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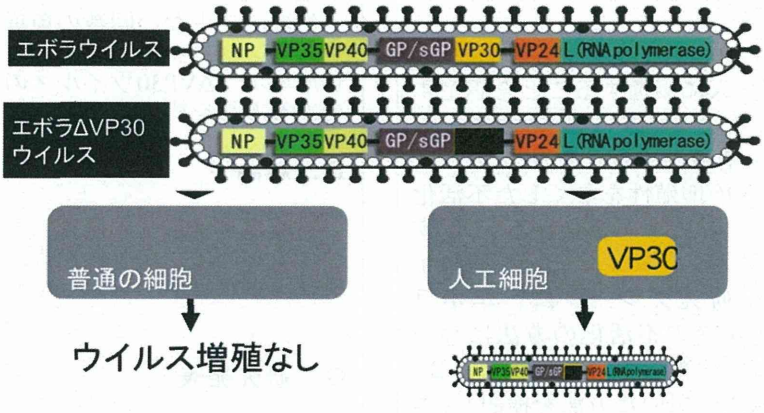

図１０：増殖性を抑えたエボラΔVP30ウイルス

エボラΔVP30ウイルスとは、人工的に作製した、エボラウイルスの増殖に必須なVP30遺伝子を欠損した変異エボラウイルスである。このエボラΔVP30ウイルスは、通常の細胞では増えないが、VP30蛋白質を発現する人工細胞で効率良く増殖する。

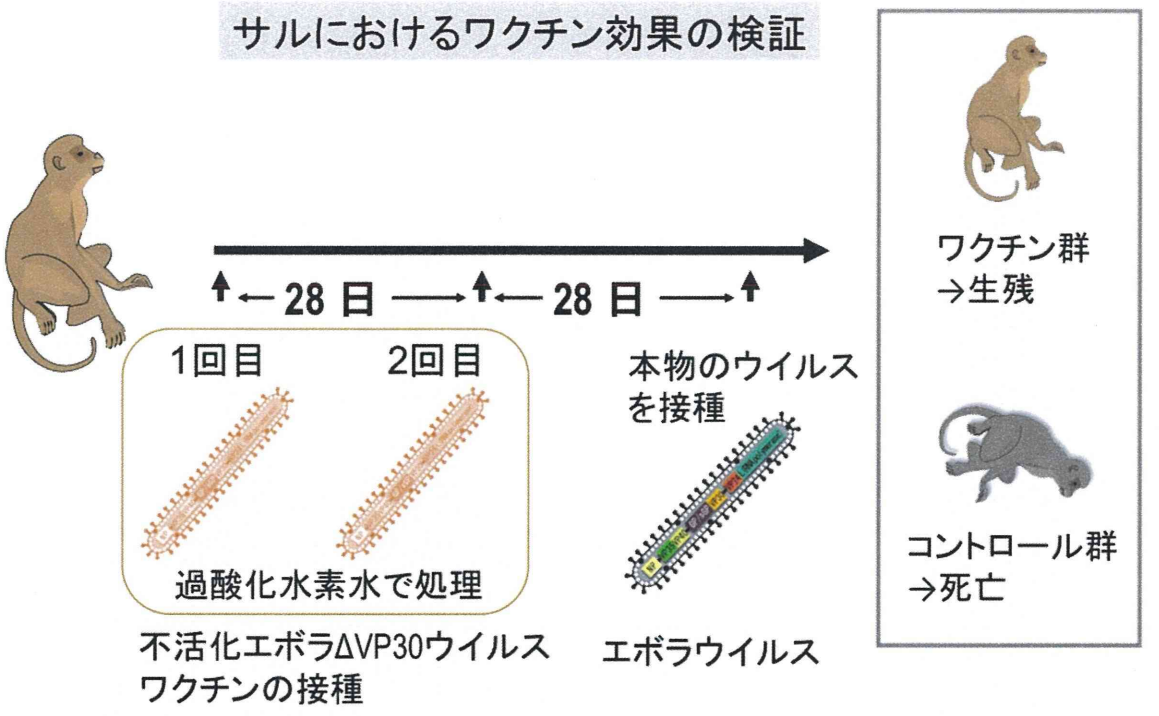

図１１：不活化したエボラΔVP30ウイルスの、サルにおけるワクチン効果の検証

過酸化水素水で不活化したエボラΔVP30ウイルスワクチンを、サルに2回接種した。その後、致死量の野生型エボラウイルスを感染させたところ、ワクチンを接種しなかったグループ（コントロール群）のサルは全て死亡したが、ワクチンを接種したグループのサルは全て生残した。

学会等発表実績

なし。

研究成果の刊行に関する一覧表

雑誌

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発表論文

REVISED VERSION #3

An Ebola Whole Virus Vaccine Is Protective In Nonhuman Primates

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Zaire ebolavirus (EBOV) is the causative agent of the current outbreak of hemorrhagic fever disease in West Africa. Previously, we showed that a whole EBOV vaccine based on a replication-defective EBOV (EBOVΔVP30) protects immunized mice and guinea pigs against lethal challenge with rodent-adapted EBOV. Here, we demonstrate that EBOVΔVP30 protects nonhuman primates against lethal infection with EBOV. Although EBOVΔVP30 is replication-incompetent, we additionally inactivated the vaccine with hydrogen peroxide; the chemically inactivated vaccine remained antigenic and protective in nonhuman primates. EBOVΔVP30 thus represents a safe, efficacious whole EBOV vaccine candidate that differs from other EBOV vaccine platforms in that it presents all viral proteins and the viral RNA to the host immune system, which might contribute to protective immune responses.

The EBOV outbreak in West Africa has already claimed more than 5,000 lives (*1*) and remains uncontrolled. One countermeasure to mitigate ebolavirus infections is vaccination. Several ebolavirus vaccine platforms have been developed over the last decades (*2*), three of which recently advanced to clinical trials: a DNA-based vaccine expressing different ebolavirus glycoproteins (GP, the major ebolavirus immunogen) (*3, 4*), a replication-incompetent chimpanzee adenovirus expressing GP (*5*), and a live-attenuated vesicular stomatitis virus (VSV) expressing GP (*5*). The DNA platform completely protects nonhuman primates (the 'gold standard' for ebolavirus research) only after multiple dosages of the DNA vaccine in combination with recombinant adenovirus (*6*), but has not been tested as a stand-alone vaccination strategy. The recombinant adenovirus platform (including the recently developed recombinant chimpanzee adenovirus) requires high vaccine doses and boosting to achieve complete and durable protection of nonhuman primates against lethal challenge with EBOV (*7, 8*). Complete protection of nonhuman primates against lethal EBOV challenge has also been accomplished with the VSV platform; however, the use of a replicating recombinant VSV (*9-12*) may be of concern due to issues related to vaccine safety. Hence, although several platforms are being tested in clinical trials, additional options should be explored.

Whole virus vaccines (either live attenuated or inactivated) have a long history as successful human vaccines, offering protection against potentially deadly viral diseases such as smallpox, influenza, mumps, and measles (*13*). Whole virus vaccines present multiple viral proteins and the viral genetic material to the host immune system, which may trigger a broader and more robust immune response than vectored vaccines that present only single viral proteins. However, initial attempts to develop a gamma-irradiated, inactivated whole EBOV vaccine failed to provide robust protection of nonhuman primates against challenge with a lethal dose of EBOV (*14*).

Previously, we developed a replication-defective EBOV (termed EBOVΔVP30) which is based on the Mayinga strain of EBOV and lacks the coding region for the essential viral transcription activator, VP30 (*15*). EBOVΔVP30 replicates to high titers in cell lines that stably express the VP30 protein, is genetically stable, and nonpathogenic in rodents (*15, 16*). Mice and guinea pigs immunized twice with EBOVΔVP30 were fully protected against a lethal challenge with mouse- or guinea pig-adapted EBOV, respectively (*16*). EBOVΔVP30 is a biosafety level-3 agent and exempt from 'Select Agent' status; an EBOVΔVP30 vaccine could therefore be manufactured in existing biosafety level-3 facilitates that operate under Good Manufacturing Practices.

To assess the effectiveness of EBOVΔVP30 a whole virus vaccine in nonhuman primates, we inoculated groups of cynomolgus macaques (Table 1) intramuscularly (i.m.) with DMEM (control, group 1), a single dose of 10^7 focus forming units (FFU) of EBOVΔVP30 (group 2), or two doses of 10^7 FFU of EBOVΔVP30 four weeks apart (group 3). Previously, we demonstrated the genomic stability of EBOVΔVP30 by carrying out three independent experiments that each comprised seven consecutive passages of the virus in VeroVP30 cells. After the last passages, we sequenced the region surrounding the VP30 deletion site and did not detect any recombination events or mutations. Moreover, the passaged viruses did not grow in wild-type cells, further indicating the lack of recombination. Despite these findings, concerns have been raised that such an event could potentially affect vaccine safety. Recently, virus inactivation with hydrogen peroxide was shown to preserve the antigenicity of lymphocytic choriomeningitis (*17, 18*), vaccinia (*17*), West Nile (*17, 19*), and influenza (*20*) viruses. To increase the biosafety profile of EBOVΔVP30, we therefore treated it with hydrogen peroxide (H_2O_2, 3% final concentration) for 4 h on ice, followed by viral plaque assays in VP30-expressing cells, which confirmed complete virus inactivation. Nonhuman primates were then vaccinated twice with 10^7 FFU of the H_2O_2-treated EBOVΔVP30 (group 4; two animals). Gamma-irradiation is an established procedure for

ebolavirus inactivation, but irradiation conditions optimized for virus inactivation (rather than for antigenic epitope preservation) may alter antigenicity and therefore protective efficacy of ebolavirus vaccines (*14*). To test these concepts, we also vaccinated macaques twice with 10^7 FFU of wild-type EBOV gamma-irradiated in BSL-4 containment (group 5); again, the irradiation conditions used here ensured virus inactivation, but were not optimized to preserve antigenicity. None of the vaccinated animals showed signs of illness, confirming our earlier data from mice and guinea pigs that EBOVΔVP30 is nonpathogenic in animals (*16*).

Four weeks after the last immunization, we challenged animals in BSL-4 containment i.m. with a lethal dose (1,000 FFU) of the heterologous Kikwit strain of EBOV. While control macaques in group 1 had to be euthanized on day 7 or 8 post-challenge according to established and approved humane endpoint criteria (*21*) (Table 1), all animals immunized once (group 2) or twice (group 3) with the EBOVΔVP30 vaccine survived the lethal challenge (Table 1). In addition, both animals immunized twice with H_2O_2-treated EBOVΔVP30 vaccine (group 4) survived infection with wild-type EBOV, indicating that H_2O_2-treated EBOVΔVP30 is immunogenic and elicits protective immune responses. In contrast, all macaques immunized with gamma-irradiated wild-type EBOV (group 5) developed signs of severe EBOV disease and had to be euthanized between days 6 and 9 post-challenge (Table 1), supporting the concept that gamma-irradiation optimized for virus inactivation alters the immunogenicity of EBOV vaccines. The macaques which had to be euthanized following challenge with EBOV (groups 1 and 5) had high virus titers in their blood post-challenge (Figure 1). In contrast, no viremia was detected in animals immunized twice with untreated (group 3) or H_2O_2-treated EBOVΔVP30 (group 4) (Figure 1), showing that H_2O_2-treated EBOVΔVP30 elicited a protective immune response. One of four animals that received a single immunization with EBOVΔVP30 (NHP 8 in group 2) was viremic on days 3 and 6 post-challenge, but cleared the virus on day 9 (Figure 1). In addition, a different animal in group 2 (NHP 7) had a fever on day 6 post-challenge (Supplementary Table 1). These data indicate that a single

112 vaccination with EBOVΔVP30 does not always prevent EBOV replication or signs of illness (fever),
113 but does protect the host from death upon EBOV challenge. Together, our findings demonstrate
114 the vaccine potential of a whole EBOV vaccine based on EBOVΔVP30.

115 To better understand the correlates of protection, we measured the immune responses
116 two and four weeks after the last immunization (i.e., two weeks and immediately prior to EBOV
117 challenge). Two weeks after the last vaccination (day -14), macaques immunized twice with
118 EBOVΔVP30 (group 3) had a high IgG antibody response to the viral GP based on a GP-specific
119 ELISA assay (Figure 2). Two immunizations with H_2O_2-treated EBOVΔVP30 (group 4) resulted in
120 a slightly lower, but still robust immune response (Figure 2). In macaques immunized once with
121 EBOVΔVP30 (group 2), we detected a low, but measurable IgG antibody response (Figure 2).
122 Serum samples from animals that succumbed to EBOV challenge, namely, those mock-
123 immunized (group 1) or immunized twice with gamma-irradiated wild-type EBOV (group 5) did not
124 possess measurable IgG titers to GP (Figure 2). The IgG titers to EBOV GP on the day of
125 challenge (day 0, Figure 2) followed the same trend, but were low. The IgG titers to EBOV GP
126 closely mirrored survival rates and virus titers (see Table 1 and Figure 1); these data indicate that
127 immunization with EBOVΔVP30 elicits an antibody response to GP that is important for protection
128 against EBOV infection. A similar correlation between a GP-specific antibody response and
129 protection has been demonstrated with other experimental EBOV vaccine platforms (*22, 23*).

130 The antibody repertoire was further characterized by assessing the levels of neutralizing
131 antibodies to GP as measured by plaque reduction neutralization (PRNT) assays. The serum
132 dilution that reduced the titer of VSV expressing EBOV GP by $\geq$ 50% (plaque reduction
133 neutralization titer 50, $PRNT_{50}$) was 1:20 – 1:40 for samples obtained from animals immunized
134 twice with EBOVΔVP30 (group 3; Supplementary Table 2); no statistically significant decline in
135 neutralizing antibody levels was detected between day -14 (two weeks before challenge) and day

0 (Supplementary Table 2). In contrast, we detected slightly lower $PRNT_{50}$ titers of ~1:10 for sera obtained from animals immunized once with untreated or H_2O_2-treated EBOVΔVP30 (groups 2 or 4, respectively; Supplementary Table 2). No neutralizing antibodies were detected in control animals or animals immunized twice with gamma-irradiated EBOV (groups 1 or 5, respectively; Supplementary Table 2). Overall, the neutralizing antibody titers were low, but similar to those detected upon vaccination of animals with VSV expressing EBOV GP (*11*).

Most experimental ebolavirus vaccine platforms provide only the viral GP as antigen, expressed from recombinant viruses or protein expression plasmids; in contrast, the EBOVΔVP30 vaccine presents all viral proteins plus the viral genetic material to the host. Early studies with EBOV-like particles (VLPs) suggested that the viral matrix protein (VP40) and nucleoprotein (NP) are also immunogenic (*24*), prompting us to carry out ELISAs specific for these two viral proteins. Two weeks after the last vaccination (day -14), macaques immunized twice with untreated (group 3) or H_2O_2-treated (group 4) EBOVΔVP30 had high NP and VP40 antibody titers (Supplementary Figure 1). Lower, but still robust NP and VP40 antibody titers were observed in macaques immunized once with EBOVΔVP30 (group 1). Contrary to the GP antibody titers, we also detected NP and VP40 antibodies in animals immunized twice with gamma-irradiated EBOV (group 5), suggesting that gamma-irradiation under conditions optimized for virus inactivation has a greater effect on the antigenicity of GP epitopes than on that of NP and VP40 epitopes. Collectively, these data demonstrate that antibodies to NP and VP40 are elicited after vaccination with EBOVΔVP30, and that the levels of these antibodies are higher in protected animals than in those that succumbed to infection. However, the significance of NP and VP40 antibodies to protection from EBOV infection is not yet known.

In addition to the antibody response, we also measured the cellular immune response by examining the number of mononuclear cells producing interferon gamma (IFNγ). On day -14 (two

160 weeks before challenge), animals in groups 2 and 3, immunized one or twice with EBOVΔVP30,
161 respectively, had the highest number of IFNγ-producing cells (Supplementary Figure 2). Although
162 treatment of EBOVΔVP30 with H_2O_2 (group 4) reduced the number of IFNγ-producing cells, more
163 IFNγ-producing cells were detected in these animals compared with those immunized twice with
164 gamma-irradiated EBOV (group 5; Supplementary Figure 2), or left untreated (group 1;
165 Supplementary Figure 2).

166 Data from Geisbert *et al.* (*14*) and our findings in this study suggest that gamma-irradiation
167 optimized to inactivate EBOV destroys the antigenicity of wild-type EBOV, particularly in EBOV
168 GP. H_2O_2-treated EBOVΔVP30, however, elicited a robust IgG response, and protected
169 nonhuman primates against lethal EBOV challenge, although H_2O_2 treatment resulted in a slight
170 reduction of antigenicity compared with untreated virus (Figure 2). Hence, H_2O_2 treatment of
171 EBOVΔVP30 appears to preserve key antigenic epitopes, as has been demonstrated for other
172 viruses (*17-20*). To examine potential differences in antigenicity between gamma-irradiated and
173 H_2O_2-treated virus, we performed an ELISA-based assay utilizing a panel of 19 monoclonal
174 antibodies directed against GP. Most monoclonal antibodies showed comparable levels of binding
175 to GP; however, four (#12, 21, 226, and 662) reacted more efficiently with H_2O_2-treated than with
176 gamma-irradiated virus (Figure 3). Most likely, gamma-irradiation affected the conformation of the
177 epitopes recognized by these antibodies, resulting in the lack of protection upon immunization
178 with gamma-irradiated virus. Hence, the epitopes recognized by monoclonal antibodies #12, 21,
179 226, and 662 may play an important role in antibody-mediated protection in immunized macaques
180 and potentially in humans; in fact, monoclonal antibody #226 is known to have virus neutralizing
181 properties (*25*). One monoclonal antibody (#1031) interacted more efficiently with gamma-
182 irradiated than with H_2O_2-treated virus, while a polyclonal antiserum reacted similarly with both
183 virus preparations tested (Figure 3).

When EBOV was first discovered over 35 years ago, whole virus vaccines inactivated by formalin or gamma-irradiation were tested, but failed to elicit complete protection in nonhuman primates (*14*). The development of whole virus vaccines was therefore abandoned and VLPs composed of GP, VP40 (and NP) were explored as a safe and immunogenic platform to present several viral proteins to the host immune system (*2, 26-28*). These VLPs are immunogenic, but three vaccinations with adjuvanted VLPs were required to achieve protective efficacy in nonhuman primates (*24*). Here, we present a novel vaccine strategy that offers several advantages: (*i*) it provides protection from a lethal challenge of EBOV in nonhuman primates after a single immunization, although one animal became viremic and another animal developed a fever; (*ii*) it is highly immunogenic as shown by robust antibody responses elicited upon vaccination; (*iii*) it is amenable to large-scale production since EBOVΔVP30 grows to titers of $>10^7$ FFU/mL in VP30-expressing cells (*15*); (*iv*) it is safe due to its inability to replicate outside of VP30-expressing cells (*15*); and (*v*) it presents all viral proteins and its genomic RNA to the host, similar to whole virus vaccines and VLPs. It should be noted that NHPs immunized once with EBOVΔVP30 (group 2) were protected from a lethal EBOV challenge, although two of the four animals showed signs of illness (fever was detected in NHP 7, and viremia was detected in NHP 8; Supplementary Table 1). However, all four animals in group 2 (NHPs 5–8) showed similar immune responses (Supplementary Table 2 and summarized in Supplementary Table 3).

To address any potential concerns over recombination events that would restore the replicative ability of EBOVΔVP30, we also chemically inactivated it with H_2O_2. Hydrogen peroxide treatment causes breaks in single- and double-stranded DNA or RNA (*17*) and thus inactivates viruses without affecting their antigenicity. By contrast, gamma-irradiation (used to generate the first experimental whole EBOV vaccine) causes the (de)hydroxylation of amino acids, the cleavage of polypeptide backbones (*29*), and the generation of free radicals that could cause the destruction of the antigenic properties of some epitopes. These differences in mechanism may

explain why viruses treated with H_2O_2 are more immunogenic than those irradiated with gamma rays; however, optimization of irradiation conditions may improve the immunogenicity of vaccine candidates.

In summary, our data indicate that EBOVΔVP30 is an effective whole EBOV vaccine that warrants further assessment.

Supplementary Materials

Materials and Methods

Table S1–S3

Figures S1–S2

Acknowledgements

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Raw data can be found at the following website:
https://docs.google.com/spreadsheets/d/1dBgzt5_z4rpqOuxXcI_FbUz8wNqMvHy6kVP_tpW0MY/edit?usp=sharing

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Table 1. Overview of vaccination and challenge strategy

Group	Vaccine	Inactivation	Vaccination		Protection	Euthanasia
			Prime	Boost		
Group 1	Mock*	---	---	---	0%** (n=4)	Days 7-8***
Group 2	EBOVΔVP30	---	1 x 10^7 FFU	---	100% (n=4)	N/A
Group 3	EBOVΔVP30	---	1 x 10^7 FFU	1 x 10^7 FFU	100% (n=4)	N/A
Group 4	EBOVΔVP30	Hydrogen peroxide	1 x 10^7 FFU	1 x 10^7 FFU	100% (n=2)	N/A
Group 5	EBOV	Gamma-irradiation	1 x 10^7 FFU	1 x 10^7 FFU	0% (n=4)	Days 6-9

* DMEM; **Percentage of animals that survived challenge with a lethal dose of EBOV; ***Days post-challenge.

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251 **Figure Legends**

252 Figure 1. Virus titers in the blood of infected nonhuman primates. Animals were immunized as
253 shown in Table 1. Four weeks after the last immunization, animals were infected with a lethal
254 dose of EBOV. Shown are EBOV titers in the blood of individual nonhuman primates from each
255 group.

256

257 Figure 2. Immune responses in vaccinated nonhuman primates. IgG antibody responses to EBOV
258 GP two weeks after the last vaccination (day -14) and on the day of challenge (day 0). Antibody
259 titers were measured using an ELISA specific for EBOV GP. Titers shown are the highest
260 reciprocal dilution that resulted in an optical density ≥ 0.2.

261

262 Figure 3. Effects of H_2O_2-treatment and gamma-irradiation on the antigenicity of EBOV GP. Using
263 a panel of 19 monoclonal antibodies (1 µg/mL) directed against EBOV GP, we performed an
264 ELISA to examine the antigenicity of gamma-irradiated EBOV (blue) and H_2O_2-treated
265 EBOVΔVP30 (red).

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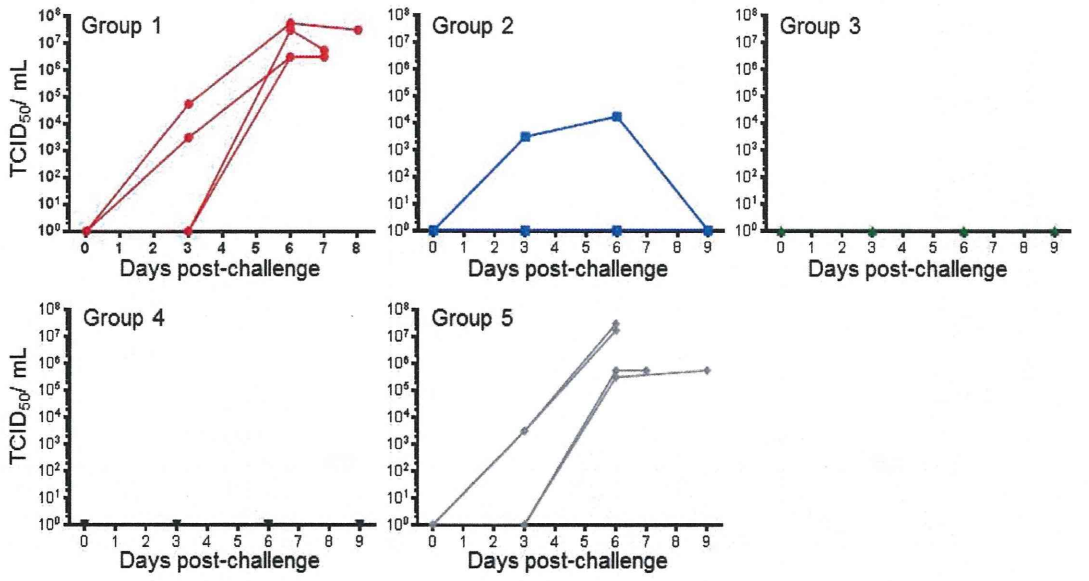
Figure 1


Group 1
Group 2
Group 3
Group 4
Group 5
TCID50/ mL
Days post-challenge