Veterinary Microbiology 237 (2019) 108405

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Henipavirus-like particles induce a CD8 T cell response in C57BL/6 mice

Eileen Stroh[a,1], Kerstin Fischer[a], Theresa Schwaiger[b], Lucie Sauerhering[c], Kati Franzke[d], Andrea Maisner[c], Martin H. Groschup[a], Ulrike Blohm[e], Sandra Diederich[a,*]

[a] Institute of Novel and Emerging Infectious Diseases, Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Greifswald-Insel Riems, Germany
[b] Department of Experimental Animal Facilities and Biorisk Management, Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Greifswald-Insel Riems, Germany
[c] Institute for Virology, Philipps-University Marburg, Marburg, Germany
[d] Institute of Infectology, Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Greifswald-Insel Riems, Germany
[e] Institute of Immunology, Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Greifswald-Insel Riems, Germany

ARTICLE INFO

Keywords:
Nipah virus
CD8 T cell response
Virus-like particles
C57BL/6 mice
Th1 cytokines

ABSTRACT

Nipah virus (NiV), a BSL-4 pathogen, belongs to the genus *Henipavirus* within the family *Paramyxoviridae*. To date, no effective vaccine is available. Although most of the current vaccine studies aim to induce a neutralizing antibody response, it has become evident that a promising vaccine should target both, humoral and cell-mediated immune response. Virus-like particles (VLPs) have been shown to activate both arms of the adaptive immune response. In our study, VLPs composed of the NiV surface glycoproteins G and F and the matrix protein of the closely related Hendra virus (HeV M) induced both, a neutralizing antibody response and an antigen-specific CD8 T cell response with proliferation, IFN-γ expression and Th1 cytokine secretion in C57BL/6 mice. In contrast, in BALB/c mice only a neutralizing antibody response was observed. All three viral proteins included in the VLPs were shown to harbor CD8 T cell epitopes; however, the combination of all three proteins enhanced the magnitude of the CD8 T cell response. To conclude, VLPs represent a promising vaccine candidate, as they induce humoral as well as CD8 T cell-mediated immune responses.

1. Introduction

Nipah virus (NiV), an emerging zoonotic paramyxovirus within the genus *Henipavirus*, causes fatal encephalitis in humans and severe respiratory disease in pigs (Chua et al., 2000). Due to the broad host range, the high fatality rates (up to 80%), and the lack of antiviral therapy, NiV has been classified as a biosafety level 4 (BSL-4) pathogen. After virus contact, administration of the monoclonal antibody m102.4 is thought to be the most suitable strategy as therapeutic treatment so far (Geisbert et al., 2014). However, rapid replication and spread of the virus limit the timeframe for application of therapeutics. Thus, preventive vaccination remains an important alternative, especially in an outbreak scenario when people who may be in contact with infected individuals, infected animals and susceptible livestock need protection. A pig vaccine that is able to prevent shedding of infectious virus is of veterinary and human health significance, including the importance to reduce the severe economic consequences of a NiV outbreak. Yet, currently no licensed vaccine against NiV is available.

Development of effective antiviral strategies and vaccination requires a deep insight into how NiV interacts with the immune system. Regarding the adaptive immune response, various approaches to find a vaccine candidate *in vivo* revealed that humoral CD4 T helper cell (Th-) dependent immunity with the development of neutralizing antibodies is a critical factor in surviving NiV infection and for future protection (DeBuysscher et al., 2014; Guillaume et al., 2004). The receptor-binding G protein and the fusion protein F have been shown to be the major targets for the generation of neutralizing antibodies (Tamin et al., 2002). Thus, diverse vaccination studies focused on the induction of a neutralizing antibody response using one or both surface glycoproteins as antigens (summarized in Satterfield et al., 2016). Several studies suggest the activation of T cells upon NiV infection or vaccination in different hosts (Cong et al., 2017; Kong et al., 2012; Prescott et al., 2015). However, one recent study in pigs suggests an additional role of the cell-mediated immune response for full protection against NiV challenge (Pickering et al., 2016).

In recent years, virus-like particles (VLPs) have been used increasingly in vaccine development against several viral diseases. In general, VLPs can be produced by *in vitro* expression of individual viral

* Corresponding author at: Friedrich-Loeffler-Institut, Südufer 10, 17493 Greifswald Insel Riems, Germany.
E-mail address: sandra.diederich@fli.de (S. Diederich).
[1] Present address: SensID GmbH, Schillingallee 68, 18057 Rostock, Germany.

https://doi.org/10.1016/j.vetmic.2019.108405
Received 16 July 2019; Received in revised form 1 September 2019; Accepted 2 September 2019

structural proteins, such as the envelope or the matrix proteins of the original virus. These proteins may have the intrinsic ability to self-assemble spontaneously into highly organized particles, that can be purified from the cell culture supernatant, and resemble viable virus particles morphologically in size and shape, but lack the viral genome. In contrast, to obtain soluble expressed protein, parts of the protein such as the transmembrane region may have to be deleted or modified, eventually resulting in structural modifications of the protein. Overall, VLPs offer great advantages: they are not infectious and thus can be handled safely under low containment conditions; they mimic the overall structure of native virus with preservation of native antigen conformation, and they have been shown to induce antibody- and cell-mediated immune responses as exogenous antigens *via* cross-presentation for various pathogens (summarized in Diederich et al., 2015). NiV-based VLPs composed of the two glycoproteins F and G and the matrix protein M have been shown to induce an antibody response with the production of neutralizing antibodies in rabbits, BALB/c mice and Syrian hamsters (Schmidt et al., 2018; Walpita et al., 2011, 2017). However, to our knowledge, it has not been investigated whether NiV-based VLPs are able to induce a CD8 T cell mediated response in any animal model. C57BL/6 mice react preferentially with a T-helper type 1 (Th1) biased immune response, which ultimately is a requirement for a CD8-mediated T cell response, whereas BALB/c mice tend to a Th2-dominated immune response after infection or immunization (summarized in Sellers et al., 2012). The aim of our study was therefore to use VLPs, consisting of NiV G, NiV F and the Hendra virus (HeV) M protein, to investigate that immunization with VLPs induces a CD8-mediated T cell response in Th1-biased C57BL/6 mice, but not in Th2-biased BALB/c mice, and finally, to demonstrate that the investigated viral proteins are able to induce a T-cell mediated immune response.

2. Materials and methods

2.1. Plasmids

The open reading frame for NiV F-HAtag (in the following designated as NiV F) was subcloned from a pczCFG5-NiV F-HAtag plasmid (Moll et al., 2004) into the pCAGGS vector. The NiV G coding sequence (GenBank accession number No. AF212302) was codon optimized for human cells, and was synthesized and cloned into pCAGGS vector (GeneArt AG/Thermo Fisher Scientific Inc.). pCAGGS HeV M was provided by Stefan Finke, Friedrich-Loeffler-Institut (Bauer et al., 2014). Complete sequences can be obtained on request.

2.2. Cells

HEK-293 T (human embryonic kidney 293 T) cells and Vero cells (African green monkey kidney cells) (Collection of Cell Lines in Veterinary Medicine, Friedrich-Loeffler-Institut, FLI) were cultivated in Minimal Essential Medium (Earl's and Hank's salts 1:1) supplemented with 1% non-essential amino acids, 0.125% sodium hydrogen carbonate, 0.012% sodium pyruvate and 10% fetal calf serum (FCS). Isolated murine splenocytes were cultivated in Iscove's Modified Dulbecco's Medium and Ham's F12 (1:1), supplemented with 10% FCS and 1% penicillin/streptomycin. All cells were cultured at 37 °C, 5% CO_2, and in a humidified atmosphere.

2.3. Mice

C57BL/6 mice were obtained from a breeding stock at FLI. BALB/c mice were purchased from Charles River (Schweinfurt, Germany). Female mice were used at 6 weeks of age. Mice were bred under SPF conditions and kept in a conventional animal house facility.

2.4. Preparation of VLPs

For antigen production, 2×10^6 HEK-293 T cells were either co-transfected or single transfected with a total of 20 µg plasmid DNA encoding for NiV F, NiV G, or HeV M mixed 1:1 with polyethylenimine. Eight hours post transfection (p.t.), medium was replaced by serum-free medium. VLPs were purified by ultracentrifugation as described earlier (Diederich et al., 2008). Briefly, at 48 h p.t., supernatants were clarified and then purified through a 20% sucrose cushion at 96,000 x g for 2 h at 4 °C. Pellets were washed at 155,000 x g for 30 min at 4 °C and re-suspended in Tris-Sodium Chloride (TN) buffer. Antigen preparation was stored at −80 °C until usage. To establish an equivalent control (control supernatant), HEK-293 T cells were transfected with empty pCAGGS vector and supernatant was treated as described before. Protein concentration was determined by bicinchoninic acid assay (BCA; Uptima) according to the manufacturer's instructions.

To verify incorporation of the viral proteins, purified VLPs were analyzed by Western Blot using primary antibodies: NiV G was detected by the mouse monoclonal antibody 5G1B1 (Fischer et al., 2018), NiV F was detected by a polyclonal rabbit anti-HA serum (H6908; Sigma-Aldrich), and HeV M was detected by a serum produced in a rabbit after immunization with a NiV M peptide (Ac-YLDKVEPEIDENGSMIPKY-KIC-NH2; immunoGlobe GmbH). Detection antibodies were goat anti-mouse AlexaFluor 488 and/or goat anti-rabbit AlexaFluor 568 (Life-Technologies). Immune complexes were visualized with the Molecular Imager VersaDoc MP 4000 system (BioRad) and were processed with Quantity One analysis software (BioRad).

2.5. Transmission electron microscopy

For analysis by transmission electron microscopy (TEM), 20 µl (c = 1 µg/µl) of VLPs were adsorbed to formvar coated nickel grids for 7 min, then stained with 1% phosphotungstic acid (pH 6.0). Analysis was performed with a Tecnai-Spirit TEM (FEI) at an accelerating voltage of 80 kV.

2.6. Immunization of animals and sampling

C57BL/6 mice were used in comparison to BALB/c mice as a methodical approach to prove the CD8-mediated T cell response. Mice were immunized intraperitoneally three times at a three-week interval with 7.5 µg VLPs mixed 1:1 with complete Freund's adjuvant for prime or incomplete Freund's adjuvant for boosts. Control mice were immunized with the same amount of control supernatant. At 21 days post boost 1 (d21pb1) and 21 days post boost 2 (d21pb2), mice were sacrificed according to animal care regulations and splenocytes were isolated for *in vitro* experiments (see Table 1). Serum was collected after heart puncture for serological assays.

The animal experiments were evaluated by the ethics committee of the State Office for Agriculture, Food Safety, and Fisheries in Mecklenburg - Western Pomerania (LALLF M-V) and gained legal governmental approval.

Table 1
Experimental design for immunization in C57BL/6 and BALB/c mice.

d0	d21 post prime	d21 post boost 1	d21 post boost 2
		(d21pb1)	(d21pb2)
Immunization (prime)	*boost 1*	*boost 2* CD8 proliferation IFNγ-production	*End of study* CD8 proliferation IFNγ-production Antibody response Cytokine detection

2.7. Indirect immunofluorescence assay

For indirect immunofluorescence assay (IFA), 2×10^4 Vero cells were transfected with 1 µg plasmid DNA encoding for either NiV F, NiV G, or HeV M with TransIT-293 Transfection Reagent according to the manufacturers' protocol. At 24 h p.t., cell monolayers were fixed with 4% paraformaldehyde (PFA) and permeabilized with 0.2% Triton-X100 in PBS^{++} (PBS with 0.5 mM MgCl, 1 mM $CaCl_2$). Cells were incubated with mouse sera at a 1:50 dilution in 5% BSA / PBS^{++} for 1 h at 4 °C, washed and then incubated with goat anti-mouse AlexaFluor 488 secondary antibodies. Cell nuclei were counterstained with 4′,6-Diamidin-2-phenylindol (DAPI). Images were acquired with an Eclipse Ti-S inverted microscope system and were processed with the NIS-Elements BR 4.00.07 software (Nikon).

2.8. Foci reduction assay

All experiments with live NiV were performed under BSL-4 conditions at the Institute of Virology, Philipps University Marburg, Germany. The NiV-induced foci reduction assay was carried out as described elsewhere (Fischer et al., 2016). Briefly, recombinant NiVeGFP (2×10^4 50% tissue culture infection dose, $TCID_{50}$) was preincubated for 45 min with mouse sera collected at d21pb2 and then Vero cells were incubated with the sera - virus - mixture. After 45 min of incubation, the inoculum was removed and cells were cultured for further 24 h. Then, cells were fixed and inactivated with 4% PFA for 48 h. Following permeabilization with methanol-acetone, NiV-induced syncytia were stained with a polyclonal guinea pig anti-NiV serum (Ringel et al., 2019).

2.9. In vitro restimulation assays

To determine a CD8 T cell response, splenocytes isolated at d21pb1 and d21pb2 were seeded in 48-well plates for *in vitro* restimulation assays and then treated with VLPs or single-protein VLPs (G-VLPs, F-VLPs or M-VLPs) at 10 µg/ml final concentration or the equivalent amount of control supernatant (stimulation control).

For proliferation assays, splenocytes were stained with 5-(and 6)-Carboxyfluorescein diacetate succinimidyl ester (CFSE) according to the manufacturers' instructions. Per well, 6×10^6 cells/ml were incubated with antigen for 5 d before analysis. For calculation of VLP-specific cell proliferation, the percentage of proliferating cells after stimulation with control supernatant was subtracted from the percentage of proliferating cells after VLP restimulation.

To detect intracellular IFN-γ, 4×10^6 cells/ml were restimulated overnight and 0.021 mM Monensin was added for the last 10 h of incubation. Subsequently, the ratio of IFN-γ^+CD8^+ cells after antigen-specific restimulation to IFN-γ^+CD8^+ cells after control supernatant stimulation was calculated based on control.

CD8 T cell-derived proliferation, IFN-γ expression as well as detection of T cell phenotypes were performed by flow cytometry (FCM) analysis.

2.10. Flow cytometry analysis, antibodies

For FCM analysis, cells were stained for 15 min at 4 °C in FACS buffer. For intracellular cytokine staining, cells were fixed and permeabilized with True-Nuclear Transcription Factor Buffer Set (BioLegend) according to the manufacturers' guidelines. A FACSCanto™ II flow cytometer (BD Biosciences) was used for analysis. Data were processed by FACSDiva software v8.0.1 (BD Biosciences) and FlowJo v10.1 (FLOWJO, LLC Data Analysis Software). Antibodies used were obtained from BioLegend and are summarized in Table 2.

Table 2
Antibodies used for FCM analysis.

Antigen	Clone	Isotype	Fluorochrome	Labelling strategy
CD8α	53-6.7	IgG2a	PerCP	Directly conjugated
IFN-γ	XMG1.2	IgG1	APC	
CD3	17A2	IgG2b	PE	
CD279 (PD1)	29 F.1A12	IgG2a	APC/Cy7	
NK1.1	PK136	IgG2a	PE	
KLRG1	2 F1/KLRG1	IgG2	BV510[(a)]	Biotin-Streptavidin

(a)Streptavidin-BV510.

2.11. Cytokine ELISA

Murine splenocytes isolated at d21pb2 were stimulated as described above for 4 d. Then, cell culture supernatants were tested in cytokine ELISAs for murine IFN-γ, TNF-α, IL-2 and IL-4 (PeproTech) according to the manufacturer's instructions.

2.12. Statistics

GraphPad prism 7 (GraphPad Software) was used to generate graphs. Statistical analyses were performed by Mann-Whitney U test using R. Differences were considered significant at $p \leq 0.05$.

3. Results

3.1. Generation and characterization of VLPs

VLPs were generated in 293 T cells using plasmids encoding for NiV G, NiV F and HeV M, since it has been shown that the incorporation of HeV M instead of NiV M leads to improved budding of particles (Yun et al., 2015). Incorporation of all three viral proteins was confirmed by Western Blot analysis (Fig. 1A). Protein concentrations for antigen preparations were determined by BCA assay with yields of about 1–1.5 µg/µl. Analysis of the produced VLPs by TEM revealed pleomorphic particles ranging in size from 100 to 500 nm, thus resembling the overall structure of the native virus (Fig. 1B).

3.2. VLPs induce a CD8 cell-mediated immune response in C57BL/6 but not in BALB/c mice

To analyze the CD8 cell response in mice, C57BL/6 and BALB/c mice were immunized three times at a three-week interval with VLPs or control VLPs. At d21pb1 and d21pb2, splenocytes were isolated and restimulated *in vitro* to assess antigen-specific proliferation by CFSE labeling and FCM analysis. We analyzed all CD8 cells without further discrimination between natural killer (NK) cells and CD8 T cells. As shown in the histograms in Fig. 2A, a proliferation was observed in CD8 cells from VLP-immunized C57BL/6 mice after VLP restimulation. Neither CD8 cells isolated from VLP-immunized C57BL/6 mice after stimulation with control supernatant, nor CD8 cells from control mice responded with proliferation independently of the stimulating antigen. For better visualization, the proliferation of CD8 cells from all C57BL/6 mice is summarized in a dot chart. At both time points, an antigen-specific proliferation after VLP restimulation was detected with a maximum of 52% of CD8 cells proliferating with high individual variation. Nevertheless, the difference between VLP-immunized C57BL/6 mice and control mice is significant at d21pb2. No or just marginal proliferation was observed in CD8 cells from VLP-immunized BALB/c mice or control mice as shown in the example histogram and the dot chart in Fig. 2B.

Aside from proliferation, IFN-γ expression is a hallmark for CD8 T cell-mediated response. Therefore, intracellular IFN-γ expression in CD8 splenocytes from C57BL/6 and BALB/c mice was measured after restimulation with VLPs or control supernatant by FCM analysis.

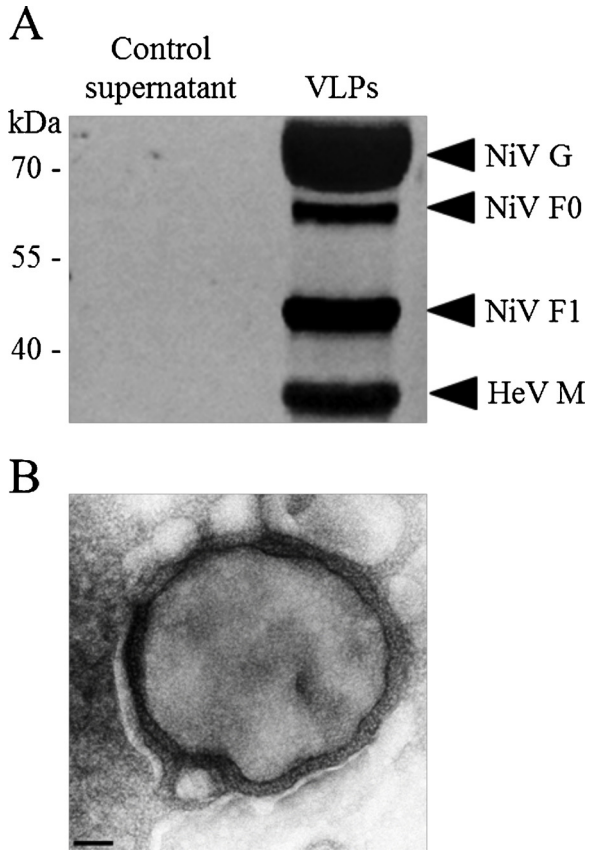

Fig. 1. Characterization of purified VLPs. (A) Western blot analysis of VLPs containing NiV G, NiV F (precursor protein F0 and subunit F1) and HeV M. (B) Morphology of VLPs was analyzed by transmission electron microscopy. Bar, 50 nm.

Numerous IFN-γ^+ events from CD8 cells of VLP-immunized C57BL/6 mice were observed after homologous restimulation, but not after stimulation with control supernatant or from control mice (Fig. 2C). The dot chart in Fig. 2C summarizes the fold changes of IFN-γ^+CD8 cells from all C57BL/6 mice after specific VLP restimulation based on control. Overall, median IFN-γ expression after antigen-specific stimulation of CD8 cells from VLP-immunized C57BL/6 mice was 7–8 times higher than expression after unspecific stimulation. At d21pb2, the difference between VLP-immunized mice compared to control mice was significant. In contrast to C57BL/6 mice, antigen-specific CD8 cell-derived IFN-γ expression was not detected in VLP-immunized BALB/c mice (Fig. 2D).

To further examine whether stimulation with VLPs induced the production of Th1-driven cytokines, splenocyte supernatants were tested for murine IFN-γ, TNF-α and IL-2 in cytokine ELISAs. As a control for a Th2 cytokine, IL-4 secretion was determined. As shown in Fig. 2E, significant differences in the Th1 cytokines in splenocyte supernatants from VLP-immunized C57BL/6 mice (median concentration IFN-γ = 18,629 pg/ml, cTNF-α = 699 pg/ml, cIL-2 = 125 pg/ml) *versus* control mice (median cIFN-γ = 1249 pg/ml, cTNF-α = 36 pg/ml, IL-2 not detectable) were measured while IL-4 was not detected. In contrast, in supernatants of VLP-immunized BALB/c mice, however, IL4 was the predominant cytokine detected (cIL-4 = 65 pg/ml) after antigen-specific restimulation (Fig. 2F). Neither IFN-γ nor IL-2 were detected in supernatants from BALB/c mice. TNF-α secretion was similarly detected in immunized and control BALB/c mice (cTNF-α = 210 pg/ml and cTNF-α = 208 pg/ml, respectively). Thus, none of the Th1-driven cytokines was found to be specifically induced in splenocytes from VLP-immunized BALB/c mice.

3.3. VLPs induce production of neutralizing antibodies in C57BL/6 and BALB/c mice

To determine if VLPs also serve as antigen-inducing specific antibodies, sera from both mouse strains collected at d21pb2 were tested for neutralizing antibodies in a NiV-induced foci reduction assay (Fig. 3). Sera from VLP-immunized C57BL/6 mice (Fig. 3A) and BALB/c mice (Fig. 3B) completely inhibited NiV-induced syncytium formation at a serum dilution of 1:10 and partially inhibited syncytium formation at serum dilutions of 1:100. Only marginal neutralizing effects where visible at a serum dilution of 1:1000. Thus, VLPs act as immunogens in both mouse strains resulting in the induction of a humoral immune response with generation of neutralizing antibodies.

3.4. Combination of NiV G, NiV F and HeV M proteins enhances CD8 T cell-mediated immune response

To investigate if one of the three viral proteins incorporated in the VLPs is sufficient for induction of a cell-mediated response, VLPs expressing only one viral protein were generated and confirmed in Western blot analysis (Fig. 4A). In order to demonstrate that all three viral proteins incorporated into VLPs harbor distinct B cell epitopes that induce a humoral immune response with generation of antibodies following immunization, an IFA against all three henipavirus proteins was performed. Collected mouse sera clearly showed to react with the respective viral proteins expressed in Vero cells (Fig. 4B).

Then, splenocytes from immunized C57BL/6 mice were restimulated with VLPs, one of the three single-protein VLP preparations, or control supernatant, and proliferation of CD8 T cells was analyzed. At both indicated time points, CD8 T cells from VLP-immunized C57BL/6 mice showed significant antigen-specific proliferation to different degrees after restimulation with the different VLP preparations (Fig. 4C). Restimulation with VLPs only containing the F or the M protein (F-VLP, M-VLP) had a minor effect, while incubation with VLPs composed of all three viral proteins, or only containing the NiV surface glycoprotein G (G-VLP) resulted in high numbers of proliferating CD8 T cells with medians of 15.3% and 15.9% at d21pb2, respectively. Additionally, a CD8 T cell proliferation (median 12.5%) was also measured within the control group at d21pb2 after G-VLP restimulation.

With regard to intracellular IFN-γ expression determined by FCM, median IFN-γ expression after stimulation of CD8 T cells from VLP-immunized C57BL/6 mice with G, F and M-containing VLPs was 7x higher than compared to unspecific stimulation (Fig. 4D). As shown before (Fig. 2C), the difference between stimulated VLP-immunized mice and VLP-stimulated control mice was significant at d21pb2. In contrast to VLPs containing all three viral proteins, there was only a minor increase in antigen-specific IFN-γ expression in CD8 T cells after restimulation with VLPs containing only G, F or M protein (Fig. 4D); although incubation with these single VLPs had induced a T cell proliferation (Fig. 4C). Thus, restimulation of splenocytes from VLP-immunized mice with G, F and M-containing VLPs resulted in a stronger CD8 T cell response than restimulation with any single-protein VLP.

4. Discussion

NiV has caused sporadic outbreaks in the past years with the most recent one in southern India in 2019 (ProMED-mail, 2019). Despite the importance of NiV as an emerging zoonotic pathogen, there currently is a lack of approved therapeutics and vaccines for use in livestock and humans. Most current vaccination strategies using soluble G subunits (Bossart et al., 2012; Middleton et al., 2014; Pallister et al., 2013), NiV-based VLPs (Walpita et al., 2011, 2017) or replicative vector-based vaccines (DeBuysscher et al., 2014; Defang et al., 2010; Guillaume et al., 2004) aim to induce neutralizing antibodies that are known to play a critical role in the protection against henipavirus infection (DeBuysscher et al., 2014; Guillaume et al., 2004). However, few recent

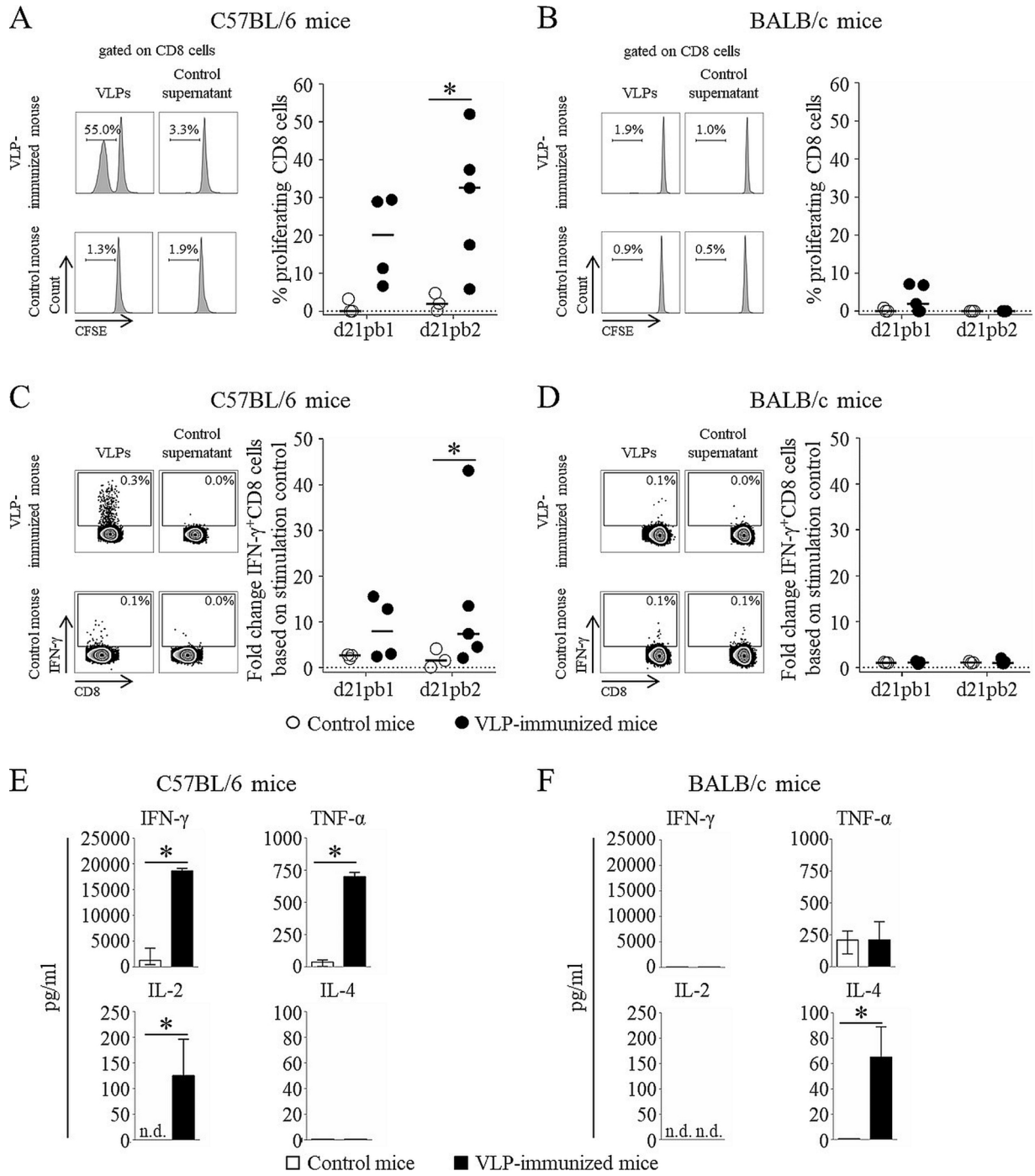

Fig. 2. CD8 cell-mediated response to VLPs in splenocytes from C57BL/6 (left panel) and BALB/c (right panel) mice. CD8 cell proliferation for (A) C57BL/6 and (B) BALB/c mice was measured using CFSE at d5 after *in vitro* restimulation of splenocytes with VLPs, and is shown exemplarily as histogram (d21pb2), and is summarized in dot charts. IFN-γ^+CD8 cells after VLP restimulation for (C) C57BL/6 mice and (D) BALB/c mice are shown exemplarily as contour plot, and is summarized as x-fold change based on stimulation control in dot charts. Black lines show median values. The zero level is indicated by the dotted horizontal line. Median (± IQR) concentrations of indicated cytokines from supernatants of splenocytes isolated at d21pb2 from (E) C57BL/6 mice and (F) BALB/c mice are control corrected. Bars represent results from 3 VLP-immunized mice or 3 control mice. * indicates significance with $p \leq 0.05$ n.d. = not detectable.

studies suggest that the cell-mediated immune response, in particular the response of CD8 T cells, is also critical for protection against henipavirus infection (Pickering et al., 2016; Ploquin et al., 2013). In this study, we showed for the first time that Henipavirus-based VLPs are not only able to stimulate a humoral response with generation of neutralizing antibodies, but also to induce a cell-mediated response in C57BL/6 mice with VLP-specific proliferation and IFN-γ expression by CD8 T cells. Moreover, the antigen-specific secretion of IFN-γ, TNF-α and IL-2 into supernatants from splenocyte cultures of VLP-immunized C57BL/6 mice after homologous restimulation revealed the induction of a Th1 immune response.

Immunization of both C57BL/6 and BALB/c mice with VLPs elicited NiV G, NiV F and HeV M-specific antibodies with neutralizing activity indicating an effective B cell response. Previous studies using NiV-based VLPs for immunization of BALB/c mice, Syrian hamsters and rabbits similarly reported the induction of anti-NiV IgG as well as significant

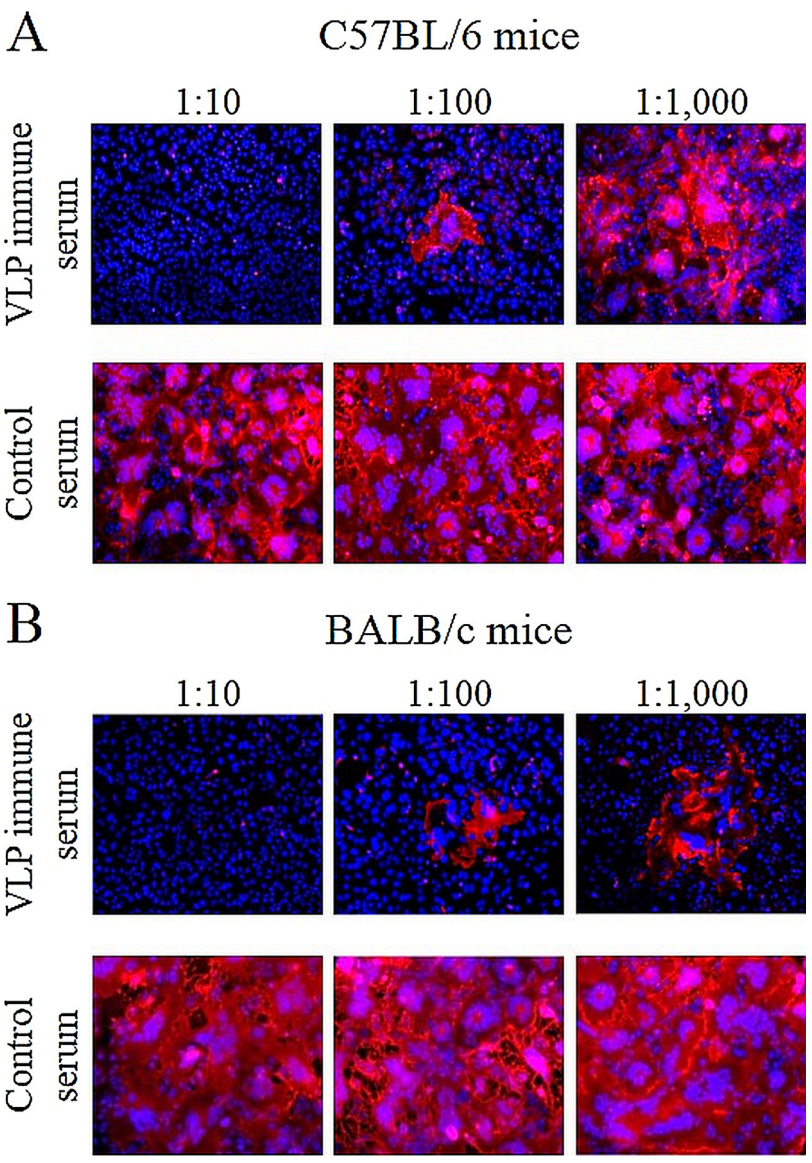

Fig. 3. Analysis of neutralizing antibodies after immunization with VLPs in C57BL/6 and BALB/c mice. Foci reduction assay with live NiV using serum collected at d21pb2 from (A) C57BL/6 mice or (B) BALB/c mice. NiV-induced syncytia were visualized with a polyclonal guinea pig anti-NiV serum and rhodamine-conjugated secondary antibodies. Nuclei were counterstained with DAPI. Magnification, 10×.

NiV neutralizing antibody titers that protected hamsters against subsequent henipavirus challenge (Schmidt et al., 2018; Walpita et al., 2011, 2017). Similarly, immunization of hamsters with a recombinant adeno-associated virus (AAV) vaccine candidate encoding for NiV G led to full protection of all hamsters during homologous NiV challenge, while at least 50% of the immunized hamsters were protected against heterologous HeV challenge (Ploquin et al., 2013). Due to very low HeV cross-neutralizing antibody titers, the authors discussed whether an involvement of the cell-mediated immune response might account for the observed survival (Ploquin et al., 2013). Vaccination and challenge studies in pigs confirm this idea and indicate that a vaccine does not necessarily work in all species. Whereas the HeV G subunit vaccine proved to be effective in horses (Middleton et al., 2014), and further showed cross-protection against NiV challenge in ferrets and non-human primates (Bossart et al., 2012; Pallister et al., 2013), immunization of pigs using the soluble HeV G subunit failed to induce a Th1 response in swine accompanied by a lack of protection with shedding of infectious virus (Pickering et al., 2016). Interestingly, pigs previously immunized with ALVAC-NiV G / NiV F showed to be protected in challenge experiments with reported IFN-γ and TNF-α secretion from PBMCs upon restimulation with NiV (Weingartl et al., 2006). Therefore, immunization with non-replicative VLPs that induced proliferation and IFN-γ expression by CD8 T cells as well as secretion of Th1 cytokines in C57BL/6 mice represents a promising vaccine alternative.

In contrast to replication competent viral vectors or live-attenuated vaccines, VLPs are noninfectious and can be produced easily in different cell lines at a large scale without biosafety concerns. Furthermore, due to their ordered and repetitive antigenic epitopes, VLPs appear to be much stronger immunogens than protein subunits or DNA vaccines leading to optimal B cell induction (summarized in Jennings and Bachmann, 2008). Their virion-like size and overall authentic morphology enables VLP uptake and cross-presentation as exogenous antigens to stimulate cellular immune responses (summarized in Crisci et al., 2012).

While this is, to our knowledge, the first report of Henipavirus-based VLP-specific CD8 T cell response in mice, an increased proliferation and IFN-γ expression in CD8 T cells after VLP restimulation has been reported before for Ebola virus (EBOV) VLP-immunized C57BL/6 mice (Warfield et al., 2005). In addition, Prescott et al. observed an increased

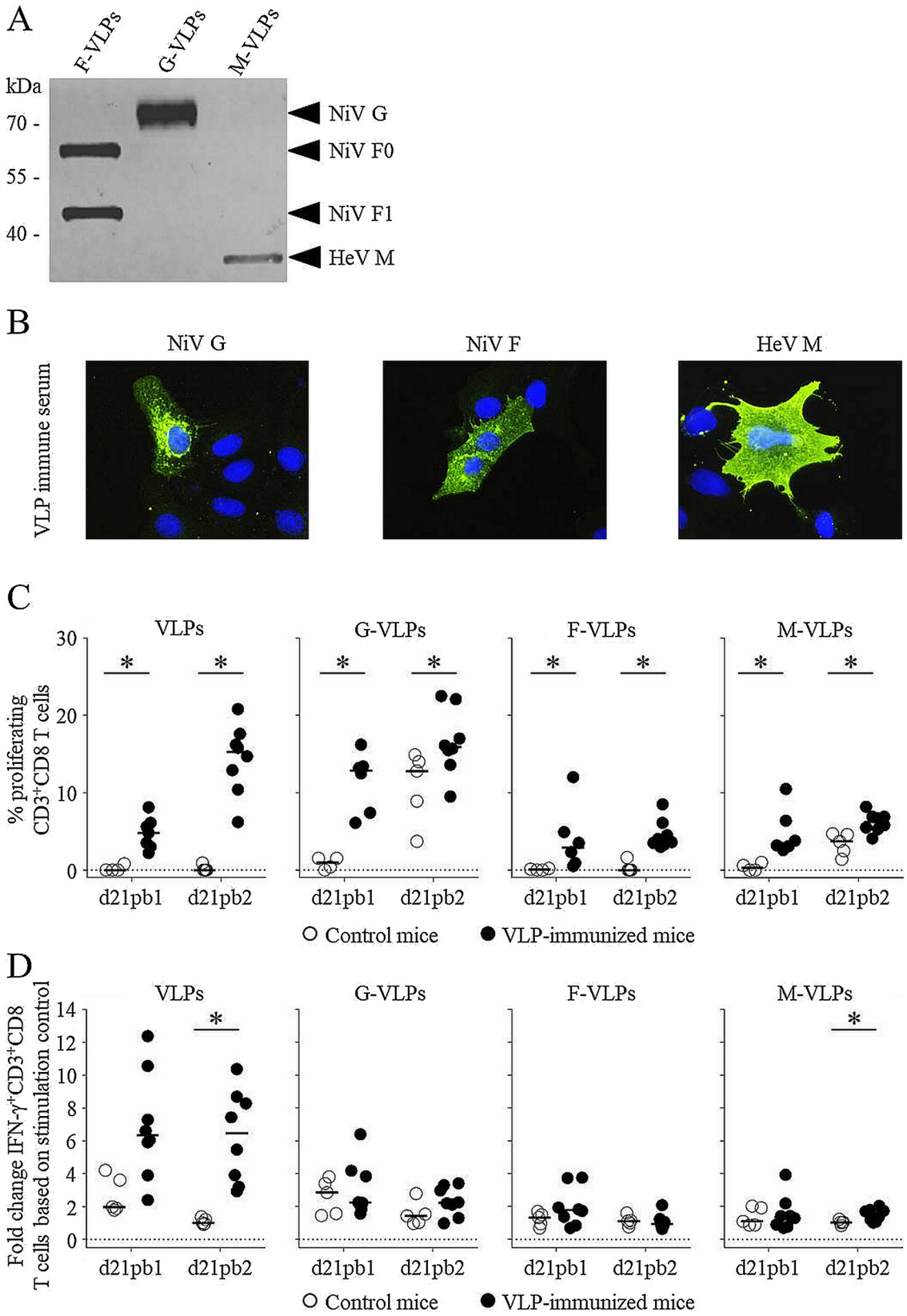

Fig. 4. CD8 T cell and antibody response to VLPs and single VLPs in splenocytes from C57BL/6 mice. (A) Western blot analysis of F-VLPs, G-VLPs and M-VLPs. (B) Immunofluorescence analysis exemplarily shown for one VLP-immunized C57BL/6 mice. Vero cells expressing NiV G, NiV F or HeV M were incubated with serum collected at d21pb2. Immune complexes were detected by a goat anti-mouse AlexaFluor488 antibody, and cell nuclei were counterstained with DAPI. Magnification, 63 × . (C) CD8 T cell proliferation was measured at d5 after respective *in vitro* restimulation of splenocytes. (D) Fold changes of IFN-γ^+CD8 T cells after restimulation with the indicated antigen. Black lines show median values. The zero level is indicated by the dotted horizontal line. * indicates significance with $p \leq 0.05$.

expression of IFN-γ and granzyme B from CD8 T cells from VSV-EBOV-GP-NiV G immunized non-human primates (NHP) after restimulation (Prescott et al., 2015). However, it was not further specified whether the induced CD8 T cell response was NiV G-specific since restimulation was only carried out in a polyclonal approach using phorbol 12-myristate 13-acetate / ionomycin. In contrast, we detected a CD8 T cell response after homologous, antigen-specific restimulation.

Mouse strains with C57BL/6 background appear to have a genetic predisposition towards a Th1 response, whereas mice with BALB/c background tend to develop a Th2 response (summarized in Sellers et al., 2012). In accordance with that, we detected secretion of the Th1 cytokines IFN-γ, TNF-α and IL-2 into supernatants from splenocyte cultures of VLP-immunized C57BL/6 but not BALB/c mice after homologous restimulation. In contrast, a previous study reported that CD8 T cells from BALB/c mice immunized with a recombinant NDV expressing the NiV F protein showed IFN-γ expression after NiV F peptide-specific *in vitro* restimulation (Kong et al., 2012). However, these differences might result from a higher antigen concentration used for restimulation as well as the use of a replicative system for immunization of BALB/c mice in the published study. Although replicative vaccines could lead to stronger humoral and cell-mediated immune responses due to a continuous antigen presence, our study indicates that in C57BL/6 mice repeated exposure to the antigen in its native conformation in VLPs is sufficient to induce neutralizing antibody titers as well as a memory CD8 T cell response. However, repeated stimulation after primary response or persistent overstimulation can lead to senescent ($KLRG1^+$) and exhausted ($PD1^+$) T cells with deterioration of T cell function and inefficient control of persisting infections (summarized in Wherry and Kurachi, 2015). Remarkably, no functional impairment of CD8 T cells was measured after repeated exposure to VLPs and only negligible amounts of $KLRG1^+$ or $PD1^+$ events from CD8 T cells from VLP-immunized mice were counted (Suppl. Fig. I). This indicates that a repetition of three immunizations and an additional restimulation *in vitro* with VLPs did not render antigen-specific CD8 T cells into functionally impaired phenotypes as senescent or exhausted T cells.

Our results further indicate that all of the VLP incorporated proteins indeed harbor CD8 T cell epitopes that induce proliferation, and marginal induction of IFN-γ expression in CD8 T cells. Hence, our results support experimental *in silico* studies reporting potential CD8 T cell epitopes within the henipavirus glycoproteins and the M protein for epitope-based vaccine development (Parvege et al., 2016; Sakib et al., 2014). Importantly, the combination of all three proteins (NiV G, NiV F and HeV M) enhanced CD8 T cell response in C57BL/6 mice, suggesting that VLPs comprised of all three proteins might be the more potent vaccine than single-protein VLPs.

In conclusion, we have demonstrated that Henipavirus-based VLPs are potent immunogens that induce a humoral response with generation of neutralizing antibodies, as well as a memory CD8 T cell response in C57BL/6 mice. These findings provide the basis for future challenge studies under BSL4-conditions to prove that Henipavirus-based VLPs lead to protection with sterile immunity. Most importantly, the promising vaccine characteristics of VLPs need to be analyzed in and proven for the intermediate host swine.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Declaration of Competing Interest

Authors declare no conflict of interest.

Acknowledgements

The authors wish to thank Dr. Christoph Staubach for help with statistical analyses and Dr. Stefan Finke for providing the HeV M plasmid. Furthermore, many thanks to Bärbel Hammerschmidt and the animal keepers for help with the animal studies. Great thanks to Carolin Rüdiger and Stefanie Knöfel for excellent technical assistance.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.108405.

References

Bauer, A., Neumann, S., Karger, A., Henning, A.K., Maisner, A., Lamp, B., Dietzel, E., Kwasnitschka, L., Balkema-Buschmann, A., Keil, G.M., Finke, S., 2014. ANP32B is a nuclear target of henipavirus M proteins. PLoS One 9, e97233.

Bossart, K.N., Rockx, B., Feldmann, F., Brining, D., Scott, D., LaCasse, R., Geisbert, J.B., Feng, Y.R., Chan, Y.P., Hickey, A.C., Broder, C.C., Feldmann, H., Geisbert, T.W., 2012. A Hendra virus G glycoprotein subunit vaccine protects African green monkeys from Nipah virus challenge. Sci. Transl. Med. 4, 146ra107.

Chua, K.B., Bellini, W.J., Rota, P.A., Harcourt, B.H., Tamin, A., Lam, S.K., Ksiazek, T.G., Rollin, P.E., Zaki, S.R., Shieh, W., Goldsmith, C.S., Gubler, D.J., Roehrig, J.T., Eaton, B., Gould, A.R., Olson, J., Field, H., Daniels, P., Ling, A.E., Peters, C.J., Anderson, L.J., Mahy, B.W., 2000. Nipah virus: a recently emergent deadly paramyxovirus. Science 288, 1432–1435.

Cong, Y., Lentz, M.R., Lara, A., Alexander, I., Bartos, C., Bohannon, J.K., Hammoud, D., Huzella, L., Jahrling, P.B., Janosko, K., Jett, C., Kollins, E., Lackemeyer, M., Mollura, D., Ragland, D., Rojas, O., Solomon, J., Xu, Z., Munster, V., Holbrook, M.R., 2017. Loss in lung volume and changes in the immune response demonstrate disease progression in African green monkeys infected by small-particle aerosol and intratracheal exposure to Nipah virus. PLoS Negl. Trop. Dis. 11, e0005532.

Crisci, E., Barcena, J., Montoya, M., 2012. Virus-like particles: the new frontier of vaccines for animal viral infections. Vet. Immunol. Immunopathol. 148, 211–225.

DeBuysscher, B.L., Scott, D., Marzi, A., Prescott, J., Feldmann, H., 2014. Single-dose live-attenuated Nipah virus vaccines confer complete protection by eliciting antibodies directed against surface glycoproteins. Vaccine 32, 2637–2644.

Defang, G.N., Khetawat, D., Broder, C.C., Quinnan Jr, G.V., 2010. Induction of neutralizing antibodies to Hendra and Nipah glycoproteins using a Venezuelan equine encephalitis virus in vivo expression system. Vaccine 29, 212–220.

Diederich, S., Gedvilaite, A., Zvirbliene, A., Kazaks, A., Sasnauskas, K., Johnson, N., Ulrich, R.G., 2015. Virus-like particles: a versatile tool for basic and applied research on emerging and reemerging viruses. In: Nanotechnology, Viral (Ed.), Khudyakov, Y., Pumpens, P., CRC Pr., Hoboken. pp. 137–160.

Diederich, S., Thiel, L., Maisner, A., 2008. Role of endocytosis and cathepsin-mediated activation in Nipah virus entry. Virology 375, 391–400.

Fischer, K., Diederich, S., Smith, G., Reiche, S., Pinho Dos Reis, V., Stroh, E., Groschup, M.H., Weingartl, H.M., Balkema-Buschmann, A., 2018. Indirect ELISA based on Hendra and Nipah virus proteins for the detection of henipavirus specific antibodies in pigs. PLOS ONE 13, e0194385.

Fischer, K., dos Reis, V.P., Finke, S., Sauerhering, L., Stroh, E., Karger, A., Maisner, A., Groschup, M.H., Diederich, S., Balkema-Buschmann, A., 2016. Expression, characterisation and antigenicity of a truncated Hendra virus attachment protein expressed in the protozoan host Leishmania tarentolae. J. Virol. Methods 228, 48–54.

Geisbert, T.W., Mire, C.E., Geisbert, J.B., Chan, Y.P., Agans, K.N., Feldmann, F., Fenton, K.A., Zhu, Z., Dimitrov, D.S., Scott, D.P., Bossart, K.N., Feldmann, H., Broder, C.C., 2014. Therapeutic treatment of Nipah virus infection in nonhuman primates with a neutralizing human monoclonal antibody. Sci. Transl. Med. 6, 242ra282.

Guillaume, V., Contamin, H., Loth, P., Georges-Courbot, M.C., Lefeuvre, A., Marianneau, P., Chua, K.B., Lam, S.K., Buckland, R., Deubel, V., Wild, T.F., 2004. Nipah virus: vaccination and passive protection studies in a hamster model. J. Virol. 78, 834–840.

Jennings, G.T., Bachmann, M.F., 2008. The coming of age of virus-like particle vaccines. Biol. Chem. 389, 521–536.

Kong, D., Wen, Z., Su, H., Ge, J., Chen, W., Wang, X., Wu, C., Yang, C., Chen, H., Bu, Z., 2012. Newcastle disease virus-vectored Nipah encephalitis vaccines induce B and T cell responses in mice and long-lasting neutralizing antibodies in pigs. Virology 432, 327–335.

Middleton, D., Pallister, J., Klein, R., Feng, Y.R., Haining, J., Arkinstall, R., Frazer, L., Huang, J.A., Edwards, N., Wareing, M., Elhay, M., Hashmi, Z., Bingham, J., Yamada, M., Johnson, D., White, J., Foord, A., Heine, H.G., Marsh, G.A., Broder, C.C., Wang, L.F., 2014. Hendra virus vaccine, a one health approach to protecting horse, human, and environmental health. Emerg. Infect. Dis. 20, 372–379.

Moll, M., Kaufmann, A., Maisner, A., 2004. Influence of N-glycans on processing and biological activity of the nipah virus fusion protein. J. Virol. 78, 7274–7278.

Pallister, J.A., Klein, R., Arkinstall, R., Haining, J., Long, F., White, J.R., Payne, J., Feng, Y.R., Wang, L.F., Broder, C.C., Middleton, D., 2013. Vaccination of ferrets with a recombinant G glycoprotein subunit vaccine provides protection against Nipah virus disease for over 12 months. Virol. J. 10, 237.

Parvege, M.M., Rahman, M., Nibir, Y.M., Hossain, M.S., 2016. Two highly similar LAEDDTNAQKT and LTDKIGTEI epitopes in G glycoprotein may be useful for

effective epitope based vaccine design against pathogenic henipavirus. Comput. Biol. Chem. 61, 270–280.
Pickering, B.S., Hardham, J.M., Smith, G., Weingartl, E.T., Dominowski, P.J., Foss, D.L., Mwangi, D., Broder, C.C., Roth, J.A., Weingartl, H.M., 2016. Protection against henipaviruses in swine requires both, cell-mediated and humoral immune response. Vaccine 34, 4777–4786.
Ploquin, A., Szecsi, J., Mathieu, C., Guillaume, V., Barateau, V., Ong, K.C., Wong, K.T., Cosset, F.L., Horvat, B., Salvetti, A., 2013. Protection against henipavirus infection by use of recombinant adeno-associated virus-vector vaccines. J. Infect. Dis. 207, 469–478.
Prescott, J., DeBuysscher, B.L., Feldmann, F., Gardner, D.J., Haddock, E., Martellaro, C., Scott, D., Feldmann, H., 2015. Single-dose live-attenuated vesicular stomatitis virus-based vaccine protects African green monkeys from Nipah virus disease. Vaccine 33, 2823–2829.
ProMED-mail, 2019. Nipah virus – India (08): (Kerala). ProMED-mail, pp. 25 Jul: 20190725.6588116. < http:www.promedmail.org > . Accessed 19 August 2019.
Ringel, M., Heiner, A., Behner, L., Halwe, S., Sauerhering, L., Becker, N., Dietzel, E., Sawatsky, B., Kolesnikova, L., Maisner, A., 2019. Nipah virus induces two inclusion body populations: Identification of novel inclusions at the plasma membrane. PLoS Pathog. 15 (4), e1007733.
Satterfield, B.A., Dawes, B.E., Milligan, G.N., 2016. Status of vaccine research and development of vaccines for Nipah virus. Vaccine 34, 2971–2975.
Sakib, M.S., Islam, M.R., Hasan, A.K., Nabi, A.H., 2014. Prediction of epitope-based peptides for the utility of vaccine development from fusion and glycoprotein of nipah virus using in silico approach. Adv. Bioinformatics 2014, 402492.
Schmidt, R., Beltzig, L.C., Sawatsky, B., Dolnik, O., Dietzel, E., Krahling, V., Volz, A., Sutter, G., Becker, S., von Messling, V., 2018. Generation of therapeutic antisera for emerging viral infections. npj Vaccines 3, 42.
Sellers, R.S., Clifford, C.B., Treuting, P.M., Brayton, C., 2012. Immunological variation between inbred laboratory mouse strains: points to consider in phenotyping genetically immunomodified mice. Vet. Pathol. 49, 32–43.
Tamin, A., Harcourt, B.H., Ksiazek, T.G., Rollin, P.E., Bellini, W.J., Rota, P.A., 2002. Functional properties of the fusion and attachment glycoproteins of Nipah virus. Virology 296, 190–200.
Walpita, P., Barr, J., Sherman, M., Basler, C.F., Wang, L., 2011. Vaccine potential of Nipah virus-like particles. PLoS One 6, e18437.
Walpita, P., Cong, Y., Jahrling, P.B., Rojas, O., Postnikova, E., Yu, S., Johns, L., Holbrook, M.R., 2017. A VLP-based vaccine provides complete protection against Nipah virus challenge following multiple-dose or single-dose vaccination schedules in a hamster model. npj Vaccines 2, 21.
Warfield, K.L., Olinger, G., Deal, E.M., Swenson, D.L., Bailey, M., Negley, D.L., Hart, M.K., Bavari, S., 2005. Induction of humoral and CD8+ T cell responses are required for protection against lethal Ebola virus infection. J. Immunol. 175, 1184–1191.
Weingartl, H.M., Berhane, Y., Caswell, J.L., Loosmore, S., Audonnet, J.C., Roth, J.A., Czub, M., 2006. Recombinant Nipah virus vaccines protect pigs against challenge. J. Virol. 80, 7929–7938.
Wherry, E.J., Kurachi, M., 2015. Molecular and cellular insights into T cell exhaustion. Nat. Rev. Immunol. 15, 486–499.
Yun, T., Park, A., Hill, T.E., Pernet, O., Beaty, S.M., Juelich, T.L., Smith, J.K., Zhang, L., Wang, Y.E., Vigant, F., Gao, J., Wu, P., Lee, B., Freiberg, A.N., 2015. Efficient reverse genetics reveals genetic determinants of budding and fusogenic differences between Nipah and Hendra viruses and enables real-time monitoring of viral spread in small animal models of henipavirus infection. J. Virol. 89, 1242–1253.