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Intraperitoneal injection with dengue virus type 1-infected K562 cells results in complete fatality among immunocompetent mice

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ABSTRACT

Dengue is one of the most important mosquito-borne viral diseases. Over half of the world's population is living in dengue endemic countries, where 100 million cases are estimated to occur annually. Although one dengue vaccine is currently available commercially, unfortunately its safety and efficacy has not been demonstrated for seronegative populations. Therefore, other vaccine candidates as well as antivirals are urgently required to control dengue diseases. To contribute to the development of preventative measures, in the present study we established an immunocompetent-mouse infection model using dengue virus type 1 Mochizuki strain. Following intraperitoneal injection with K562 cells infected with Mochizuki strain, all mice injected with $\geq 1 \times 10^6$ cells were killed within 7–11 days. Mice injected with $\geq 1 \times 10^7$ cells showed viremia ($\sim 10^4$–10^5 FFU/ml) within 24 h of injection. Since a higher infective titer was detected in the mouse brain, this suggested that viruses were transmitted from the blood circulation into the brain. In further experiments, mice immunized with two types of DNA vaccines were challenged with virus. In contrast to the non-immunized control mice, all vaccinated mice survived after challenge. This immunocompetent-mouse infection model using dengue virus type 1 Mochizuki strain may be a useful tool to evaluate vaccines and preventive medicines against dengue virus.

Dengue fever, one of the most important mosquito-borne viral diseases in humans, is caused by infection with dengue virus (DENV) (Guzman and Harris, 2015; World Health Organization, 2018). DENV belongs to the family *Flaviviridae*, genus *Flavivirus*, and comprises four distinct serotypes (DENV-1, DENV-2, DENV-3 and DENV-4) (Pierson and Diamond, 2013). Approximately 3.9 billion people are at risk of infection (Brady et al., 2012), and the estimated annual number of infections with DENV is 390 million, of which 100 million people have shown clinical symptoms (Bhatt et al., 2013). Epidemiological studies have suggested that secondary heterotypic infection correlates with severe forms of the disease (Burke et al., 1988; Guzman et al., 1990).

Attempts at developing vaccines and antiviral agents against DENV have been ongoing for over 70 years since the first DENV (DENV-1, Mochizuki strain) was isolated in 1943 (Zulkarnain et al., 1994). The first dengue vaccine, Sanofi's Dengvaxia, has now been approved for use in 20 endemic countries (World Health Organization, 2018). Evaluations of clinical trials indicated that vaccine efficacy was partially limited, and that vaccine-induced severity was demonstrated in some dengue-seronegative populations (Sridhar et al., 2018). Other advanced vaccine candidates, TAK-003 and TV003/TV005, have reached phase 3 clinical studies (Swanstrom et al., 2018; Magnani et al., 2017). However, the continued development of alternative vaccine strategies is important. To enable further progress, the development of a suitable animal model is vital.

Non-human primates are the most reliable animal model for DENV infection and can be used for the evaluation of vaccines and therapeutic agents (Halstead et al., 1973; Scherer et al., 1978). Drawbacks include low incidence of clinical manifestations observed in humans and the cost of maintaining a large number of experimental animals. Rodents have been most widely used for *in vivo* experiments. However, immunocompetent mice are essentially resistant to DENV infection. Only injection with a very high titer of DENV can induce a detectable viral load in the blood and some tissues (Chen et al., 2004). Recently, immunocompromised mice have been used as DENV infection models

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(Chan et al., 2015). AG129 mice, lacking interferon alpha/beta and gamma receptors, showed susceptibility to DENV and exhibited some clinical manifestations similar to human patients (Shresta et al., 2006). While these immune-modified mouse models are useful for the study of DENV pathogenesis and vaccine development, an immunocompetent animal model would undoubtedly be preferable.

It is generally accepted that higher levels of viremia relate to more severe disease (Vaughn et al., 2000; Libraty et al., 2002). Conversely, reduction of viremia is associated with decreased disease severity and transmission efficiency, suggesting that the level of viremia is a surrogate marker to estimate pathogenesis or protection in DENV infection. Therefore, an animal model exhibiting a detectable level of viremia after challenge is critical to evaluating the efficacy of vaccines and antiviral agents, as well as assessing pathogenesis. In our previous study, a DENV-2 mouse infection model showing viremia was established in immunocompetent mice by intraperitoneal injection with DENV-2-infected K562 cells (Yamanaka and Konishi, 2008). Other groups also employed this method and demonstrated viremia in other DENV serotype infection mouse models (Chiang et al., 2014, 2016; Chen et al., 2015; Hu et al., 2016).

In the present study, we established a DENV-1 infection mouse model using immunocompetent mice. We also investigated whether the present challenge system was useful for evaluating the efficacy of vaccines using two types of DNA vaccine-immunized mouse model.

Based on our previous study describing the establishment of a DENV-2 mouse infection model (Yamanaka and Konishi, 2009), K562 cells were inoculated with DENV-1 Mochizuki strain in the presence of an infection-enhancing monoclonal antibody (MAb) D1-IV-3B8 (hereafter 3B8). After two days incubation, > 100-fold enhancements were observed in the presence of 3B8 ranging from 6.25 to 100 μg/ml IgG, compared with the cell count in the control (10^2 infected cells) in the absence of 3B8, as indicated in Fig. 1A (dotted line). Although the numbers of infected cells were similar in this range, the yield titers determined on Vero cells during 1–4 days incubation varied depending on the IgG concentration, with higher IgG concentrations (100 μg/ml) displaying higher yield titers (Fig. 1B). Furthermore, the highest yield titers were observed in the supernatant at 3 (or 4) days incubation in this range. Therefore, "100 μg/ml of 3B8" and "usage of infected K562 cells at 2 (or 3) days incubation" were applied in further experiments to prepare DENV-1-infected K562 cells. For the preparation of a large number of DENV-1-infected K562 cells, we followed the second round of infection method described previously (Yamanaka and Konishi, 2009). The highest yield titer (6.4 $\log_{10}$ focus forming units [FFU]/ml) was detected in the culture medium at day 3 (Fig. 1C). Therefore, for all subsequent experiments, infected K562 cells 2 days after the mixture of infected and uninfected cells, which were expected to produce a high yield (> 6.0 $\log_{10}$ FFU/mL) in the following incubation period (days 3, 4 and 7), were used for the intraperitoneal injection of mice.

DENV-1-infected K562 cells were prepared following the above conditions, and 3×10^6–3×10^7 cells were intraperitoneally injected into ICR mice. Mice injected with 3×10^7 and 1×10^7 cells showed similar average viremia levels of 4.7 and 4.5 $\log_{10}$ FFU/ml, respectively, 2 h after injection, followed by those injected with 3×10^6 cells (3.6 $\log_{10}$ FFU/ml) and infected mouse brain homogenate (2.9 $\log_{10}$ FFU/ml) (Fig. 2A). The viremia levels significantly decreased with time between 2 and 24 h ($P < 0.05$) during incubation in the peritoneal cavity. Titers in mice challenged with infected mouse brain homogenate were undetectable. Furthermore, we observed mouse survival until 21 days after injection (Fig. 2B). Mortalities were observed among the mice injected with 3×10^7 cells from 2 days after injection, with 100% mortality within 7 days. Similarly, other groups injected with infected cells showed 100% mortality within 8–10 days. In contrast, all mice injected with DENV-1-infected brain homogenate survived. Considering the simplicity and ease of preparing infected cells, as well as the viremia levels and survival rates, 1×10^7 K562 cells were chosen for injection into mice in subsequent experiments.

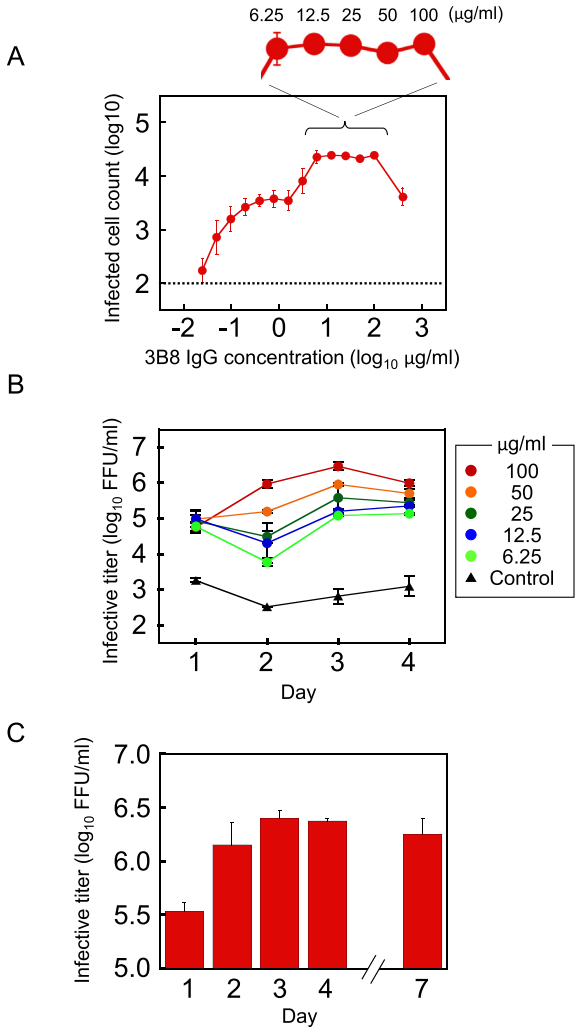

Fig. 1. Growth of DENV-1 in the presence of 3B8 in K562 cells. (A) Enhancing activity of 3B8 against DENV-1. In brief, 1×10^5 K562 cells were infected with DENV-1 at a multiplicity of infection of 0.001, in the presence of 3B8 at a final IgG concentration range of 0.02–400 μg/ml. The abscissa indicates the final concentration of IgG in the virus antibody-cell mixture in the 96-well plate, the ordinate indicates the infected cell counts (in $\log_{10}$). Assays were performed in duplicate, and each datum represents an average obtained from two separate assays with SDs indicated by error bars. (B) Yield infective titers obtained from infection-enhanced K562 cells. Culture medium containing DENV-1 and a greater range of 3B8 concentrations (6.25–100 μg/ml; see Fig. 1A) was harvested 1–4 days after infection. The abscissa indicates the day of harvest, the ordinate indicates the infective titer ($\log_{10}$ FFU/ml) determined on Vero cells. (C) Viral growth on the second round of infection. A small number (1×10^5 cells) of DENV-1-infected K562 cells, which were enhanced in the presence of 3B8 (100 μg/ml), were mixed with a large number of uninfected K562 cells (1×10^7 cells) and incubated in the presence of 3B8 again for the second round of infection. The cell mixture was cultured in 75-cm^2 flasks, and the infective titers were determined in the supernatant. The infective titers of the two flasks were averaged, and each datum represents the average.

To determine the effect of the mouse strain and age on viremia levels in our system, ICR and BALB/c mice were injected with DENV-1-infected K562 cells (Fig. 3A). Individual viremia levels 2 h after injection were similar between these two groups (4.3–5.0 $\log_{10}$ FFU/ml). However, at 24 h after injection, the average viremia level in BALB/c mice (2.8 $\log_{10}$ FFU/ml) was significantly lower than that in ICR mice (4.1 $\log_{10}$ FFU/ml). Furthermore, we monitored mouse survival after injection (Fig. 3B). Despite the lower viremia level at 24 h after injection, mortality was observed among BALB/c mice from 4 days after

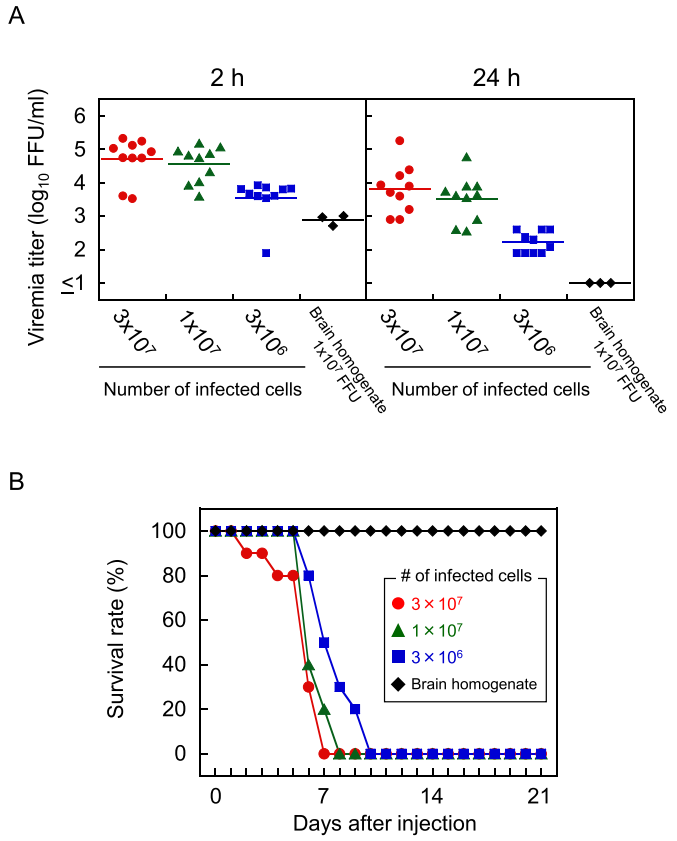

Fig. 2. Injection of DENV-1-infected K562 cells into ICR mice. (A) Effect of the number of infected cells on viremia level. DENV-1-infected K562 cells [3×10^7 (red circle), 1×10^7 (green triangle), 3×10^6 (blue square)] were injected intraperitoneally into groups of 10 4-week-old male ICR mice. As a reference, three 4-week-old male ICR mice were injected intraperitoneally with infected mouse brain homogenate containing 7.0 $\log_{10}$ FFU of DENV-1 Mochizuki strain (black diamond). Mice were bled 2 and 24 h after injection, and infective titers in the blood were determined on Vero cells. The individual plasma virus titer ($\log_{10}$ FFU/ml) was obtained from 10 or three mice. The average is indicated by bars. (B) Survival curve after challenge. ICR mice injected with DENV-1-infected K562 cells or DENV-1-infected mouse brain homogenate were observed for 21 days to record neurologic symptoms and/or death.

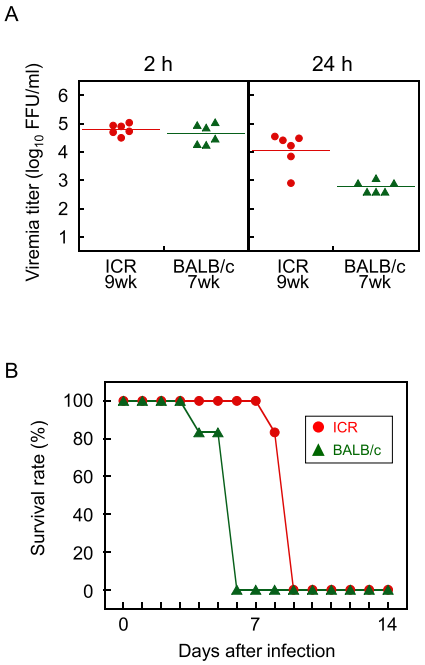

Fig. 3. Comparisons of mouse strains. (A) Comparison of the abilities of different mouse strains to develop viremia following K562 injection. Six 9-week-old male ICR mice (red circle) and six 7-week-old male BALB/c mice (green triangle) were injected intraperitoneally with 1×10^7 DENV-1-infected K562 cells and were bled at 2 and 24 h after injection. The individual plasma virus titer ($\log_{10}$ FFU/ml) was obtained from six mice, with the average indicated by bars. (B) Survival curve after challenge. ICR and BALB/c mice injected with DENV-1-infected K562 cells were observed for 21 days to record neurologic symptoms and/or death.

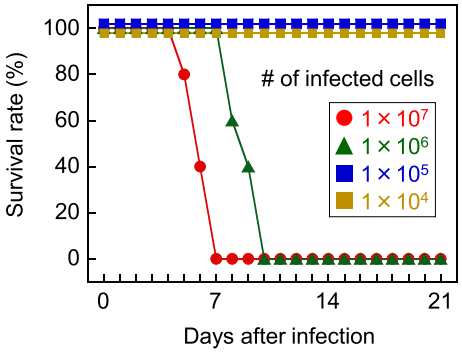

Fig. 4. Determination of the LD_{50}. Various numbers of DENV-1-infected K562 cells (1×10^4, 1×10^5, 1×10^6 and 1×10^7) were injected intraperitoneally into six 4-week-old male ICR mice per group. They were observed for 21 days to record neurologic symptoms and/or death.

injection. Complete mortality was observed within 6 and 9 days in BALB/c and ICR mice, respectively. Older (9-week-old) ICR mice showed prolonged survival compared with younger (4-week-old) ICR mice (Figs. 2B and 3B). These results suggest that both BALB/c and ICR mouse strains, including older ICR mice, are suitable for this injection system with some variations in viremia duration and survival period.

To determine the LD_{50} value of our challenge system, the correlation between the number of infected K562 cells injected (1×10^4–1×10^7) and the survival rates were investigated (Fig. 4). The survival rate was clearly dependent on the number of injected cells, that is, 100% with 1×10^4 and 1×10^5 cells compared with 0% with 1×10^6 and 1×10^7 cells. The LD_{50} was determined as 3.2×10^5 DENV-1-infected K562 cells in ICR mice.

The present DENV-1 injection system would be expected to induce a high rate of mortality with neurological symptoms in most challenged mice. Thus, to investigate whether virus circulating in mouse blood can reach the central nervous system and propagate there, whole brains were collected from ICR mice at 5 days after injection with infected K562 cells. Individual infectivity in each whole brain homogenate was determined on Vero cells (Fig. 5). All mouse brains contained DENV-1 ranging from 4.8 to 6.0 $\log_{10}$ FFU/brain, with an average of 5.5 $\log_{10}$ FFU/brain.

To assess the suitability of the present mouse challenge system for evaluating vaccine efficacy, mice were immunized with two types of DNA plasmid vaccines against DENV-1 (each 100 μg/dose): (i) p7F4G3, a pFUSE-based plasmid mixture expressing MAb 7F4 neutralizing antibody against DENV-1 (Yamanaka et al., 2015), and (ii) pcD1ME, a pcDNA3-based plasmid expressing the prM and E proteins of the DENV-1 Mochizuki strain (Konishi et al., 2006). ICR mice immunized with p7F4G3 were bled on days 5–9 and their pooled serum samples showed approximately 100% and 50% plaque reduction at dilutions of 1:2 and 1:20, respectively (Fig. 6A). In contrast, mice immunized with control vector did not show any neutralizing activity. Ten days after immunization, all mice were challenged with DENV-1-infected K562 cells and survival was monitored (Fig. 6B). All p7F4G3-vaccinated mice survived during the observation period, whereas those in the control

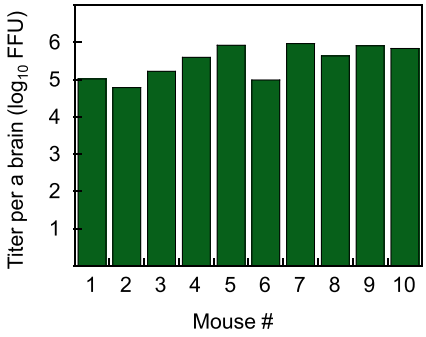

Fig. 5. Measurement of infectivity in mouse brains. Ten 4-week-old male ICR mice were injected intraperitoneally with 1×10^7 DENV-1-infected K562 cells. At 5 days after injection, mice were euthanized and whole brains were collected. Individual infectivity ($\log_{10}$ FFU/brain) in the brain homogenate was determined.

vector group all died within 11 days of challenge. Furthermore, pooled serum samples from ICR mice immunized with pcD1ME showed a neutralizing antibody titer of > 1:1280 (75% plaque reduction) a week before challenge. All mice survived during the observation period (Fig. 6B). These results demonstrated that our challenge system was useful for evaluating vaccine efficacy, and that two types of DNA vaccines (p7F4G3 and pcD1ME) were effective at protecting from lethal DENV-1 challenge.

In the present study, we have demonstrated a simple challenge system involving intraperitoneal injection of DENV-1-infected K562 cells using immunocompetent mice. The challenge induced lethal viremia, resulting in fatality in all mice within 1–2 weeks. Although detectable levels of viremia quickly decreased and were cleared from mouse blood, high infective titers (approximately 5 $\log_{10}$ FFU/brain) were still detectable in mouse brains 5 days after challenge. In contrast, no fatalities with low level and short duration of viremia were observed following intraperitoneal injection with a DENV-1-infected mouse brain homogenate. These suggested that a large number of viruses, which continuously disseminated from infected K562 cells into the mouse circulation, had broken through the mouse blood–brain barrier, and then successfully propagated in the brain, causing high mortality in mice. However, lethality was caused by neurologic symptoms, which are not the major cause of clinical disease in humans, and therefore may be a limiting factor in use of this model for studies on the pathogenesis and pathophysiology of dengue diseases. DENV-1 Mochizuki strain has historically undergone more than 100 passages in mouse brains with approximately 100% lethality for immunocompetent mice (Hotta, 1952). Therefore, our simple intraperitoneal injection system might enable passage of DENV into the brain, thereby replacing the typical intracerebral injection route. Although we did not investigate the replication of DENV in other organs except for brain, Lee et al. reported that intracranial challenge of DENV into suckling ICR mice induced strong infection in brains, and antigen was detectable in liver, but not spleen, lung or kidney (Lee et al., 2005).

Preparation of high infective titer inoculum to achieve 100% mortality via intraperitoneal injection into mice (Sarathy et al., 2015a; Sarathy et al., 2015b) is technically difficult to achieve with some DENV strains. Our simple intraperitoneal injection system using infected cells might overcome this problem. For example, considering that 1.0×10^6 infected K562 cells, which are able to achieve 100% mortality among immunocompetent mice in the present injection system, can be cultured in 1 ml of medium, approximately 20 shots of mouse inoculum could be prepared in a single 75 cm^2 tissue culture flask. This suggests that our challenge system may reduce the labor and time required for preparation of challenge virus.

MAb 7F4 shows strong neutralizing activity against DENV-1 *in vitro* (Yamanaka et al., 2013). Although only a limited neutralizing antibody

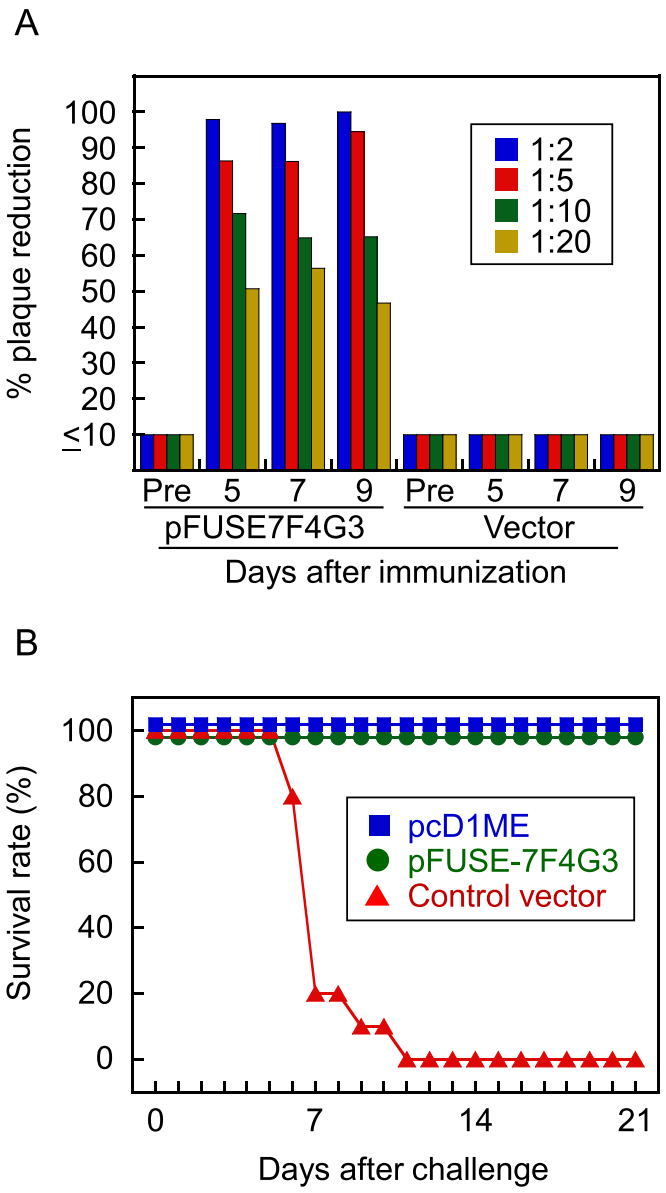

Fig. 6. Evaluation of vaccine protection against lethal challenge. (A) Neutralizing activity of p7F4G3 in mice. Ten 3-week-old ICR male mice were inoculated with p7F4G3 (100 μg) or control vector (100 μg). The percent plaque reduction at a range of dilutions (1:2, 1:5, 1:10, 1:20) of pooled sera was indicated on 1 day before, and 5, 7 and 9 days after vaccination. (B) Survival curves in vaccinated mice after challenge with DENV-1-infected K562 cells. Two weeks after the third immunization with pcD1ME (five 3-week-old male ICR mice) or 10 days after the single immunization with p7F4G3, mice were challenged with 1×10^7 DENV-1-infected K562 cells. Vaccinated mice were observed for 21 days to record neurologic symptoms and/or death.

titer was detected in mouse serum in the present study, all p7F4G3-vaccinated mice were completely protected from lethal challenge. In contrast, all mice in the control vector group died after challenge, indicating that antibody-expressing plasmid p7F4G3 may represent a new effective vaccine strategy for dengue virus. Moreover, immunization with a conventional DNA vaccine, expressing the prM and E proteins of DENV-1, also completely protected mice from subsequent lethal challenge with infected-K562 cells. These results indicated that our challenge system was useful for evaluating some types of dengue vaccine candidates.

Conflicts of interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.104560.

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