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Protective efficacy of monovalent and trivalent recombinant MVA-based vaccines against three encephalitic alphaviruses

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Vaccine 36 (2018) 5194–5203
Date of Publication from Ext Publisher: July 2018

Defence Research and Development Canada
External Literature (P)
DRDC-RDDC-2018-P108
August 2018

Canada

IMPORTANT INFORMATIVE STATEMENTS

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In conducting the research described in this report, the investigators adhered to the 'Guide to the Care and Use of Experimental Animals, Vol. I, 2nd Ed.' published by the Canadian Council on Animal Care.)

Vaccine 36 (2018) 5194–5203

Contents lists available at ScienceDirect

Vaccine

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

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ARTICLE INFO

Article history:
Received 26 July 2017
Received in revised form 24 June 2018
Accepted 27 June 2018
Available online 20 July 2018

Keywords:
MVA-BN
Vaccines
Encephalitic alphaviruses
Monovalent
Mixture of vaccines
Trivalent
Protective efficacy

ABSTRACT

The three encephalitic alphaviruses, western, eastern, and Venezuelan equine encephalitis viruses (WEEV, EEEV, and VEEV) are potential biothreat agents due to high infectivity through aerosol exposure, ease of production in large amounts, and relative stability in the environment. Currently, there is no licensed vaccine for human use to these three encephalitic alphaviruses, and efforts to move vaccine candidates forward into clinical trials have not been successful. In this study, the modified vaccinia Ankara-Bavarian Nordic (MVA-BN®) vaccine platform was used to construct and produce three monovalent recombinant MVA-BN-based encephalitic alphavirus vaccines, MVA-BN-W, MVA-BN-E, and MVA-BN-V. Additionally, a MVA-BN-based construct was designed to produce antigens against all three alphaviruses, the trivalent vaccine MVA-BN-WEV. The protective efficacy of these vaccines was evaluated *in vivo*. Female BALB/c mice were immunized with two doses of each monovalent MVA-BN-based alphavirus vaccine, a mixture of the three monovalent vaccines, MVA-BN-W + E + V, or the trivalent vaccine MVA-BN-WEV at a four-week interval. Two weeks after the booster immunization, the mice were instilled intranasally with 5×10^3 to 1×10^4 plaque forming units of WEEV, EEEV, or VEEV. All mice immunized with monovalent vaccines survived the respective virus challenge without any signs of illness or weight loss, while all the control mice died. The triple mixture of vaccines or the trivalent vaccine also provided 90 to 100% protection to the mice against WEEV and VEEV challenges, and 60% to 90% protection against EEEV challenge. These data suggest that each monovalent MVA-BN-W, MVA-BN-E, and MVA-BN-V is a potential vaccine candidate against respective encephalitic alphavirus and the three monovalent vaccines can be given in a mixture (MVA-BN-W + E + V) or the trivalent vaccine MVA-BN-WEV can serve as a true multivalent vaccine without significantly reducing efficacy against WEEV and VEEV despite slightly reduced efficacy against EEEV challenge.

1. Introduction

Alphaviruses comprise a group of about 31 enveloped viruses with a positive sense, non-segmented single-stranded RNA genome [1,2]. They share basic structural, sequence, and functional similarities, including a genome with two polyprotein gene clusters [2]. The three encephalitic alphaviruses, western, eastern, and Venezuelan equine encephalitis viruses (WEEV, EEEV, and VEEV) are highly pathogenic for both equines and humans and have caused periodic epizootics throughout North, Central, and South America [1,2].

Although the three viruses are naturally transmitted by mosquitoes, accidental laboratory infections [3] and experimental studies in animals [4] have demonstrated that all three alphaviruses are highly infectious by the aerosol route. They can easily be produced in large quantities and unlike many other pathogenic viruses, they are relatively stable (either liquid or dry) in the environment [5]. These characteristics have made the three viruses suitable for weaponization, and as such, they are potential agents of biological warfare interest [6]. No vaccine for human use is currently available for any of the three encephalitic alphaviruses. Live attenuated or formalin-inactivated vaccines used in horses are not suitable due to their high reactogenicity or low immunogenicity in humans respectively.

Modified vaccinia Ankara (MVA) is an attenuated vaccinia virus [7] that is adapted to chicken embryo fibroblasts. MVA-Bavarian Nordic (MVA-BN®), non-replicating in humans and in other mammals [8], is approved as a smallpox vaccine in Canada and in the EU (under the trade names IMVAMUNE® and IMVANEX®

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https://doi.org/10.1016/j.vaccine.2018.06.064
0264-410X/

respectively). Some of the features that make MVA-BN an excellent vaccine platform include its outstanding safety profile in humans, which was demonstrated in several clinical trials [9–14], and its intrinsic adjuvant capacities to induce both humoral and cellular immune responses [15,16]. Finally, the impact of pre-existing vector immunity to MVA is limited, unlike other viral vectors such as adenovirus-based vaccines [17]. MVA-BN therefore has been used as a vector for many different vaccines ranging from infectious diseases to various cancers [18–21].

In this manuscript, monovalent recombinant MVA-BN vaccines for the three encephalitic alphaviruses, WEEV, EEEV, and VEEV were constructed and produced along with a trivalent construct which expresses the antigens of all three viruses. The protective efficacy of vaccines in monovalent, triple mixture (of the three monovalent vaccines), and trivalent formats was evaluated *in vivo*.

2. Materials and methods

2.1. Reagents

All cell culture reagents were purchased from Gibco (Fisher Sci., Ottawa, ON). Virus DNA for PCR was purified using the NucleoSpin Blood QuickPure Kit (Macherey und Nagel, Düren, Germany) and RNA for reverse transcription (RT)-PCR was purified using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). For PCR and RT-PCR amplification of the inserted transgenes, One Taq Polymerase (NEB, Frankfurt, Germany) and the 5'Polymerase (VWR, Darmstadt, Germany) were used.

2.2. Cells and viruses

Vero (CCL-81) and HeLa (CCL-2) cells were obtained from the American Type Culture Collection (ATCC Manassas, VA). Cells were maintained in Dulbecco's modified Eagle media (DMEM) containing 5% (Vero) or 10% (HeLa) heat-inactivated fetal bovine serum (FBS). WEEV Fleming was purchased from ATCC. VEEV Trinidad Donkey (TrD) and EEEV PE6 were kindly provided by Dr. George Ludwig (U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD). Seed stocks of WEEV or EEEV were made by inoculation of Vero cell monolayers with WEEV or EEEV at a multiplicity of infection of 0.1. Stocks of VEEV TrD were made by inoculation of suckling mice and harvesting the brain as a 10% suspension. Supernatants from infected cells were aliquoted and stored at −70 °C. The titers of the alphaviruses were determined by plaque titration on Vero cells. MVA-BN®, deposited at the European Collection of Cell Cultures, Salisbury (UK) under number V00083008 was used for generation of the vaccine candidates. MVA-BN and derived recombinant vaccines were grown in primary chicken embryo fibroblast (CEF) cells (Thermo Fisher/Life Technologies, Darmstadt, Germany) at serum free conditions.

2.3. Construction of MVA-BN-based alphavirus vaccines

The recombinant vaccines encoding the structural proteins E3-E2-6 K-E1 of WEEV (strain 71 V-1658 for vaccine MVA-BN-W), EEEV (strain FL93-939NA for vaccine MVA-BN-E), and VEEV (strain TrD for vaccine MVA-BN-V), respectively, or of all three alphaviruses for the trivalent vaccine MVA-BN-WEV were constructed using the MVA-BN vector.

The cDNAs for the structural protein genes were codon optimized and adapted to avoid large stretches of identity or repeated sequences in or between the genes and synthesized by GeneArt, Regensburg, Germany. For optimal expression, the individual cDNAs were combined with suitable vaccinia early or early/late promoters. For the expression of WEEV and EEEV envelope proteins, the native MVA-BN tandem repeat promoter Pr13.5-long was used [22], whereas for the expression of VEEV envelop protein, the synthetic PrHyb promoter was applied [23]. The trivalent vaccine MVA-BN-WEV was constructed by inserting the envelope genes with their respective promoters of the monovalent constructs into the respective intergenic regions (IGRs) in MVA-BN. This made it necessary to insert both, the WEEV and VEEV envelope cDNAs as a tandem into one IGR, as illustrated in Fig. 1. cDNAs coding for the respective alphavirus envelope proteins E3-E2-6 K-E1 were inserted into the MVA-BN genome following standard methods [24]. The genetically pure stocks were used for produc-

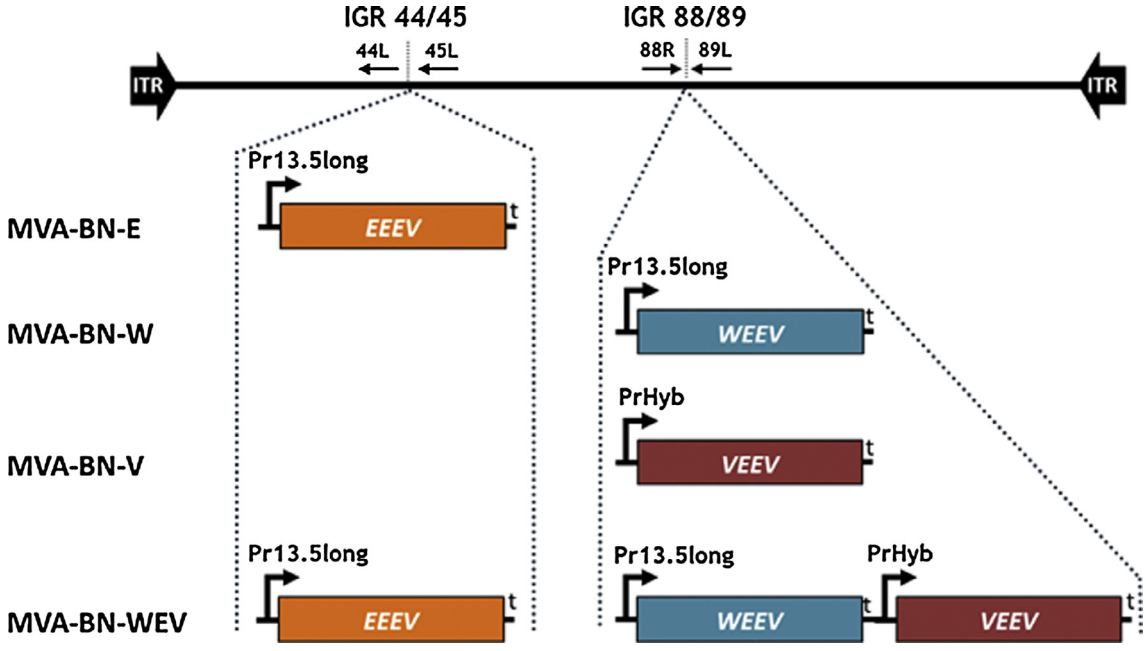

Fig. 1. Overview of vaccines. Four MVA-BN based vaccines were generated using IGR as insertion sites with the flanking MVA genes and directions indicated. The codon optimized sequence of the E3-E2-6 K-E1 envelope proteins of WEEV, EEEV or VEEV was inserted with the pox promoters indicated. t = early transcription termination signal; Pr13.5long = promoter of the MVA 13.5 gene; PrHyb = synthetic hybrid promoter.

tion of research grade material in CEF cells. Infectious titers were determined on primary CEF cells as tissue culture infective dose $_{50}$ ($TCID_{50}$)/ml.

2.4. Antigen expression characterization of MVA-BN-based alphavirus vaccines

Expression of alphavirus structural proteins by the recombinant MVA-BN-based alphavirus vaccines were analyzed in HeLa cells (passage < 50) by flow cytometry using standard methods. In brief, HeLa cells were infected with $10 \times TCID_{50}$ per cell; surface staining was performed 20 hrs post infection with antibodies that were confirmed to be specific for the respective vaccine structural proteins (WEEV, EEEV or VEEV). The mouse anti-WEEV monoclonal antibody (mAb) 11D2 (1:2,000) against the E1 of WEEV strain B11 was used to detect the expression of the E1 protein from MVA-BN-W. The mouse anti-EEEV polyclonal antibody (pAb) was purified from mouse ascites (ATCC VR1242AF) by protein G affinity column in accordance to the manufacturer's instructions. The pAb (1:500) was used to detect the expression of structural proteins E1 and E2 from MVA-BN-E. The mouse anti-VEEV mAb 1A4A1 from DRDC SRC, 1:2,000 against the E2 protein was used to detect its expression on the surface of cells infected with MVA-BN-V. A goat anti-mouse antibody conjugated to Allophycocyanin (Jackson Immuno Research Laboratories, 1:500) was used as a secondary detection antibody. Infected HeLa cells were additionally stained with 4′,6-diamidino-2-phenylindole for live/dead discrimination. Control cells were infected with MVA-BN empty vector or MVA-BN-red fluorescent protein (RFP). The stained cells were gated on live and infected cells, which were RFP positive or green fluorescent protein (GFP) positive, as the recombinant alphavirus vaccines contain the marker used for selection during generation of the vaccines. The flow cytometric analysis was performed on a BD LSR II Flow Cytometer (Becton Dickinson, Heidelberg, Germany).

2.5. Serum anti-alphavirus neutralization titration assay

All the experiments with live alphavirus were carried out in the Containment Level 3 laboratory at Defence Research and Development Canada (DRDC) Suffield Research Centre (SRC) in compliance with guidelines set by Health Canada and the Canadian Food Inspection Agency. All serum samples were incubated at 56 °C for 30 mins to inactivate the complement system in serum. The alphavirus neutralization test was carried out in flat bottomed 96 well micro titre plates (VWR) starting with a 1:30 dilution of serum followed by a series of two-fold dilutions in 50 μl per well. Subsequently, virus (100 $TCID_{50}$ per well) diluted in DMEM was added to each well in a volume of 50 μl. Serum and virus were pre-incubated at 37 °C for 1 hr to allow neutralization of the virus. Thereafter, 10,000 Vero cells per well were added in a volume of 50 μl. After incubation for 3 days at 37 °C and under 5% CO_2, the plates were examined by microscopy. The antibody neutralizing titer (NT), expressed as the reciprocal of the dilution and given on a log scale, was identified as the highest dilution that resulted in 50% inhibition of cytopathic effect.

2.6. Efficacy evaluation of MVA-BN-based alphavirus vaccines

Female BALB/c mice (16–18 g) were obtained from Charles River Canada. All procedures for mouse experiments were approved by the Animal Care Committee at DRDC SRC and complied with guidelines set by the Canadian Council on Animal Care. Experiments were carried out to evaluate the efficacy of monovalent MVA-BN-based alphavirus vaccines, a triple mixture of monovalent vaccines, or a trivalent MVA-BN-based alphavirus vaccine. Groups of 5 mice were given two doses of 1×10^8 $TCID_{50}$ of monovalent MVA-BN-based alphavirus vaccines or MVA-BN vector control subcutaneously (S.C.) or intramuscularly (I.M.) 28 days apart. Blood samples were taken on days -1, 14 and 41 after the initial immunization. Mice were anesthetized through injection with 50 mg/kg sodium pentobarbital or exposure to isoflurane aerosol [25]. The anesthetized mice were instilled intranasally (I.N.) with 5×10^3 or 1×10^4 plaque forming units (pfu) of WEEV Fleming, EEEV PE6, or VEEV TrD diluted in Hank's balanced salt solution (HBSS) in a volume of 50 μl, 14 days after the booster immunization. Mice were examined daily for 14 days for body weight and signs of illness using a clinical scoring system [26]. The studies were repeated with the triple mixture of vaccines given together at a dose of 1×10^8 $TCID_{50}$ per individual vaccine administered S.C. in a total volume of 150 μl as well as for the trivalent vaccine. The volume was adjusted using HBSS. Replicate experiments were performed for each study.

2.7. Statistical analyses

All data were analyzed with GraphPad Prism software (GraphPad Software, Inc). Kaplan-Meier survival curves were analyzed by the log-rank (Mantel-Cox) test. The reciprocal antibody neutralization titers were log transformed and analyzed by the two-tailed Student's *t*-test. Differences were considered to be statistically significant at $P < 0.05$.

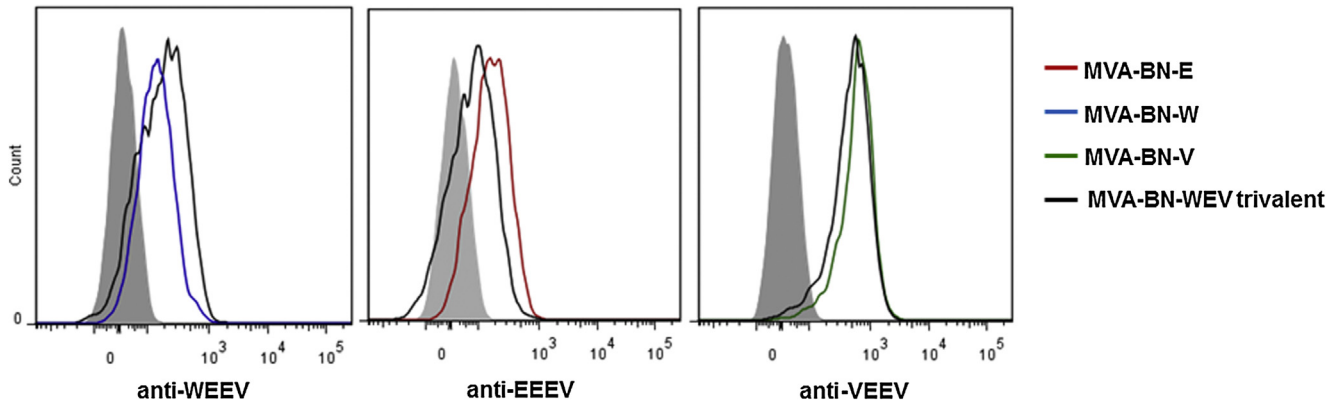

Fig. 2. Antigen expression analysis by flow cytometry. HeLa cells were infected with, MVA-BN-W (blue), MVA-BN-E (red), MVA-BN-V (green), or MVA-BN-WEV trivalent vaccine (black) and stained with mouse antibodies that were specific for the respective vaccine structural proteins E1 and E2 (anti-WEEV, anti-EEEV, or anti-VEEV respectively) at 20 hrs post infection. The stained cells were gated on live and infected cells, which were RFP positive or GFP positive. MVA-BN-RFP without primary antibody (grey) was used for comparison to the background fluorescence of the vaccines. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Anti-alphavirus NTs in the serum of immunized mice.

Vaccines	Immunization routes	Neutralized alphaviruses	NTs*		
			Preserum	14 days	41 days
MVA-BN-V	S.C.	VEEV	<1.78	<1.78	3.58 ± 0.30[a,b]
MVA-BN-V	I.M.		<1.78	<1.78	3.77 ± 0.20[a,b]
MVA-BN-W + E + V Mixture	S.C.		<1.78	<1.78	<1.78
MVA-BN-WEV Trivalent	S.C.		<1.78	<1.78	<1.78
MVA-BN	S.C.		<1.78	<1.78	<1.78
MVA-BN-W	S.C.	WEEV	<1.78	2.28 ± 0.17[a]	2.88 ± 0.17[a,b]
MVA-BN-W + E + V Mixture	S.C.		<1.78	1.88 ± 0.17[a]	2.53 ± 0.32[a,b]
MVA-BN-WEV Trivalent	S.C.		<1.78	<1.78	2.58±0.17[a,b]
MVA-BN	S.C.		<1.78	<1.78	<1.78
MVA-BN-E	S.C.	EEEV	<1.78	1.88 ± 0.17[a]	2.88 ± 0.17[a,b]
MVA-BN-W + E + V Mixture	S.C.		<1.78	<1.78	2.28 ± 0.36[a,b]
MVA-BN-WEV Trivalent	S.C.		<1.78	<1.78	<1.78
MVA-BN	S.C.		<1.78	<1.78	<1.78

* The NT, expressed as the reciprocal of the dilution and given on a log scale, was identified as the highest dilution that resulted in 50% inhibition of CPE.
[a] $P < 0.05$, or $P < 0.01$ compared to preserum.
[b] $P < 0.05$, or $P < 0.01$ compared to 14 days.

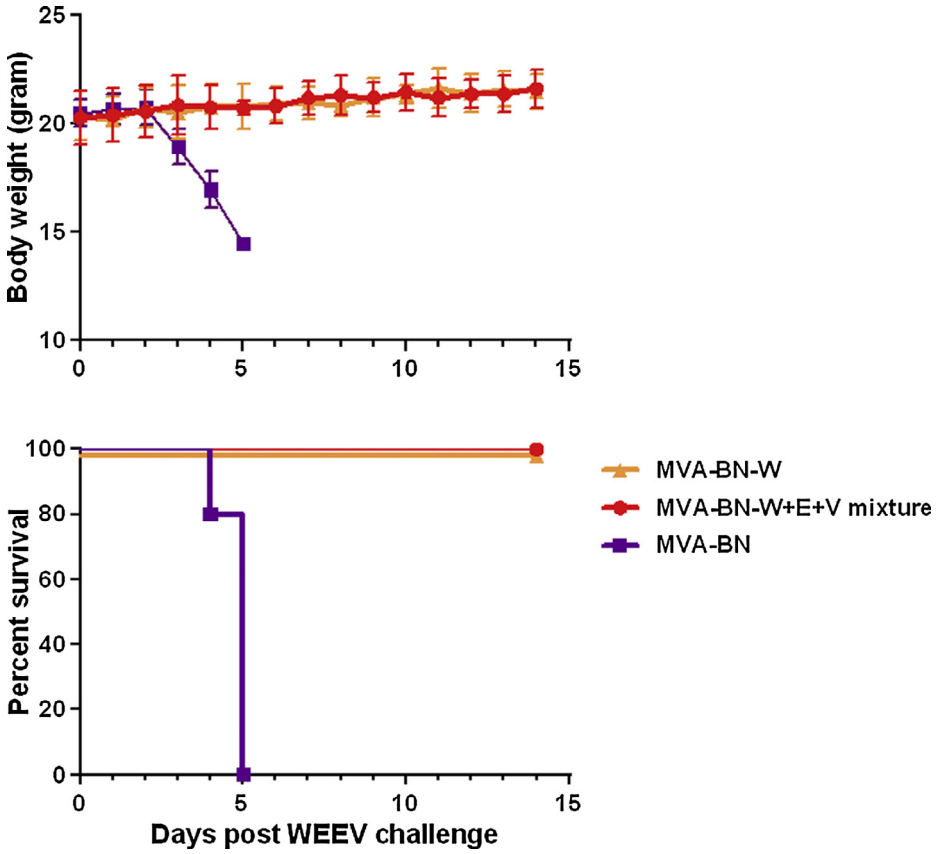

Fig. 3. MVA-BN-W versus MVA-BN-W + E + V against 5×10^3 pfu of WEEV Fleming challenge. A group of 5 mice were immunized S.C. with two doses of MVA-BN-W (▲), MVA-BN-W + E + V triple mixture of vaccines (●), or MVA-BN control (■) 28 days apart, and challenged I.N. with WEEV Fleming (5×10^3 pfu) 14 days after the booster vaccination.

3. Results

3.1. Construction and characterization of MVA-BN-based alphavirus vaccines

Three monovalent recombinant MVA-BN-based vaccines against WEEV, EEEV, and VEEV were created by insertion of the cDNA for the respective codon optimized E3-E2-6 K-E1 envelope polyprotein into the MVA-BN vector. The trivalent recombinant MVA-BN-based vaccine was assembled by insertion in tandem of the WEEV and VEEV inserts with their respective promoters into MVA-BN. Subsequently cells were co-infected with this intermediate construct and MVA-BN-E to obtain the trivalent MVA-BN-WEV (Fig. 1). The same promoters utilized in the monovalent constructs,

MVA-BN-W, MVA-BN-E and MVA-BN-V were utilized for expression in the same IGRs of MVA in the trivalent vaccine. Nucleotide sequence homology was reduced between WEEV and VEEV to 54% after codon optimization for human expression and by using different MVA promoters. A start codon was placed at the 5′ end of each of the E3-E2-6 K-E1 transgenes, as the capsid proteolytic cleavage normally produces the N terminus of the E3-E2-6 K-E1 polyprotein. The expression of encoded envelope protein in recombinant MVA-BN infected cells was demonstrated by flow cytometry after staining with alphavirus species specific antibodies. The antigen and species specificity of the antibodies were confirmed for the monovalent vaccines, as the antibodies did not cross-react with the other expressed antigens in HeLa cell infection (data not shown). The expression of the monovalent and trivalent vaccines was compared and revealed similar expression levels for the WEEV and VEEV antigens, and a trend for slightly lower expression of the EEEV envelope protein by the trivalent vaccine (Fig. 2).

3.2. Neutralizing antibody response

Alphavirus NTs measured prior to vaccination and two weeks after prime and boost respectively are summarized in Table 1. Initially the I.M. and S.C. routes of immunization were compared. For the serum from monovalent MVA-BN-V vaccinated mice, the VEEV NTs were only detected at day 41 after the initial vaccination (two weeks after the booster vaccination) in both S.C. and I.M. immunization routes. The NT in the I.M. group was higher, but not significantly higher ($P > 0.05$) than that in the S.C. group.

Unlike monovalent MVA-BN-V, the serum samples from the triple mixture (MVA-BN-W + E + V) and trivalent vaccine (MVA-BN-WEV) groups did not show measurable VEEV NTs on any day tested. For the serum from the monovalent vaccine MVA-BN-W and triple mixture groups, the WEEV NTs were detected at both days 14 and 41 after the initial immunization. The NTs at day 41 in both groups were higher than those at day 14 groups ($P < 0.05$), indicating a booster effect induced by the second vaccination. The trivalent vaccine group only showed WEEV NT after the booster immunization (day 41). MVA-BN-E also induced EEEV neutralizing antibodies that were detected at day 14 after the initial immunization and boosted higher by day 41 ($P < 0.01$). However, NT in the triple mixture group was only detected after the booster immunization (day 41). Although at day 41, the NT in the MVA-BN-E was higher than that in the triple mixture group, the difference was not significant ($P > 0.05$). The trivalent vaccine group did not show any detectable level of EEEV neutralizing antibodies at any days tested.

3.3. Protective efficacy study

3.3.1. Monovalent vaccine versus triple mixture of vaccines

Initially, the protective efficacy of MVA-BN-V was evaluated using two immunization routes. Mice were immunized with MVA-BN-V by either S.C. or I.M. route and then challenged (I.N.) with VEEV TrD (1×10^4 PFU). All MVA-BN-V immunized groups (S.C. or I.M.) survived the challenge without any signs of illness, independent of the vaccination route used, while all control mice showed signs of illness including body weight loss and were euthanized/died within 10 days after challenge ($P < 0.01$, as compared to the control; data not shown). The S.C. route of immunization was then chosen for all subsequent efficacy studies.

Next, the three monovalent vaccines (1×10^8 $TCID_{50}$) were compared with the triple mixture of monovalent vaccines (each

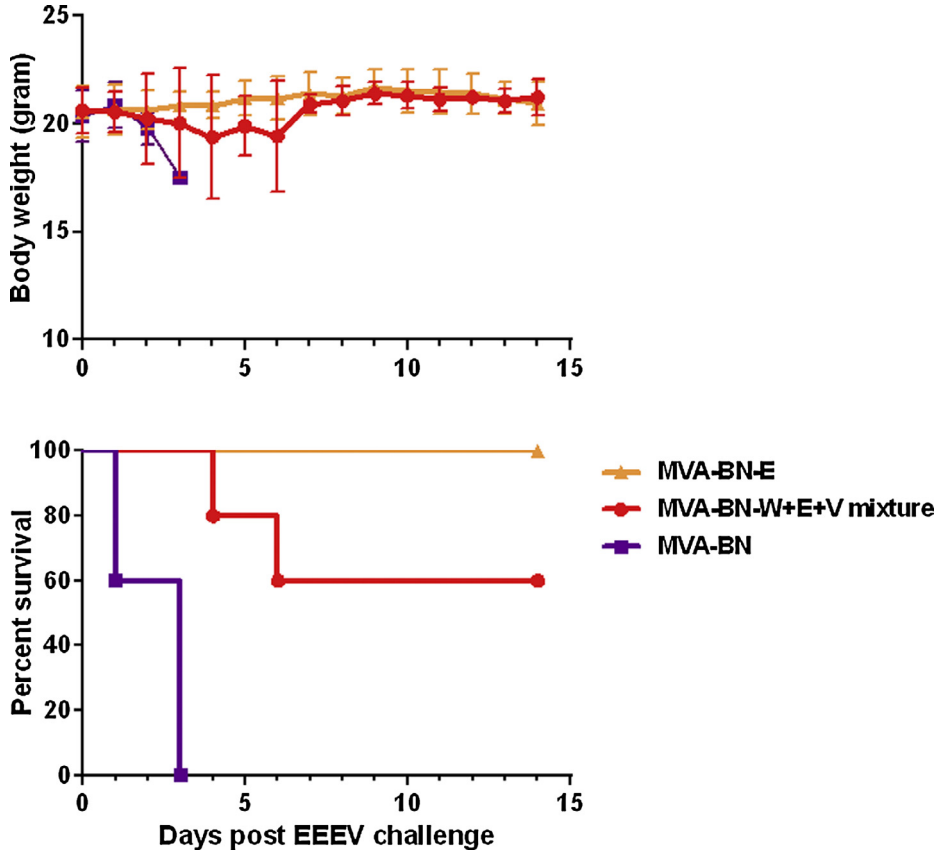

Fig. 4. MVA-BN-E versus MVA-BN-W + E + V against 5×10^3 pfu of EEEV PE6 challenge. A group of 5 mice were immunized S.C. with two doses of MVA-BN-E (▲), MVA-BN-W + E + V triple mixture of vaccines (●), or MVA-BN control (■) 28 days apart, and challenged I.N. with EEEV PE6 (5×10^3 pfu) 14 days after the booster vaccination.

at a dose of 1×10^8 $TCID_{50}$ per individual vaccine) respectively. As shown in Fig. 3, all MVA-BN-W and MVA-BN-W + E + V vaccines immunized mice survived the 5×10^3 pfu of WEEV Fleming challenge without any signs of illness ($P < 0.01$, as compared to the control), while all the MVA-BN empty vector control mice showed symptoms such as ruffled hair, hunched back, reduced mobility, paralysis, and weight loss and were euthanized/died within 5 days after challenge. Similarly, MVA-BN-E vaccine provided 100% protection to the mice without any signs of infection ($P < 0.01$, as compared to the control). All control mice showed signs of infection and died as early as 3 days after EEEV PE6 challenge (5×10^3 pfu), indicating the stringency of this EEEV challenge model. However, the triple mixture of vaccines only provided 60% protection to the mice in this study ($P < 0.01$, as compared to the control) (Fig. 4). MVA-BN-V vaccine or the triple mixture of vaccines provided 100% protection against 5×10^3 pfu of VEEV TrD with no signs of illness ($P < 0.01$, as compared to the control), all MVA-BN empty vector control mice died within 9 days after challenge (Fig. 5).

3.3.2. Triple mixture of vaccines versus trivalent vaccine

The triple mixture of monovalent vaccines, MVA-BN-W + E + V (again each at a dose of 1×10^8 $TCID_{50}$ per individual vaccine) was compared with the MVA-BN-WEV trivalent vaccine (1×10^8 $TCID_{50}$). Against WEEV Fleming, protection of 100% was afforded by the MVA-BN-WEV and 90% by the MVA-BN-W + E + V (Fig. 6) ($P < 0.01$, as compared to the control). With EEEV PE6, survival with the trivalent vaccine was 60% ($P < 0.01$, as compared to the control), while the triple mixture of vaccines shown 90% protection ($P < 0.01$, as compared to the control) (Fig. 7). Both triple mixture and trivalent vaccines protected equally well against VEEV TrD with 90% protection observed ($P < 0.01$, as compared to the control) (Fig. 8). All control mice (MVA-BN empty vector vaccinated) succumbed to infection with their respective challenge virus strain and were then euthanized/died.

4. Discussion

Currently, personnel at risk of exposure to VEEV are recommended by CDC, to be vaccinated with a live attenuated vaccine TC-83 (an Investigational New Drug, IND) followed by booster vaccination with formalin-inactivated TC-83 vaccine (also known as C-84) [27,28]. Formalin-inactivated WEEV and EEEV vaccines were also developed and can be used as INDs for at-risk personnel. The major drawback of TC-83 is its high adverse reactogenicity rate (about 20% in vaccinees) [27], which is unacceptable for being licensed as a human vaccine. The formalin-inactivated vaccines for WEEV, EEEV, and VEEV have better safety profiles; however, the immunogenicity is low for these vaccines, requiring three doses for immunization and annual boosters to maintain protective immune response. Several approaches have been used to develop safer and more effective vaccines for WEEV, EEEV, and VEEV [27,28]. A reverse genetics approach to introduce specific mutations to the viral sequence was used by Davis *et al.* to develop a live-attenuated VEEV vaccine candidate. This vaccine candidate was designated as V3526 from a full-length cDNA clone of the TrD strain by deleting a furin cleavage site from the envelope protein precursor 2 and inserting a single amino acid mutation in E1 [29]. V3526 was protective against aerosol or subcutaneous challenge of various subtypes of VEEV in rodents and nonhuman primates [30–32]. The success of pre-clinical studies made V3526 a leading candidate of VEEV vaccines to move forward to the safety and immunogenicity study in a Phase 1 clinical trial. Unfortunately,

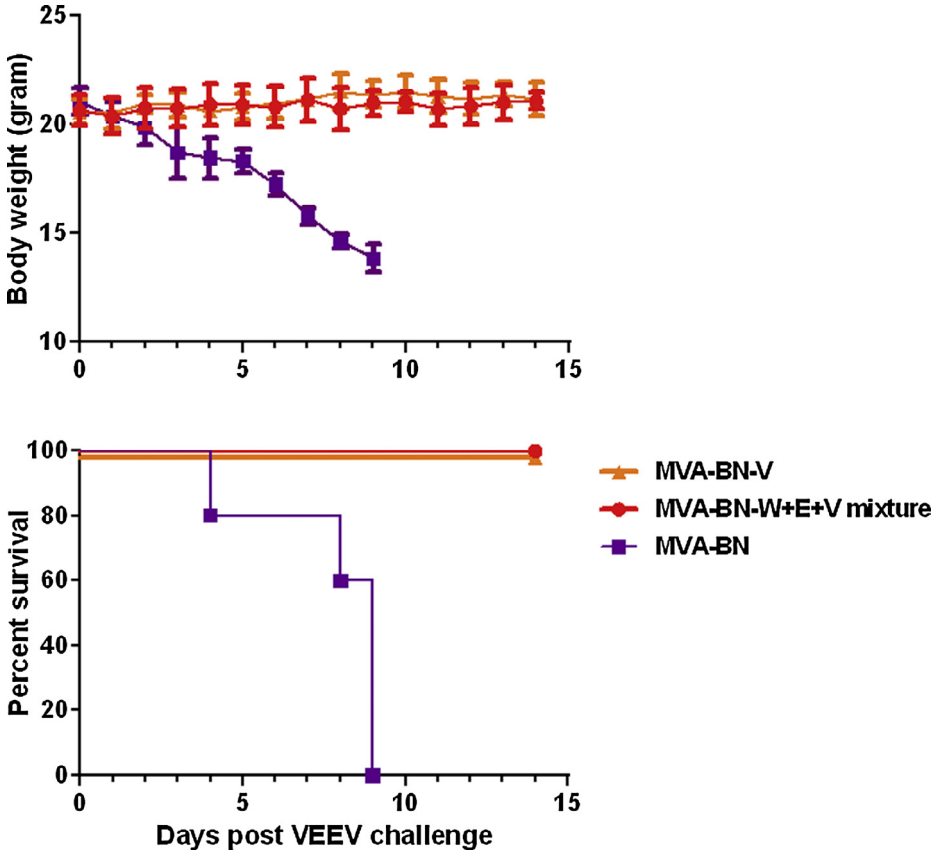

Fig. 5. MVA-BN-V versus MVA-BN-W + E + V against 5×10^3 pfu of VEEV TrD challenge. A group of 5 mice were immunized S.C. with two doses of MVA-BN-V (▲), MVA-BN-W + E + V triple mixture of vaccines (●), or MVA-BN control (■)28 days apart, and challenged I.N. with the VEEV TrD (5×10^3 pfu) 14 days after booster vaccination.

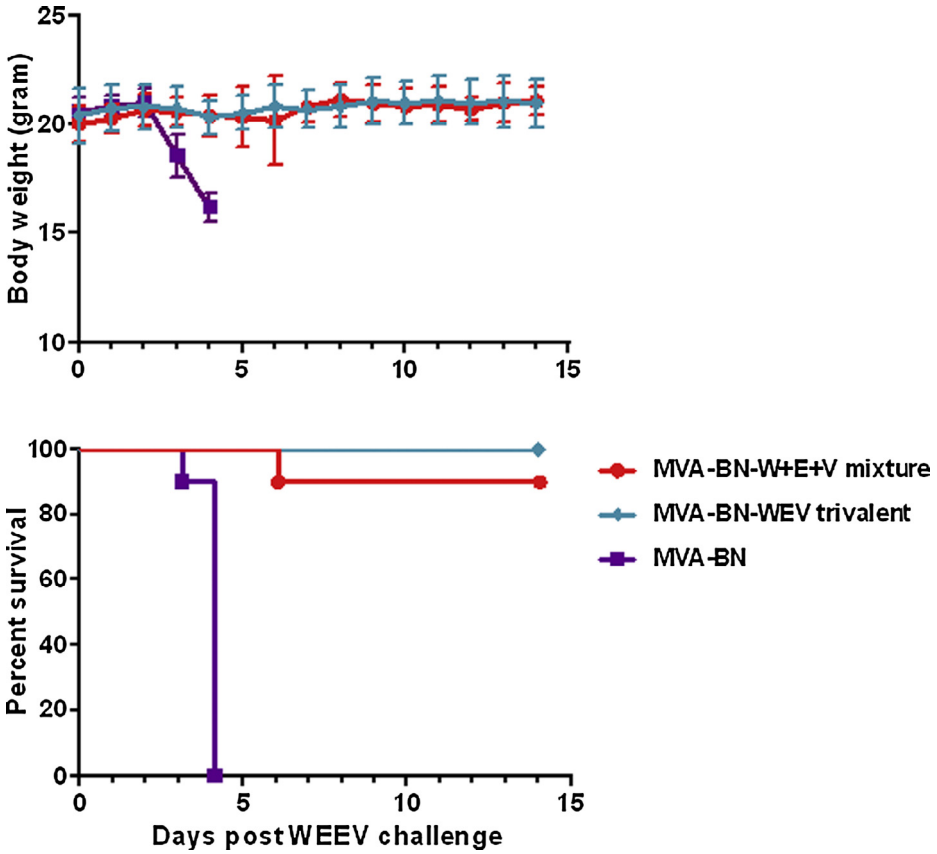

Fig. 6. MVA-BN-W + E + V versus MVA-BN-WEV against 5×10^3 pfu of WEEV Fleming challenge. A group of 5 mice were immunized S.C. with two doses of MVA-BN-WEV trivalent vaccine (◆), MVA-BN-W + E + V triple mixture of vaccines (●), or MVA-BN control (■) 28 days apart, and challenged I.N. with WEEV Fleming (5×10^3 pfu) 14 days after booster vaccination.

V3526 caused headache, fever, malaise and sore throat in a significant number of vaccinees although the vaccine induced strong immune responses [33]. These adverse effects prompted the discontinuation of the clinical trial for V3526 as a live attenuated vaccine for VEEV. Another approach for overcoming the problems of the traditional live attenuated VEEV vaccines was through the construction of chimeric Sindbis virus (SINV) expressing structural proteins of WEEV, EEEV, and VEEV. To construct live attenuated chimeric SIN/VEE viruses, the genes encoded the replicative enzymes and the *cis*-acting RNA elements of SINV were ligated with the genes encoding the structural proteins of VEEV TC-83 strain. Mouse studies showed the chimeric virus was highly attenuated and immunogenic [34,35]. A similar approach was used to make chimeric SIN/EEE and SIN/WEE viruses conferring complete protection against intraperitoneal challenge of a homologous strain of EEEV [36], and WEEV [37], respectively in a mouse model, and against EEEV in nonhuman primates [38]. Alternative approaches utilized the structural proteins by themselves to elicit immune protection either as subunit vaccines, or expressed from viral vectors. Dupuy *et al.* demonstrated that codon optimization and intramuscular electroporation delivery improved immunogenicity and efficacy of a VEEV DNA vaccine, where the structural polyprotein was expressed from a mammalian promoter [39]. Mice injected with the vaccine by intramuscular electroporation generated a similarly high level of VEEV-neutralizing antibody that was observed in mice given the live-attenuated VEEV vaccine TC-83 [40]. Viruses, such as vaccinia virus and adenovirus, can be modified to deliver genes encoding antigens of WEEV, EEEV, and VEEV. Several studies demonstrated that an adenovirus-vectored WEEV vaccine encoding E3-E2-6 K-E1 structural proteins of the 71 V-1658 strain of WEEV conferred rapid and complete protection [25,41]. However, pre-existing immunity to the human adenovirus vector was thought to reduce the immune response in human adenovirus-vectored vaccines. Another vaccine was made based on a vaccinia virus vector expressing the structural proteins of VEEV TrD [42]. The vaccine provided protection of mice against peripheral challenge of various subtypes of VEEV. However, only partial protection was achieved against intranasal challenge. Moreover, replicating vaccinia virus was utilized, which poses a safety concern for human use.

In the studies described here, the encephalitic alphavirus vaccines were designed and constructed to include the E3-E2-6 K-E1 coding sequences for WEEV, EEEV, or VEEV respectively based on the non-replicating MVA-BN vector. The codon usage was adapted for optimal expression in humans. The choice of promoters for expression of antigens in a recombinant vector plays a critical role for the success of a vaccine. In all three monovalent, recombinant MVA-BN-based alphavirus vaccines, strong early promotors were selected for the expression of antigens to start very early after infection of cells with the vaccines [22,23]. Taking advantage of the large coding capacity of MVA for extraneous transgenes, a trivalent vaccine MVA-BN-WEV was constructed. The same promoters were utilized in the trivalent vaccine with their respective antigens. All three monovalent vaccines were confirmed to express the E3-E2-6 K-E1 transgenes of WEEV, EEEV, and VEEV respectively in HeLa cells by flow cytometry analysis. As well, the trivalent vaccine expressed all three transgenes to similar levels to the monovalent vaccines as determined by flow cytometry with the EEEV envelope proteins slightly lower than that of the monovalent MVA-BN-E.

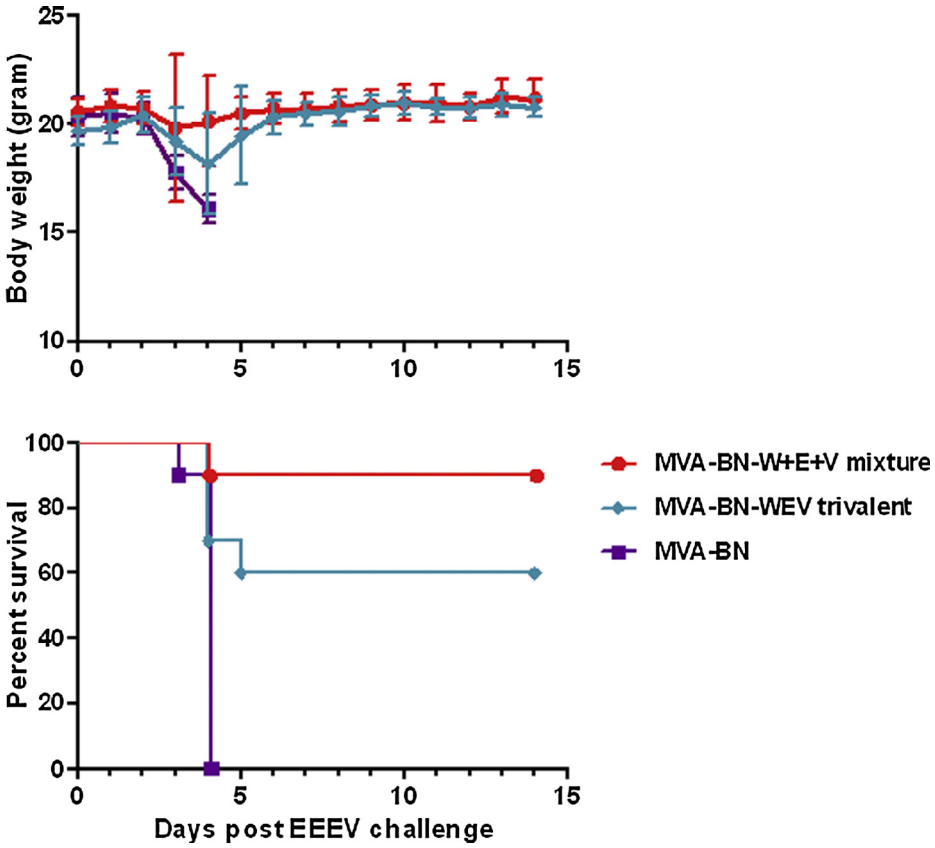

Fig. 7. MVA-BN-W + E + V versus MVA-BN-WEV against 5×10^3 pfu of EEEV PE6 challenge. A group of 5 mice were immunized S.C. with two doses of MVA-BN-WEV trivalent vaccine (◆), MVA-BN-W + E + V triple mixture of vaccines (●), or MVA-BN control (■) 28 days apart, and challenged I.N. with EEEV PE6 (5×10^3 pfu) 14 days after booster vaccination.

Each monovalent vaccine, the triple mixture of monovalent vaccines, or the trivalent vaccine was then evaluated *in vivo* against the respective virus. In general, monovalent MVA-BN-based vaccines protected mice from lethal alphavirus I.N. challenge and disease. Surviving mice showed no signs of illness or weight loss. For MVA-BN-V, the immunization routes of S.C. and I.M. were compared. No significant difference was observed between the two routes of administration. Both gave 100% protection against even a high challenge dose of VEEV TrD (10^4 pfu). The triple mixture provided 90–100% protection against WEEV or VEEV challenge without any signs of illness, indicating no interference between MVA-BN-W and MVA-BN-V when administered together. However, when evaluated in the EEEV challenge model, the triple mixture provided 60–90% protection. This may reflect a variability of the model and/or the higher virulence of the EEEV challenge strain causing death of non-vaccinated animals in 3–4 days in the two experiments, but it could also be at least partially attributed to six amino acid differences in the E1 and E2 sequences between the vaccine antigen (from EEEV FL93-939NA) and the challenge strain (PE6). Further, potential immune interference between WEEV and/or VEEV affecting EEEV protection levels cannot be ruled out, when the vaccines were administered in a mixture of vaccines. A study on a virus like particle vaccine in sheep bluetongue virus, demonstrated a certain degree of immune interference between closely related antigens could happen in a regimen of the mixture of vaccines possibly due to immunodominance of one antigen over another [43], although some reports demonstrated that the protective immunity against multiple antigens could be induced through simultaneous inoculation with various vaccines without evidence of immune interference [44–46]. The trivalent vaccine MVA-BN-WEV demonstrated similar high levels of protection to the monovalent and the triple mixture of vaccines against WEEV and VEEV (90–100% protection). However, like the triple mixture, the trivalent vaccine provided slightly lower protection against EEEV (60%) as compared to the monovalent MVA-BN-E (100%). Flow cytometry analysis of alphavirus antigens revealed a lower expression of EEEV structural proteins by HeLa cells infected with the trivalent MVA-BN-WEV compared to the expression induced by infection with the monovalent MVA-BN-E vaccine. Inside the virosome or virus factory of a cell, the trivalent vaccine would be required to express the three antigens simultaneously, which may account for the reduced expression of EEEV antigen observed by FACS expression. A potentially reduced level of expression of the EEEV antigens may have a more drastic reduction in the level of protection against the more virulent EEEV (40–50% case mortality), as opposed to the less virulent WEEV (15% case mortality) and VEEV (<1% case mortality). Detailed studies on the production of the three alphavirus transgenes in the trivalent vaccine may give us better insight into how these polyproteins are synthetized within the MVA virus factory in a cell [47,48]. Multivalent vaccines have been generated from a single MVA vector and successfully tested against multiple viruses of Filovirus family [49].

In order to investigate the mechanism of protective efficacy of MVA-BN-based alphavirus vaccines, the serum samples from the vaccinated mice were evaluated for NT. Previous studies indicated the utility of antibody NT as a correlate of protection [28,40]. MVA-BN-based monovalent alphavirus vaccines elicited anti-alphavirus neutralizing antibodies. A booster immunization increased the titers. However, the triple mixture of vaccines only elicited neutralizing antibodies against WEEV and EEEV, not against VEEV, albeit

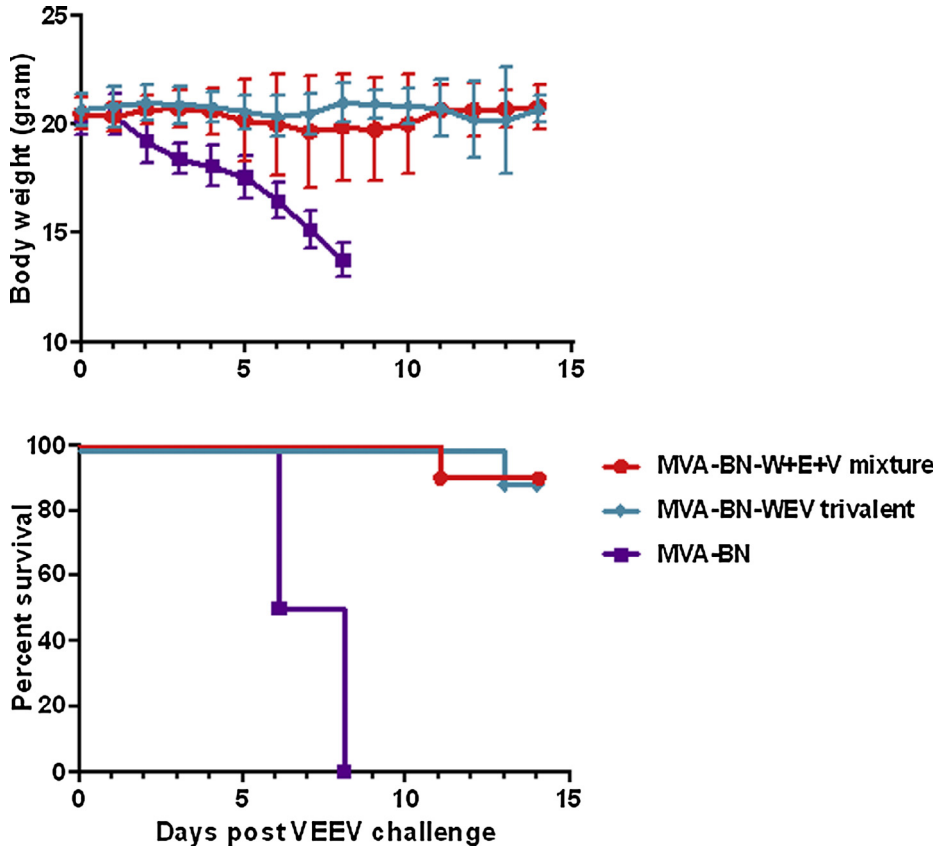

Fig. 8. MVA-BN-W + E + V versus MVA-BN-WEV against 5×10^3 pfu of VEEV TrD challenge. A group of 5 mice were immunized S.C. with two doses of MVA-BN-WEV trivalent vaccine (◆), MVA-BN-W + E + V triple mixture of vaccines (●), or MVA-BN control (■) 28 days apart, and challenged I.N. with VEEV TrD (5×10^3 pfu) 14 days after booster vaccination.

conferring full protection. Further, the triple mixture of vaccines underperformed monovalent vaccines in terms of the magnitude of neutralizing antibodies. The trivalent vaccine only induced neutralizing antibodies against WEEV.

Although neutralizing antibodies seem to play a pivotal role in protective efficacy against alphaviruses [25,50,51], T-cell immunity was shown to also participate in the protection [52]. The MVA vector is good at eliciting both humoral and T-cell immunities [53] and has been reported as a vaccine vehicle for another member of alphaviruses, Chikungunya virus (CHIKV) [18–20]. An MVA vaccine expressing the structural envelope cassette E-E2-6 K-E1 induced a high level of neutralizing antibodies, which correlated with protection against lethal CHIKV challenge in AG129 mice [19]. In contrast, another MVA vaccine expressing E3-E2 proteins protected AG129 mice from challenge with CHIKV with low or undetectable levels of neutralizing antibodies [20]. A third MVA vaccine, encoding C-E3-E2-6 K, and E1 structural proteins, induced strong, broad, highly polyfunctional, and long-lasting CHIKV-specific $CD8^+$ T cell responses, together with neutralizing antibodies against CHIKV infections in mice [18].

In our study, anti-alphavirus neutralizing antibodies are likely to play a role in the protection against encephalitic alphavirus-mediated infections in the mice vaccinated with MVA-BN-based alphavirus vaccines, but T-cell and innate immunity could also contribute to the protection [54]. This becomes particularly apparent in mice immunized with the triple mixture of vaccines, since they were equally protected against VEEV as mice immunized with the monovalent vaccine, yet no VEEV NT was detected in triple mixture vaccinated animals. The lack of alphavirus NTs induced by the trivalent vaccine, apart from WEEV NT at 41 days also lends support that the protection is not solely based on neutralizing antibodies. Additional studies are required before further conclusions can be made.

The studies described here demonstrated that MVA-BN-based monovalent alphavirus vaccines were fully protective against lethal I.N. challenge with the respective alphaviruses. Further, a mixture of the three monovalent vaccines and for the first time a single vector trivalent vaccine were shown to provide protection against all three encephalitic alphaviruses. These data warrant further development of MVA-BN vectored alphavirus vaccines as safe multivalent vaccines for human use.

Conflict of interest

Robin Steigerwald, Marcus Kalla, Ariane Volkmann, and David Noll, as employees of BN have a vital interest in the success of BN. Robin Steigerwald and Marcus Kalla are inventors on a patent application regarding recombinant MVA-based alphavirus vaccines. The other authors indicated no potential conflicts of interest.

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4. AUTHORS (Last name, followed by initials – ranks, titles, etc., not to be used)

Hu, W.-G.; Steigerwald, R.; Kalla, M.; Volkmann, A.; Noll, D.; Nagata, L.P.

5. DATE OF PUBLICATION (Month and year of publication of document.)	6a. NO. OF PAGES (Total pages, including Annexes, excluding DCD, covering and verso pages.)	6b. NO. OF REFS (Total references cited.)
July 2018	10	54

7. DOCUMENT CATEGORY (e.g., Scientific Report, Contract Report, Scientific Letter.)

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8. SPONSORING CENTRE (The name and address of the department project office or laboratory sponsoring the research and development.)

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MVA-BN; Vaccines; Encephalitic alphaviruses; Monovalent; Mixture of vaccines; Trivalent; Protective efficacy

13. ABSTRACT/RÉSUMÉ (When available in the document, the French version of the abstract must be included here.)

The three encephalitic alphaviruses, western, eastern, and Venezuelan equine encephalitis viruses (WEEV, EEEV, and VEEV) are potential biothreat agents due to high infectivity through aerosol exposure, ease of production in large amounts, and relative stability in the environment. Currently, there is no licensed vaccine for human use to these three encephalitic alphaviruses, and efforts to move vaccine candidates forward into clinical trials have not been successful. In this study, the modified vaccinia Ankara-Bavarian Nordic (MVA-BN®) vaccine platform was used to construct and produce three monova lent recombinant MVA-BN-based encephalitic alphavirus vaccines, MVA-BN-W, MVA-BN-E, and MVA-BN-V. Additionally, a MVA-BN-based construct was designed to produce antigens against all three alphaviruses, the trivalent vaccine MVA-BN-WEV. The protective efficacy of these vaccines was evaluated *in vivo*. Female BALB/c mice were immunized with two doses of each monovalent MVA-BN-based alpha- virus vaccine, a mixture of the three monovalent vaccines, MVA-BN-W + E + V, or the trivalent vaccine MVA-BN-WEV at a four-week interval. Two weeks after the booster immunization, the mice were instilled intranasally with 5×10^3 to 1×10^4 plaque forming units of WEEV, EEEV, or VEEV. All mice immunized with monovalent vaccines survived the respective virus challenge without any signs of illness or weight loss, while all the control mice died. The triple mixture of vaccines or the trivalent vaccine also provided 90 to 100% protection to the mice against WEEV and VEEV challenges, and 60% to 90% protection against EEEV challenge. These data suggest that each monovalent MVA-BN-W, MVA-BN-E, and MVA-BN-V is a potential vaccine candidate against respective encephalitic alphavirus and the three monovalent vaccines can be given in a mixture (MVA-BN-W + E + V) or the trivalent vaccine MVA-BN-WEV can serve as a true multivalent vaccine significantly reducing efficacy against WEEV and VEEV despite slightly reduced efficacy against EEEV challenge.