naive mouse sera were employed as a negative control at the same concentration. The plates underwent three washes with PBST and were then incubated at 37 °C for one hour with 100 µL/well of a 1/3000 dilution of HRP-linked goat anti-mouse IgG antibody (Cell Signaling Technology, 7076). After three additional washes with PBST, 100 µL of TMB substrate (Abcam, Cambridge, UK) was added to each well. The reaction was stopped by adding 100 µL of 2 N H_2SO_4 to each well after 10 min of incubation at 37 °C in the dark. Absorbance at 450 nm was measured using an iMarkTM microplate reader (Bio-Rad Laboratories, Inc). All washing steps were performed using the Wellwash™ Versatile Microplate Washer (Thermo Fisher Scientific, Waltham, USA). The results, including the mean and standard deviation (SD) of three independent experiments performed in duplicate, are presented.

Cytokine profiling

Mouse lymphocytes and splenocytes were stimulated with various combinations of rNPs, following the protocol outlined in LPA. Subsequently, the cells were collected. The transcriptional levels of cytokines IL-1B, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12B, IL-17 A, TNF-A, IFN-γ, GAPDH, and GM-CSF were assessed using RT-qPCR. The primer set sequence is presented at Table 2.

Reverse-transcriptase qPCR and primer design

Total RNA isolation from stimulated cells was carried out using the GenJET RNA purification kit in accordance with the manufacturer's instructions. One µg of total RNA was translated into cDNA by using SensiFAST cDNA Synthesis Kit (Bioline, UK) after which 1 µl each of cDNAwas used to perform Real-time PCR with cytokine-specific primers. Mouse cytokine-specific primers for qPCRs were designed using the PrimerBank website and synthesized by Sentebiolab (Sentebiolab Biotechnology, Türkiye) commercially. qPCR reaction consisted of 10 µl of 2x SensiFAST SYBR® No-ROX Mix (Bioline, UK), 1 µl of mouse cytokine-specific forward and reverse primer, 1 µl of cDNA and 7 µl of ddH_2O. qPCR reactions were performed on Rotor-Gene 6000® real-time system (Qiagen AG Germany) according to the following parameters: initial activation at 95 °C for 5 min followed by 40 cycles of 95 °C for 10 s and 60 °C for 15 and 72 °C 20 s. Data was evaluated using software of Rotor-Gene 2.1.0.9. The relative mRNA amount was quantified and normalized using via the ΔCq and $2-\Delta\Delta Cq$ methods. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was chosen as a reference gene for this study, given its stability and validity [40].

Statistical analyses

The statistical analyses were done using the Prism 6for OS X Version 9.2.0 (GraphPad Software, San Diego, CA) program. Significance between grouped EIA data was determined by Mann–Whitney U or a two-tailed Welch's t-tests using Prism 9.2.0 (GraphPad). DTH assay data were expressed as mean ± SEM of the left and right hind footpad thickness difference. For statistical analysis two-way ANOVA and Bonferroni post-tests were used. Significance for all comparisons was accepted at $p < 0.05$ value.

Ethical clearance

The ethical approval for the animal experiments outlined in this study has been granted by the Ethics Committee for Animal Studies at Bezmialem Vakif University in Istanbul, Turkey, with the approval number 4.2019/12.

Results

Study design

The study was designed as shown at Fig. 1. As shown at Fig. 1 and described in methods, Balb/C mice were immunized with different combinations of NP's HAZV and of CCHFV to assess cell-mediated immunity (CMI). Later,

Table 2 Primer set used in cytokine profiling

Cytokine	5'-3' Primer Sequence
INTERLEUKIN 1-BETA	FW: TGGACCTTCCAGGATGAGGACA RV: GTTCATCTCGGAGCCTGTAGTG
INTERLEUKIN 2	FW: TGAGCAGGATGGAGAATTACAGG RV: GTCCAAGTTCATCTTCTAGGCAC
INTERLEUKIN 4	FW: ATCATCGGCATTTTGAACGAGGTC RV: ACCTTGGAAGCCCTACAGACGA
INTERLEUKIN 5	FW: GATGAGGCTTCCTGTCCCTACT RV: TGACAGGTTTTGGAATAGCATTTCC
INTERLEUKIN 6	FW: TACCACTTCACAAGTCGGAGGC RV: CTGCAAGTGCATCATCGTTGTTC
INTERLEUKIN 10	FW: CGGGAAGACAATAACTGCACCC RV: CGGTTAGCAGTATGTTGTCCAGC
INTERLEUKIN 12-BETA	FW: TTGAACTGGCGTTGGAAGCACG RV: CCACCTGTGAGTTCTTCAAAGGC
INTERLEUKIN 17A	FW: CAGACTACCTCAACCGTTCCAC RV: TCCAGCTTTCCCTCCGCATTGA
IFN-GAMA	FW: GCCACGGCACAGTCATTGA RV: TGCTGATGGCCTGATTGTCTT
TNF-ALPHA	FW: GGTGCCTATGTCTCAGCCTCTT RV: GCCATAGAACTGATGAGAGGGAG
GM-CSF	FW: GGCCTTGGAAGCATGTAGAGG RV: GGAGAACTCGTTAGAGACGACTT
GAPDH	FW: CATCACTGCCACCCAGAAGACTG RV: ATGCCAGTGAGCTTCCCGTTCAG

the immunized mice were stimulated with either CCHFV rNP or HAZV rNP through footpads, to evaluate DTH response. Following the measurement of DTH reactions, the mice were euthanized, and blood was collected from the heart. Lymph nodes and spleens were then harvested and employed in the lymphoproliferation assay (LPA).

Production of NPs and confirmation of authenticities

CCHFV rNP and HAZV rNP were produced in *E. coli* with a C-terminal His-tag as described earlier by us (19,38). These highly purified protein antigens were used in DTH and LPA experiments. Western blot analysis using an anti-His antibody was performed to verify the identity of rNP's (Fig. 2A).

To assess the antigenic competency and authenticity of CCHFV NP and of HAZV NP, it was crucial to confirm that both NPs induce a humoral immune response in mice. After the final booster immunization with both NP's, mouse sera were obtained from the facial vein and subjected to Enzyme Immuno Assay (EIA) to assess the humoral immune responses against the relevant antigens. The EIA confirmed the antigenic competence of rNP's and their ability to induce a humoral immune response, as shown in Fig. 2B.

CCHFV rNP and HAZV rNP both induce cell-mediated immunity

Delayed Type Hypersensitivity reactions constitutes a crucial component of CMI and is considered to be primarily a function of Th1 lymphocytes [41]. Here, we assessed and contrasted the capacities of NPs in inducing DTH. The group of mice immunized with CCHFV rNP was stimulated with CCHFV rNP (CICS) or HAZV rNP (CIHS), while the other group immunized with HAZV rNP was stimulated with HAZV rNP (HIHS) or CCHFV rNP (HICS) via footpad for assessment of DTH. The stimulation involved administering the relevant proteins in saline to the mice's right hind footpad, subsequent to their immunization with both rNPs. PBS was injected into the left hind footpad as a negative control. The study measured the differences of thickness between the right and left footpads caused by inflammation over a period of 72-hours, with 24-hours intervals. The increase in mean thickness caused by inflammatory infiltration reveals the intensity of the DTH response [42]. At the 24th hour of measurement, CICS group mice exhibited 42.7%±14.3% mean increase in thickness over control group ($p < 0.0001$), while HIHS group mice showed a mean thickness increase of $31.8 \pm 6.9\%$ ($p < 0.0001$) compared to the negative control group of mice immunized with PBS. At 48 h, the CICS group exhibited a mean difference in DTH response of $39.23 \pm 4.7\%$ ($p < 0.01$), while the in HIHS

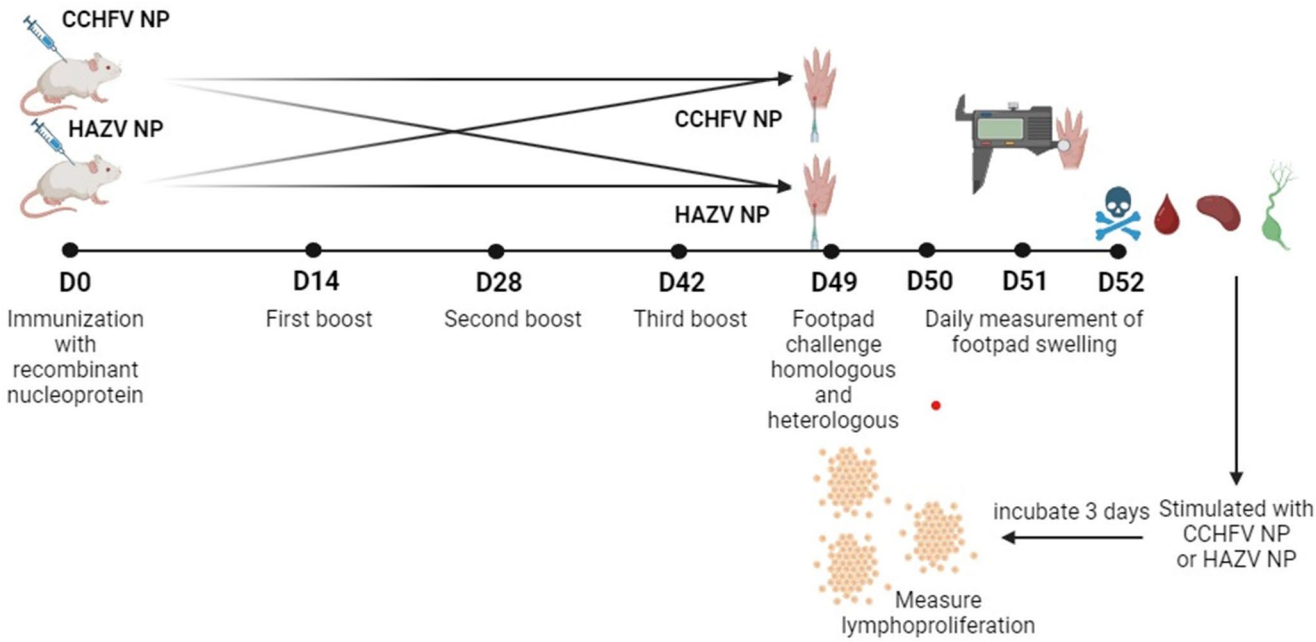

Fig. 1 Study design. In this study, the nucleoproteins from the Hazara JC280 strain (HAZV rNP) and the Crimean-Congo Hemorrhagic Fever Virus Kelkit06 strain (CCHFV rNP) were produced recombinantly. Mice were immunized with various combinations of these proteins to assess cell-mediated immunity (CMI). Subsequently, the immunized mice were subjected to stimulation with either CCHFV rNP or HAZV rNP, administered via the footpad, to evaluate delayed-type hypersensitivity (DTH) activity. Following the measurement of DTH reactions, the mice were euthanized, and blood was collected from the heart. Lymph nodes and spleens were then harvested and employed in the lymphoproliferation assay (LPA)

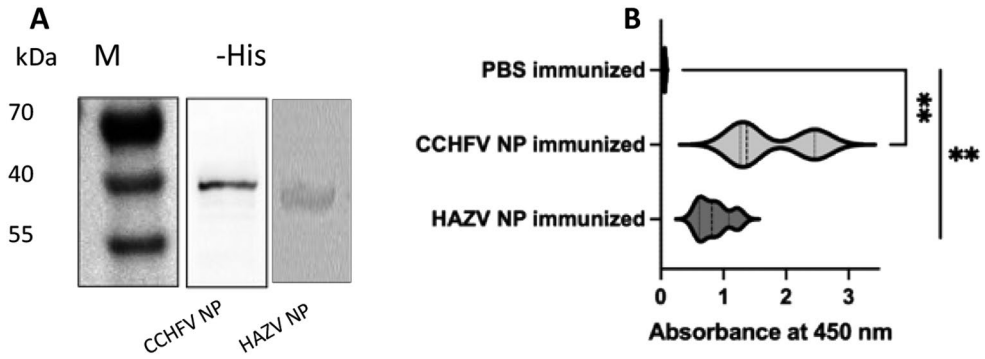

Fig. 2 Production, generation and confirmation of antigenicity of NPs of CCHFV and HAZV. M: marker (Cat. #24052, Intron Biotechnology). (**A**) Western blot analysis of purified rNP using goat polyclonal anti-His tag-HRP at 1:1000 dilution. Demonstration of antigenic fidelity of recombinant NP's in mice. (**B**) Plates were also coated with 1 µg/well purified CCHFV-rNP or HAZV rNP and analyzed against 100-fold-diluted CCHFV-rNP or HAZV rNP immunized mouse sera. PBS immunized mouse sera were used at the same dilutions as negative controls. The antibodies specific to NP were identified using an HRP-conjugated anti-mouse IgG antibody. All mice sera were taken and used after complete immunization with relevant antigens. Each sample was tested duplicate and expressed as mean optical density values at an absorbance value of 450 nm. Each data point represents mean ± SEM. EIA data were compared with two-tailed unpaired *t* test with Welch's correction and found to be significant

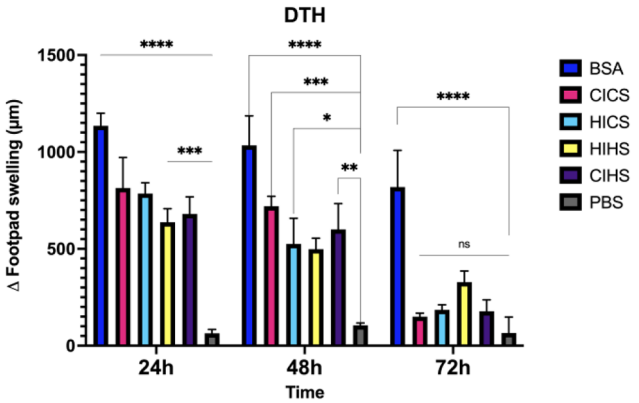

Fig. 3 The DTH responses induced by rNP in mice demonstrated cross-reactivity. Mice were immunized with CCHFV rNP or HAZV rNP, as positive control with BSA and as negative control with PBS as described. One week after last immunization, mice were stimulated by injection of relevant proteins (15 µg in final) or PBS into footpads. The thickness increase was measured with 24-hour interval till 72. hour via electronic caliper gauge. Each data point represents mean values ± SEM of immunized groups (5 mice for each group). The footpad thickness difference of mice was compared to corresponding negative and positive controls and found statistically significant (*, $p < 0.1$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; and ns, not significant). Significance for all comparisons was accepted at $p < 0.05$ value. The data from each group from at the same time point were analyzed with Two-way ANOVA and Bonferroni's multiple comparison tests. ΔFootpad Swelling: The thickness difference between antigen injected and PBS injected footpad. *BSA* Bovine serum albumin, *NP* nucleoprotein, *PBS* Phosphate buffered saline

group of the difference was 26.7 ± 6.07% ($p < 0.1$). There was no significant difference in the incrassation between the two groups at 72 h ($p > 0.05$). The mean food pad swelling results demonstrate that both proteins elicit a strong DTH reaction in comparison to the control mice (Fig. 3).

Similar anti-NP CMI indicating cross reactive splenocytes

In the next set of experiments, cross-reactivities of splenocytes to the NPs were determined through DTH. The thickness of footpads in the CIHS mice group increased significantly due to DTH response, with a mean increase of 34.8 ± 8.99% ($p < 0.01$) at 24 h compared to the control group. This increase persisted at 31.9 ± 14.17% ($p < 0.01$) at 48 h, as shown in Fig. 3. The HICS mice group exhibited a significant increase in footpad swelling due to DTH response and these responses were compared to the control group. The mean increase was 38.7 ± 4.7% ($p < 0.001$) at 24 h and persisted to 27.6 ± 12.13% ($p < 0.1$) at 48 h (Fig. 3). These findings were interpreted as a confirmation that both NPs induce a robust DTH response which recognize antigenic epitopes on the NP of the compared virus in an in vivo milieu.

Additional to DTH assay, cross-reactivity of splenocytes was investigated by the LPA test. The validity of the LPA test was assessed by using relevant antigens in immunized mice with the control groups. Stimulation of CICS mice group splenocytes with 100 ng of CCHFV rNP induced a 165% greater lymphoproliferative response while stimulation of HIHS-immunized mice splenocytes with 100 ng of HAZV rNP induced 139% more lymphoproliferative response compared to unstimulated splenocytes (Table 3). Furthermore, in CIHS group, 116% more lymphoproliferative response were detected compared to unstimulated control splenocytes. In the HICS group cells stimulated with 100 ng of antigen, this rate was 59% more compared to the unstimulated control group splenocytes (Table 3). These results suggest that in NP-immunized mice with either

Table 3 Lymphoproliferative responses induced by NPs at different doses in mice. To detect the lymphoproliferative response in mice, lymphocytes and splenocytes of mice immunized with rNP's were collected one week after last immunization. A total of 1.5×10^5 cells/well (lymphocytes and splenocytes were pooled in 1:0,5 ratio and were stimulated with different concentrations of rNP's (1 and 0.5 µg/ml in 100 µl) and with concanavalin A (1 µg/ml in 100 µl) as positive control. After a two days incubation, cells were labeled for 24 h with 10µM BrdU labeling solution. The increases in cell numbers were assessed by the amount of the BrdU-labeled DNA. Anti-BrdU antibody and incorporated BrdU reactions were measured colorimetrically at 450 nm. Percent response is calculated as follows: [(mean OD of the antigen-stimulated group–mean OD of the negative control group)/mean OD of the negative control group] x100. *SE* standard error of the mean, *OD* optical density, *con A* concanavalin A

GROUP	Stimulant	Amount of antigen (µg/ml)	LYMPHOPROLIFERATON SI	SE	% Response
CICS	CCHFV NP	1	2.65	0.0025	165
		0.5	1.66	0.038	66
		0		0.04	0
	ConA	1	1.38	0.068	38
HIHS	HAZV NP	1	2.35	0.0025	139
		0.5	2.70	0.038	170
		0		0.04	0
	ConA	1	5.021	0.068	402
CIHS	HAZV NP	1	1.72	0.0025	72
		0.5	2.16	0.038	116
		0		0.04	0
	ConA	1	3.95	0.068	295
HICS	CCHFV NP	1	1.59	0.0025	59
		0.5	1.096	0.038	9
		0		0.04	0
	ConA	1	4.68	0.068	68

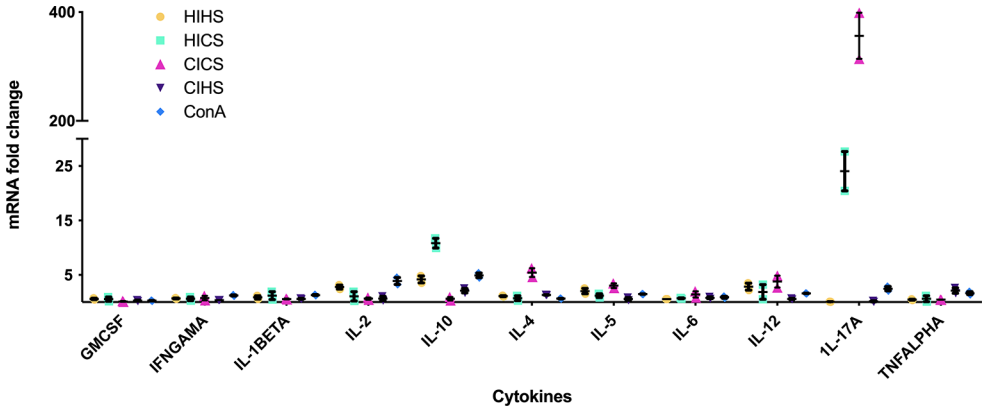

Fig. 4 Assessment of the quantity of cytokine mRNAs produced by splenocytes in vitro. Mouse splenocytes immunized with rNPs of CCHFV or HAZV were stimulated in vitro with rNPs of CCHFV or HAZV, and the levels of cytokine mRNAs was measured by RT-qPCR. Data for the final analysis from three independent experiments are presented and expressed as the mean ± standard error (SE). Mean group values were compared by one-way ANOVA and nonparametric

protein, splenocytes responded to the NP of the comparator virus in vitro indicating the presence of cross-reactive antigenic epitopes on both proteins. These results confirm that LPA is an appropriate in vitro setup to test the immune recognition as well as to determine the cross reactivities of splenocytesagainst NPs.

Detection of high-level IL-17 a response to CCHFV NP of CCHFV

An alternative method for assessment of cellular immunity to any given antigen is the detection of cytokine response by splenocytes to the stimulation. In the present study, we opted to analyze the transcripts of cytokines in LPA. Quantitative RT-qPCR was employed to measure mRNA levels for various cytokines. The levels of cytokine mRNAs were found to be comparable among the groups that were stimulated with HAZV rNP, as depicted in Fig. 4. The most remarkable results in qPCR assay were related to IL-17 A mRNA levels. The data on IL-17 A specific mRNAs indicated that splenocytes stimulated with CCHFV NP produced an outstanding level of IL-17 A mRNA. In the CICS test group, 356-fold more IL-17 A mRNAs were detected compared to the control cells and in HICS animals, the increase in the quantity of IL-17 A mRNA was 24-fold (Fig. 4). These findings imply that the two NPs elicit distinct splenocytes reactions, specifically with regards to IL-17 A mRNA transcripts.

Discussion

This study aimed to explore whether the NPs of CCHFV and HAZV exhibiting significant homology at the protein level, induce cross-reactive CMI. The choice of viral NPs for investigation was largely based on their structural similarity. The RNA hybridization test was performed to show a 60% similarity in the NP sequences of the two viruses [18]. Additionally, their abundance in the virions of CCHFV and related viruses suggests potential significant roles played in the virus life cycle as well as in stimulating immune responses with common features during infection.

While the precise implications of viral NPs in natural infections are not fully elucidated, vaccine studies strongly indicate their crucial role. Some studies ascribed a fully protective function to NP and others disputed this claim. For example, NP-based vaccines for bunyaviruses, including Rift valley virus, have been reported to demonstrate that NP is effective in preventing disease by stimulating a strong memory T cell response [43]. Despite generating strong humoral and cellular immune responses, the vaccine against CCHFV constructed using Modified Vaccinia virus Ankara to express the CCHFV NP, failed to confer protection in mice during testing in the mouse model [44]. However, in a recent mouse study, it was reported that a CCHFV virus-like replicon particle vaccine provided protection characterized by a robust anti-NP humoral and cellular immune responses [45]. Similarly in another investigation, a DNA-based vaccine against CCHFV containing NP and full glycoprotein GPC, demonstrated efficacy in protecting against mortality in Cynomolgus macaques [46]. In these studies, T and B cell responses against NP were considered to be functional and therefore, authors suggested that a combined T and B cell responses should be aimed in vaccinations against CCHFV rather than just neutralizing function alone [47–49]. Consequently, a comprehensive understanding of the contribution of CCHFV NP to cellular immunity might bring some clarity to this important aspect of CCHFV immunology. To investigate the impact of NP in the overall immune response, the cellular arm of immunity to CCHFV NP was addressed here and comparing the response to HAZV NP adds additional information related to the question of surrogacy of HAZV for CCHFV.

In CCHFV survivors, virus-specific T cell responses have been identified to be predominantly against the NP epitopes [29]. Considering this rationale, our objective was to evaluate the existence and potency of CMI response to rNPs, both in vivo and in vitro. To validate successful immunization, the humoral immune response in vaccinated animals was initially assessed through EIA. Animals immunized with CCHFV NP demonstrated significantly elevated humoral immune responses compared to those immunized with HAZV NP. While the notion that CCHFV NP might possess higher immunogenicity was proposed, this remains a hypothesis warranting additional evidence. Despite the precise cause of this phenomenon eluding complete understanding, our study emphasized that generating a humoral immune response specifically against the relevant protein proved adequate for the study's efficacy, independent of the degree of animal immunization. To illustrate the in vivo manifestation of CMI, we assessed the DTH test, which serves as a prominent arm of such response [50]. This test involves sensitizing an individual to a specific antigen through prior exposure or immunization. Upon subsequent exposure to the same antigen, a delayed inflammatory reaction occurs. This reaction is primarily orchestrated by the activation of T cells, specifically CD4 + and CD8 + T cells, which release cytokines and other mediators, resulting in inflammatory swelling at the site of antigen exposure. The characteristic late onset of Delayed Type Hypersensitivity (DTH) reactions, typically within 24 to 72 h, distinguishes them from immediate hypersensitivity reactions observed in allergies, where allergic responses happen earlier and manifest more rapidly. Following the stimulation from of the footpad of immunized mice with HAZV NP or CCHFV NP, a substantial enlargement in foot pad swelling was noted after 24 h, persisting for three days. These findings demonstrate that both CCHFV NP and HAZV NP could trigger a T cell immune response and lead to possible T cell cross-reactivity in vivo (Fig. 3). Our results on similar robust DTH responses to both NPs as correlates CMI indicate that NPs might constitute a significant viral antigenic entity which is functional during the immune responses induced by protective candidate vaccines as well as during recovery from the natural infection in surviving patients. Our results additionally show that HAZV NP as a cross reactive antigen in inducing DTH in CCHFV NP immunized animals could deserve to be further scrutinized as a surrogate for CCHFV NP in vaccine and immunology investigations.

The LPA test is another mean to assess the existence and strength of the CMI in the presence of stimulating antigen in vitro, whereas DTH reveals T cell response and potential T cell cross-reaction in vivo [51]. The Lymphoproliferative Assay (LPA), similar to DTH assay, involves stimulating splenocytes using the relevant NP antigens from mice immunized with either CCHFV rNP or HAZV rNP. Our LPA findings revealed a noteworthy increase in the number of splenocytes across all tested groups when exposed to the stimulating antigens. These results suggest that splenocytes from mice immunized with the respective proteins can recognize the epitopes of both CCHFV NP and HAZV NP. This implies that these NPs may share similar epitopes for splenocytesproviding further confirmation that HAZV NP

could constitute a proxy molecule for CCHFV NP in more detailed studies.

To investigate CMI, the analysis cytokines response upon exposure to a stimulating antigen provides valuable insights. This in vitro analysis allows for the identification of specific T cell subsets that mediate the immune response. In a previous study, the cytokine analysis in an immunocompetent mouse model revealed consistently higher levels of IL-2, IL-4, IL-6, IL-10, TNF-α, and IFN-γ. Furthermore, the immune response induced by a plasmid-based vaccine, where the CCHFV NP was cloned into Sindbis virus vector, resulted in increased release of IFN-γ, IL-2, and TNF-α [52]. In the present study, cytokine analyses were performed by RT-qPCR after LPA by using stimulated splenocytes. There were notable differences in the levels of cytokines transcribed in response to CCHFV rNP. Among the cytokines transcribed through in vitro stimulation of HAZV rNP immunized mouse splenocytes with HAZV rNP (HIHS) during LPA, the level of IL-10 and IL-12 were particularly noteworthy. The level of interleukin-10, which plays a critical role in dampening excessive immune responses and maintaining immune balance [53], was found to be four-fold higher transcribed compared to the control group. Although qPCR does not fully represent actual cytokine levels, it provides valuable insights into gene expression. The observation that IL-10 levels were lower in the CCHFV NP immunized groups compared to the HAZV NP immunized groups may indicate a differential immune modulation by these viral proteins. IL-10 is a key regulator in immunity, often involved in controlling inflammation and immune responses [54, 55]. This suggests that HAZV NP might induce a more regulatory or anti-inflammatory response, potentially influencing the overall immune response differently compared to CCHFV NP. Further studies measuring protein levels are necessary to fully understand these dynamics. In the HIHS group once again, the level of IL-12, which promotes the development of a Th1 immune response [56], was 2.8-fold higher transcribed, compared to GAPDH. Additionally, in HIHS group, IL-17 A mRNA level remained at the basal level (0,06-fold). However, when HAZV rNP-immunized splenocytes were stimulated with CCHFV rNP (HICS), the mRNA level of IL-17 A showed a substantial increase (24-fold) (Fig. 4B). In this group, the mRNA levels of IL-10 were increased by approximately 10-fold, while IL-12 levels stayed similar to the HIHS group. In the case of CCHFV-immunized mouse splenocytes stimulated with CCHFV rNP (CICS) during LPA, the mRNA levels of IL-4, a cytokine that stimulates B cells [57], and IL-12 were found to be 5.4-fold and 3.7-fold higher compared to GAPDH, respectively. Notably, the mRNA level of IL-17 A in the same group exhibited an extraordinary 356-fold increase. When the same splenocytes were stimulated with HAZV rNP (CIHS), the mRNA level of IL-10 increased by 2.1 times, while IL-17 A levels remained within the normal range (0.01-fold). These findings highlight distinct cytokine responses associated with HAZV rNP and CCHFV rNP stimulation, indicating possible differences in immune modulation and activation pathways. It is likely that CCHFV NP has additional elements favoring IL-17 dominant response tilting the immunity towards an inflammatory reaction. Furthermore, Cross et al. reported elevated levels of IL-17 in the serum of CCHFV-infected macaques and indicated that this factor could potentially contribute to vascular leakage in CCHF patients through the mediation of high circulating nitric oxide [58–60]. The results observed in our study that elevated IL-17 A mRNA level observed after CCHFV NP stimulation might imply a potential association between IL-17 and hemorrhagic illnesses. Additional investigations are warranted to elucidate the relationship between IL-17 levels and hemorrhagic symptoms of CCHF disease. In this study, the examination of IL-17 levels has been confined only to the mRNA level. Therefore, it is imperative to explore the IL-17 levels through diverse methodologies to obtain a comprehensive and more accurate information. Another factor to be considered in addressing the differential cytokine responses induced by two NPs is the fact our set up is an in vitro one and at our experiments NPs are introduced to the immune elements as exogenous protein. Therefore, it might be possible that the nature of the immune response in viral infection settings where viral proteins are primarily cytosolic might differ affecting the types and subsets of immune splenocytes activated. Additionally, the sources of increased IL-17 A cytokine levels are not addressed in this study and this point deserves to be scrutinized.

While the consistent production and purification of proteins using identical methodologies contribute to the reliability of our results, it is important to acknowledge potential limitations. The reliance on bacterial expression systems and the subsequent denaturation and purification steps may introduce variations in the protein structure or conformation, impacting their biological activities. Another limitation pertains to the measurement of cytokine levels via RT-qPCR. In the study, cytokine analyses were carried out at mRNA level. Even though determination of cytokines at mRNA level is a well-accepted methodology, it would be desirable to assess cytokines as secreted proteins.

In conclusion, our study represents the first comprehensive investigation that compares the cellular immune responses between CCHFV and HAZV. Overall results of this study provide a basis that both NPs might have similar antigenic epitopes in inducing CMI in experimental animals and potentially serve as surrogate for each other. It is important to highlight that this study focused solely on the immune response of cells to NP from both viruses. To gain

a comprehensive understanding of the utility of HAZV in investigating the immunology and pathogenesis of CCHFV infection, it will be crucial to compare the functions and antigenicity of each CCHFV protein with those of HAZV proteins. Furthermore, the investigation of the different subsets T cells specific to NPs should be performed in infection models as well as in CCHF patients. These additional studies might contribute to a more comprehensive characterization of the immune responses against CCHFV and HAZV.

Author contributions MKY and MZD designed the concept of the project. MKY wrote the manuscript. MKY, EK, MZD: writing—reviewing and editing. MKY, EK, NSGC performed the experiments. All the authors have read and agreed to the published version of the manuscript.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Competing interests The authors declare no competing interests.

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