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Bovine Herpesvirus type 4 (BoHV-4) Vector Delivering Nucleocapsid Protein of Crimean Congo Hemorrhagic Fever Virus Induces Comparable Protective Immunity against Lethal Challenge in IFNAR$^{-/-}$ Mice Model

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Abstract

Crimean-Congo hemorrhagic fever virus (CCHFV) is the causative agent of a tick-borne infection with significant mortality rate of up to 40% in the endemic areas, with evidence for geographical expansion. Lacking effective therapeutics and control measures, the development of protective CCHFV vaccine remains a crucial public health task. This manuscript describes, for the first time, a Bovine herpesvirus type 4 (BoHV-4) based viral vector (BoHV4-ΔTK-CCHFV-N) and its immunogenicity and protection potential in BALB/c and IFNAR$^{-/-}$ mice models in comparison with Adenovirus type 5 (Ad5-N) and pCDNA3.1 myc/His A (pCD-N1), two widely used vaccine platforms. All constructs expressing viral nucleocapsid (N) protein successfully elicited cytokine and total/specific antibody responses in BALB/c mice. BoHV4-ΔTK-CCHFV-N and Ad5-N constructs further produced 100% protection in IFNAR$^{-/-}$ mice during CCHFV Ank-2 strain lethal challenge. Despite elevated specific antibody responses in both animal models, the produced antibodies were unable to neutralize the virus in vitro. A comparison of delivery platforms was not possible, due to similar protection rates in IFNAR$^{-/-}$ mice. In conclusion, vector-based CCHFV N protein expression proved to constitute an effective approach for the vaccine development pipeline and BoHV-4 emerged as a strong alternative to previously-used virus vectors.

Keywords: Crimean-Congo hemorrhagic fever, Nucleocapsid, Bovine Herpesvirus type 4, IFNAR$^{-/-}$ mice, Lethal dose

Introduction

Crimean Congo hemorrhagic fever virus (CCHFV), a member of Orthonairovirus genus and Nairoviridae family, is a major tick-borne human pathogen, causing hemorrhagic fever disease with considerable mortality rates up to 40% in endemic areas [1, 2]. The widespread distribution, significant mortality rates and the lack of specific treatment or control measures make CCHFV an urgent target for vaccine development [3].

In general, viral membrane and glycoproteins are targeted in vaccine development efforts, as they directly interact with host immune cells and stimulate neutralizing antibodies. However, these regions also mutate rapidly and unexpectedly, to escape host defense and may not be practical as vaccine targets when the circulating strains demonstrate significant sequence diversity.

CCHFV nucleocapsid (N) protein is an important structural component, stimulating humoral and cellular responses following natural or experimental infections and has been targeted for vaccine development by using DNA and viral vector platforms in several studies [5-7]. During challenge experiments in different modified mice models such as IFNAR$^{-/-}$, Immune suppressed (IS) and STAT1$^{-/-}$, vaccinations based on this protein have demonstrated protective effect in lethal challenges despite lack of neutralizing antibodies [6]. A recent report employing vectors in combination with virus-like particles has demonstrated the impact of Th_1 response for the survival of IFNAR$^{-/-}$ mice. Interestingly, neutralizing antibodies were not produced against nucleocapsid protein of CCHFV and has negligible effect on protection against lethal dose challenge [8]. Adenovirus and modified Vaccinia Ankara (MVA) based vectors were previously used to deliver N protein with inconclusive results. A MVA vector produced adequate immune responses, yet without protection from lethal challenge [7]. Considering other disadvantages like pre-existing adenovirus immunity [9], exploration of new platforms for N protein delivery is a credible approach and likely to accelerate CCHFV vaccine development efforts.

Bovine herpesvirus type 4 (BoHV-4), a member of gamma herpesvirinae subfamily and rhadinovirus genus, is prevalent among a range of ruminants [10]. This virus can be detected in healthy as well as sick animals and is mainly considered as a secondary pathogen in cattle [11, 12]. The potential of BoHV-4 to infect humans in vivo is obscure and it is categorized it in the Biosafety level (BSL) 2 agent in international biosafety regulations [13,14]. This virus has been explored as a novel herpesviral vector in targeting different virus antigens such as Ebola surface glycoprotein (BoHV-4-syEBOVgD106ΔTK), Peste des Petits Ruminants virus hemagglutinin (H) (BoHV-4-A-PPRV-H-ΔTK) and various glycoproteins (A29L, M1R and

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B6R) of Monkeypox virus (BoHV-4-A-CMV-A29LgD$_{106}$ΔTK, BoHV-4-A-EF1α-M1RgD$_{106}$ΔTK and BoHV-4-A-EF1α-B6RgD$_{106}$ΔTK). Its easy propagation in cell culture, relatively simple genome, large capacity for insertion, availability of an animal model (rabbit) and lack of documented cell transformation makes BoHV-4 a promising delivery vector. [15-19], already tested in vaccine and cancer therapy experiments [20].

In this study, we generated a recombinant Bovine herpesvirus type 4, expressing the N protein of CCHFV (BoHV4-ΔTK-CCHFV-N) and evaluated its immunogenicity and protection potential in BALB/c and IFNAR$^{-/-}$ mice models as a novel viral vector. To support this hypothesis, we further compared BoHV4-ΔTK-CCHFV-N findings with widely used delivery platforms including Adenovirus type 5 (Ad5-N) alongside with a DNA vector (pCD-N1) expressing the same antigen.

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). All 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). During these studies, human endpoints scores were considered. Multiple observations per day were conducted to confirm animals' welfare. Constant access to sterilized water and food was provided. Mice were euthanized by CO_2 exposure and cervical dislocation. BALB/c and IFNAR$^{-/-}$ mice provided from B&K Universal Ltd, (Marshall Bioresouces, Hull, East Yorkshire, UK).

Cells and Viruses

Bovine herpesvirus type 4 (BoHV-4) carrying bacterial artificial chromosome vector (BoHV4-BAC) was developed from Movar33/63 European strain through insertion of a F plasmid (pBeloBAC11) containing cassette (loxp-BAC-EGFP-loxp) between ORF2 and 3 genes (GenBank Accession Number: NC002665), not essential for virus replication by in vitro homologous recombination and named as infectious BAC (iBAC). CCHFV Ank-2 strain (GenBank Accession Number: MK309333) was used as the challenge virus. CCHFV and BoHV4-BAC were cultivated in Scott and White No. 13 (SW-13) and Madin Darby bovine kidney (MDBK) cells, respectively. SW-13 and MDBK cells cultured in Eagle's Minimum Essential Medium (Sigma, St. Louis, MO, USA) and Leibovitz's L-15 (Thermo Fisher

Scientific, Waltham, MA, USA) media supplemented by 10% fetal bovine serum (Biological Industries, Kibbutz Beit-Haemek, Israel), 2 mM L-glutamine (Biological Industries, Kibbutz Beit-Haemek, Israel), 100 U penicillin and 0,1 mg/ml streptomycin (Thermo Fisher Scientific, Waltham, MA, USA), respectively. Besides, baby hamster kidney (BHK21-C13) and MDBK-cre (MDBK stably expressing Cre recombinase enzyme) cells were used in transfection assays and loxp-BAC-EGFP-loxp elimination from BoHV4-BAC construct, respectively. Cultivation of these lines were the same as MDBK cells. In addition, we have performed preliminary experiments using human embryonic kidney (HEK293 and HEK293A) cells for generation and cultivation of recombinant adenovirus type 5 expressing CCHFV N protein. These cells cultured and maintained at 37°C/5% CO_2 in Minimum Essential Medium alpha (Thermo Fisher Scientific, Waltham, MA, USA). Final N expressing constructs including BoHV4-ΔTK-CCHFV-N and Ad5-N were propagated and tittered in MDBK and HEK293A cells, respectively and stored at -80°C. All viruses and cells were obtained from departmental collection. All biological assays including virus cultivation and animal experiments with infectious virus particles were performed in biosafety level 3 plus (BSL3+) and animal biosafety level 3 plus (ABSL3+) facilities of Virology Department, Veterinary Faculty of Ankara University, Turkey.

Recombinant BoHV4-ΔTK-CCHFV-N

CCHFV genomic RNA extraction was performed using QIAamp Viral RNA Mini Kit (QIAGEN, Germantown, MD, USA) according to manufacturer's instructions and Superscript IV reverse transcriptase (Thermo Fisher Scientific, Waltham, MA, USA) was employed for cDNA synthesis. The primer sets, used to amplify complete N coding gene of CCHFV based on Turkey-Kelkit06 complete sequence (GenBank Accession Number: GQ337053), TK-CMV-N-TK homologous cassette to BoHV-4 Movar 33/63 strain and N PCR product containing 50 bp homologous arms of pCDNA3.1 myc/HisA vector, are given in Table 1. In the first step, pCD-N1 construct was generated by insertion of gel purified N PCR product between EcoRI and XhoI sites of pCDNA3.1 myc/His A plasmid (Invitrogen, Carlsbad, CA, USA). The final construct was verified by sequencing. In the next step, to propagate TK-CMV-N-TK cassette from pCD-N1, primer sets (TM1 and TM2) containing 50 bp homologous arms of thymidine kinase gene (GenBank Accession Number: AF318573) of BoHV4-BAC were used. This cassette was used to perform recombineering in SW102 bacterial cells containing BoHV4-loxp-BAC-CMV-EGFP-loxp (iBAC) following gel purification according to the standard protocol that can be found elsewhere

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(https://redrecombineering.ncifcrf.gov/protocols/) [21]. This bacterial strain is derived from DY380 (DH10B-derived strain) and therefore contains a defective λ prophage with recombination proteins *exo*, *bet*, and *gam* being controlled by the temperature-sensitive repressor *cI*857. To perform recombineering, TK-CMV-N-TK cassette was electroporated into heat induced SW102 (42ºC for 15 minutes) containing iBAC in 0.1 cm cuvette using Micropulser electroporator (BioRad, Hercules, CA, USA) in a concentration of 100 ng and subsequently plated on Luria-Bertani (LB) agar containing 12.5µg/µL of chloramphenicol. Following extraction of the verified BoHV4-BAC-ΔTK-CCHFV-N DNA from SW102 by alkaline lysis method, MDBK cells transfected by using Lipofectamine 3000 reagent (Thermo Fisher Scientific, Waltham, MA, USA) to generate infectious BoHV4-BAC-ΔTK-CCHFV-N. To eliminate loxp-BAC-EGFP-loxp from final construct, recombinant BoHV4-BAC-ΔTK-CCHFV-N was propagated in MDBK-cre cells to create BoHV4-ΔTK-CCHFV-N.

Table 1: Primer sets used in PCR amplification reactions

Name	Primer Sequence (5'→3')[a]	Purpose
CCHFV-N-F[b]	gaattcatggaaaacaagatcgagg	N-ORF amplification
CCHFV-N-R[b]	ctcgagaggaggagaaaagctgaa	
BoHV4-N-F[c]	*tggaagggtagagaggattgtctttgtgtccttctgtttgagagcaatgg*gggattttggtcatgaga	BoHV4-ΔTK-CCHFV-N construction
BoHV4-N-R[c]	*tgctttgttgcagtttacaatacgtggagactcctgcaatattttacagc*gatttagagcttgacggg	
p516-SLiCE-N-F[d]	**tctccacaggtgtccactcccaggtccaaccgaattcccc**atggaaaacaagatcgagg	Ad5-N construction
p516-SLiCE-N-R[d]	**aaacaagttgctcgaagtcgacgagctcaagcttagatctccc**aggaggagaaaagctgaa	

a: Turkey-Kelkit06 complete sequence (Accession # GQ337053)
b: Underlined regular letters indicate restriction endonuclease recognition sites (EcoRI and XhoI)
c: Underlined italic letters indicate up-and downstream flanking sequences of TK gene of BoHV-4 to use in recombineering (Accession # AF318573). Regular letters are primers to pCDNA3.1 vector to amplify CMV-insert-polyA cassette.
d: Underlined bold letters indicate homologous arms to pDC516 vector to use in SLiCE cloning assay.

Recombinant Ad5-N

AdMax™HI-IQ Kit J (MICROBIX Biosystems, ON, Canada) was used to generate Ad5-N. Basically, S gene was cloned into the pDC516 vector containing E3 homologous arms to pBHGfrtΔE1 plasmid. Cloning method of Seamless Ligation Cloning Extract (SLiCE) which includes PCR amplification by primers targeting N with 50 bp homologous arms to 5' and 3' sides of EcoRI of pDC516 vector was used according to the standard protocol [20]. The employed primer set is provided in Table 1. SLiCE extract was prepared from PPY bacteria (DH10B derived *E.coli* strain:F⁻ *end*A1 *rec*A1 *gal*E15 *gal*K16 *nup*G *rps*LΔ*lac*X74*Φ80lac*ZΔ

M15*ara*D139Δ(*ara,leu*)7697 *mcr*AΔ(*mrr-hsd*RMS-*mcr*BC) *cyn*X: [*ara*CpBAD-*redα* EM7-*redβ*Tn5-*gam*] λ^{-}) as described elsewhere [22]. The final construct (pDC516-N) was confirmed by colony PCR, restriction enzyme analysis and sequencing of the target gene. Homologous recombination occurred with co-transfection of pDC516-N (5µg) and pBHGfrtΔE1 (3µg) plasmids, by using Lipofectamine 3000 reagent in T25 cell culture flask contained HEK293 cells at a confluency of 90%. Twenty-four hours post transfection, the cells were harvested by trypsin and transferred to a T75 culture vessel. The viruses were collected after 12-14 days when cytopathic effects (CPEs) including rounded and detached cells were visualized in 90% of the monolayer. After 3 rounds of virus passages in HEK293 cells, sufficient titer of the recombinant viruses was obtained. Recombinant viruses were titered in HEK293A cells by Adeno-X™ Rapid Titer Kit (Clontech, CA, USA). In addition, Ad5-wt virus was produced by transfection of pFG140 vector in HEK293 cells. Following titer determination, virus stocks were stored at -80°C.

In vitro Detection of N Protein Expression

To confirm in vitro expression of N protein from pCD-N1 and pDC516-N constructs, indirect immunofluorescence assay (IIFA) was performed in BHK21-C13 cells as previously described [23].

For Western blotting (WB), BoHV4-ΔTK-CCHFV-N and Ad5-N infected MDBK and HEK293A cells scraped 96-hour post infection, and the assay was performed based on a previous study [23]. Anti-Beta Actin antibody (St John's Laboratory, London, UK) and CCHFV Ank-2 infected SW-13 cells (0.1 moi) on 3 days post-inoculation were used as controls.

Immunization Schedule

A total of twenty-four female BALB/c (8-10 weeks old) mice were used in the study (Table 2). Animals were randomly divided into six groups of four individuals, comprising BoHV4-ΔTK-CCHFV-N, Ad5-N, Movar33/63, Ad5-wt, pCD-N1 and pCDNA3.1 myc/His A and negative control (normal saline). The study groups received 100$TCID_{50}$ /0.3 mL dose of the relevant viruses through the intra-peritoneal route while pCD-N1 and pCDNA3.1 myc/His A groups injected by 50µg of the constructs through tight muscle site. Booster injections were given after 2 weeks at the same regime and serum samples have been collected on days 0, 14 and 28 and stored at -80°C until further analysis. In addition, splenocytes from individual mice collected on day 28 for cytokine analysis.

Table 2: Immunization schedule

Group	Number	Inoculation dose/volume	Injection route	Interval (days)
BoHV4-ΔTK-CCHFV-N	4	100TCID$_{50}$/300µl	i.p.*	0-14
Movar33/63	4	100TCID$_{50}$/300µl	i.p.	0-14
Ad5-N	4	100TCID$_{50}$/300µl	i.p.	0-14
Ad5-wt	4	100TCID$_{50}$/300µl	i.p.	0-14
pCD-N1	4	50 µg/100µl	i.m.**	0-14
pCDNA3.1 myc/His A	4	50 µg/100µl	i.m.	0-14
Normal saline	4	300 µL	i.p.	0-14
T cell adoptive and antibody passive transfer	12	Splenocyte (2x10^5) + IgG (500µg)	i.p.	-

* intraperitoneal, ** intramuscular

Challenge Virus

CCHFV Ank-2 strain, lethal for IFNAR$^{-/-}$ mice, was used in intraperitoneally challenge experiment [23]. The immunization schedule (vaccine constructs) of IFNAR$^{-/-}$ mice was the same of BALB/c mice. On day 28, each groups received 300µl of lethal dose of the virus (100LD$_{50}$=1000TCID$_{50}$). The experiment continued for 13 days and daily observation of clinical signs including nasal or ocular discharges, appearance changes, weight losses, depression and death were recorded. Euthanasia criteria for survived groups were based on the complete recovery from clinical signs and stability of body weight for a minimum of 5 days. Sera were collected on days 0, 14, 28 and prior to sacrifice and stored at -80ºC.

Total Antibody Isotyping Assay from Immunized BALB/c Mice

Total antibody Isotyping ELISA kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to determine total immunoglobulin types in immunized BALB/c mice sera, collected on days 0 and 28 as previously described [23].

233 **ELISA for CCHFV N-Protein Specific IgG Detection**

234 Infected SW-13 cells with 1 moi of Ank-2 strain were collected 2 days post inoculation (dpi)
235 and cell lysates were used as ELISA antigens. A total of 45 µg of cell lysate was added to
236 each well of Nunc MaxiSorp flat-bottom (Thermo Fisher Scientific, Waltham, MA, USA) in
237 bicarbonate buffer (pH8) and incubated overnight at 4ºC. The next day, 1/1000 diluted serum
238 samples from challenged IFNAR$^{-/-}$ mice were added to each well and incubated for 2 hours at
239 room temperature (RT). Subsequently, anti-mouse IgG-HRP antibody (Abcam, Cambridge,
240 MA, USA) at 1/10000 dilution added and further incubated at RT for 1 hour followed by
241 adding 3, 3', 5, 5' - Tetramethylbenzidine (TMB ELISA Peroxidase) substrate. Finally, the
242 reaction stopped by adding 2N H2SO4. The results of unvaccinated mice serum samples were
243 subtracted from cell lysate as background.

244 **Antibody Passive Transfer and T cell Adoptive Transfer**

245 Splenocytes and serum samples from immunized BALB/c mice were harvested on day 28.
246 After staining with trypan blue, 200,000 splenocytes combined with 100-300 µl of sera
247 containing 500 µg of IgG (measured by mouse anti-IgG kit, Wuhan Fine Biotech Co., Ltd.
248 Wuhan, Hubei, China) injected through intraperitoneal route to IFNAR$^{-/-}$ mice which each
249 group contained 4 (Table 2). Twenty-four hours later, the mice were challenged by
250 1000TCID$_{50}$ of Ank-2 strain and survival rates were recorded for a maximum of 15 days.

251 **Virus Neutralization Assay (VNA)**

252 VNA was employed to elucidate neutralizing ability of actively or passively immunized
253 animals. Actively immunized BALB/c and IFNAR$^{-/-}$ mice serum samples on days 14 and 28
254 and serum samples on day 4 from passive immunized IFNAR-/- mice were serially diluted in
255 Dulbecco's Modified Eagle's medium (DMEM) and mixed with an equal volume of
256 100TCID$_{50}$ of the virus in duplicate. After 1 hour of incubation at 37°C, serum-virus mixture
257 was inoculated in 1-day-old SW-13 cells at 90% confluency, prepared in 24-well plates.
258 Infected cells were further incubated under the same conditions for 4-5 days, with daily
259 observation via inverted microscope for the virus-induced cytopathogenic effects.

260 **Cytokine assays**

261 The collected supernatants from splenocytes of BALB/c and serum samples of BALB/c and
262 IFNAR$^{-/-}$ mice were subjected to cytokine measurements. Briefly, immunized BALB/c mice
263 were euthanized and spleens were aseptically removed to prepare single cell splenocytes.
264 After cell dissociation using sterile strainer, red blood cell lysed by red blood cells (RBC)

lysis buffer (BI, Israel) and splenocytes were cultured in 24-well plates. Immediately, 10 moi of Ank-2 strain was added to each well and supernatants collected after 48 and 72 hours. Quantitation of cytokines was performed using LEGENDplex™ Mouse Th1/Th2 Panel 8-plex kit (BioLegend, San Diego, CA, USA) by FacsCanto II Flow Cytometer (BD Bioscience, Franklin Lakes, NJ, USA) and results were analyzed by LEGENDplex™ data analysis software.

Statistical Analysis

Antibody isotype 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). One-way ANOVA (Tukey's *post hoc* correction) was also employed for the analysis of neutralizing antibody production. Graphs were produced using GraphPad Prism version 7.0 (GraphPad Software, San Diego, CA, USA; www.graphpad.com). Data were considered to be statistically significant when $p < 0.05$. All molecular biology procedures were simulated using SnapGene Viewer software (www.snapgene.com).

Results

Recombineering and Homologous Recombination

Generation of BoHV4-ΔTK-CCHFV-N was accomplished in SW102 bacteria containing BoHV4-BAC (Figure 1A). The correct colonies were selected and construct verification was performed via restriction enzyme analysis by comparing to Movar33/63 strain. The stability of construct within the bacteria was determined by 10 bacterial passages. Following transfection of pDC516-N and pBHGfrtΔE1 plasmids, the recombination occurred in HEK293 cells and recombinant Ad5-N viruses were collected after about 12-14 days (Figure 1B). The recombinant viruses showed the expected pattern after plaque purification and restriction enzyme analysis.

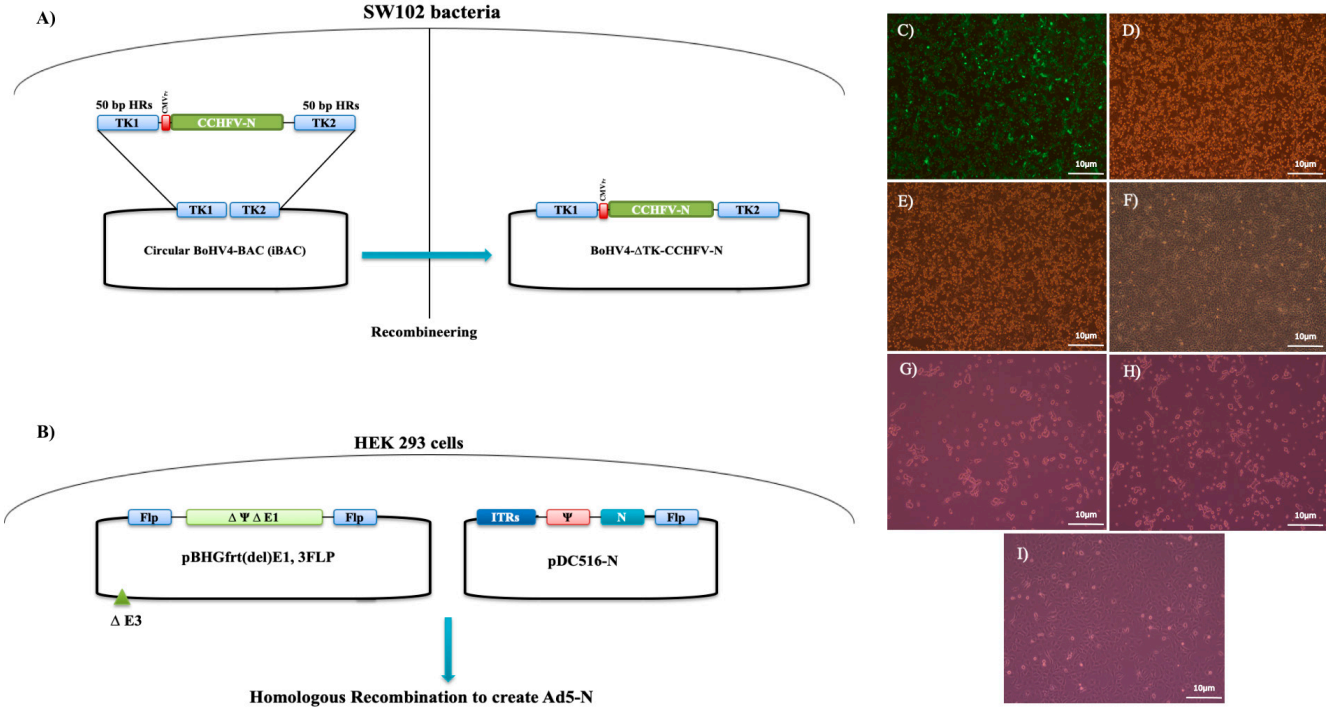

Figure 1. A, B) Schematic figure of recombineering in SW102 bacteria to create BoHV4-ΔTK-CCHFV-N (A) and homologous recombination in HEK293A to obtain Ad5-N (B). **C-I)** BoHV4-ΔTK-CCHFV-N, Movar33/63, Ad5-N and Ad5-wt propagation in cells. C) BoHV4-ΔTK-CCHFV-N infected MDBK cells on day 5 (fluorescence). D) BoHV4-ΔTK-CCHFV-N infected MDBK cells on day 5 (phase contrast). E) Movar33/63 infected MDBK cells on day 5. F) Uninfected MDBK cells on day 5. G) Ad5-N infected HEK293 cells on day 5. H) Ad5-wt infected HEK293 cells on day 5. I) Uninfected HEK293 cells on day 5 (x400).

In vitro Verification of N Protein Expression

N protein expression verified in pCD-N1 and pDC516-N transfected BHK21-C13 cells on day 2 post DNA delivery by IIFA (Figure 2C-F). Besides, presence of N protein (~52 kDa) in infected/transfected cells by N expressing constructs (BoHV4-ΔTK-CCHFV-N, Ad5-N and pCD-N1) confirmed by immunoblotting (Figure 2A).

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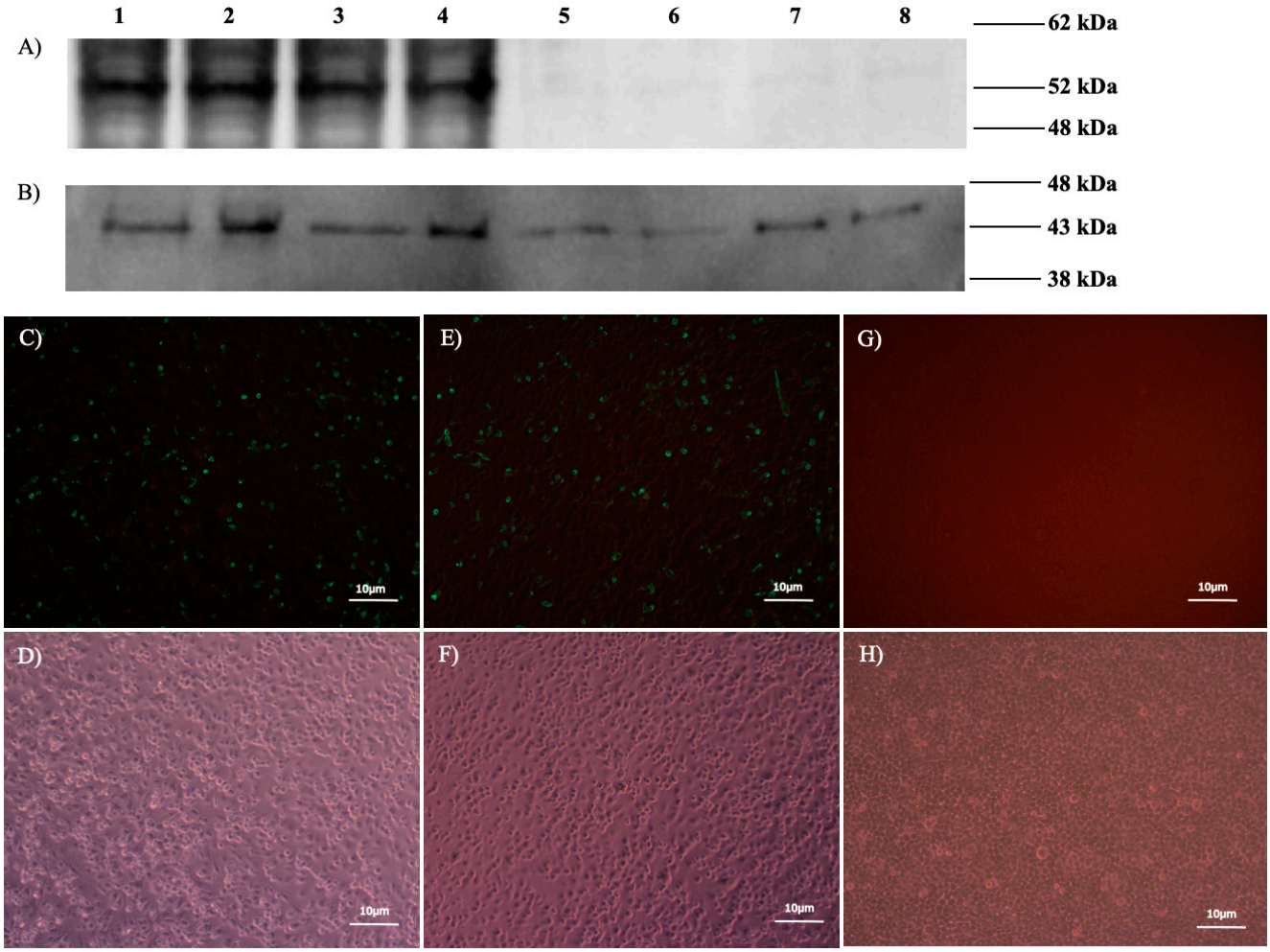

Figure 2. In vitro expression of N protein. A, B) WB. A) Positive band of 52 kDa detected in lane 1 (CCHFV Ank-2 infected SW-13 cells), lane 2 (BoHV4-ΔTK-CCHFV-N infected MDBK cells), lane 3 (Ad5-N infected HEK293A cells), and lane 4 (pCD-N1 transfected HEK293A cells). We also included control groups of lane 5 (Movar33/63 infected MDBK cells), lane 6 (Ad5-wt infected HEK293A cells), lane 7 (pCDNA3.1 myc/His A transfected HEK293A cells) and lane 8 (negative control) in the experiment. B) Beta-actin protein (43 kDa) as control of WB in all mentioned groups. **C-H) IIFA 48 hours post transfection.** C) pCD-N1 transfected BHK21-C13 (fluorescence). D) pCD-N1 transfected BHK21-C13 (phase contrast). E) pDC516-N transfected BHK21-C13 (fluorescence). F) pDC516-N transfected BHK21-C13 (phase contrast). G) Negative control (fluorescence). H) Negative control (phase contrast).

Serological Assays

BALB/c mice immunized by N expressing constructs showed elevated levels of IgG1, IgG2a, IgG2b and IgG3, compared to their relative backbones (Figure 3A-D). As demonstrated, pCD-N1 construct has more potential for IgG1 and IgG3 production. Besides, this construct alongside with BoHV4-ΔTK-CCHFV-N, are predominant in inducing IgG2a. BoHV4-ΔTK-CCHFV-N immunized mice showed a higher level of IgG2b in comparison to the N

expressing constructs. The IgG_{2a}/IgG_1 ratio reflected a shift towards Th2 responses in the immunized BALB/c mice (Figure 3E). In the ELISA assay, all the sera from immunized $IFNAR^{-/-}$ mice on day 28 with N expressing constructs showed a relatively high amount of IgG antibodies. The differences were not significant among the vaccine groups (Figure 3F). Despite the high level of antibody production in the immunized BALB/c and $IFNAR^{-/-}$ mice, cytopathic effects were observed in SW-13 cells in the neutralization assays, indicating that produced antibodies are non-neutralizing.

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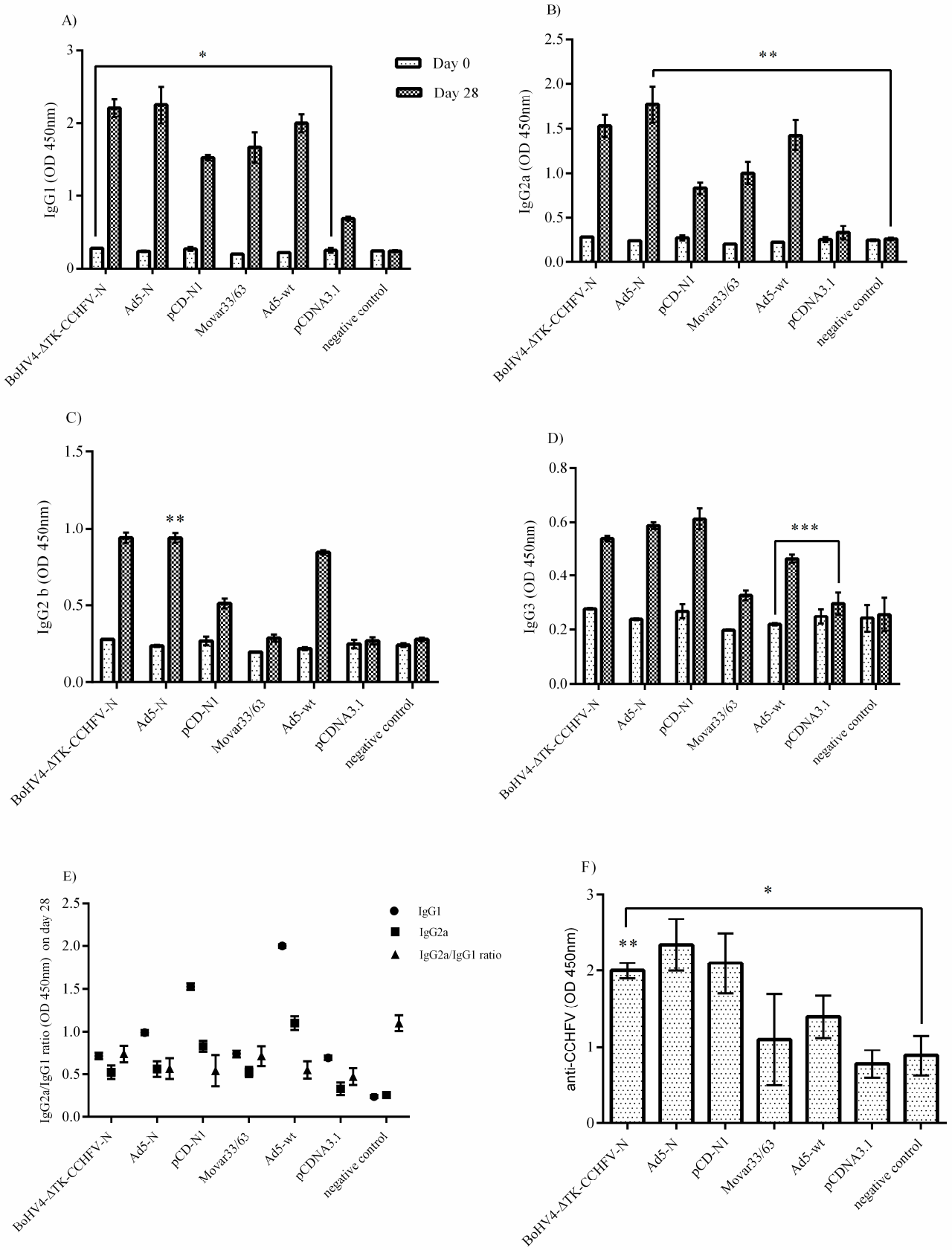

Figure 3. Serological Assays. Total antibody responses and ELISA assays were performed in BALB/c and IFNAR$^{-/-}$ mice sera, respectively. A) IgG1 response: pCD-N1 immunized mice showed a higher amount of this antibody compared to other N expressing constructs. B) IgG2a response: By considering the backbones, BoHV4-ΔTK-CCHFV-N and pCD-N1 groups had more potential to stimulate IgG2a production. C) IgG2b response: By considering the N expressing groups and related backbone subtraction, BoHV4-ΔTK-CCHFV-N group became eminent. D) IgG3 response: The results were comparable to IgG1 findings. E) IgG2a/IgG1 ratio: All groups except normal saline demonstrated a ratio < 1, indicating a shift towards Th2 responses. F) ELISA assay: Both viral vectors expressing N protein could produce specific IgG antibodies in comparison to DNA vaccine construct. All of the data are shown as mean ± SD.*$p < 0.05$; **$p < 0.01$ and ***$p < 0.001$ versus backbones.

Cytokine Assays

Cytokine responses in stimulated splenocytes of immunized BALB/c mice were analyzed. Evaluation of the major Th_1 cytokines in the supernatants revealed significantly higher levels of IFN-gamma in the pCD-N1 and IL-2 in Ad5-N at both 48- and 72-hours post-stimulation. Other constructs expressing N protein were unable to stimulate significant IL-2 production (Figure 4A, B). Ad5-N group was predominant in IL-4, IL-5 and IL-13 as an indicator of Th2 response. Cytokine production via other constructs expressing N protein were hardly detectable, when compared with their relative backbones (Figure 4C-E). In addition, Ad5-N and BoHV4-ΔTK-CCHFV-N groups showed potential to produce adequate amounts of IL-10 and TNF-alpha. Ad5-N was also eminent in IL-6 production (Figure 4F-H).

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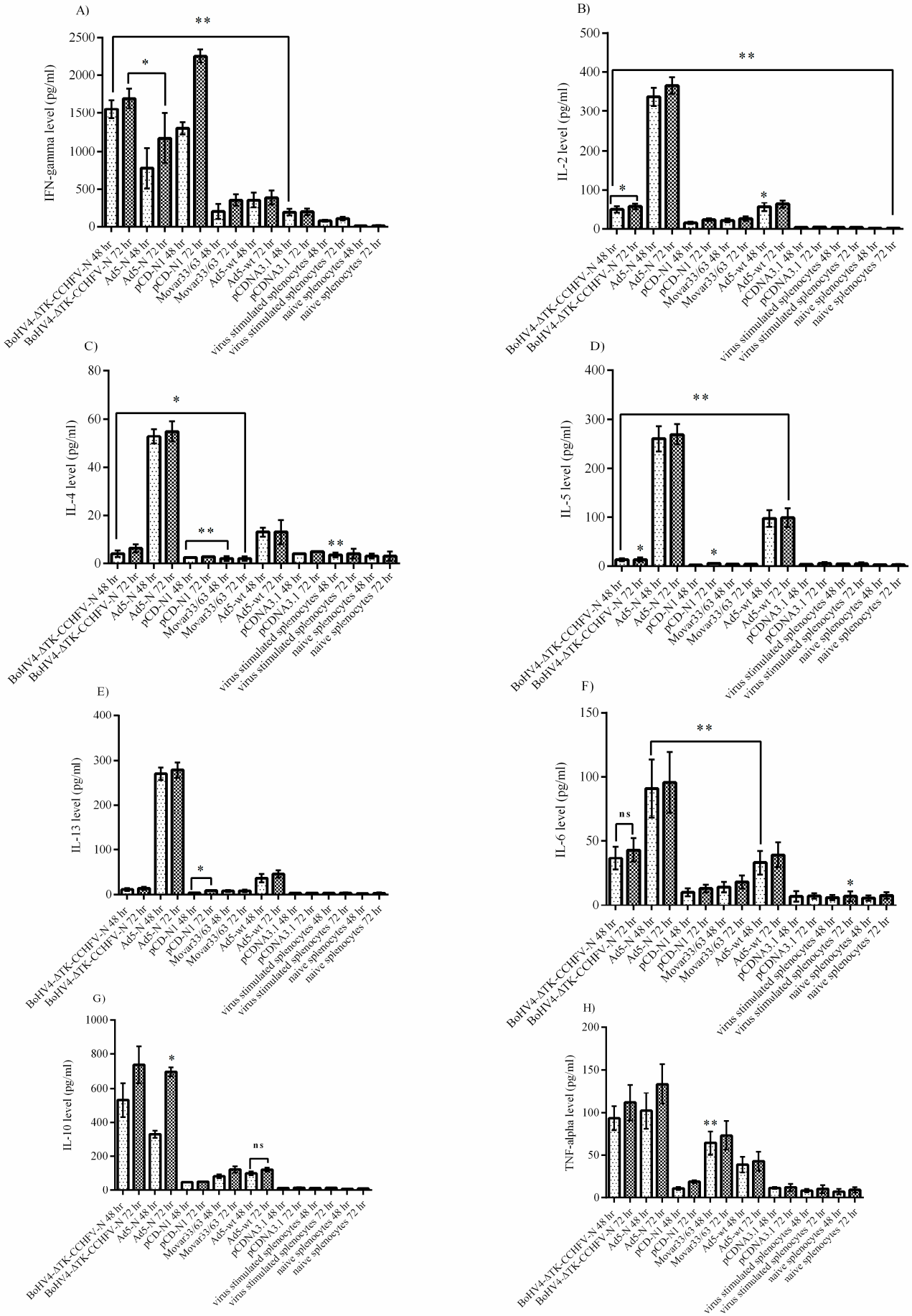

Figure 4. Cytokine responses of virus stimulated splenocytes from BALB/c mice after 48 and 72 hours. A) IFN-gamma response: As demonstrated here, pCD-N1 group was higher in comparison to other N expressing groups. B) IL-2 response: Ad5-N was dominant. Other groups were unable to stimulate adequate production. C) IL-4 response: The results were similar to IL-2. D) IL-5 response: Ad5-N has more potential to secrete IL-5. E) IL-13 response: The results were similar to IL-2, IL-4 and IL-5. F) IL-6 response: While Ad5-N group was elevated compared to BoHV4-ΔTK-CCHFV-N, sufficient levels were also stimulated by this construct. G) IL-10 response: Both BoHV4-ΔTK-CCHFV-N and Ad5-N groups induced significant amounts as compared with their relative backbone and pCD-N1. H) TNF-alpha response: The results were similar to IL-10. All of the data are shown as mean ± SD. $*p < 0.05$; $**p < 0.01$ and $***p < 0.001$ versus relative backbones.

Analysis of cytokine responses in BALB/c mice sera indicated that pCD-N1 construct induced significant IL-2, IL-13 and TNF-alpha. On the other hand, Ad5-N demonstrated more potential for IFN-gamma induction in comparison to other N expressing constructs. For IL-10, BoHV4-ΔTK-CCHFV-N induced response was pronounced. Ad5-N and BoHV4-ΔTK-CCHFV-N produced sufficient amounts of IL-4 and IL-6 (Figure 5A-H).

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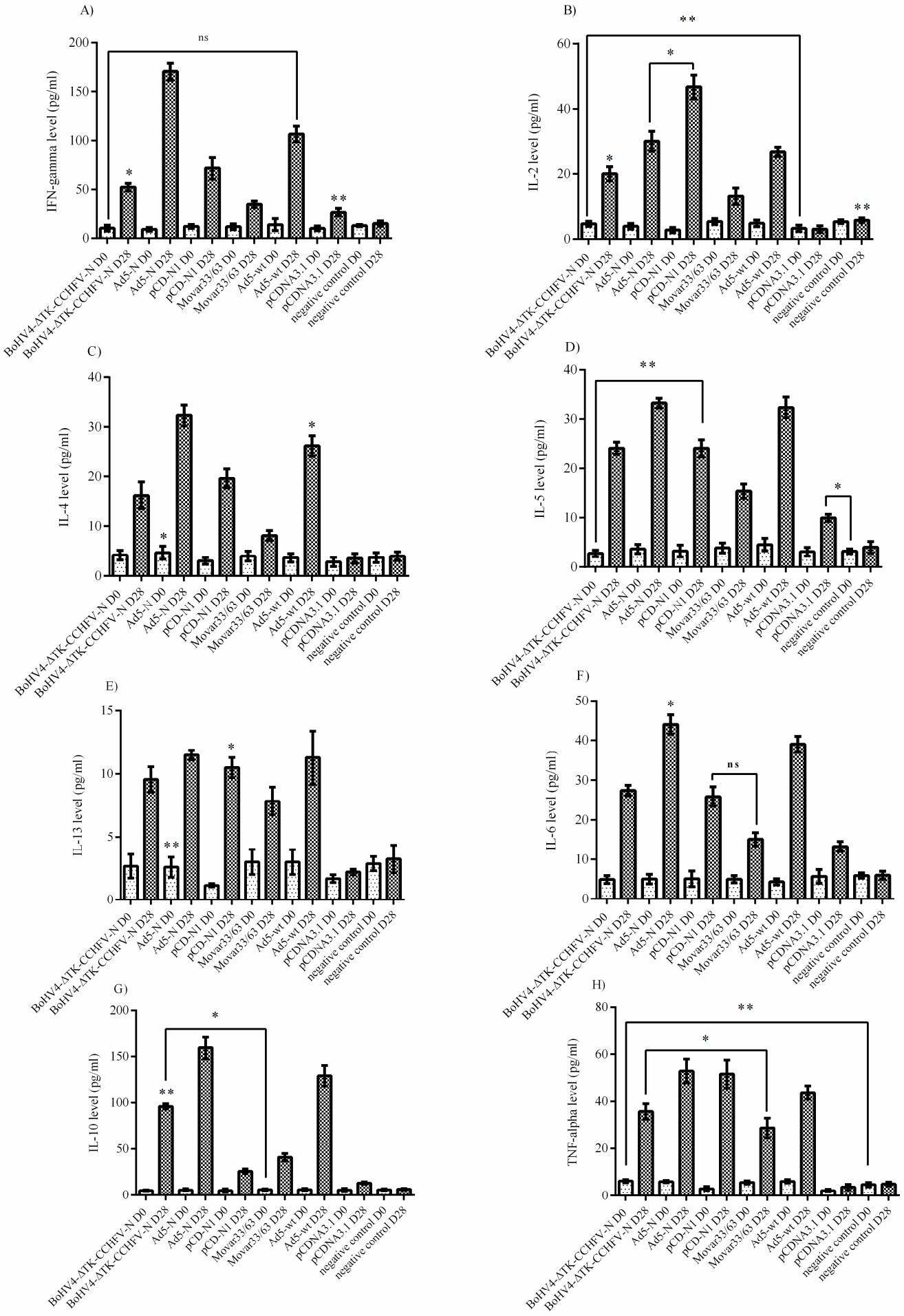

Figure 5. Cytokine responses in BALB/c mice sera. **A)** IFN-gamma response: Ad5-N construct was dominant in comparison to other N expressing ones. **B)** IL-2 response: By considering the relative backbones, pCD-N1 construct showed increased potential to elicit this cytokine's production. **C)** IL-4 response: Similar to IL-2 response, pCD-N1 group showed elevated levels. IL- 4 was further significant in BoHV4-ΔTK-CCHFV-N group. **D)** IL-5 response: identical to IL-4. **E)** IL-6 response: BoHV4-ΔTK-CCHFV-N and pCD-N1 groups showed higher levels **F)** IL-10 response: BoHV4-ΔTK-CCHFV-N has showed a higher potential in stimulation. **G)** IL-13 response: similar to IL-2. **H)** TNF-alpha response: By subtracting backbones, it is obvious that pCD-N1 is dominant in this kind of response. *$p < 0.05$; **$p < 0.01$ and ***$p < 0.001$ versus pVAX-1 group. All of the data are shown as mean ± SD.

Analysis of IFNAR$^{-/-}$ mice sera (performed on day 0 and survival days for N expressing constructs or days before death for control groups) showed that none of the N expressing groups could stimulate IFN-gamma, IL-2 and IL-5 production (Figure 6A, B, D). Despite production in all three constructs (BoHV4-ΔTK-CCHFV-N, Ad5-N and pCD-N1), the protective effect of IL-4 was prominent only in BoHV4-ΔTK-CCHFV-N and Ad5-N groups (Figure 6C). In addition, BoHV4-ΔTK-CCHFV-N, Ad5-N and pCD-N1 caused relatively high IL-6 and TNF-alpha induction, while negligible levels were observed in the control groups (Figure 6E, F).

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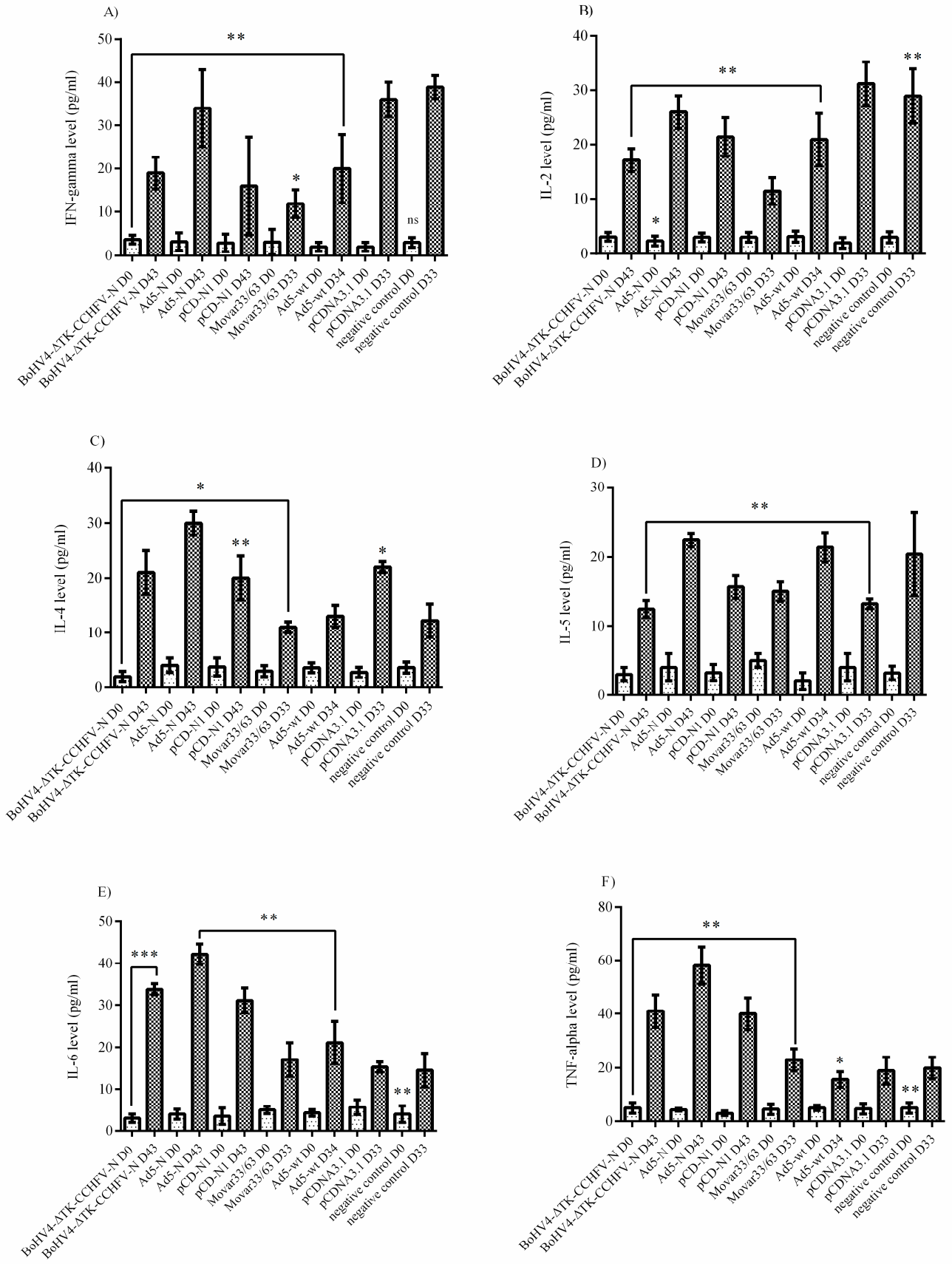

382 **Figure 6.** Cytokine responses in IFNAR$^{-/-}$ mice sera. **A)** IFN-gamma response: All N expressing constructs were
383 unable to stimulate adequate levels. **B)** IL-2 response: The results were similar to IFN-gamma. **C)** IL-4 response:
384 BoHV4-ΔTK-CCHFV-N, Ad5-N and pCD-N1 groups' responses were elevated. However, by considering the
385 relative backbone, only BoHV4-ΔTK-CCHFV-N and Ad5-N groups were significant. **D)** IL-5 response: All 3
386 groups of BoHV4-ΔTK-CCHFV-N, Ad5-N and pCD-N1 showed no potential of IL-5 stimulation. **E)** IL-6
387 response: BoHV4-ΔTK-CCHFV-N, Ad5-N and pCD-N1 groups demonstrated high levels. **F)** TNF-alpha
388 response: The results were the similar to IL-6. *$p < 0.05$; **$p < 0.01$ and ***$p < 0.001$ versus pVAX-1 group.
389 All of the data are shown as mean ± SD.

390 **Challenge Experiment, Antibody Passive Transfer and T cell Adoptive Transfer**

391 After challenge with the Ank-2 strain, N expressing groups including BoHV4-ΔTK-CCHFV-
392 N and Ad5-N showed a 100% protection rate, while one mouse in the pCD-N1 group died due
393 to virus replication confirmed by $TCID_{50}$ assay from infected brain, spleen and liver. All
394 control groups (Movar33/63, Ad5-wt, pCDNA3.1 myc/His A and normal saline) died within
395 6 days post-challenge (Figure 7A). In the survived mice, clinical symptoms including body
396 weight loss (Figure 7B), ruffled fur, appearance changes, depression and nasal/ocular
397 discharge started on day 1 of challenge and persisted for 3-4 days in different groups,
398 followed by recovery in all survivors. The survived mice stabilized after day 10 and the
399 experiments were carried on for additional 3 days to guarantee the observations. The virus
400 replication in the control groups were also verified by $TCID_{50}$ assay in SW-13 cells. Body
401 weight percentage were recorded in all challenge groups as an indicator of clinical disease or
402 recovery. Besides, 3 groups of IFNAR$^{-/-}$ each containing 3 individuals used in antibody
403 Passive and T cell adoptive transfer showed different protective rates. In BoHV4-ΔTK-
404 CCHFV-N group 2 mice survived while in Ad5-N and pCD-N1, only one mouse recovered
405 from the challenge experiment (Figure 7C).
406

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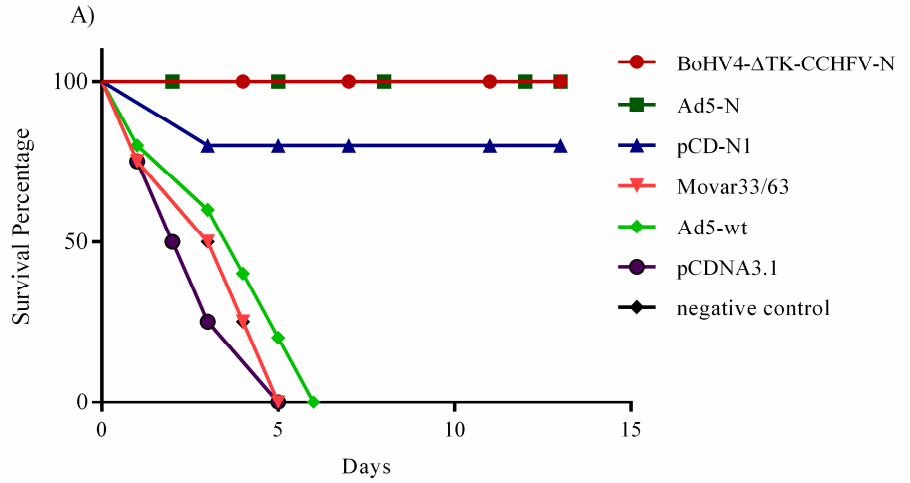

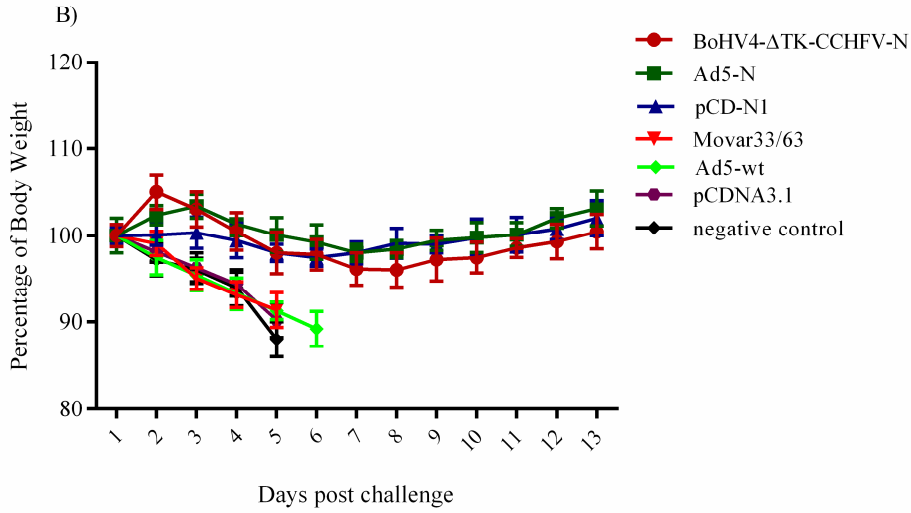

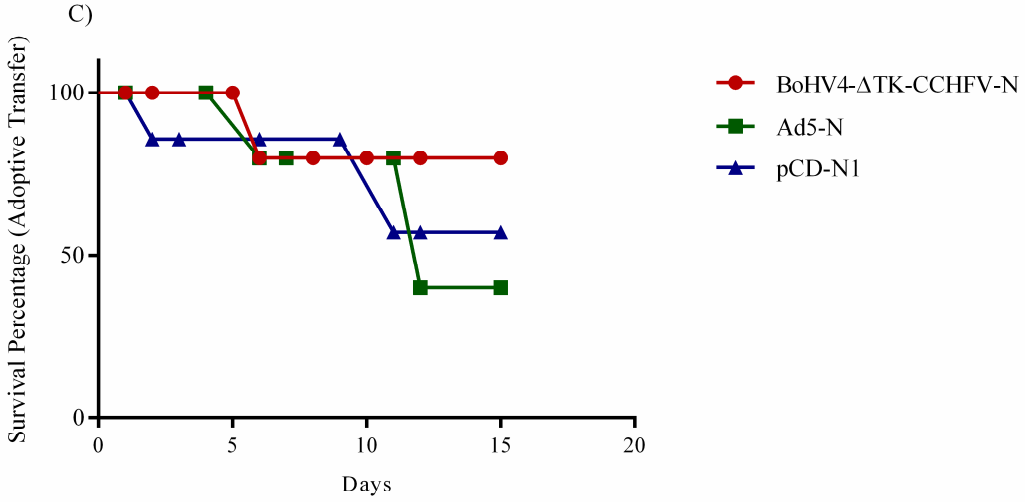

Figure.7. Challenge experiment. **A)** Survival rate in challenge assay: Survival rate of 100% observed in BoHV4-ΔTK-CCHFV-N and Ad5-N groups. The results of pCD-N1 are also satisfactory with one death (survival rate of 75%). Control groups of Mover33/63 (death on day 5 post-challenge), Ad5-wt (death on day 6 post-challenge), pCDNA3.1 myc/His A (death on day 5 post-challenge) and normal saline (death on day 5 post-challenge) were also included in the experiment. **B)** Percentage of body weight: Despite lethal challenge, an almost stable body weight range was observed in BoHV4-ΔTK-CCHFV-N, Ad5-N and pCD-N1 groups. All of the data are shown as mean ± SD. C) Survival rates in antibody passive and T cell adoptive transfer experiment: 24 hours after transfer of splenocytes plus serum samples of BALB/c immunized mice, IFNAR$^{-/-}$ mice challenged and after 13 days BoHV4-ΔTK-CCHFV-N showed a higher survival rate (75%) in comparison to other groups of Ad5-N and pCD-N1 (survival rate of 50%).

Discussion

In parallel with the incidence of tick-borne pathogens, an expansion of CCHFV activity is evident and the virus is reported from several previously unaffected regions [24]. Due to the lack of licensed vaccine and effective therapeutics, development of new approaches to ensure protective immunity to CCHFV in susceptible individuals became imperative. Previous efforts to develop immunity in indigenous populations of endemic regions include the Russian/Bulgarian vaccine, that contains chloroform inactivated virus, heated at 58 °C, and absorbed on $Al(OH)_3$ [25]. This vaccine has been thoroughly evaluated in 2012 and reported to have the potential to induce sufficient IFN-gamma response in the volunteers, given that the required booster doses could be administered [26].

In addition to employing inactivated viruses, various strategies have been explored for an effective CCHFV vaccine. Viral glycoproteins and nucleocapsid encoded by the M segment and S segment, respectively, are potential targets for several DNA or virus-like particle-based vaccines [6, 27, 28].

The CCHFV nucleocapsid protein (N), an essential component for intracellular virus replication, provides a prominent target for vaccine development. N-based vaccines of closely-related viruses have already been reported to elicit sufficient immune responses for protection [29, 30]. Being well-conserved among global viral lineages and bearing T cell epitopes, N protein becomes an attractive target for CCHFV vaccination as well [31]. Besides, in almost all vaccine development efforts based on N protein, protective rate of 100% were observed in different expressing platforms and mice models [7,8,23,27]. Therefore, we set out to focus on N protein to be used in different immunization platforms: adenoviral (Ad5-N) and newly-developed Bovine herpesvirus type 4 viral (BoHV4-ΔTK-CCHFV-N) vectors alongside with a DNA-based plasmid (pCD-N1). It has been previously demonstrated that BoHV-4 based viral vectors could stimulate immune responses when expressing

immunogenic dominant antigens of diverse viral diseases like Ebola and Bovine herpesvirus type 1 [32]. The crucial requirement of newly developed vaccine vectors is their capability to specifically stimulate immune responses in the animal model. For the first time, we have tried this viral vector expressing an antigen from Bunyaviridae to examine its immunological potential against N protein of CCHFV in BALB/c and IFNAR$^{-/-}$ mice models. Following successful in vitro generation of the expression constructs (BoHV4-ΔTK-CCHFV-N, Ad5-N and pCD-N1), we immunized BALB/c mice with a single booster dose after 14 days. The immune responses were evaluated via cytokine assays in virus-stimulated splenocytes and serum samples and total/specific immunoglobulin subtypes in serum samples. The neutralization potential of the produced immunoglobulins was further assessed via virus neutralization assay. We observed that N expressing systems (BoHV4-ΔTK-CCHFV-N, Ad5-N and pCD-N1) could induce prominent cytokine responses in both splenocytes and serum samples of BALB/c mice. Overall, Ad5-N dominated in the all cytokine responses among other expression constructs in the supernatant of virus stimulated splenocytes. Besides, IL-6, IL-10 and TNF-alpha were the cytokines that BoHV4-ΔTK-CCHFV-N demonstrated more potential to elicit secretion. The only significantly induced cytokine via pCD-N1 was IFN-gamma. On the other hand, pCD-N1 construct showed high potential for IL-2, IL-4, IL-5, IL-13, IL-6 and TNF-alpha stimulation in BALB/c mice sera. BoHV4-ΔTK-CCHFV-N construct further resulted in elevated IL-4, IL-6 and IL-10 responses in BALB/c mice. For a deeper investigation in protection against lethal challenge, we analyzed cytokine responses in IFNAR$^{-/-}$ mice sera on days 0, 28 (2 weeks after booster and before challenge) and different days post-challenge (death days in control mice and day 43 for the survivor groups). We have observed negligible potential of BoHV4-ΔTK-CCHFV-N, Ad5-N and pCD-N1 to stimulate IFN-gamma, IL-2 and IL-5. In contrast, all three constructs showed high levels of IL-6 and TNF-alpha which can be considered as associated with survival in the challenged mice.

In serological assays, BoHV4-ΔTK-CCHFV-N, Ad5-N and pCD-N1constructs could trigger production of all evaluated total IgG immunoglobulin subtypes in BALB/c mice sera. In human infections, IgM and IgG are serological markers of exposure and they become detectable during or after the first week of the infection in survivors and in fatal cases, a lack of IgG and IgM response has been documented, indicating the impact of antibodies (although non-neutralizing) in immune control of the virus replication [28, 33, 34].

We further evaluated the specific IgG antibody responses in IFNAR$^{-/-}$ mice by developing an in-house ELISA assay using CCHFV infected cells. Here, we observed pCD-N1 to possess more potential for humoral response stimulation. The other constructs also elicited remarkable

IgG levels on day 28. However, the total and specific antibody responses elicited by our N expressing constructs failed to neutralize CCHFV in vitro, as assessed by the virus neutralization assay. Inability of the N protein to produce sufficient amounts of specific neutralizing antibodies were previously documented in animal models and non-neutralizing antibodies were also reported to protect mice from lethal CCHFV challenges [23,35, 36].

A recombinant DNA-based CCHFV-N expression system was previously developed, using Modified Vaccinia Ankara vector as delivery system. However, the immune responses in vaccinated mice were not evaluated in detail, lacking cytokine and antibody response analyses. Although this system seemed to have induced an antigen-specific immunogenicity in mice, it failed to exert any protective effect upon virus challenge [7].

In this study, we have shown the role of T cell and passive antibody transfer of immunized mice in CCHFV lethal challenge protection. As previously shown that a balanced Th1 and Th2 responses are necessary to elicit protection in challenge experiments, we decided to try a combination of T cell and antibody in this assay [8]. Antibody plus T cells of BoHV4-ΔTK-CCHFV-N immunized BALB/c mice elicited 75% protection rate in IFNAR$^{-/-}$ mice. This phenomenon was previously demonstrated in vaccinated mice by MVA-GP and no protection was documented [37].

Particular shortcomings of the delivery systems we employed for the expression of CCHFV-N in this study must be addressed. A major obstacle of the adenovirus-based vectors is the pre-existing immune response which may cause inefficient expression following human vaccination. Employing rare virus serotypes such as 2 and 5, as performed in this study, may overcome or alleviate this problem [36]. Besides, adenovirus-based vectors are also highly immunogenic and booster doses in individuals may induce serious side effects [38].

We have further investigated BoHV-4 as a novel viral vector and evaluated its potential as a carrier of CCHFV N protein via comparisons with adenoviral and DNA vector platforms. Our findings indicate that despite the variation among the cytokine and antibody responses in BALB/c mice, immunization with CCHFV-N expressing BoHV-4 has significant protection potential against the lethal challenge in IFNAR$^{-/-}$ mice. Therefore, we set out to employ BoHV-4 viral vector as the main delivery system for CCHFV N protein towards the development of the effective vaccine. However, due to similar protection rates observed in BoHV4-ΔTK-CCHFV-N and Ad5-N, a detailed comparison of these platforms could not be performed. The clinical findings following challenge further suggest that Ad5-N induced immunity may be effective in alleviating diseases symptoms for a faster recovery in the IFNAR$^{-/-}$ mice. However, as mentioned above, the main obstacle of Ad5-wt is the high

potential for immune stimulation. Overall, BoHV-4 based viral vector have certain advantages over the DNA and adenoviral vectors [15]. One of the most important characteristics of BoHV-4 is its persistency in macrophages and monocytes cells. This may result in the elimination of booster dose requirements due to frequent virus reactivation in persistence sites. In addition, macrophages and monocytes further act as antigen presenting cells which can facilitate the presentation of antigens carried by the vector. However, a probable suppression of insert expression due to persistency, resulting in insufficient immune stimulation must also be considered. Lack of preexisting immune response in humans and neutralizing antibodies in cattle, the main host of the virus, constitute additional advantages of BoHV-4 based delivery [15]. Therefore, BoHV-4 should be explored in detail as a new potential viral vector for CCHFV vaccination.

In conclusion, our findings indicate that N protein of CCHFV can be considered as the main target in vaccine development regardless of the expressing platform. We also established a new viral vector expressing viral N protein (BoHV4-ΔTK-CCHFV-N), which showed considerable potential as an antigen delivery system. The next step will be to focus on the immunological aspects of this newly developed platform in mice or even in other animal models like goat or sheep which are considered as playing important role in virus epidemiology in the nature.

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

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