

Research Article

***Deinococcus* Mn²⁺-Peptide Complex: A Novel Approach to Alphavirus Vaccine Development**

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Short title: Alphavirus Vaccine and antioxidant Mn-peptide complex

Abstract

Chikungunya virus (CHIKV), an Old World alphavirus and Venezuelan equine encephalitis virus (VEEV), a New World alphavirus are the causative agents of debilitating diseases in humans. Over the past decade, CHIKV caused numerous outbreaks in the Asian and European countries and Americas making it an emerging pathogen bearing great global health importance. VEEV, on the other hand, has been developed as a bioweapon in the past due to its ease of preparation, aerosol dispersion and high lethality in aerosolized form. Currently, there are no FDA approved vaccines against these viruses. Live attenuated vaccines (V3526 and CHIKV 181/25) were developed against both VEEV and CHIKV but had to be retracted due to residual virulence in spite of being highly immunogenic during the clinical trials.

Classical gamma irradiated virus vaccine development approach is limited by immunogenicity loss due to oxidative damage to the surface proteins at the high doses of radiation required for complete virus inactivation. Thus, we have used an *in vitro* reconstituted radioprotective antioxidant complex (MDP, consisting of Mn^{2+} , peptide and phosphate, derived from radioresistant bacteria *Deinococcus radiodurans*) in the present study which selectively protects proteins but not the nucleic acid from the radiation-induced oxidative damage as desired for safe and efficacious vaccine development.

In this study, we demonstrate that V3526 and CHIKV 181/25 irradiated in the presence of MDP have significant epitope preservation even at supra-lethal doses of radiation. Irradiated viruses were found to be completely inactivated via several *in vitro* and *in vivo* (neonatal mice) assays. V3526 inactivated in the presence of MDP elicited significantly higher antibody response upon immunization and also resulted in drastically

improved protective efficacy *in vivo*. Thus, the novel MDP-based gamma inactivation approach described here can easily be applied for developing vaccines against any pathogen of interest in a fast and cost effective manner.

Author Summary

Inactivated vaccines form the primary defense strategy against an emerging disease or an outbreak like Ebola, since live attenuated vaccine development is time consuming, requires detailed genetic study, and rigorous testing. For irradiated whole-virus vaccines, ionizing radiation (IR)-induced destruction of a virus' genome is desired, while radiation-induced damage to epitopes is counterproductive to the preservation of antigenic potency. We report a novel IR-inactivation strategy for alphaviruses using the Mn-peptide antioxidant complex (MDP) that prevents the oxidation of envelope proteins at IR doses that render their genomes non-infectious. This approach was successfully tested on Venezuelan equine encephalitis and chikungunya viruses (V3526 and CHIK 181/25 respectively). Viruses inactivated by gamma-radiation in presence of MDP were shown to be safe *in vivo*. Immunization of mice with V3526 vaccine preparations yielded a more protective and immunogenic vaccine. Overall our results suggest that this approach can easily be extended to any pathogen of interest.

Introduction

The discovery and commercial development of a licensed lead vaccine candidate often requires decades of basic research followed by years of pre-clinical and clinical studies of safety and efficacy. Recent experiences with many pathogens including Ebola have highlighted the importance and requirement of developing vaccines much more rapidly. Due to the significantly greater time required for the discovery and testing of a subunit or live attenuated vaccine, the use of whole, inactivated preparations appear to be the most promising strategy for speedy deployment of first line of viral vaccines [1, 2].

Gamma-irradiation is a classical virus inactivation method that is simple, economical and requires no additional purification steps [1-12]. It is also an FDA-approved method widely applied to sterilizing medical, dental, food and household products. An irradiated-cryopreserved malaria vaccine, PfSPZ, is also currently under clinical trials [13]. Yet, the long-standing tenet of radiobiology that protein epitope destruction during irradiation is inevitable at doses needed to kill viruses has caused a setback to the development of irradiation-based approaches to vaccine production [1-3]. During ionizing radiation (IR) exposure, the energy of the photons induces direct damage whereas the radiolysis of aqueous solution generating free radicals inflicts indirect damage [14]. IR induced direct damage indiscriminately targets both nucleic acids and proteins. However, it has been discovered that some extremely radiation resistant organisms have evolved mechanisms to specifically protect proteins from the far more damaging indirect effects of gamma (γ)-rays in aqueous preparations. Mn^{2+} -peptide antioxidants that can uncouple protein and nucleic acid damage caused by γ -radiation were first reported in *Deinococcus radiodurans*, a naturally occurring radiation resistant extremophilic bacterium capable of surviving 12-16 kGy [15]. *D. radiodurans*, accumulates high concentration levels of manganese intracellularly in complex with other small metabolites and these complexes selectively preserve the intracellular proteins from oxidative damage under extreme environmental conditions leaving nucleic acids open to attack by reactive oxygen species (ROS) [15-17]. Subsequently, rational design of the peptide-component of these Mn antioxidants yielded a synthetic complex named MDP, which forms spontaneously when the synthetic decapeptide DEHGTAVMLK, orthophosphate (Pi) and Mn^{2+} are combined. Under aqueous conditions, MDP preserved

the function of irradiated enzymes exposed to massive doses of γ -radiation (>50 kGy), but did not significantly protect DNA [18]. The recent discovery that MDP can protect the structural integrity of antigens at IR doses far above those which abolish infectivity of bacteriophage lambda has provided a new perspective to virus inactivation method that had been previously discounted due to its nonspecific destruction of the protein component [18, 19].

For irradiated whole-virus vaccines, IR-induced destruction of a virus' genome is desired, while IR-induced damage to epitopes is counterproductive to the preservation of antigenic potency. In this study, the unique ability of MDP to uncouple protein damage from nucleic acid damage has been harnessed in alphaviruses exposed to supralethal doses of gamma-radiation (50 kGy). Alphaviruses are globally distributed arboviruses and are classified into New World and Old World alphaviruses [20]. Venezuelan equine encephalitis virus (VEEV), a New World alphavirus, is a potential bioweapon that is highly infectious in an aerosolized form. [21-23]. VEEV causes biphasic disease with initial replication in lymphoid and myeloid tissues followed by central nervous system infiltration initiating neuropathology and sometimes-fatal encephalitis [24]. In the past decades, VEEV has caused periodic enzootic outbreaks in Central and South America and Southern parts of North America that had rapidly progressed to epidemic scale due to lack of timely diagnostic and therapeutic measures [25, 26]. Chikungunya virus (CHIKV), an Old World alphavirus, is an emerging pandemic pathogen that causes severe arthralgia and maculopapular rashes. Historically, chikungunya has been a tropical disease prevalent in Africa and South-east Asian countries, however, recently several outbreaks have been reported in many Asian countries, France, Italy, Caribbean Islands

and the Americas [27-30]. In 2014, thousands of CHIKV cases were reported throughout the United States with 11 locally transmitted cases in Florida, making it an emerging virus of significant public health importance [31]. At present, the lack of FDA-approved vaccines against either of these two arboviruses underscores the urgency for vaccine development. Earlier, V3526, a live-attenuated strain of VEEV, was developed by site-directed mutagenesis based on the furin cleavage site deletion from PE2 glycoprotein and incorporation of a single amino acid mutation in the E1 glycoprotein [32, 33]. CHIK 181/25, a live-attenuated vaccine candidate for CHIKV, was developed by plaque-to-plaque passaging [34, 35]. Although both of these live-attenuated vaccines yielded high-level immunogenicity in human clinical trials, a fraction of the vaccinees developed adverse effects [33, 35]. Live-attenuated vaccines have an inherent risk of reversion to the wild type as well as transmission potential by the vectors. Therefore, inactivation of V3526 and CHIK 181/25 in a manner, which renders them non-infectious without compromising their immunogenic potential, might result in safe and efficient vaccine candidates [1]. Here we show that the unique MDP-irradiation method yielded such non-infectious, safe, highly immunogenic alphaviral vaccine candidates.

Results

Gamma irradiated virus gets inactivated in a radiation dose dependent manner.

Purified aqueous preparations of V3526 and CHIK 181/25 were mixed with or without MDP and exposed to increasing doses of gamma-irradiation (0-50 kGy) on ice. Virus inactivation was confirmed by serially passaging the irradiated virus preparations on monolayers of Vero cells (10 times to amplify any escaped viruses). No virus-induced

cytopathic effect (CPE) was observed in cells infected with viruses irradiated to 20 kGy or greater (Figs 1A, 1D, S1A and S1B). This finding was confirmed by viral gene-specific RT-PCR amplification of cellular RNA and virus titration on the cell supernatants collected at the end of the 10th passage (Figs 1B, 1C, 1E and 1F). Virus-specific immuno-staining to localize V3526 and CHIK 181/25 antigens within the infected cell cytoplasm also revealed no detectable viral proteins in the cells infected with viruses that were irradiated to 20 kGy or greater (Figs 2). Our results demonstrate that the viruses were completely inactivated on exposure to 20 kGy irrespective of the presence of MDP complex.

Gamma irradiation of virus in presence of MDP complex preserved the structural integrity of the viral epitopes.

Preserving the structural integrity of viral epitopes during vaccine development is crucial to mounting a robust antibody response upon immunization and was one of the major aims of this study. Resolving the viral proteins on polyacrylamide gels demonstrated that MDP protected the viral proteins from IR induced degradation even at the highest IR dose tested (50 kGy) whereas, in the absence of MDP no viral proteins were visible at the IR dose of 5 kGy (CHIK 181/25) or 20 kGy (V3526) (Figs 3A and 3D). To determine the antibody-binding capacity of the critical viral neutralizing epitopes, western blot analyses were performed using monoclonal antibodies against the neutralizing epitopes of the viruses (Figs 3B and 3E). As observed for the total viral proteins, the neutralizing epitopes on the viral surface were significantly protected by MDP, even at the highest IR dose tested (50 kGy). In contrast, when virus was irradiated

in absence of MDP, the bands corresponding to the same protein epitopes were lost at IR doses as low as 20 kGy. These results confirmed the IR dose-dependent inactivation of viruses treated with MDP, but without significant loss in epitope binding integrity.

To evaluate whether or not MDP selectively protects envelope proteins over the single-stranded RNA (ssRNA) alphaviral genome, BHK-21 cell cultures were transfected with RNA isolated from irradiated virus preparations. The positive-sense ssRNA alphaviral genome is capable of generating infectious and replicating virions when transfected *in vitro*. The absence of virus-specific staining in the BHK-21 cells transfected with RNA from viruses irradiated to 20 kGy or greater doses of IR in the presence or absence of MDP (Figs 3C and 3F) indicated that MDP discriminately preserved the viral proteins, but not the viral genome from IR-induced damage. RT-PCR amplification of the RNA genome from inactivated V3526 preparations demonstrated that the 11.4 kb ssRNA genome was degraded to ≤ 1 kb fragments at irradiation doses >20 kGy (data not shown); therefore, sufficiently degraded to prevent host-cell reactivation mediated by recombinogenic processes. Thus, our results show that exposure of alphaviruses to supralethal IR doses in the presence of MDP abolished infectivity by genome destruction, yet preserved viral proteins required for the development of safe protective vaccine candidates.

Gamma irradiated virus preparations caused no virulence in neonatal mice model.

The safety of the irradiated virus (+/- MDP) preparations was interrogated *in vivo* using the gold standard assay via intracranial inoculation in neonatal mice [4, 36]. The IR dose was chosen based on the *in vitro* results as only these doses were to be further tested

for protective efficacy. This confirmed the absence of mouse adaptability and re-activation of the irradiated viruses exposed to IR doses of 20-50 kGy. The animals were daily monitored for morbidity as well as any clinical symptoms of the disease such as, circular movement, shrunken appearance, shivering and inability to stay upright. All the animals that received the inactivated virus preparations developed normally, without any clinical signs of disease (Figs 4A-4D, 5A-5B and 6A-6B). The brain homogenate of mice injected with virus irradiated to 40 kGy with or without MDP complex were passed into a subsequent set of neonatal mice that were observed similarly. Samples irradiated to 40 kGy were additionally passaged in a second set of neonatal mice. The absence of replication-proficient particles was validated by virus titration (Figs 4E-4F, 5E and 6G) as well as virus-specific RT-PCR (Figs 5C-5D and 6C-6F) in brain tissue for V3526, and brain as well as knee tissues for CHIK 181/25 at the end of both the passes. Collectively, the results from these assays demonstrate that the inactivated virus preparations were safe *in vivo*.

V3526 gamma-inactivated in the presence of MDP confers significant protection against aerosol challenge.

The study was concluded by evaluating the *in vivo* protection and immunity conferred on mice by V3526 inactivated at 30 and 40 kGy in the presence of MDP (i_{30} V3526-MDP and i_{40} V3526-MDP, respectively) when the mice were challenged with a lethal aerosol exposure to the wild-type VEEV-Trinidad Donkey (TrD) strain. As expected, animals immunized with V3526 irradiated in the presence of MDP were significantly healthier (total body weight), (Figs S2A and S2B) and displayed substantially increased survival following exposure to VEEV-TrD (even when compared

to the current IND, C84) (Figs 7A and 7B). Two immunizations with i_{40} V3526-MDP administered at 5 μ g resulted in a 70% survival, which increased to 80% protection when administered with alum as adjuvant. This was in contrast to a mere 10% survival when immunized with V3526 irradiated at 40 kGy without MDP (Fig 7B). Two immunizations with i_{30} V3526-MDP administered at 1 μ g also resulted in a 70% survival in contrast to 40% survival when immunized with V3526 irradiated at 30 kGy without MDP (Fig 7A). Indeed, the presence of MDP during V3526 irradiation not only resulted in a higher rate of survival, but also improved the antibody response. Moreover, immunizations with 5 μ g of i_{30} V3526-MDP or 1 μ g of i_{40} V3526-MDP both gave significantly improved antibody titers (Figs 7C and 7D). Even a single immunization with i_{30} V3526-MDP or i_{40} V3526-MDP resulted in significantly higher antibody response unlike V3526 irradiated without MDP that failed to trigger any antibody response after the primary immunization. However, no correlation was observed between the protective efficacy and the neutralizing antibody response (Figs S3A and S3B). This finding was similar to other previously published studies which did not demonstrate a correlation of protection with neutralizing antibody response (REF). Thus, our results clearly demonstrate the superiority of the irradiated viral vaccines prepared in the presence of MDP over conventional irradiated vaccine approaches.

Discussion

IR has long been exploited for vaccine development against a broad range of infectious agents [1, 3-12]. Although, the simplicity and absence of additional purification steps makes IR-based vaccine development attractive, the IR induced

oxidative damage to the epitopes results in generation of poorly immunogenic vaccines [1, 4]. A very high dose of IR is needed to sufficiently degrade the smaller genome of a virus (11.4Kb for alphaviruses). Unfortunately, such high IR doses also result in oxidative damage of the viral surface proteins required to generate a robust antibody response after vaccination. Reduction of IR dose in order to prevent the protein oxidation accompanies an inherent risk of incomplete inactivation of the virus. This becomes even more important with viruses having positive-sense, ssRNA genomes. In the past, incomplete chemical inactivation of viral vaccines have led to several outbreaks [37-41]. Thus, it is critical to be able to inactivate the viruses at supralethal doses of IR to confirm proper inactivation, but protect the antigenic proteins at the same time.

Studies on the underlying mechanisms of the exceptional radiation resistance of *D. radiodurans*, revealed the crucial role played by the intracellular Mn- small metabolite complexes as non-enzymatic ROS scavenging system specifically protecting the proteome [15, 16, 42]. Ultrafiltrates from this bacterium are enriched in such complexes and retain their radical oxygen scavenging properties (ROS) both *in vitro* and *ex vivo* [18, 43]. Mn- small metabolite complexes (such as MDP) when reconstituted *in vitro* based on their intracellular stoichiometric concentrations, retain their exceptional ROS scavenging ability and can be applied to IR-based vaccine development [19, 44].

Therefore, we exploited this unique strategy of developing IR-inactivated vaccines, where MDP was used during IR-inactivation of V3526 and CHIK 181/25 to uncouple the IR induced proteome damage from genome damage. The rationale behind using these live-attenuated strains of VEEV and CHIKV was that the elimination of their residual instability by IR in presence of MDP complex would result in the development

of highly efficacious inactivated vaccine candidates [45-48]. Additionally, a number of earlier studies have already evaluated the underlying mechanisms and immune responses triggered by both these vaccines in detail [47, 49-53].

In contrast to other recent gamma inactivation attempts where viruses were inactivated on dry ice, the viruses were kept on wet ice during gamma irradiation in our study [4, 54]. Although, dry ice reduces the IR-induced indirect damage mediated by ROS generation, this significantly increases the IR dose threshold required for complete genome inactivation [15, 54]. MDP complex is an excellent ROS scavenger that selectively protects the protein addressed the issue of preserving the epitope integrity required to mount an effective immunological response without compromising the genome inactivation. Both the viruses were completely inactivated from IR dose of 20 kGy and upwards. Additional *in vitro* assays including virus titration, immunofluorescence of cells infected with irradiated virus and virus-specific RT-PCR on the cellular RNA verified virus inactivation. No live virus was detected in samples treated with ≥ 20 kGy in the serial passing of the inactivated virus on Vero cell monolayer. The novelty of this approach was the preservation of the viral epitopes from ROS-induced oxidative damage by MDP complex even at IR doses as high as 50 kGy. Neutralizing epitopes on VEEV and CHIKV E2 surface glycoproteins are implicated in protective immunological response [55, 56]. Our results from silver staining as well as neutralizing antibody specific western blots confirmed that the antigenic epitopes on the surface glycoproteins of V3526 and CHIKV181/25 were significantly preserved in presence of MDP complex even at the highest IR dose of 50 kGy. In contrast, for similar IR doses the epitopes were completely degraded when the viruses were irradiated alone.

The preservation of the structural integrity of the viral epitopes was an indicator of the potential of these irradiated vaccines in mounting neutralizing immune response. To verify the IR-induced genome damage, Northern blot and transfection of the genome of the irradiated virus as well as viral gene specific PCR were performed. The genome of the irradiated virus appeared to have been degraded to a size below 1kb that have facilitated it being rendered non-infectious, thus implicating the role of MDP complex in the uncoupling of IR-induced protein damage from genome damage.

The FDA recommends safety evaluation assessment of vaccine candidates by intracranial inoculation in neonatal mice and this is a gold standard assay for evaluating the safety of alphavirus vaccines [4, 36, 57]. Thus, the safety of the irradiated V3526 and CHIKV181/25 was tested by two consecutive, intracranial serial passages in neonatal mice. Although, all the doses deemed safe from *in vitro* studies were tested in the first passage, brain homogenate of only the doses to be further used in protective study were used in the second passage. No mortality or clinical symptoms of disease were observed in any of the mice infected with the irradiated virus. This implied that addition of MDP complex to the virus preparations did not interfere with the inactivation process and thus the viruses irradiated to 20 kGy and higher doses were completely inactivated irrespective of presence or absence of MDP complex.

MDP-irradiated V3526 to 30 kGy or 40 kGy was further tested *in vivo* for protective efficacy against challenged with VEEV TrD via aerosol route in comparison to V3526 irradiated in absence of MDP complex. I₄₀V3526-MDP improved the survival by 60% over i₄₀V3526 whereas; the survival improvement was by 30% for i₃₀V3526-MDP over i₄₀V3526. Addition of alum did not significantly improvement the survival. Thus, at

comparable antigen dosage, MDP-based irradiated V3526 had significantly improved survival protection over V3526 irradiates without MDP as well as C-84 (the current IND). For all the antigen doses, MDP-based irradiated V3526 caused significantly higher total IgG titers and also attenuated the disease symptoms. Thus, the present study yielded non-infectious, safe, highly immunogenic alphaviral vaccine candidates.

Importantly, our non-viable VEEV and CHIKV vaccine candidates were derived from live-attenuated counterparts that failed clinical trials due to residual-infectivity-dependent-pathogenesis (RIDP). It follows that MDP-irradiation offers a path to ‘vaccine rescue’ for a growing pool of whole-virus vaccines shelved because of RIDP [2, 33, 45, 58, 59]. Moreover, the absence of any toxic chemicals during the inactivation process negates the requirement for additional purification steps. The simplicity and robustness of this approach as well as its non-specificity for any pathogen makes it a rapid and potentially universal inactivation strategy in the production of protective irradiated vaccines. Thus, the MDP-irradiation approach could facilitate approval of existing and future whole virus vaccine candidates aimed at emerging and established viruses for which safe vaccines do not yet exist.

Figures

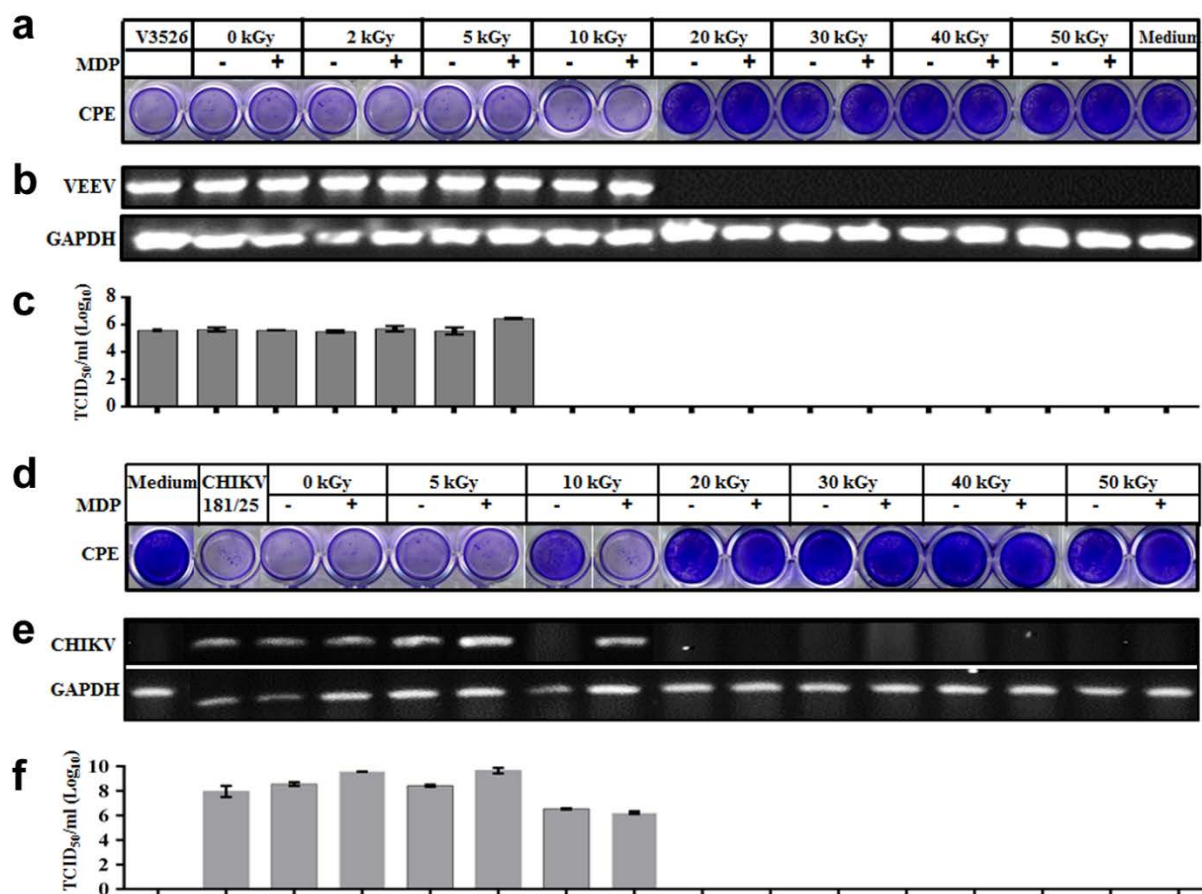


Fig 1. Dose-dependent alphavirus-inactivation by acute gamma-irradiation. Viruses were completely inactivated upon exposure to gamma-radiation at 20 kGy or higher dose of exposure. (A) V3526-induced cytopathic effect (CPE), (B) V3526-specific PCR amplification on the cellular RNA, and (C) V3526 titration by TCID₅₀ evaluation (Log₁₀TCID₅₀/ml) in the supernatant of Vero cells infected with the irradiated V3526 preparations. (D) CHIK 181/25-induced cytopathic effect, (E) CHIK 181/25-specific RT-PCR amplification on the cellular RNA, and (F) CHIK 181/25 titration by TCID₅₀ evaluation (Log₁₀TCID₅₀/ml) in the supernatant of Vero cells infected with irradiated CHIK 181/25 preparations. Results are representative of at least four technical replicates.

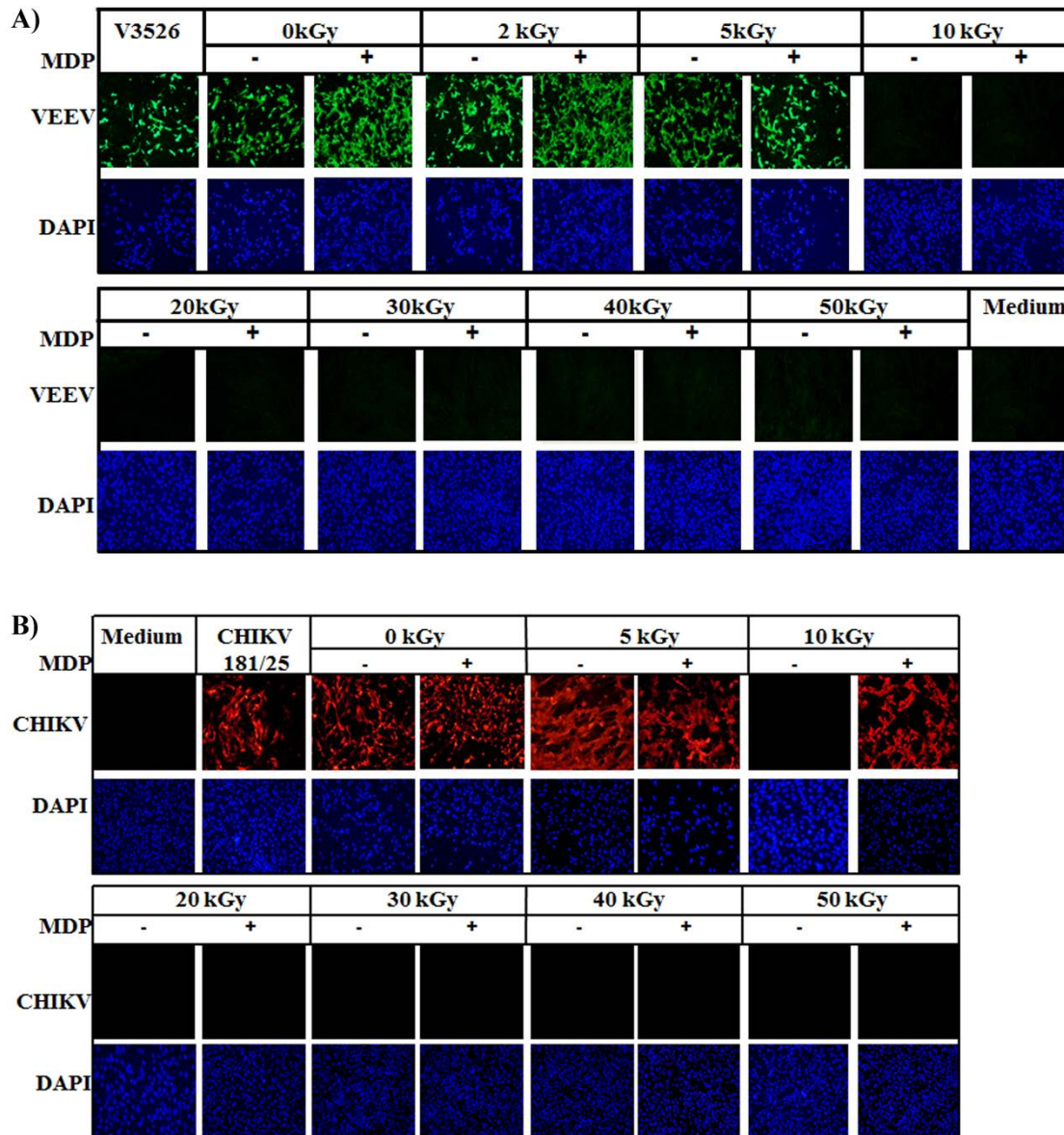


Fig 2. Gamma-irradiation abolishes virus infectivity in a dose-dependent manner.

Vero cells infected with control and irradiated (A) V3526 and (B) CHIK 181/25 preparations followed by virus-specific immuno-staining at 72 h post-infection to determine the localization of viral antigens within the cell cytoplasm. Results are representative of at least four technical replicates.

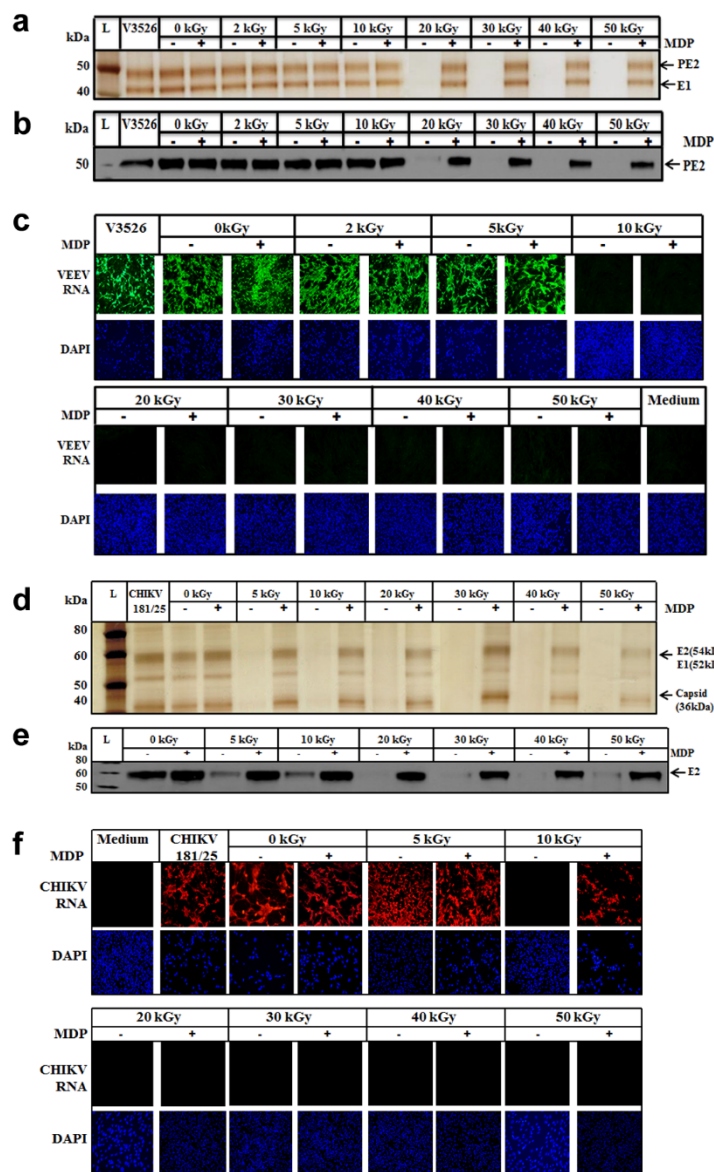


Fig 3. During aqueous gamma-irradiation, MDP selectively protects alphavirus epitopes, but not their ssRNA genomes. (A) Polyacrylamide gel electrophoresis of the viral proteins, and (B) western blot analysis of the neutralizing epitope E2^c show protection of V3526 surface proteins and epitopes from IR-induced oxidation in presence of MDP. (C) *In vitro* transfection of BHK cells with the viral RNA from irradiated V3526 preparations shows significant degradation of viral genome due to irradiation both in absence and presence of MDP at 20 kGy or higher doses of exposure and thus preventing any active virus replication (10 kGy dose showed faint staining in isolated patches at least once in the technical replicates). (D) Polyacrylamide gel electrophoresis of the viral

proteins and, (E) western blot analysis of the neutralizing epitope E2 show protection of CHIK 181/25 surface proteins and epitopes from IR-induced oxidation in presence of MDP. (F) *In vitro* transfection of BHK cells with the viral RNA from irradiated CHIK 181/25 preparations shows sufficient degradation of viral genome due to irradiation both in absence and presence of MDP at 20 kGy or higher doses of exposure. L = Protein ladder. Results are representative of at least four technical replicates.

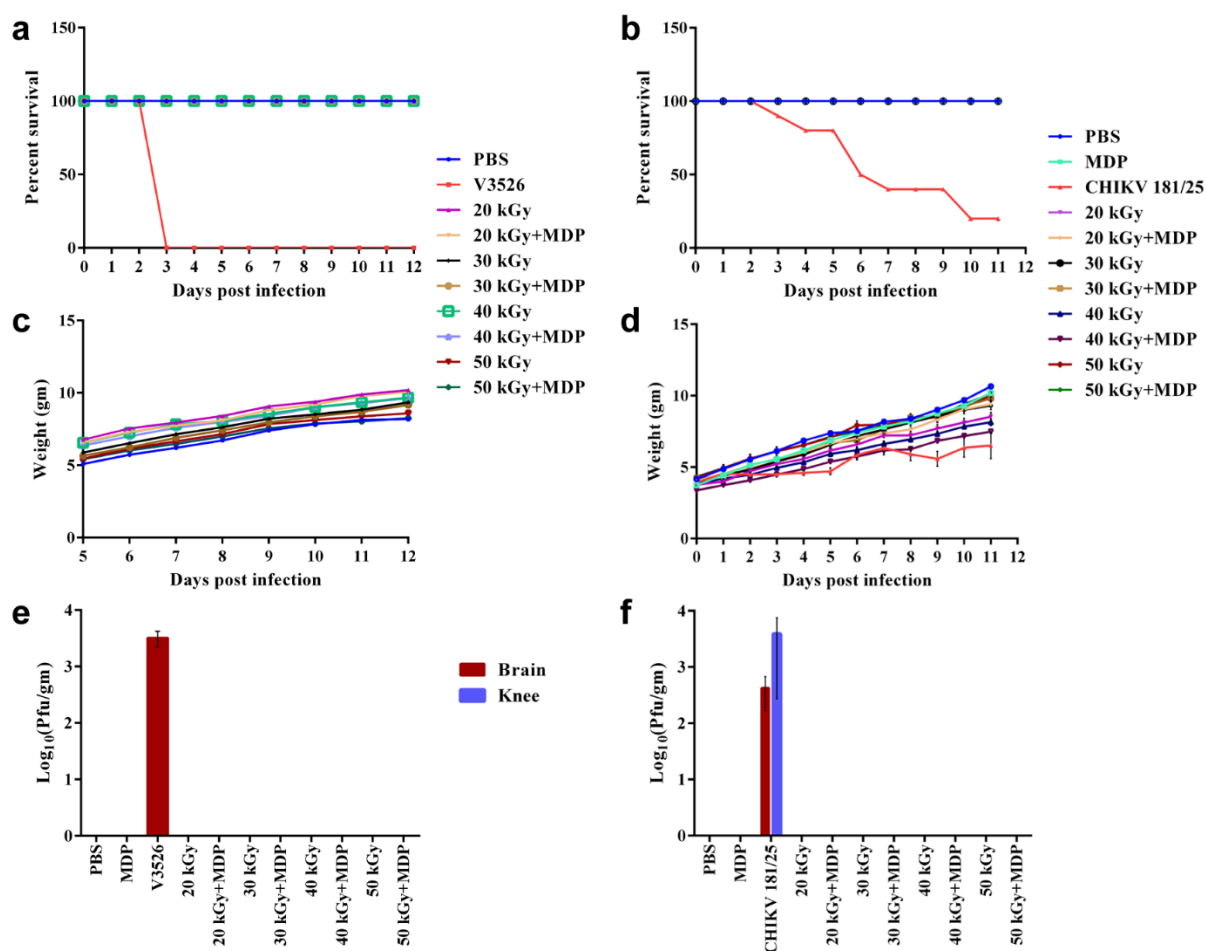


Fig 4. Gamma-irradiated alphaviruses caused no disease symptoms in neonatal mice. Percent survival for animals receiving gamma-inactivated (A) V3526 and (B) CHIK 181/25 preparations showing inactivated virus preparations are safe *in vivo*. No significant change was observed in the total body weight of the neonatal mice injected with irradiated (C) V3526 and (D) CHIK 181/25 preparations. (E) V3526 titration from the brain tissues collected at the end of the 1st passage, and (F) CHIK 181/25 titration

from the brain and knee tissues collected at the end of the 1st passage confirmed absence of any actively replicating virus after irradiation.

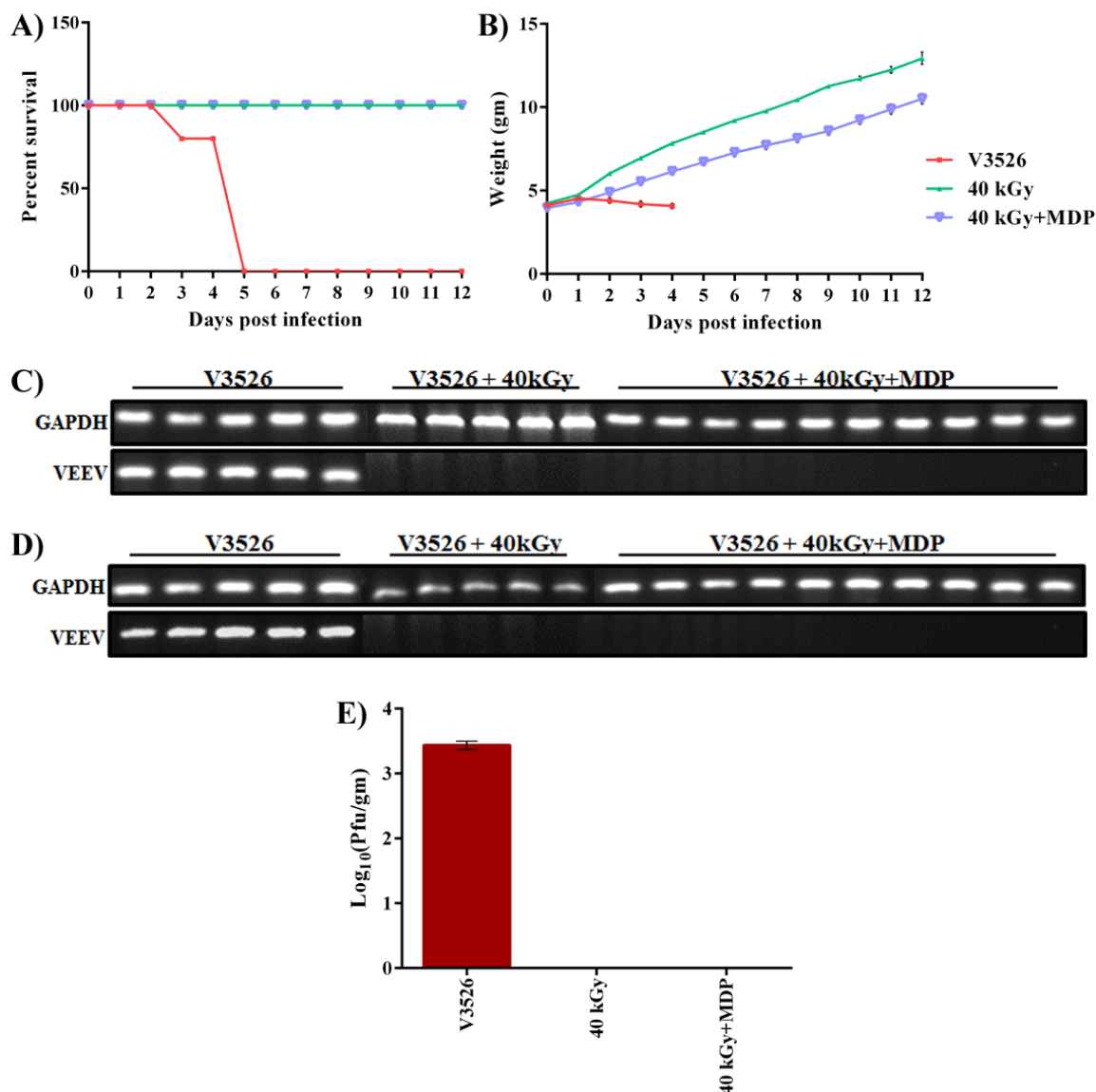


Fig 5. Gamma-irradiated V3526 does not cause disease in the neonatal mouse model.

(A) Percent survival, (B) average loss in total body weight of the neonatal mice injected with irradiated V3526 at the end of the 2nd passage. VEEV-specific PCR amplification in the brain samples collected from the neonatal mice injected with irradiated V3526 at the end of (C) 1st and (D) 2nd passages. (E) Virus titration from the brain tissues of the neonatal mice injected with irradiated V3526 at the end of the 2nd passage.

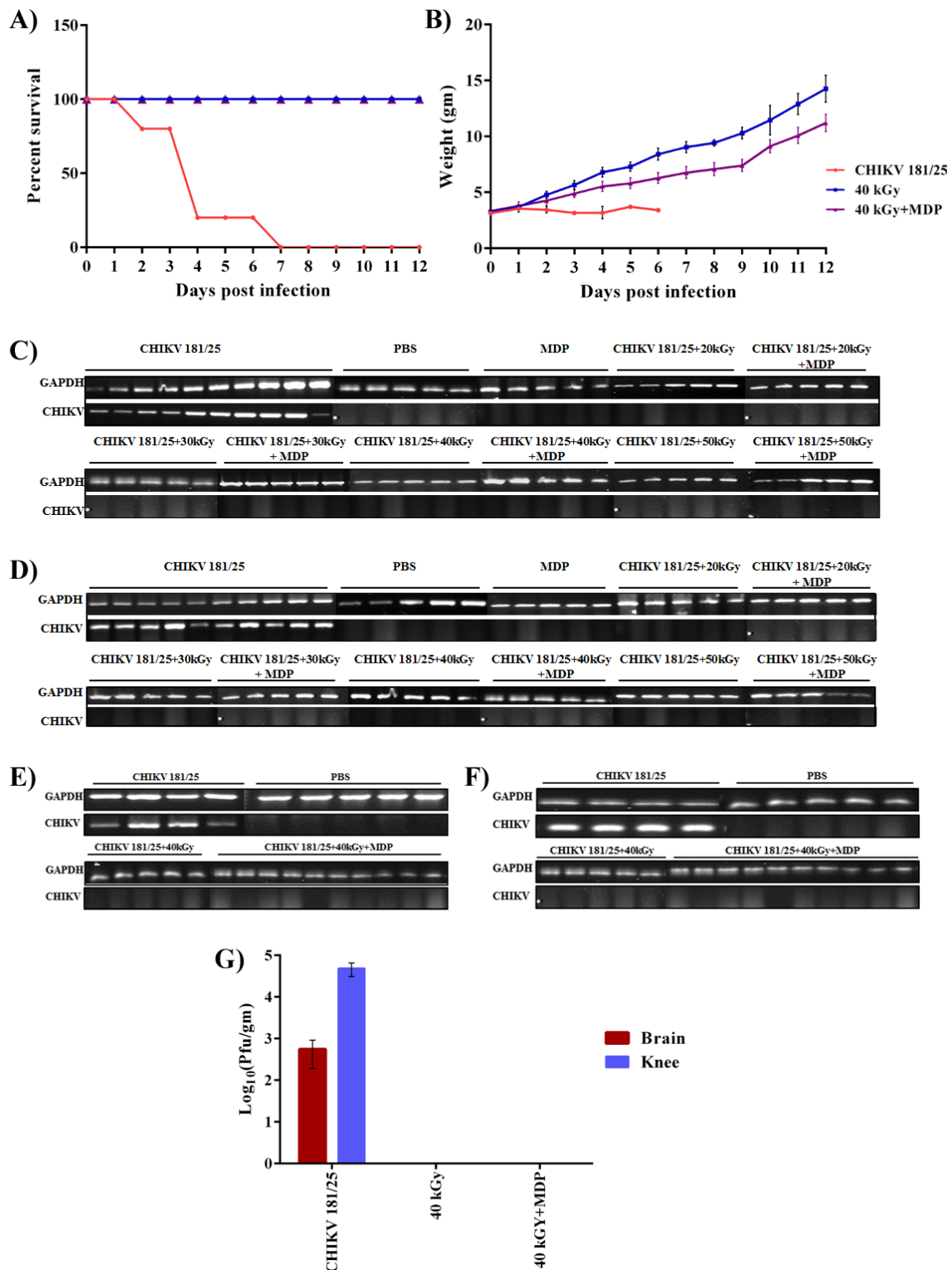


Fig 6. Gamma-irradiated CHIK 181/25 does not cause disease in the neonatal mouse model. (A) Percent survival, (B) total body weight of the neonatal mice injected with

irradiated CHIK 181/25 at the end of the 2nd passage. CHIKV-specific RT-PCR amplification in the (C) brain and (D) knee tissues collected at the end of 1st passage; and (E) brain and (F) knee tissues collected at the end of 2nd passage from the neonatal mice injected with irradiated CHIK 181/25. (G) Virus titration from the brain and knee tissues of the neonatal mice injected with irradiated CHIK 181/25 at the end of the 2nd passage.

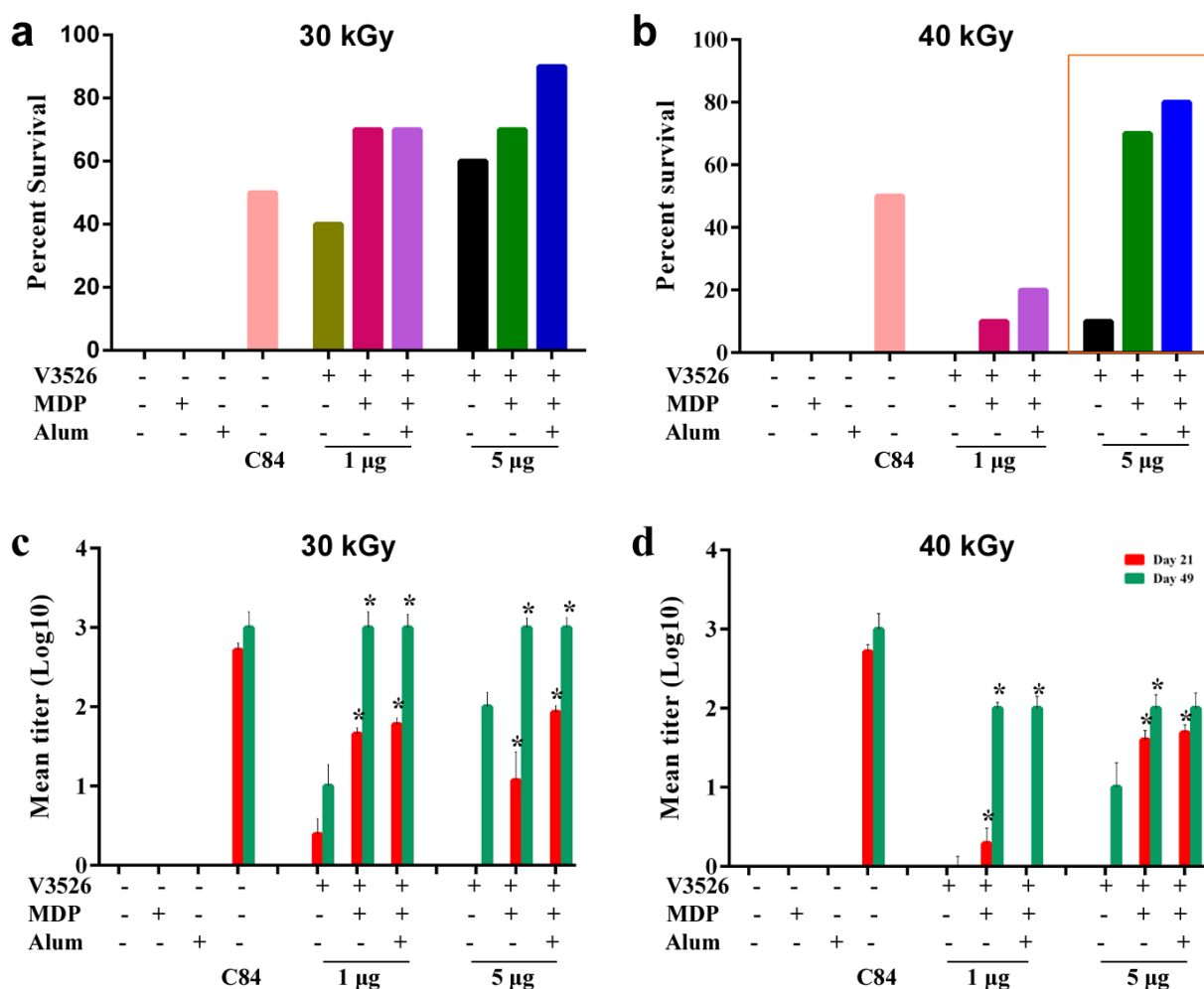


Fig 7. V3526 gamma-inactivated in the presence of MDP confers significant protection against aerosol challenge. Percent survival for animals immunized with V3526 preparations irradiated to (A) 30 kGy and (B) 40 kGy and challenged with aerosol exposure to VEEV-TrD show significantly higher protective efficacy of virus inactivated in presence of MDP. Total antibody response against VEEV in mice immunized with V3526 inactivated at (C) 30 kGy and (D) 40 kGy in the presence and absence of MDP

complex also show significantly higher antibody response against virus preparations inactivated in presence of MDP. (Mean antibody titers $\pm$ standard deviation; * p-value $\leq$ 0.05 in comparison to V3526 irradiated without MDP).

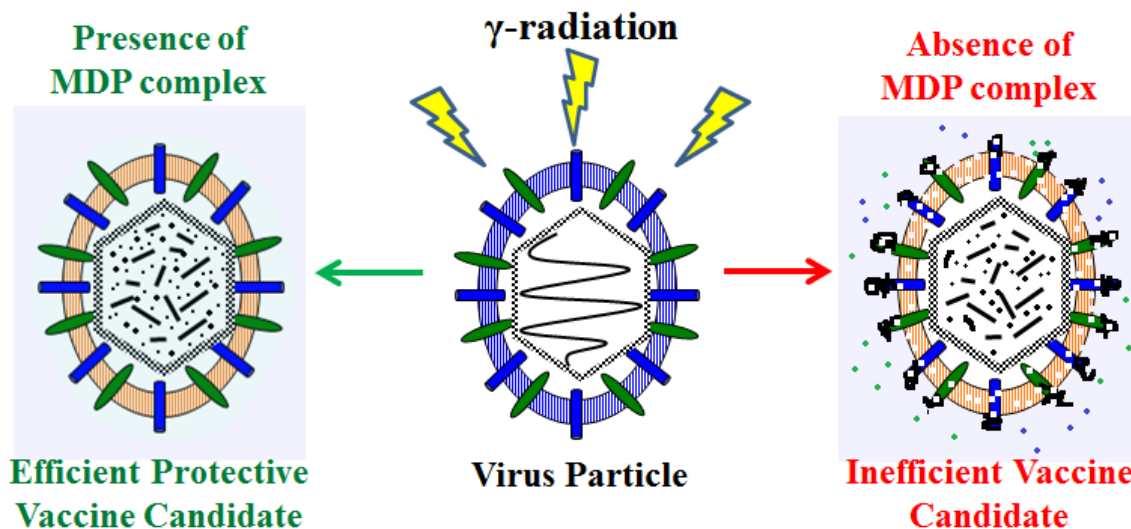


Fig. 8 Schematic representation of gamma-inactivation approach in the presence of MDP complex. Gamma-irradiation of virus in presence of ROS scavenging MDP complex protects the viral epitopes but the viral genome is degraded due to IR damage unlike degradation of both surface epitopes and viral genome in absence of MDP complex.

Materials and Methods

Virus preparation

V3526 was purified from supernatants of baby hamster kidney cells (BHK) infected with V3526 working stock virus (BHK p2) at a multiplicity of infection equal to 10. At 48 h post-infection, supernatants were harvested, clarified by low-speed centrifugation ($10,000 \times g$, Sorvall RC-5C, GSA rotor) and concentrated by polyethylene glycol (PEG) precipitation (7% PEG, 2.3% NaCl, w/v) at 4°C overnight. Virus was pelleted at $10,000 \times g$ for 30 min (Sorvall GSA rotor) and suspended in 10 ml $1 \times$ TNE (10 mM Tris, 0.2 M NaCl, 1 mM EDTA, pH 7.4). Continuous sucrose gradients were

generated using the Gradient Master (Biocomp Instruments, Fredericton, NB, Canada) as per the manufacturer's protocol. Virus was purified on 20-60% continuous sucrose gradients in $1 \times \text{TNE}$ at $100,000 \times g$ (Beckman SW 32 Ti) at 4°C for 4 h. To change the buffer, the purified virus was diluted in $1 \times \text{D-PBS}$ (Invitrogen Corporation, Grand Island, NY), pelleted ($100,000 \times g$ at 4°C for 4h) and suspended in D-PBS.

CHIK 181/25 was purified from supernatants of Vero cells infected with CHIK 181/25 working stock virus at a multiplicity of infection equal to 10. At the onset of infection, cell supernatant was harvested at $3,600 \times g$, for 30 min at 4°C . The supernatant was clarified through a $0.45 \mu\text{m}$ vacuum filter (Corning Incorporated, Corning, NY, USA) prior to ultracentrifugation at $141,000 \times g$, 4°C for 5 h. The pellet was dissolved in Tris-NaCl buffer pH 7.8 and then subjected to 15%-50% sucrose gradient purification at $139,000 \times g$, 4°C for 5 h. The final concentrated virus band was diluted in $1 \times \text{PBS}$ (Calbiochem, Billerica, MA) and stored at -80°C . Viral titers were determined by standard plaque assay on Vero cells, and protein concentrations were determined by BCA Assay (Pierce, Thermo Scientific, Grand Island, NY).

MDP complex and virus preparation for gamma-irradiation

The synthetic decapeptide (DP) H-Asp-Glu-His-Gly-Thr-Ala-Val-Met-Leu-Lys-OH was custom-synthesized with a peptide-purity of 95%. The net peptide content for each batch was determined by amino acid analysis performed at American Peptide Co. Inc. (American Peptide Co. Inc., Sunnyvale, CA). Stock solutions of 30 mM DP (in H_2O) were stored at -80°C . The radioprotective ability of each new stock solution of 30 mM DP prepared for virus protection was determined by post-irradiation activity assay for *Bam*HI [18]. Prior to irradiation, V3526 and CHIK 181/25 virus stocks were mixed with Pi buffer (250 mM) (pH 7.4) (KH_2PO_4 and K_2HPO_4 , Sigma), 30 mM DP, and 100 mM MnCl_2 (Sigma) to achieve a final concentration of 25 mM Pi buffer (pH7.4), 3 mM DP, 1 mM MnCl_2 . Stock solutions were prepared in H_2O from Barnstead Nanopure ultrapure water purification system (Thermo Scientific, Rockford, IL). The final concentration of V3526 and CHIK 181/25 in MDP were (1×10^{10} pfu/ml and 0.3 mg/ml) and (1.375×10^9 pfu/ml and 0.22 mg/ml), respectively. Virus-MDP suspensions were prepared immediately prior to irradiation and held on ice prior to and during irradiation. All

irradiations were performed in air on wet ice with ^{60}Co in an irradiation unit model 109-68, J. L. Shepherd and Associates, San Fernando, CA. V3526 was irradiated to 0, 2, 5, 10, 20, 30, 40 and 50 kGy at 3 kGy/hour whereas CHIK 181/25 irradiation doses were 0, 5, 10, 20, 30, 40 and 50 kGy at 16 kGy/hour. The assays reported here included V3526 and CHIK 181/25 virus stocks stored at -80°C as well as virus preparations held on wet ice during irradiation.

Cytopathic effect

Irradiated viruses were tested for residual infectivity by serial passage on Vero cells. Briefly, 50% confluent Vero cell monolayer was infected with 20-30 μl of inactivated virus and was observed daily for virus induced cytopathic effect (CPE). Cells were fixed and stained simultaneously with 2% neutral buffered formalin containing 0.1% crystal violet at 72 h post-infection. The collected cell supernatant (200 μl) was subsequently passaged onto a newly prepared Vero cell monolayer and CPE was observed similarly.

Virus titration

Virus titration was performed using the standard TCID_{50} assay that determines the dilution at which 50% of cells in a culture are infected. Cell supernatant (10 μl) was subjected to ten-fold serial dilution in MEM/NCS media and 100 μl from each dilution was used for infection of 75% confluent Vero cell monolayer in 96 well plate. The 50% endpoint dilution was evaluated at 72 h post-incubation as described previously [60].

Electrophoresis and western blot

Virus from control or test samples was resolved on 4-12% Bis-Tris gels (Invitrogen, Grand Island, NY) and transferred to nitrocellulose membranes (Amersham Biosciences, Pittsburgh, PA). Membranes were blocked with 6% non-fat dry milk (Santa Cruz Biotechnology, Dallas, TX) in $1 \times \text{TBST}$ (20 mM Tris-HCL, 150 mM NaCl, 0.1% Tween 20) prior to primary antibody incubation, VEEV envelope glycoprotein E2^{c} specific 1A4A-1 monoclonal antibody (1:15000) [55] or CHIKV envelope glycoprotein E2 specific CHK-42 monoclonal antibody (1:25000) [56]. Goat anti-mouse IgG (H+L)-HRP conjugate (1:5000) (Bio-Rad, Hercules, CA) was used as secondary antibody.

Membranes were developed using freshly prepared ECL Western blot developing agent (Western Lightning Plus, PerkinElmer, Waltham, MA). For silver staining, virus from control or test samples was resolved on 4-12% Bis-Tris gels (Invitrogen, Grand Island, NY). Silver staining was performed according to the manufacturer's instructions on the Silver stain kit (Bio-Rad, Hercules, CA).

Enzyme-linked immunosorbent assay (ELISA)

Briefly, Costar EIA/RIA 96-well high-binding plates (Corning Inc., Corning, NY) were coated with 0.3 µg of sucrose purified VEEV Trinidad Donkey strain (VEEV-TrD) per well and incubated overnight, or up to 1 week, at 4°C. The following day, plates were blocked with Dulbecco's phosphate buffered saline (DPBS) (GIBCO™ Invitrogen Corp., Grand Island, NY) containing 0.05% Tween 20 (Sigma-Aldrich, St. Louis, MO) and 5% nonfat dry milk (Becton Dickinson and Co., Sparks, MD) (PBSTM) for 2 hours at 37°C. The plates were washed 3 times with PBST using the BioTek ELx405™ microplate washer (BioTek Instruments, Inc., Winooski, VT). Mouse sera were diluted in PBSTM containing 1% heat inactivated fetal bovine serum (GIBCO™ Invitrogen Corp., Grand Island, NY), added to the plate and serially diluted 1:2 and then incubated for 1-2 hours at 37°C. Plates were washed 3 times with PBST followed by the addition of peroxidase-labeled goat anti-mouse IgG 1:40,000 (KPL, Gaithersburg, MD). The plates were incubated with the secondary antibody for 1 hr at 37°C and then washed 3 times with PBST. The ABST Peroxidase substrate (KPL, Gaithersburg, MD) was added to each well and color developed for approximately 10-15 min at which time the optical density (OD) at 410 nm was determined using a Spectramax M5 microplate reader (Molecular Devices, Sunnyvale, CA). The negative control was normal mouse serum (Sigma Aldrich, St. Louis, MO) and was diluted 1:100. The positive control was anti-VEEV mouse monoclonal antibody 1A4A-1 (1mg/mL). 1A4A-1 was diluted to a starting concentration of 1:750 then serially diluted 1:2. Endpoint titers were determined using a cutoff of 0.2.

Plaque reduction neutralization assay

One hundred microliter aliquots of virus containing 100 PFU were combined with an equal volume of Hank's Buffered Saline Solution containing the serially diluted antibody indicated and held overnight at 4°C. The virus antibody mixtures were then added to confluent monolayers of Vero cells and incubated at 37°C, 5% CO₂ for one hour with gentle rocking every 15 minutes. The monolayers were then overlaid with 2 ml of 0.6% agarose in DMEM containing 10% heat inactivated fetal bovine serum. After 24 hours, a second overlay of 0.6% agarose in DMEM containing 5% fetal bovine serum and neutral red was added and the plates were incubated at 37°C, 5% CO₂. Plaques were enumerated the following day. Virus infectivity was considered neutralized if the number of plaques was decreased by 80% compared to controls that contained fetal bovine serum.

RNA extraction and PCR amplification

Viral RNA isolation was done using PureLink Viral RNA/DNA Minikit (Invitrogen, Grand Island, NY) and cellular and tissue RNA was isolated using Trizol reagent (Invitrogen, Grand Island, NY) as per the manufacturer's protocol. The RNA pellet was dissolved in Nuclease free water (Bioworld, Bioplus Fine Chemicals, Dublin, OH) and stored at -80°C. cDNA synthesis was done using SuperScript III First Strand Synthesis Supermix for RT-qPCR (Invitrogen, Grand Island, NY). 2X Go Taq Green Master Mix (Promega, Madison, WI) was used for PCR amplifications. Primers used were: (Gapdh: 5'-GGGTGAGGCCGGTGCTGAGT-3' and 5'-CACCCTTCAAGTGGGCCCCG-3'); (V3526 nsp4: 5'-GCTAACAGAAGCAGATACCAG 3' and 5'-GCAGCCGAATCCAATACGGGC 3'); (V3526 nsp3: 5'-ATGCCATGTGGCCCGTTGCA 3' and 5'-CACCGGTCGCGCCAGAACT3'); (CHIK 181/25 nsp4: 5'-GCCAGACACGGAGACGCCAAC3 and 5'-TGACCCTACTGAGAACAGCAC3') and (CHIK 181/25 nsp1: 5'-ATCCCGACTCAACCATCCTG3' and 5'-GGGTGCGTGTACAGCATAGA3').

RNA transfection and immuno-staining

Genome from the irradiated virus was transfected into 80% confluent BHK-21 cell monolayer in 8-well glass chamber slides (Lab-Tek, Thermo Scientific, Grand Island, NY) using Lipofectamine 2000 (Invitrogen, Grand Island, NY) according to the manufacturer's protocol. Cell fixation was performed at 72 h post-transfection with 1:1 v/v chilled acetone:methanol at room temperature for 1 min and blocked with 1% BSA (Sigma-Aldrich, St. Louis, MO). Primary antibody incubation used either polyclonal rabbit anti-VEEV antibody (kindly provided by Dr. Franziska B. Grieder, USUHS, Bethesda, MD) (1:200) or CHIKV immune ascitic fluid (1:100) (ATCC, Manassas, VA) in blocking buffer. The secondary antibodies used for V3526 and CHIK 181/25 were FITC-labeled affinity purified goat antibody to rabbit IgG (H+L) (1:300) (Kirkegaard and Perry Laboratories, Gaithersburg, MD) and goat anti-mouse TRITC-conjugated IgG (1:80) (Millipore, Billerica, MA) respectively. The cells were mounted with Vectashield mounting medium with DAPI (Vector Laboratories Inc, Burlingame, CA).

Animals

Swiss CD-1 mice (3–6 days old) along with dams were purchased from Charles River Laboratories, Wilmington, MA for the safety efficacy study. Female BALB/c mice (6-8 weeks) were purchased from National Cancer Institute, Frederick, MD for the protective efficacy study. Mice were housed in microisolator cages and were provided food and water *ad libitum* with a 12 h light/dark cycle.

Ethics statement

Animals were housed in a facility fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)–International and treated in accordance with the guidelines from the *Guide for the Care and Use of Laboratory Animals* of the Institute for the Laboratory Animal Research, National Research Council. Safety efficacy evaluation was conducted in a BSL-2 facility at Uniformed Services University of The Health Sciences (USUHS), Bethesda, MD and the protective efficacy evaluation was conducted at the ABSL-3 containment facility at United States Army Medical Research Institute of Infectious Diseases (USAMRIID), Frederick, MD.

Research was conducted under an IACUC approved protocol in compliance with the Animal Welfare Act, PHS Policy, and other Federal statutes and regulations relating to animals and experiments involving animals. The facility where this research was conducted is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 2011.

Safety efficacy

Groups of neonatal CD-1 mice (n=5 to 10) were intracranially (i.c.) injected with 5×10^4 pfu of viruses. Controls used for the study were non-irradiated virus, saline, and MDP complex. Mice were monitored daily for their weight and morbidity for a period of 12 days post-injection. The brain homogenate from the first passage (only V3526 irradiated to 40 kGy along with the V3526 control was selected for further testing) was intracranially passaged in a second set of suckling mice for similar observations. Animals were CO₂ euthanized followed by cervical dislocation at the end of study prior to collection of tissues (V3526: brain; CHIK 181/25: brain and knee).

Protective efficacy

Groups of female BALB/c mice (n=10) were intramuscularly (i.m.) immunized with 1 or 5 µg of the inactivated V3526 virus preparations on D0 and D28. Alhydrogel (alum) was also used as an adjuvant. Animals in control groups were administered with either saline, MDP complex or alum. VEEV C-84 IND was administered subcutaneously (s.c.) at 4 µg per mouse on D0, D7 and D28 as described previously [54]. Blood samples were collected on D(-2), D21 and D49. All the animals were challenged with 5×10^4 PFU of VEEV Trinidad strain (VEEV-TrD) by aerosol route on D56. Estimated presented concentration of viruses were calculated using Guyton's formula and virus quantitation values were determined by back-titration of samples collected during the aerosol exposures. Daily monitoring and clinically scoring was performed following challenge. Mice with a clinical score of 5 and higher, displaying neurological signs like hind limb paralysis/circling or unresponsive when stimulated were considered unprotected and were

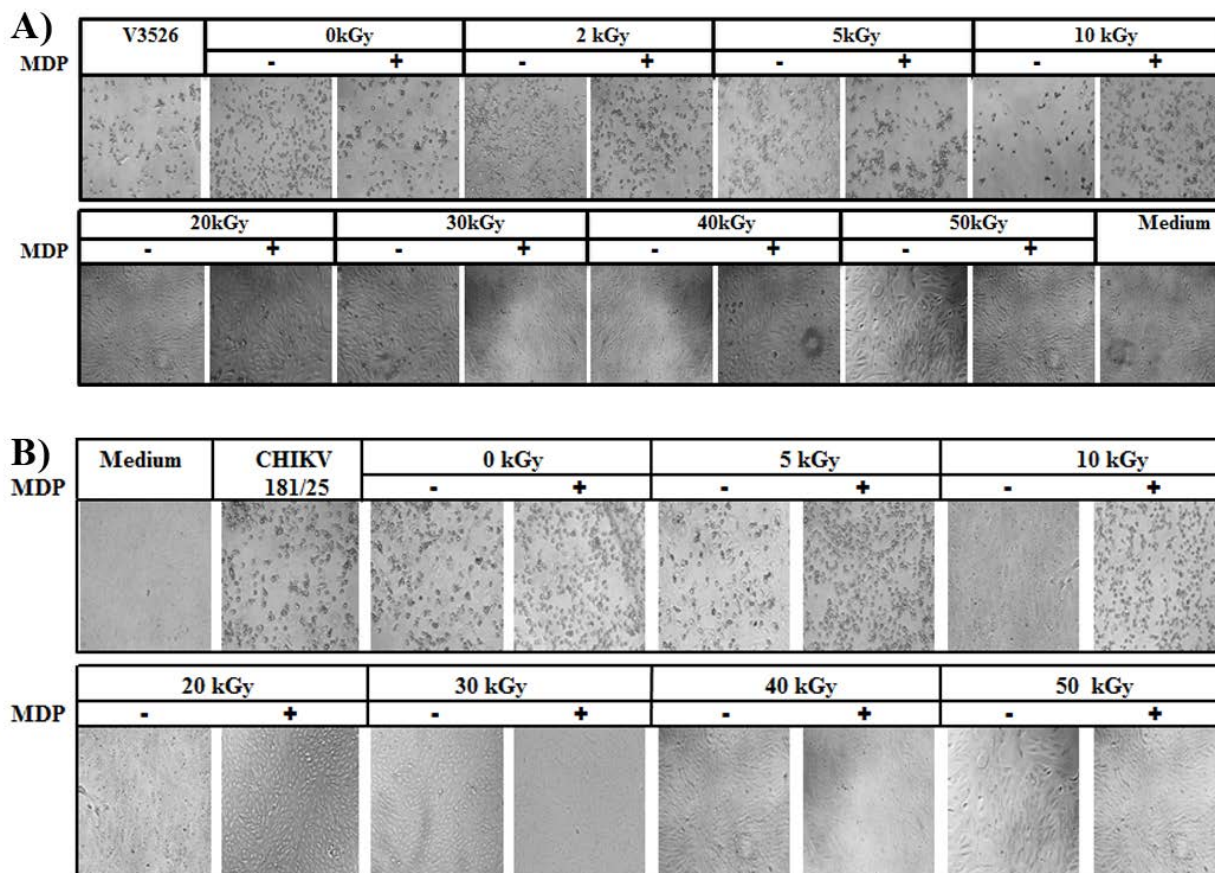
euthanized. Mice that survived for 3 weeks after challenge were considered protected and were euthanized at the end of the study.

Statistical analysis

Student's t- test was used for comparison between all the groups. For all data, statistical significance was accepted at $p \leq 0.05$.

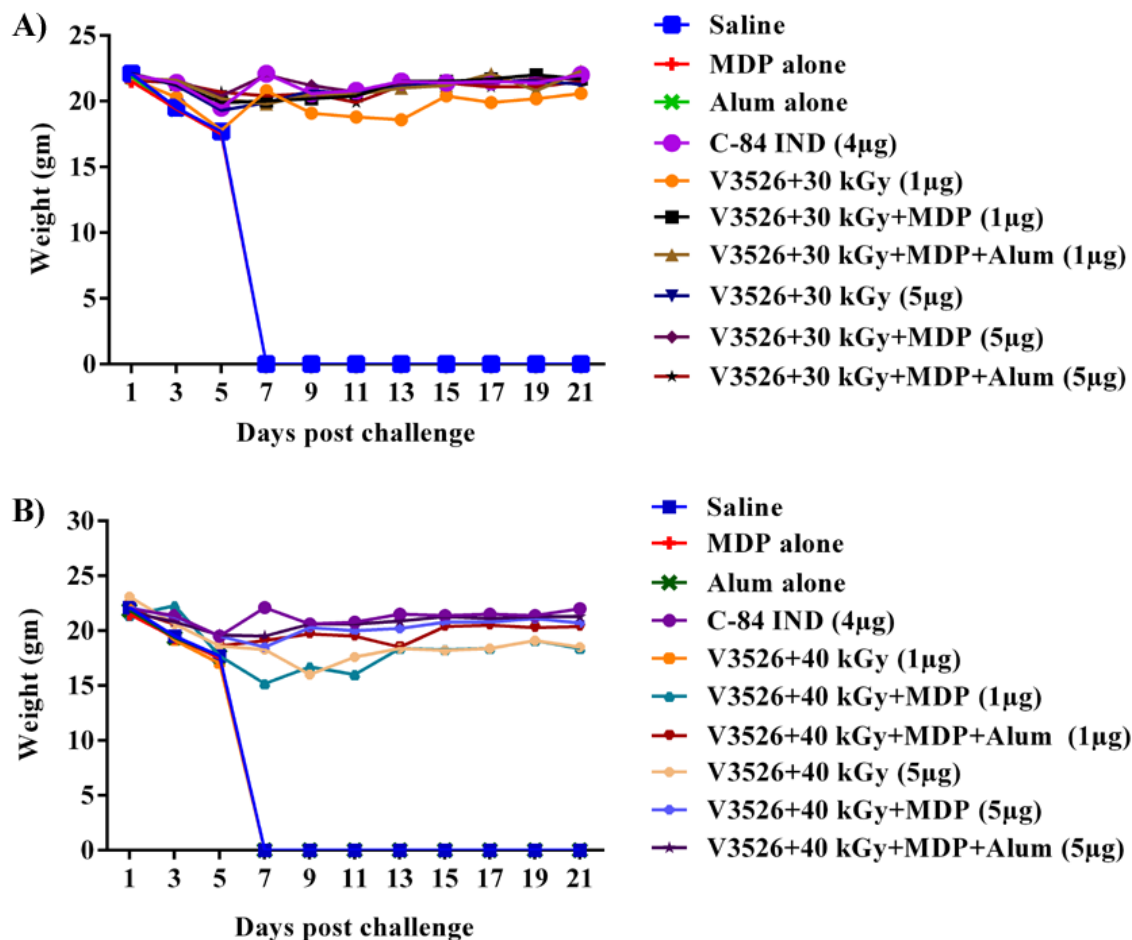
Supporting Information

S1_Fig



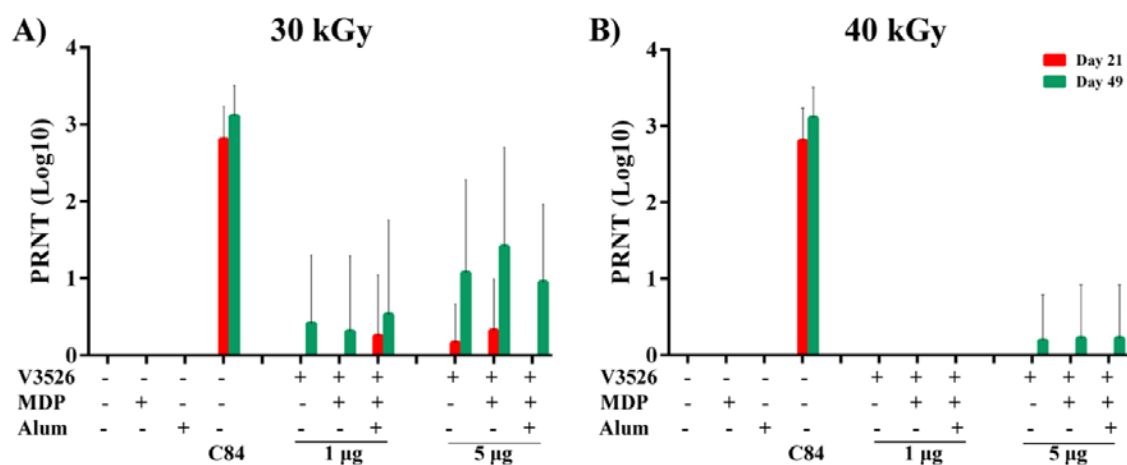
S1 Fig. Virus infectivity is abolished *in vitro* upon gamma-irradiation. Vero cells infected with control and irradiated (A) V3526 and (B) CHIK 181/25 preparations at the end of 10th passage. Results are representative of at least four technical replicates.

S2_Fig



S2 Fig. Body weight loss in immunized mice after aerosol exposure. Average body weight of mice immunized with V3526 inactivated by (A) 30 kGy and (B) 40 kGy in the presence and absence of MDP after exposure to VEEV-TrD is shown for each test and control group.

S3_Fig



S3 Fig. Neutralizing antibody response in immunized mice. Neutralizing antibody titers in the serum of mice immunized with V3526 inactivated by 30 kGy and 40 kGy, in the presence or absence of MDP. Data is shown for each test and control group.

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Author Contributions

Conceived and designed the experiments: RKM PG EG MJD PJG. Performed the experiments: MG PG EG EM PJG. Analyzed the data: MG PG. Contributed reagents/materials/analysis tools: RKM MJD PJG. Wrote the paper: MG PG MJD RKM.

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