Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 27 November 2018 doi:10.20944/preprints201811.0603.v1

Peer-reviewed version available at *Viruses* **2019**, *11*, 75; doi:10.3390/v11010075

Adjuvant Potential of CD24 on Immunogenicity and Lethal Challenge Protection of a DNA vector Expressing Nucleocapsid Protein of Crimean Congo Hemorrhagic Fever Virus

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Abstract

Crimean Congo hemorrhagic fever virus (CCHFV) is the causative agent of a globally-spread tick-borne zoonotic infection with an eminent risk of fatal human disease. Imminent public health threat posed by disseminated virus activity and lack of an approved therapeutic make CCHFV an urgent target for vaccine development. We described the construction of a DNA vector expressing nucleocapsid protein (N) of CCHFV (pV-N13) and investigated its potential to stimulate cytokine and total/specific antibody responses in BALB/c and challenge experiment in IFNAR$^{-/-}$ mice. Due to lack of sufficient antibody stimulation towards N protein, we have selected CD24 protein as a potential adjuvant which has proliferative effect on B and T cells. Overall, our N expressing construct when administered solely or in combination with pCD24 vector elicited significant cellular and humoral responses in BALB/c, despite variations in particular cytokines and total antibodies. However, the stimulated antibodies produced due to expression of N protein have shown no neutralizing ability in VNA. Furthermore, challenge experiments were revealed protection potential of N expressing construct in IFNAR $^{-/-}$ mice model. In conclusion, we have shown that CD24 has prominent effect as a genetic adjuvant when co-delivers with a synergic foreign gene expressing vector. Besides, targeting of S segment of CCHFV can be considered as a practical way in developing vaccine against this virus due to its ability to induce immune response which leads to protection in challenge assays in IFN-gamma defective mice models.

Key words: CCHFV, CD24, nucleocapsid, genetic adjuvant, immunogenicity, IFNAR$^{-/-}$ mice

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Introduction

Crimean Congo Hemorrhagic Fever (CCHF) is a tick-borne viral infection with a risk of fatal hemorrhagic disease in endemic areas. Symptomatic CCHF in humans presents as a febrile disease with severe hemorrhagic manifestations and a mortality rate of up to 40%, depending on various viral and host factors [1]. The aetiological agent is Crimean Congo Hemorrhagic Fever Virus (CCHFV), classified in Orthonairovirus genus of the Nairoviridae family. Similar to the other members of the *Nairoviridae* family, viral genome consists of 3 negative sense single-stranded RNAs, frequently referred as small (S), medium (M) and large (L) segments [2].

As a highly contagious and potentially-lethal infection that is difficult to treat, prevent and control, with the potential to cause nosocomial spread, CCHFV is an eminent target for vaccine development [3]. In general, envelope glycoproteins are the initial and most obvious viral structure considered for novel vaccine design, due to their abundance in the virion and direct relation with the host immunity. However, these regions demonstrate substantial sequence diversity among different CCHFV strains, especially during propagation in ticks prior to human transmission [4]. Therefore, alternate immunization targets must also be evaluated for a broad coverage of geographically-segregated strains [5].

Nucleocapsid protein (N) of CCHFV is the main Open Reading Frame (ORF) found in the S segment and involved in virus encapsidation. This protein is well-conserved among CCHFV strains and constitutes the immunodominant antigen with several T-cell epitopes and no known B cell targeting regions and is considered as a potential target in vaccine development for some Bunyaviruses such as Rift Valley virus [6]. The lack of B cell epitopes due to internal structure of this protein results in lower levels of neutralizing antibodies. However, it has been demonstrated that there is no clear association with neutralizing antibody response and survival rates in the challenged $IFNAR^{-/-}$ mice. It seems that balanced Th1 and Th2 responses are essential for protection in CCHFV induced disease [7].

Cluster of differentiation 24 (CD24) is a highly glycosylated mucin-like cell surface protein present in B and T lymphocytes, neutrophil and macrophages. Activated B cells possessing CD24 can effectively co-stimulate the clonal expansion of CD4+ T lymphocytes [8]. As previously demonstrated, CD4+ T cells have critical role in combating viral infections directly, as well as their enhanced effect on CD8+ T cells to engage with dendritic cells [9]. The most important known function of CD4+ T cells is to promote high affinity, neutralizing antibodies production by B lymphocyte cells [10]. Recently, evidence of these cells having a

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direct effect on viral agents by stimulation of antiviral cytokine production or cytotoxicity effect have been identified. Naive CD24 T cells can recognize viral pathogens through antigen presenting cells (APCs). Subsequently, Th1 cells are generated due to type I interferon (IFN) and IL12 [11]. However, the presence of CD24 in dendritic cells has potential to down regulate T cell proliferation [12].

In this research, we assessed immunogenicity and protection potential of N expressing DNA vector (pV-N13) in BALB/c and IFNAR$^{-/-}$ mice by measuring cytokine and total/N specific antibody responses in combination with independently expressed CD24 (pCD24) as a potential genetic adjuvant. Based on this strategy, along with our immunization regime, we hypothesized that simultaneous expression of CD24 may contribute to the regulation of B and T cells proliferation to elicit the antibody production in the immunized animals.

Material and Methods

Ethics Statement

All animal experiments were performed with official permission of the Ankara University Ethical Committee for Animal Experiments (17/12/2014; 2014-23-155 and 17/10/2018; 2018-20-130). Animal samplings were conducted according to the national regulations on the operation and procedure of animal experiments ethics committees (Regulation Nr.26220, Date: 09.7.2006). Mice were humanely euthanized by CO_2 exposure and cervical dislocation. Multiple observations per day were conducted to confirm animals' welfare, with constant access to autoclaved water and food provided to each individual.

Cells

SW13 cells were used to propagate the CCHFV and perform virus neutralization assay. For indirect immunofluorescence assays (IIFA) using immunized mice sera, we used BHK-21-C13 and BHK-N (stably expressing CCHFV N) cells. Cell lines were obtained from Department's cell culture collection and cultured in EMEM (BHK21-C13 and BHK-N cells) and Leibovitz's L-15 (SW13 cells) media supplemented with 10% heat inactivated FBS (Biological Industries, Israel), 1% penicillin-streptomycin (Biological Industries, Israel) and 1% L-glutamine (Biological Industries, Israel). All steps involving infectious virus manipulation were performed in BSL3 (+) facility of the Virology Department, Faculty of Veterinary Medicine, Ankara University, Turkey.

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Virus Propagation

For virus cultivation, 90% confluent SW13 cells in T75 culture flasks were inoculated with CCHFV ANK-2 at moi of 0.1 and incubated at 37°C up to 5 days to complete the cytopathic effects, observed as cell detachment and bubbling. The viruses were subsequently harvested, tittered and stored at -80°C.

Plasmids Construction

RNA purification and cDNA synthesis from CCHFV ANK-2 infected SW13 cell supernatants were undertaken by RNeasy Mini Kit (Qiagen, Germany) and Superscript IV First Strand Synthesis kit (ThermoScientific, USA), according to manufacturer's instructions. Complete CCHFV N-coding region was amplified by using Phusion High Fidelity DNA polymerase (ThermoScientific, USA). The primers used to amplify CCHFV N-coding region were designed based on the sequence of another local isolate (Kelkit strain, Gene Bank Accession Number: GQ337053). PCR reactions were performed for 35 cycles as follows: initial denaturation at 98°C for 30 seconds, denaturation at 98°C for 10 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 40 seconds followed by a final extension of 10 minutes at 72°C. PCR products were gel purified and cloned into pVAX-1 (Invitrogen, USA) using SLiCE extract which was prepared from *Escherichia coli* strain PPY, as described previously to construct pV-N13 [13]. PCR product used in SLiCE reaction was flanked by 50 bp homologous arms to EcoRI site of pVAX-1 vector's MCS to guarantee in vitro homologous recombination (Table 1). CD24 amplification (Table 1) and subsequent pCD24 construction were performed based on cloning of CD24 in pVAX-1 as described previously [14]. pV-N13 and pCD24 constructs were verified by colony PCR, restriction enzyme digestion and next generation sequencing. Endotoxin-free plasmids were prepared by Purelink Expi Endotoxin-free Maxiprep Kit (ThermoFisher, USA) and quantitated (Nanodrop C1, UK), prior to immunization.

In vitro Protein Expression

To conduct IIFA following 24 hours of cell cultivation in 24 well plates, pV-N13 and pCD24 plasmids have been separately transfected into the cells by using Lipofectamine 3000 (ThermoScientific, USA) according to the manufacturer's instruction. Forty-eight hours post-transfection, cells were fixed with 3.7% formaldehyde followed by blocking with 5% skimmed milk (Cell Signaling, USA) in 1x TBS buffer containing 0.2% Tween-20 (1x TBST) for 90 minutes. For pV-N13 construct, anti-CCHFV-N human polyclonal antibody and for

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pCD24 vector primary antibody of CD24 FITC-antibody (ThermoScientific, USA) were diluted by 1:250 in 1x TBST buffer then added to each well followed by overnight incubation at +4°C. Incubation with secondary antibody (FITC-labelled anti-Mouse Whole IgG; Sigma, USA) was performed at room temperature for 1 hour. Immunoreactive cells were visualized by examination with Axio Vert A1 Microscope (Ziess, Germany).

For western blot assay, pV-N13 transfected BHK21-C13 cells were scraped 72 hours post-DNA delivery, collected by spinning and lyzed using PRO-PREP solution (Intron Biotechnology, Korea) according to the manufacturer's instruction. After quantitation by Bradford assay kit (ThermoScientific, USA), proteins were separated by Mini-Protean TGX Stain free precast gels (BioRad, USA) in 1x Tris/Glycine/SDS buffer (BioRad, USA) and transferred to PVD membrane using Trans-Blot Turbo Transfer System (BioRad, USA). After immobilization of bands, blocking and incubation with primary antibody were performed as described earlier. The membrane was treated with anti-human-IgG-HRP conjugate (Sigma, USA) at room temperature for one hour. The bands were visualized after incubation with Clarity Western ECL substrate solution (BioRad, USA) for 10 minutes at dark and imaged using ChemiDoc MP System (BioRad, USA).

BHK-N cells

To perform IIFA from serum samples of immunized mice, we have developed a BHK21-C13 cell line that is stably transfected by pCDNA-N plasmid. This plasmid was created by insertion of N encoding gene in pCDNA3.1 vector. For this purpose, after cell transfection, the selection steps were performed in the presence of 500µg/µl geneticin (G418) for two weeks. The survived colonies were collected and pooled and continued to grow in the DMEM media containing the selective antibiotic pressure.

Immunization of Mice

A total of twenty 8-10-week-old BALB/c mice were randomly divided into five groups. The immunization was repeated twice with an interval of 2 weeks. Blood samples were collected from tail vein on days 0, 14 and 28. Collected serums were used in neutralization, IIFA, cytokine and total/specific antibody assays. In addition, the immunized mice were humanely euthanized on day 28 and splenocytes from each individual were collected for in vitro cytokine assay (Fig. 1A-B).

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Challenge Experiments

Prior to mice challenge, the lethal doses of 4 different local CCHFV strains isolated from human cases were analyzed in IFNAR$^{-/-}$ mice. Mice were inoculated intraperitoneally with virus dilutions in triplicate and observed twice a day for symptoms including appearance changes, depression, weight loss and death. Following detection of the suitable strain and relevant lethal dose, a total of 20 IFNAR$^{-/-}$ mice were immunized based on the given schedule for BALB/c mice and animals were challenged with 100LD$_{50}$ of the virus 2 weeks after final booster. The mice were observed daily for symptoms during 13 days post challenge.

IIFA

The presence of N specific antibodies has been analyzed by IIFA in BHK-N cells. For this purpose, heat inactivated serums (taken on day 28) and 1/50 dilution of each ones added to 4 wells of a 96 well plate containing confluent BHK-N previously fixed by 3.7% formaldehyde and blocked using 5% skimmed milk in 1x TBST buffer. After a 90-minute incubation at room temperature (RT), the secondary antibody of FITC-labelled anti-Mouse IgG (whole molecule) has been added to wells. Following one-hour incubation at RT, cells were visualized by fluorescence microscope.

Virus Neutralization Assay

Neutralizing activity of anti-N antibodies in immunized mice were evaluated via virus neutralization assay (VNA), using the CCHFV ANK-2 isolate under BSL3 (+) conditions. Briefly, serum samples were inactivated at 56°C for 30 minutes, serially-diluted (two-fold in DMEM) and mixed with an equal volume of 100TCID$_{50}$ virus titer in duplicate and incubated for 1 hour at 37°C. The serum-virus mixtures were subsequently inoculated onto 1-day-old SW13 cells, grown in 24-well plates. Infected cells were further incubated under the identical conditions for 4-5 days, with daily observation via inverted microscope for the virus-induced cytopathogenic effects.

Total Antibody Isotyping

Serum samples obtained from immunized mice on day 0 and 28 were used for the determination of total antibody isotypes, using Total Antibody Isotyping ELISA kit (ThermoFischer, USA). Briefly, 50 µl of diluted serums (1:5000) was mixed with equal volume of goat anti-mouse IgG+IgA+IgM HRP conjugate and kept at room temperature for 1 hour. Following 3 washing steps, 75 µl of the TMB substrate was added. After 15 minutes, TMB stop solution was added and the reaction was read at 450 nm in ELISA reader (Titertek Multiskan, Finland).

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Cytokine assay

Individual spleens were aseptically collected and were dissociated by cell strainers. Red blood cells were lysed by RBC lysis buffer (BI, Israel), washed with 1x DPBS and final splenocyte pellet was resuspended in RPMI 1640 media (Sigma, USA). Subsequently, cells were dispersed to 24-well plates at a concentration of $2{,}5x10^5$ per well and immediately infected with 10 moi of CCHFV ANK-2. Infected splenocytes were further incubated for 72 hours at 37°C and 5% CO_2 atmosphere. Culture supernatants were collected at 48 and 72 hours of incubation and stored at -80°C until further use. Measurement of cytokines derived from both mice sera (on days 0 and 28) and splenocyte supernatants have been performed using LEGENDplex™ Mouse Th_1/Th_2 Cytokine Panel (8-plex) kit (BioLegend, USA) as described by the manufacturer using FacsCanto II Flow Cytometer platform (BD Bioscience, USA).

Statistical analysis

Antibody isotyping data and cytokine levels among groups were evaluated using and two-way (Sidak's *post hoc* correction) ANOVA by SPSS (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY, USA). All data were presented as Mean ± SD. Graphs were produced using GraphPad Prism version 7.0 (GraphPad Software, San Diego, CA, USA; www.graphpad.com). Statistical significance level was determined as 0.05. All molecular biology procedures were simulated using SnapGene Viewer software (www.snapgene.com)**.**

Results

Vector Construction and In vitro Expression of CCHFV N

After virus propagation in SW13 cells (Fig. 2B) and PCR amplification of S segment from extracted RNAs with no codon optimization, pV-N13 plasmid was constructed via SLiCE approach due to its simplicity and efficiency (Fig. 2C). In addition, pCD24 construct was generated by standard cloning method. In IIFA, CCHFV N (Fig. 2D, E) and CD24 (Fig. 2F, G) expression could be visualized as green fluorescence 48 hours post transfection. In Western blotting, the 52 kDa N protein was detected in the cells after 72 hours post-transfection (Fig. 2H).

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Serological assay

The pCD24 construct induced the highest level of total antibody isotypes among study groups except for IgM, which was significantly elevated in pV-N13 plus pCD24 group. The pV-N13 plus pCD24 and pV-N13 groups showed a relatively significant amount of IgG1, IgG2a, IgG2b and IgG3 antibodies (Figure 3A-E). The IgG2a/IgG1 ratio was also determined and exceeded 1 only in the pCD24 group (Figure 3F). Despite high amounts of antibodies produced, all sera subjected to VNA revealed a lack of detectable neutralizing activity, at 14- and 28-days post immunization. In IIFA, serum samples (days 14 and 28) from the groups of pV-N13 and pV-N13 plus pCD24 were observed to bind the stably expressed N in BHK-N cells (Figure 3G-H).

Cytokine Assay

Virus stimulated splenocytes from pV-N13 and pV-N13 plus pCD24 immunized BALB/c mice showed significant amounts of IFN-gamma and IL-2 cytokines after 48- and 72-hours cultivation. These cytokine responses were comparably elevated in pCD24 group despite the lack of in vitro viral protein expression (Fig. 4A-B). The pV-N13 plus pCD24 group also produced sufficient levels of IFN-gamma in the serum. Moreover, pV-N13 and pV-N13 plus pCD24 groups revealed a prominent potential for IL-2 stimulation (Fig. 5A-B).

pV-N13 plus pCD24 group showed the highest levels of IL-4, IL-5 and IL-13 secretion in the supernatant of virus stimulated splenocytes. pV-N13 immunized mice also revealed a potential to produce significant amounts of IL-4 and IL-13. In spite of IL-13, pCD24 group has diminished responses of IL-4 and IL-5 in the supernatants (Fig. 4C-E). Significantly elevated levels of IL-4, IL-5 and IL-13 were observed in pV-N13 plus pCD24, pV-N13 and pCD24 groups. Mice receiving pV-N13 plus pCD24 demonstrated the highest IL-5 production (Fig. 5C-E).

In the virus stimulated supernatants, the highest amounts of TNF-alpha and IL-6 cytokines were detected in the pV-N13 plus pCD24 group. The pCD24 immunized mice showed a significantly elevated IL-10 levels. Furthermore, TNF-alpha and IL-6 secretion are relatively increased in the pV-N13 group (Fig. 4G-H). IL-10 and TNF-alpha is pronounced in pV-N13 plus pCD24 immunized mice group. On the other hand, IL-6 production is markedly increased in pCD24 group (Fig. 5G-H).

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Challenge Studies

Three local CCHFV strains produced mortality in IFNAR$^{-/-}$ mice during in vivo infectivity assays (Fig. 6A). Based on the growth performance of viruses in SW13 cells and infectivity in IFNAR$^{-/-}$ mice (LD50=1:1000/0,2mL), CCHFV ANK-2 strain was selected as the challenge virus. Challenge assay was performed in immunized IFNAR$^{-/-}$ mice 2 weeks following the final booster dose on day 28. The survival rate and body weight percentage were calculated on day 13 post-challenge. The pV-N13 and pV-N13 plus pCD24 groups survived after challenge and body weight in both groups were almost stable except for the slight decrease during the first days (Fig. 6B-C). Animals immunized with saline, pVAX1 and pCD24 died on days 5, 6 and 8, respectively (Fig. 6B).

Discussion

The efforts for finding a suitable gene of CCHFV as vaccine target are well-justified, due to the imminent public health threat posed by disseminated virus activity in broad geographical regions and lack of an approved therapeutic regime. While a vaccine licensed for human use would decrease disease incidence and infection-associated morbidity and mortality, animal vaccination is likely to have an impact on vector and reservoir control, resulting in reduction of zoonotic transmission [3].

DNA vector-based delivery of immunogenic viral proteins such as glycoproteins and nucleocapsid is a practical approach for CCHFV vaccine development, since any other strategy requiring virus handling would necessitate high biological containment facilities [15]. Currently, immune response during CCHFV infection is not well understood, with scarce data on T and B cell epitopes in virally-encoded proteins [16]. Studies based on glycoproteins (precursor Gc and Gn) and nucleocapsid, have revealed contradictory results for protection in challenges of IFNAR$^{-/-}$ and STAT-1 knock-out mice. However, it is generally accepted that activation of CD4^{+} and CD8^{+} responses are involved in the immune control of infection severity and neutralizing antibodies play little role in protection. Therefore, we focused on N, suggested as an ideal target in protection assays in various platforms such as modified Vaccinia Ankara Virus (MVA), Adenovirus (AdV), Virus-like Particles (VLP) and DNA vectors [5-7]. It is also important to assess probable correlation between challenge protection and CD4^{+} and CD8^{+} responses [17].

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Despite the built-in adjuvant of DNA vectors (CpG), this gene transfer platform is less immunogenic when compared to live or subunit vaccines [18]. Co-delivery of genetic adjuvants during DNA immunization can substantially enhance the immunogenicity of the expressed antigens. Particular cytokines (GM-CSF, IL-1, TGF-b and IFN-gamma) and co-stimulatory factors (CD80, CD86 and CD40L) have been explored as genetic adjuvants in different settings [19–26]. Various factors, such as administration route and co-injection of additional potentiators have been reported to affect the outcomes of genetic adjuvants. Intradermal but not intramuscular co-injection of CD80 with herpes simplex virus antigens elicited protection from virus challenge [27]. In another study, co-administration of CD40L with b-galactosidase resulted in striking increase in antigen specific production of IFN-gamma, cytolytic T cell activity, and IgG2a antibodies, indicating a Th1 bias [26].

CD24 acts as a co-stimulatory factor of T lymphocyte homeostasis and proliferation, and involved in B lymphocyte activation and differentiation. CD24 stimulates antigen-dependent proliferation of B lymphocytes, and prevents their terminal differentiation into antibody producing cells [28].

In this study, we have evaluated an eukaryotic expression vector (pVAX-1) expressing CCHFV nucleocapsid protein (pV-N13), to conduct challenge experiment and assess total and specific immune responses against S segment of CCHFV in IFNAR$^{-/-}$ and BALB/c mice models. In addition, we have investigated potential impact of CD24 as a genetic adjuvant in this setting. The pVAX-1 based CD24 expression vector (pCD24) was delivered individually and/or in combination with N expressing construct. We have observed that individual delivery of pCD24 could induce cytokine and total antibody responses and produced increased total IgG subtype responses, surpassing the N expressing constructs in BALB/c mice. Furthermore, pCD24 demonstrated a prominent effect when administered simultaneously with the pV-N13 vector, on Th1 and Th2 cytokines induction, noted as statistically-significant elevations of IFN-gamma, IL-2, IL-4, IL-5 and TNF-alpha in stimulated splenocytes and all cytokines measured in immunized mice sera. A similar finding was also noted in total IgG1 and IgG2a production, despite a lack of statistically-significant differences. Therefore, an overall synergistic effect of pCD24 and pV-N13 construct was noted. Evaluation of total immunoglobulin isotypes in immunized mice further confirmed the capacity of the N expressing construct to induce significant amounts of total IgG1, IgG2a, IgG2b, IgG3 and IgM antibodies. Interestingly, the adjuvant expressing plasmid produced significantly higher levels of IgG subtypes when injected individually. Despite the synergistic effect of CD24 expression on the antibody production against pV-N13, we have identified that the produced

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antibodies have no in vitro neutralizing activity. These findings do not undermine N as a vaccine target as it has been previously documented in animal models that non-neutralizing antibodies could protect mice model from lethal challenges [3,6]. A similar phenomenon was also described in influenza vaccine studies with animals, where protective anti-influenza immunity is attained in the absence of measurable neutralizing antibodies [29]. In CCHFV infections in STAT-1 knockout mice, low level of neutralizing antibodies was observed and cell-mediated immunity as well as interferon induction seem to play decisive roles in outcome [17]. For a deeper investigation of this phenomenon, we conducted challenge assays in IFNAR$^{-/-}$ mice due to their defect in IFN-gamma receptors. CCHFV is considered as an IFN-gamma sensitive virus so challenge experiments were conducted in this mice breed [30]. In this experiment, protective rate of 100% was documented in pV-N13 and pV-N13 plus pCD24 groups. The adjuvant potential of CD24 in our challenge experiment was not clear in the survival assay. However it is obvious that pV-N13 plus pCD24 group showed a more stable percentage of body weight in comparison to pV-N13 group. This finding supports our other data in cytokine and total antibody responses. We are assuming that CD24 expression which lead to a general stimulation of immune response has potential of increasing specific reaction toward the virus infection in IFNAR$^{-/-}$ mice model. On the other hand, defective IFN-gamma receptors in this mice make the results' analysis more complicated so it is not easy to interpret the challenge assay and make a general hypothesis in BALB/c mice and humans.

In conclusion, due to the importance of this virus in the endemic areas like Turkey, an urgent attempt to develop an efficient vaccine must be conducted. Besides, route of transmission in the ticks which expose the virus to mutation makes the attempts to fight the disease more strenuous. Our findings along with others are emphasizing on this fact that nucleocapsid can be considered as an attractive target in immunization due to its some special characteristics like preservation and ability to stimulate balanced Th1 and Th2 responses. In addition, we have demonstrated the potential of CD24 as a new genetic adjuvant in DNA vaccination of mice model. The effect of CD24 in immune system must be investigated more to ascertain any correlation among this protein and the different arms of innate immune responses.

Acknowledgement

This project was partially supported by a grant from the Scientific and Technological Research Council of Turkey - TUBITAK (Project Number: SBAG 115 S 074). The funder had no role in the conceptualization, design, data collection, analysis, decision to publish, or manuscript preparation. Authors would like to thank to Prof. Dr. Oliver Griesbeck,

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Department of Systems and Computational Neurobiology, Max-Planck-Institute of Neurobiology, Martinsried, Germany for providing PPY bacteria.

Author Contributions: A.O. and T.A.F. designed the study; T.A.F., A.O., K.F. and A.H. conducted the experiments; T.A.F., A.O. and K.E. analyzed data and prepared the figures; T.A.F., A.O., K.E., E.Y., and F.A. wrote the manuscript. All authors read and approved the final manuscript.

Competing Interest

The authors declare no financial and non-financial interests.

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LEGEND OF TABLE AND FIGURES

Table 1: Primer sequences employed for CCHFV N amplification and plasmid construction. Underlined bold letters indicate up and downstream homologous sequences of the plasmid multiple cloning site (EcoRI) of pVAX-1 vector used in SLiCE cloning method.

Name	Sequence (5'- 3')
CCHFV-N-F [a]	atggaaaacaagatcgagg
CCHFV-N-R [a]	aggaggagaaaagctgaa
pVAX-SliCE-N-F	**taagcttggtaccgagctcggatccactagtccagtgtggtggacc**atggaaaacaagatcgagg
pVAX-SliCE-N-R	**actcgagcggccgccactgtgctggatatctgcagaatt**aggaggagaaaagctgaa
CD24-F[b]	acccacgcagatttattcca
CD24-R[b]	accacgaagagactggctgt

a: Based on the CCHFV Turkey-Kelkit06 complete sequence (GenBank accession: GQ337053).
b: Based on the CD24-expressing ORF (GenBank accession: NM013230).

A)

Number in each group	Group	Amount (50 µL)	Injection route	Interval (days)
4	pV-N13	50µg	Intramuscular	0-14
4	pV-N13 + pCD24	40µg + 10µg	Intramuscular	0-14
4	pCD24	50µg	Intramuscular	0-14
4	pVAX-1	50µg	Intramuscular	0-14
4	Negative control (saline)	50µL	Intramuscular	0-14

B)

Fig.1: Immunization scheme.

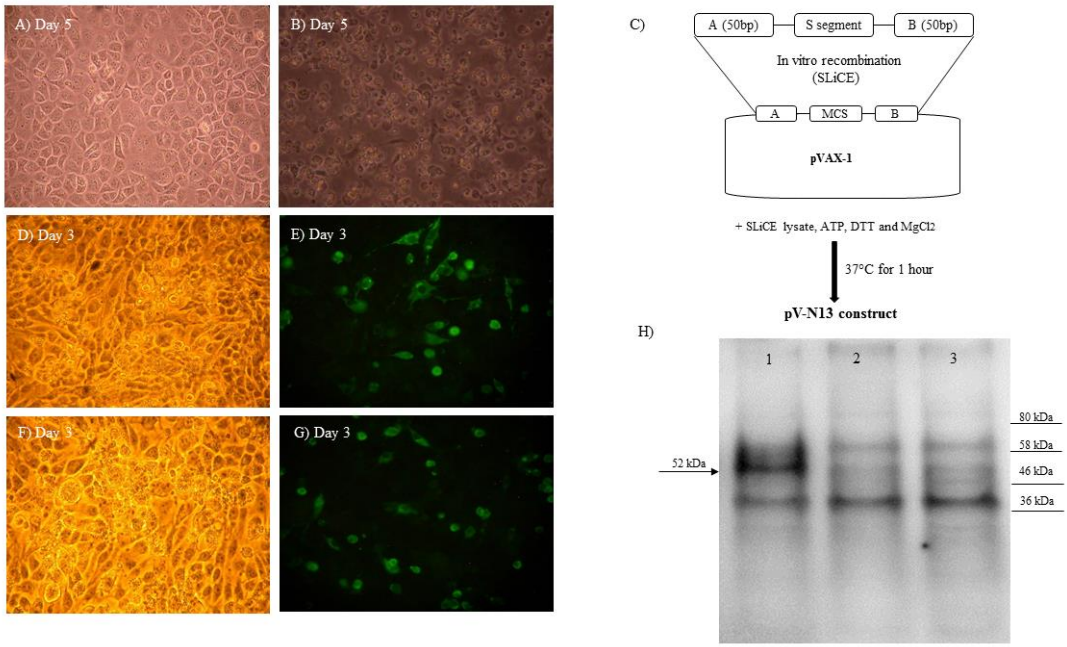

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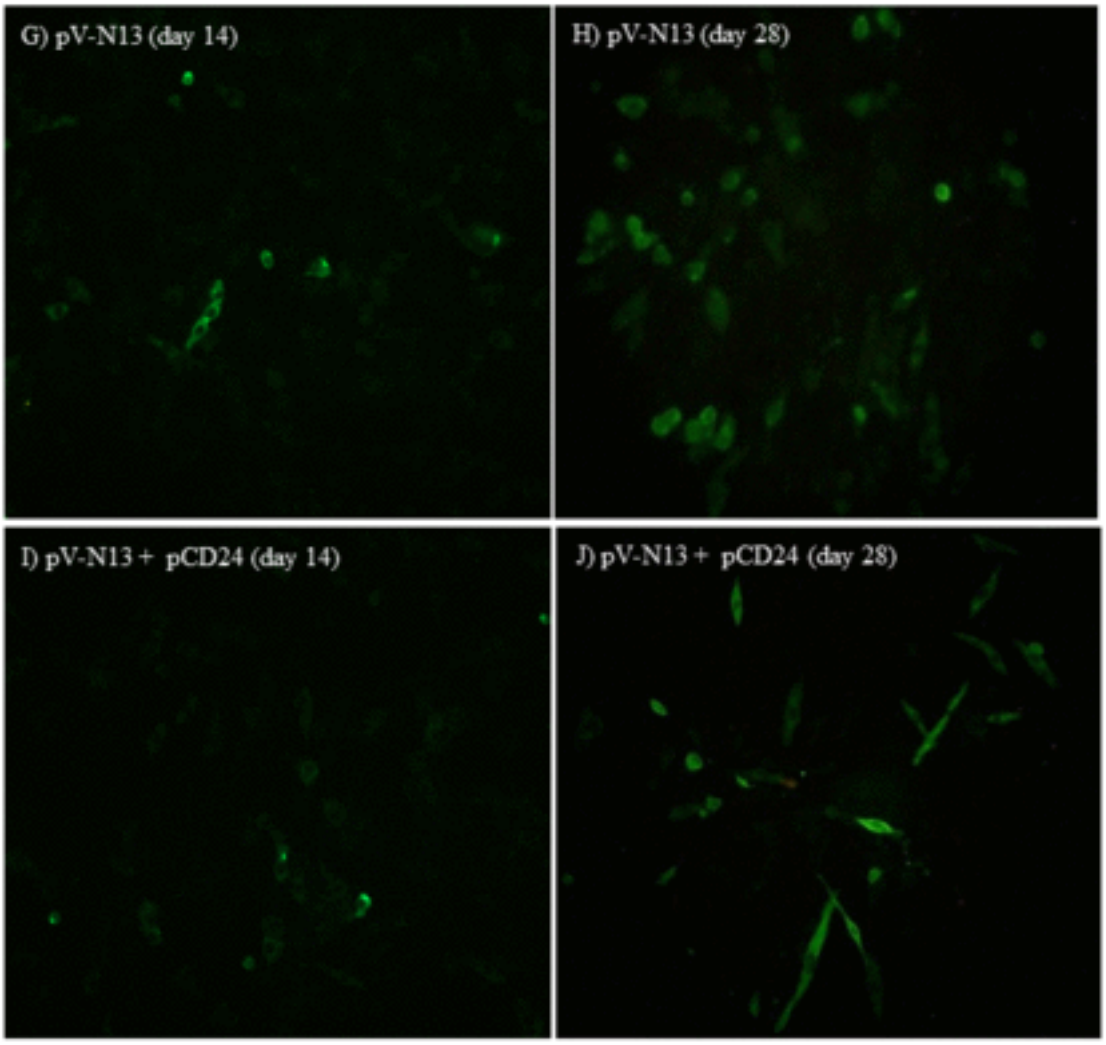
Fig. 2. **(A-B)** Propagation of ANK-2 strain of CCHFV in SW13 cells on day 5 (A: cell control, B: Virus infected cells). **(C)** Plasmid construct used for DNA vaccination in the study. In vitro homologous recombination between empty vector and amplified nucleocapsid, flanked by 50 bp homologous arms to EcoRI recognition sequence of the vector multiple cloning site, occurs in the presence of SLiCE lysate from PPY bacteria, ATP, DTT and $MgCl_2$. **(D-G)** N protein in cells transfected by pV-N13 via IIFA 72 hours post transfection (D: phase contrast, E: fluorescent contrast). CD24 protein in cells transfected by pCD24 via IIFA 72 hours post transfection (F: phase contrast, G: fluorescent contrast). **(H)** Western blot analysis of BHK21-C13 cells transfected with pV-N13. The expected protein (~52 kDa) was detected in pV-N13 transfected cells after 72 hours (lane 1). We included pVAX-1 transfected cells (lane 2) and cell control (lane 3) in the assay.



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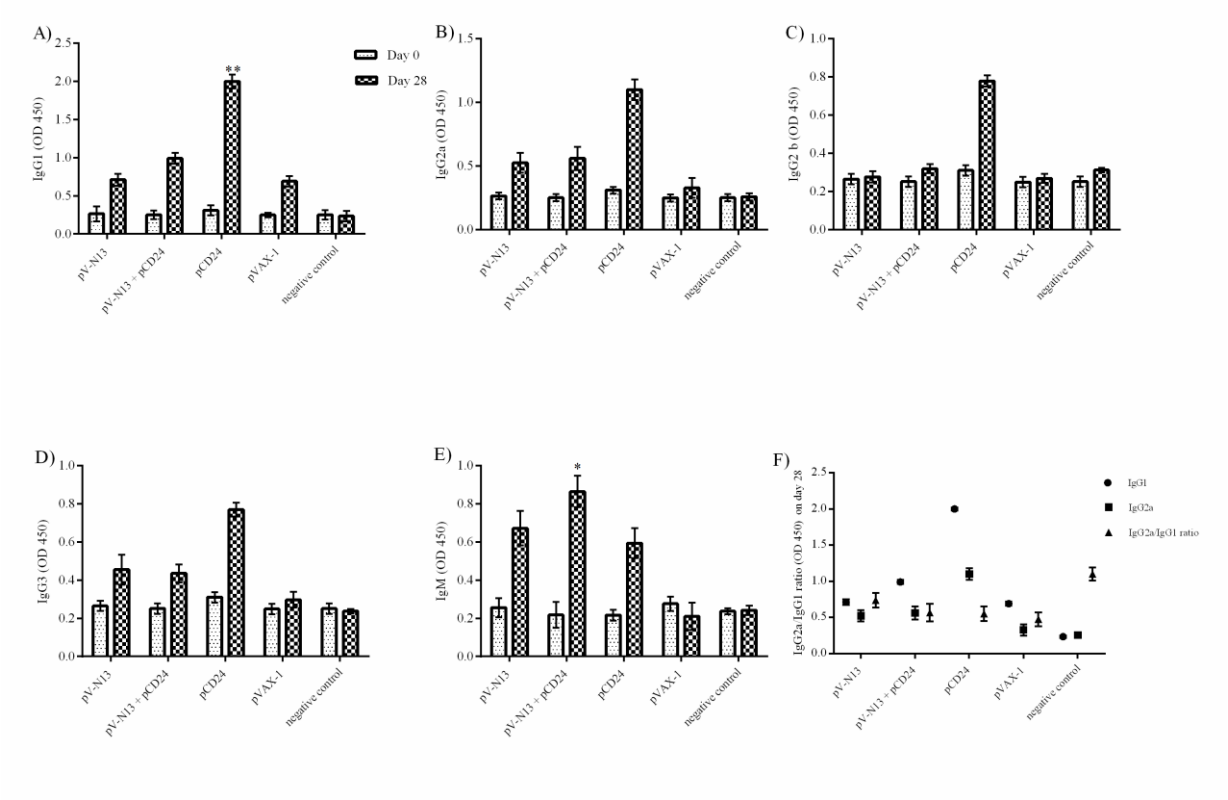

Fig. 3. Serological assays (A) IgG1 response. **(B)** IgG2a response. **(C)** IgG2b response. **(D)** IgG3 response: In all mentioned assays pV-N13 plus pCD24 and pCD24 groups are dominant. **(E)** IgM response: pV-N13, pV-N13 plus pCD24 and pCD24 groups are stimulator of IgM response. **(F)** Comparison of IgG1, IgG2a and IgG2a/IgG1 responses. *$p < 0.05$; **$p < 0.01$ and ***$p < 0.001$ versus negative control group. **(G-H)** Detection of N specific antibodies present in the serum samples in BHK-N cells (G: pV-N13 immunized mice serum samples, H: pV-N13 plus pCD24 immunized mice serum samples).

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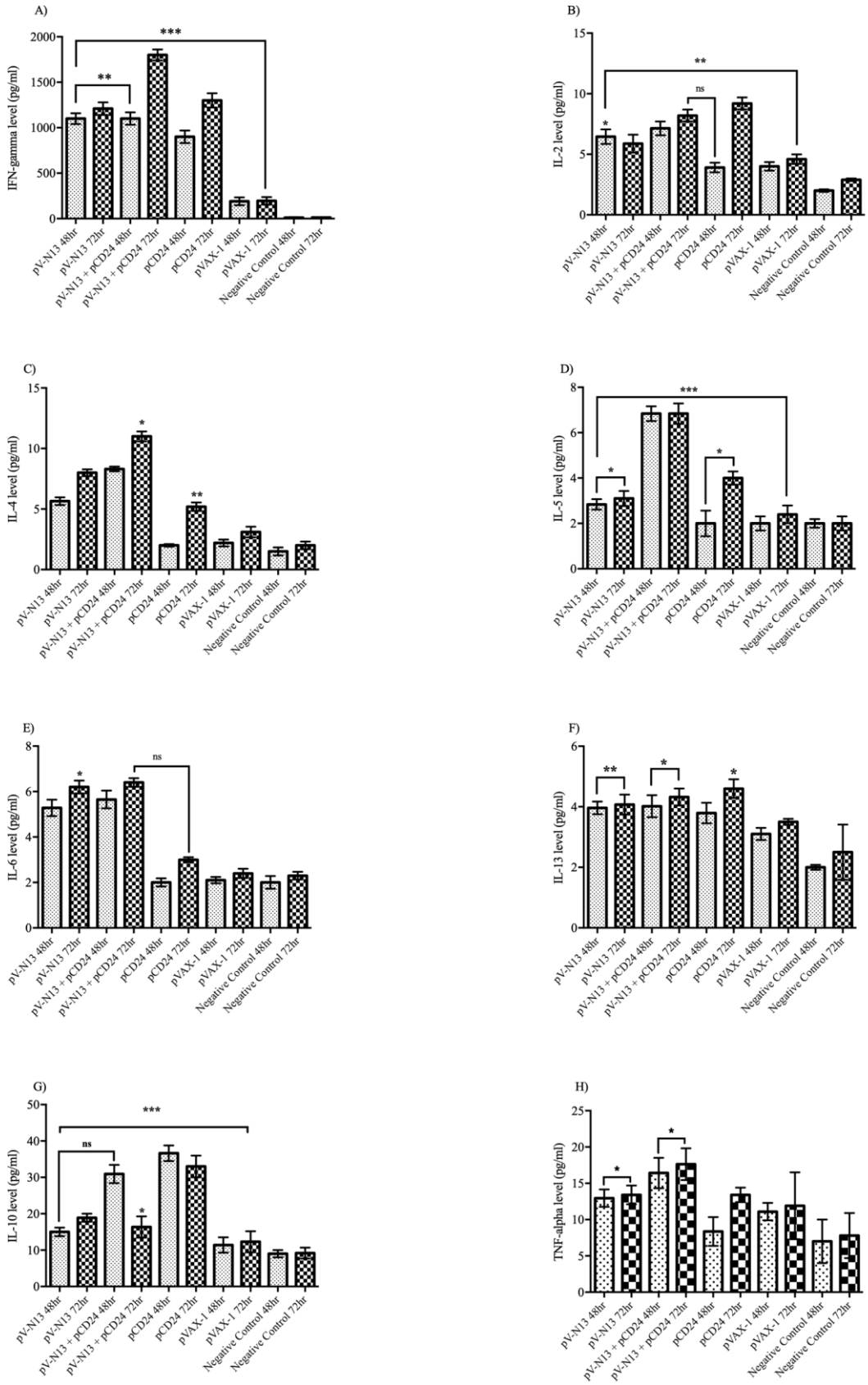

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Fig. 4. Cytokine responses in the supernatant of CCHFV stimulated splenocytes of immunized BALB/c mice. (A) IFN-gamma response: As demonstrated here, pV-N13 plus pCD24 group's result is higher in comparison to other groups. pCD24 stimulation level is also significant. **(B)** IL-2 response: IL-2 response is predominant in pV-N13 plus pCD24 and pCD24 groups. **(C)** IL-4 response: As shown, pV-N13 plus pCD24 and pV-N13 immunized BALB/c mice demonstrated the highest amount. **(D)** IL-5 response: IL-5 response is predominant in pV-N13 plus pCD24 and pCD24 groups. **(E)** IL-13 response: All the immunized mice show almost identical levels of IL-13 secretion. **(F)** IL-6 response: pV-N13 plus pCD24 and pV-N13 groups are higher in comparison to other groups. **(G)** IL-10 response: When compared N expressing groups to empty vectors, pV-N13 plus pCD24 group elicits a pronounced IL-10 response in BALB/c mice model. Also, pCD24 has potential to induce IL-10 responses. **(H)** TNF-alpha response: As shown, pV-N13 plus pCD24 group stimulated the highest amount. *$p < 0.05$; **$p < 0.01$ and ***$p < 0.001$ versus negative control group.

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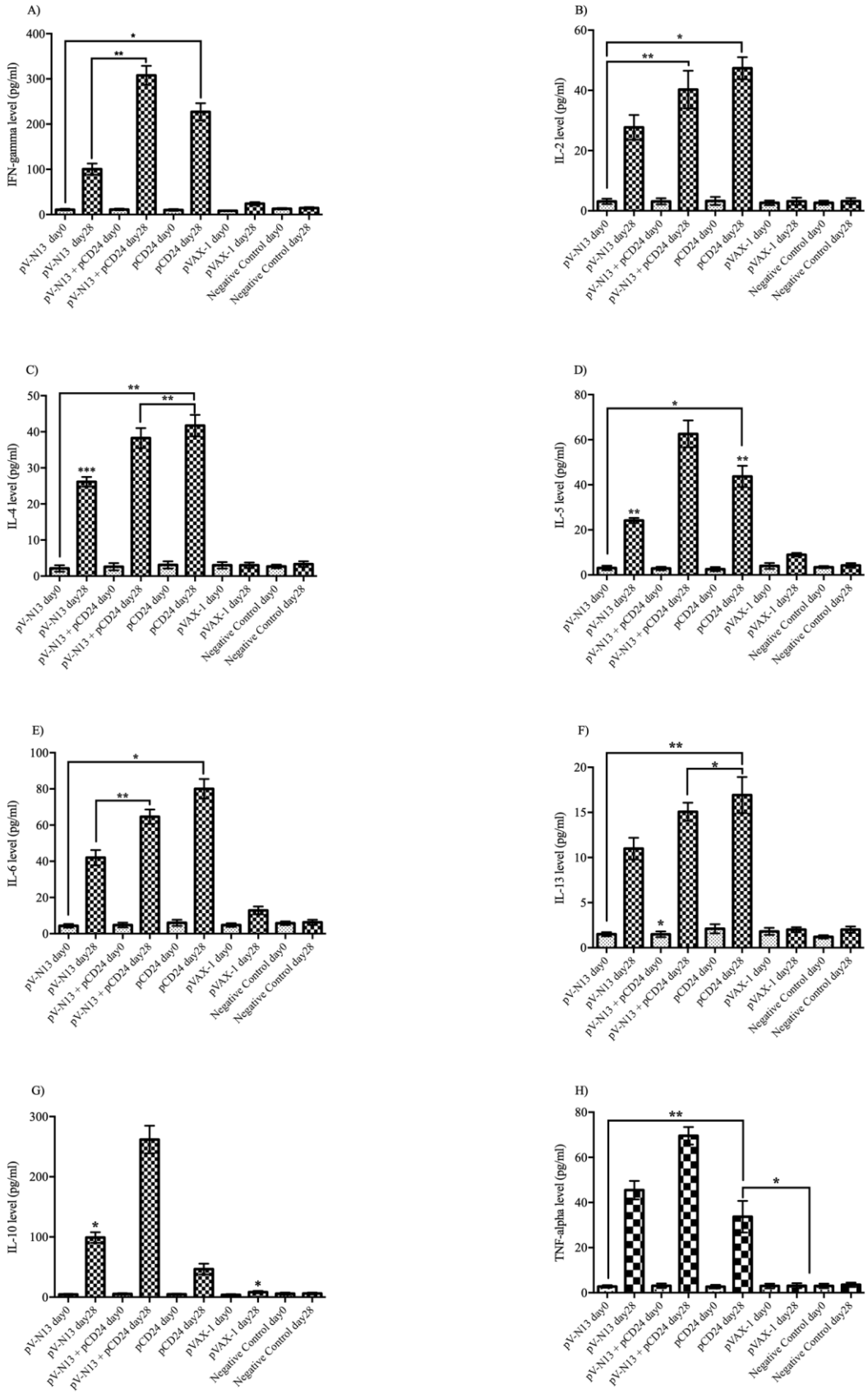

Fig. 5. Cytokine responses in the serum samples of immunized BALB/c mice. (A) IFN-gamma response: pCD24 vector (on day 28) stimulated a prominent production of IFN-gamma. pV-N13 plus pCD24 on day 28 is

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by far dominant when compared to other groups. **(B)** IL-2 response: pCD24 and pV-N13 plus pCD24 possess the highest amount. Besides, IL-2 level in pV-N13 group is also adequate. **(C)** IL-4 response: IL-4 stimulation in pCD24 and pV-N13 plus pCD24 is considerable when compared to other groups. **(D)** IL-5 response: Interestingly, pV-N13 plus pCD24 group has potential to stimulate this cytokine in immunized mice in a higher amount compared to remaining groups. Levels of IL-5 in pCD24 immunized mice is also significantly elevated. **(E)** IL-13 response: The results are comparable to those of IFN-gamma. **(F)** IL-6 response: pCD24 and pV-N13 plus pCD24 immunized mice possessed the highest level of IL-6 in the serum samples. **(G)** IL-10 response: The results indicate the predominance of pV-N13 plus pCD24 group. **(H)** TNF-alpha response: pV-N13 plus pCD24 construct stimulated the highest levels of TNF-alpha. *$p < 0.05$; **$p < 0.01$ and ***$p < 0.001$ versus negative control group.

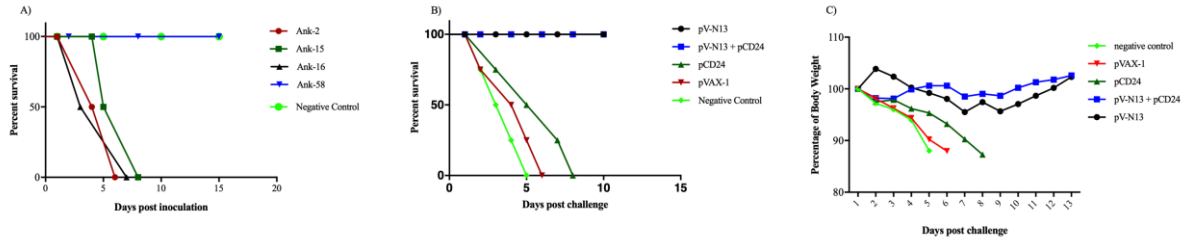

Fig.6. Challenge experiment. (A) Challenge assay to find the suitable strain of CCHFV: Four different isolates were assayed in IFNAR$^{-/-}$ to identify lethal strains. **(B**) Survival rate in challenge assay: the pV-N13 and pV-N13 plus pCD24 groups survived in lethal dose challenge of IFNAR$^{-/-}$ mice. **(C)** Percentage of body weight: Despite lethal challenge, pV-N13 and pV-N13 plus pCD24 groups showed an almost stable body weight range.