Chimpanzee adenovirus vaccine protects against Zaire Ebola virus


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Abstract

This study evaluated the use of a chimpanzee-based adenovirus vaccine in mouse and Guinea pigs models of Zaire Ebola virus (ZEBOV) infection. Vaccine vector expressing the envelope glycoprotein of ZEBOV was created from the molecular clone of chimpanzee adenovirus pan7 (AdC7). AdC7 vaccine stimulated robust T and B cell responses to ZEBOV in naïve mice inducing complete protection to an otherwise lethal challenge of ZEBOV. Complete protection to Zaire Ebola virus was also observed in Guinea pigs vaccinated with a relatively low dose of AdC7 (5 × 10⁹/kg). Pre-existing immunity to AdHu5 was generated in mice following pre-exposure to AdHu5 or administration of pooled human immune globulin. Pre-existing immunity to human adenoviruses severely compromised the efficacy of the human AdHu5 vaccine but not the chimpanzee AdC7 vaccine. These results validate further development of Chimpanzee-based vaccine and highlight the impact of pre-existing immunity to the vaccine carrier.

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Keywords: Adenovirus; Chimpanzee; Vaccine; Ebola; Mice; Guinea pig; Preexisting immunity

Introduction

Ebola virus is one of several pathogens that cause lethal hemorrhagic fever (Sanchez, 2001). Most strains of Ebola have a high fatality rate in humans. Outbreaks of Ebola infections have occurred sporadically in Africa. While these outbreaks have caused substantial fatalities, they remained well contained. Identification of the natural reservoir for Ebola viruses, which is believed to be wild animals, would help understand and potentially prevent re-emergent infections. The profile of Ebola virus infections that makes it such a dangerous pathogen also contributes to its utility as a potential agent of biological warfare. Development of an efficacious vaccine is thus considered a high priority in spite of the limited impact of Ebola virus on human morbidity and mortality world-wide.

The Ebola envelope glycoprotein (GP) is the only viral protein located on the envelope of the virus and presumably directs its tropism (Feldmann et al., 1999; Ito et al., 2001). A number of vaccine strategies including naked or lipid encapsulated DNA, vaccinia, Venezuelan equine encephalitis virus (VEE) and adenovirus have been evaluated for induction of protection against challenge with Ebola virus (Geisbert and Jahrling, 2003; Hart, 2003). All strategies have focused on a subset of Ebola virus gene products as targets for eliciting protective immune responses. Predominant work has utilized the nucleoprotein (NP) as a target antigen for cellular immunity and GP as a target antigen for both cellular and humoral immunity (Hart, 2003; Wilson and Hart, 2001). Among those strategies, an adenoviral vector was the only vaccine carrier capable of protecting macaques against an otherwise lethal dose of Zaire Ebola virus (Sullivan et al., 2000, 2003).

The use of replication-defective versions of adenoviruses in gene therapy experiments illustrated the ability of these vectors to generate vibrant T and B cell responses to both the capsid...
proteins and transgene products (Yang et al., 1994, 1995, 1996). Generating strong immunity to a transgene product such as Ebola GP is desirable in the context of vaccine development. However, immunity to adenoviral capsid proteins prevents vector re-administration and thus limits application of this vaccine carrier to a single dose per recipient (Kass-Eisler et al., 1994, 1996). Therefore, vaccination necessitating prime/boost strategy and/or protection against multiple pathogens may be problematic. In addition, virtually all experiments performed to date with adenoviral vectors utilized a construct based on human serotype 5 (AdHu5). A significant proportion of the North American population has evidence for pre-exposure to AdHu5 (i.e., 30–50%) based on the presence of high levels of neutralizing antibodies (NAB) in serum (Chirmule et al., 1999).

Recent isolation of several novel adenoviruses derived from nonhuman primates raised the question whether they could be used as vectors for vaccine application (Farina et al., 2001; Fitzgerald et al., 2003; Roy et al., 2004a, 2004b). Indeed, neutralization of chimpanzee-derived adenoviral vectors by human serum was not significant suggesting that preexisting immunity to human adenovirus could possibly be circumvented by chimpanzee adenovirus (Roy et al., 2004b). This study evaluates specific T and B cell responses to Ebola GP and their relative importance and response to lethal challenges of ZEBOV following immunization in mice and Guinea pigs with Chimpanzee AdC7 adenoviral vector expressing the Ebola GP antigen. The immune response and protection mediated by AdC7 vaccine vector is also evaluated in the presence of preexisting immunity to human adenovirus.

Results

Vectors based on human adenovirus serotype 5 (AdHu5) and the chimpanzee adenovirus pan7 (AdC7) were created to express the full-length Zaire Ebola glycoprotein (ZGP) and tested in mice for induction of T and B cell responses. Peptide specific activation of CD8+ T cells, as measured by the production of IFN-γ, was detected in both AdHu5-ZGP and AdC7-ZGP vaccinated animals at frequencies equal to 12 ± 3 and 14 ± 2%, respectively (Fig. 1). Mononuclear cells stimulated with control unspecific peptide demonstrated background levels with 0.4 to 0.5% of CD8+ T cells positive for INF-γ (Fig. 1). The B cell response to the Ebola envelope was analyzed by measuring sera from immunized mice for neutralization of a LacZ expressing HIV-based vector pseudotyped with the Zaire Ebola glycoprotein (ZGP). Neutralizing antibody (NAB) to ZGP-pseudotyped HIV vector could be detected in mice 30 days after vaccination with AdHu5-ZGP and AdC7-ZGP at levels, in terms of reciprocal dilution, equal to 90 ± 10 and 100 ± 16 (N = 5 per group, repeated 4 times), respectively.

The most direct means of evaluating the efficacy of these vaccines in mice is to assess protection against weight loss and death following lethal challenge with mouse-adapted Zaire Ebola virus (Bray et al., 1998). Mice were immunized with a single dose of 5 × 10^10 particles per animal as performed previously and vaccinated animals were challenged with 200 LD50 of the mouse-adapted strain of ZEBOV 21 days later. All control mice (vehicle and AdHu5-LacZ) died between days 5 and 9 post-challenge. In contrast, all mice vaccinated with AdHu5-ZGP or AdC7-ZGP survived the challenge with mouse-adapted ZEBOV (Fig. 2). Weight loss was observed only from control groups (vehicle and AdHu5-LacZ, data not shown). Complete protection following vaccination with 5 × 10^10 particles of AdC7-ZGP was demonstrated with challenge doses of ZEBOV up to 200,000 LD50, which was the highest dose tested (data not shown).

A number of additional parameters relevant to protection following vaccination were studied. AdC7-ZGP vaccine was administered at doses ranging from 10^6 to 10^10 particles per

![Fig. 1. B10.BR mice were vaccinated intramuscularly (I.M.) with 5 × 10^10 particles per animal of the indicated vectors and splenocytes were harvested 10 days later for frequency analysis. A specific or control non-specific peptide to ZEBOV/GP was (0.4 μg/well) incubated with 10^6 splenocytes in the presence of Brefeldin and the cells were subsequently analyzed by flow cytometry for expression of CD8 and gamma interferon. ZEBOV/GP peptide: specific to Zaire Ebola GP, Control peptide: control non-specific to Ebola GP. Groups of 3–4 mice were analyzed per vector and the experiment was repeated 4 times. Data originate from a representative experiment.](image-url)
mouse. Total protection to a lethal challenge with mouse-adapted Ebola Zaire virus was observed down to a dose equal to $5 \times 10^6$ particles per mouse while partial protection (80% survival) was obtained with $5 \times 10^7$ particles per mouse (Table 1). No protection was seen at doses equal or lower than $5 \times 10^7$ particles per mouse. Another important feature of the use of adenovirus vectors in this setting is the time course by which protection is achieved. In experiments summarized in Fig. 3, animals were immunized with AdC7-ZGP vector and challenged 5, 10, and 15 days later. Partial protection was observed as early as 5 days and total protection was achieved from day 10 on after vaccination.

A significant concern regarding the use of AdHu5 based vaccines is the potential interference of preexisting immunity to natural human adenovirus infections on vaccine efficacy. Experiments were performed to evaluate this concern. To mimic pre-existing immunity, mice were pre-exposed to AdHu5 expressing a non-relevant antigen, β-galactosidase, or administered with pooled human immune globulin. For pre-exposure, mice were administered AdHu5-LacZ vectors intramuscularly in order to activate T and B cells against the adenovirus serotype capsid and elicit humoral immunity slightly lower to what is observed in humans following natural infections. Five groups were studied including no preimmunization and no vaccine (group 1), vaccination with AdHu5-ZGP without (group 2) and with (group 3) preimmunization with AdHu5-LacZ, and vaccination with AdC7-ZGP without (group 4) and with (group 5) preimmunization with AdHu5-LacZ. Fig. 4 shows that preimmunization with AdHu5-LacZ resulted in NAB titer against AdHu5 equal to 1:320 without detectable NAB to AdC7, however, antivector NABs were not detected in mice prior to adenovirus exposure or in naïve animals (data not shown). In these experiments, vaccination of naïve mice with either AdHu5-ZGP or AdC7-ZGP vectors afforded complete protection as observed previously. In contrast, preimmunization with AdHu5-LacZ significantly diminished efficacy of the AdHu5 vaccine as evidenced by substantial weight loss (Fig. 5) and 70% mortality (Fig. 4A) without any detectable impact on efficacy of the AdC7 vaccine (Figs. 4A and 5).

In the presence of pre-existing immunity to AdHu5, T and B cell responses to the Ebola antigen expressed by AdC7 vector were slightly reduced, possibly reflecting antigenic similarity between chimpanzee and human adenoviruses. This was further addressed by passive transfer of pooled human immune globulin, containing antibodies to common human serotypes of adenovirus including AdHu5 (Casimiro et al.), into mice prior to vaccination. Intravenous injection of pooled human Ig 24 and 2 h before vaccination resulted in anti-AdHu5 titer in the blood of recipients equal to 1:1280. This approach essentially reconstitutes the full spectrum of anti-Ad humoral immunity found in humans in contrast to the experiment described above with AdHu5-LacZ pre-immunization which generates cellular and humoral immune responses against AdHu5 serotype only. As shown in Fig. 6, passive transfer of pooled human Ig seriously compromised the efficacy of the AdHu5-ZGP vaccine with no survivors following challenge and a diminished T cell response (84% down) with no detectable NAB. In contrast, the same conditions did not have a detectable impact on the protective efficacy of the AdC7-ZGP vaccine (i.e. 100% survivors) although a statistically not significant diminution in the average of T and B cell responses was observed (down by 15%, $P = 0.1$ and 19%, $P = 0.2$, respectively).

![Table 1](image)

<table>
<thead>
<tr>
<th>Survival of vaccinated mice with different doses of AdC7-ZGP following challenge with mouse-adapted Ebola virus*</th>
<th>(5 \times 10^6)</th>
<th>(5 \times 10^7)</th>
<th>(5 \times 10^8)</th>
<th>(5 \times 10^9)</th>
<th>(5 \times 10^{10})</th>
</tr>
</thead>
<tbody>
<tr>
<td>AdC7-LacZ</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>AdC7-ZEBOVGP</td>
<td>0/10</td>
<td>0/10</td>
<td>8/10</td>
<td>10/10</td>
<td>10/10</td>
</tr>
</tbody>
</table>

* Group of 10 BALB/c mice were vaccinated intramuscular and challenged with 200 LD50 of mouse-adapted ZEBOV 3 weeks later.

b Doses of AdC7 vector represent particles administered per mouse by unique injection. Numerator represents the number of surviving mice out of 10.
Several vaccine strategies were found to be protective in mice but failed in animal models such as Guinea pigs or nonhuman primates which are more representative of a ZEBOV infection in humans. Therefore, additional studies were performed in Guinea pigs to evaluate whether chimpanzee AdC7 could be protective in a more stringent model of ZEBOV infection. Groups of 6 Guinea pigs were vaccinated with AdC7-ZGP at $5 \times 10^9$, $5 \times 10^{10}$ or $5 \times 10^{11}$ particles per kilogram and challenged I.P. 28 days later with 1000 plaque-forming units of Guinea pigs-adapted ZEBOV. As shown in Table 2, 100% of vaccinated Guinea pigs survived the challenge with ZEBOV which otherwise killed all unvaccinated control animals. Protection correlated with the presence of serum NAB which significantly increased with higher vaccination doses. All vaccinated Guinea pigs demonstrated a steady weight gain that was similar among all groups (data not shown).

**Discussion**

The work of Nabel and colleagues clearly established the potential of adenovirus vaccine platform in the development of a vaccine against Ebola virus. They demonstrated protection in cynomolgus macaques to lethal challenge with ZEBOV 28 days after a single intramuscular injection of $2 \times 10^{12}$ particles of a mixture of AdHu5 vectors expressing Ebola NP and GP. While these studies are extremely encouraging, a number of issues require further evaluation in the context of the further development of this approach. This study addresses efficacy of chimpanzee adenovirus-based vaccine vector against Zaire Ebola virus.
Ebola virus and the impact of pre-existing immunity to human adenoviruses.

A number of groups have surveyed human populations for NAB against AdHu5. Over 30% of subjects in Western civilizations have substantial levels of AdHu5 NAB while the frequency of AdHu5 NAB increases to over 90% of subjects in some third world populations (Chirmule et al., 1999; Mast, 2003). Several studies have shown that AdHu5 NAB do indeed compromise AdHu5 based vaccines, including a murine model of rabies in which rabies NAB and protection to lethal dose of rabies was measured as well as murine and nonhuman primate models of HIV vaccines in which antigen specific T cell frequencies were measured (Casimiro et al., 2003; Fitzgerald et al., 2003; Xiang et al., 2002). Preliminary results from the Merck HIV vaccine study are consistent with this hypothesis in that the number of subjects responding to the AdHu5 vaccine was diminished in those with preexisting AdHu5 NAB (Emini, 2002). Some studies indicate that preexisting immunity to human adenovirus can be bypassed with high doses of AdHu5 or priming with DNA prior to immunization with AdHu5 (Gao et al., 2003b; Wu et al., 2005; Yang et al., 2003). However, the clinical relevance of these strategies remains to be demonstrated as they involve quantities of AdHu5 that are several orders of magnitude above the highest dose evaluated in humans or necessitate multiple injections which preclude rapid intervention.

To evaluate the issue of pre-existing immunity for Ad-based vaccination against Ebola virus, we focused on the murine model in which BALB/c mice were challenged with a mouse-adapted strain of ZEBOV. This leads to 100% mortality with dose of challenge as low as 1 pfu/animal (Bray et al., 1998). The murine model has been criticized because it appears less stringent a test of vaccine efficacy than the nonhuman primate model. A number of vaccine strategies that succeeded in the

![Graph](image)

**Fig. 5.** Body weight of vaccinated BALB/c mice with preexisting immunity following challenge with mouse-adapted ZEBOV. Challenged mice were weighed everyday from day 1 to day 11 post-infection. Weight is expressed as the average body weight from each mouse of a group of 10 mice.

![Graph](image)

**Fig. 6.** Impact of pre-existing immunity to AdHu5 with pooled human Ig (h-Ig) on vaccine efficacy. B10.BR (T cell assay) and BALB/c (B cell assay and challenge experiments) mice were injected intravenously with pooled h-Ig 24 and 2 h before vaccination as follow: no h-Ig and no vaccine (group 1), without (group 2) and with (group 3) h-Ig and vaccination with $5 \times 10^{10}$ particles AdHu5-ZGP, and without (group 4) and with (group 5) h-Ig and vaccination with $5 \times 10^{10}$ particles of AdC7-ZGP. (A) Survival following 200 LD50 of mouse-adapted ZEBOV ($N = 10$ /group). (B) Frequency of IFN-γ expressing CD8+ T cells to ZGP epitope at day 10 post-vaccination ($N = 4$ /group, repeated 2 times). (C) NAB to Ebo-pseudotyped HIV vector at day 30 after vaccination ($N = 10$ /group, repeated 2 times). Error bars represent the standard deviation of the data.
Table 2

Survival of AdC7-EboZ vaccinated Guinea pigs following challenge with Guinea pigs-adapted Zaire Ebola virus

<table>
<thead>
<tr>
<th>Dose of AdC7-ZGP/kg</th>
<th>Percentage survival (%)</th>
<th>NABb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5 × 10⁹</td>
<td>100</td>
<td>20 ± 0</td>
</tr>
<tr>
<td>5 × 10¹⁰</td>
<td>100</td>
<td>53 ± 23</td>
</tr>
<tr>
<td>5 × 10¹¹</td>
<td>100</td>
<td>267 ± 92</td>
</tr>
</tbody>
</table>

a Group of 6 Guinea pigs were vaccinated I.M. with the appropriated number of AdC7-ZGP particles per kilogram and challenged I.P. with 1000 pfu of Guinea pigs-adapted ZEBOV 4 weeks later. One control group of 3 Guinea pigs was administered with saline (vehicle) and challenged as described above.

b Reciprocal dilution of NAB titers to Ebo-pseudotyped HIV vector at day 21 after vaccination (N = all animals/group, repeated two times).

mice (5 × 10⁹ and 2.2 × 10¹¹ particles/kg, respectively) despite the fact that the Guinea pigs model is view by many as more stringent than the mouse model. At this point, we cannot rule out that the chimpanzee AdC7 vaccine vector is sub-optimal in mice and perform better in larger mammals. This observation also suggests that AdC7-ZGP could potentially be scalable as a single dose for application in humans since AdHu5-based vaccine was administered to individuals at doses approximating 1.3 × 10⁹ particles/kg (1 × 10¹³ particles/individual, (Emini, 2002). However, formal assessment of the optimal dose would be required as studies indicate that animals do not accurately predict toxic or effective doses of adenovirus in humans (Raper et al., 2002; Schnell et al., 2001).

In the current study, AdC7-ZGP vector was as potent as AdHu5-ZGP vector in naïve mice which developed a strong T and B cell responses with both vector. In addition, 5 × 10⁹ particles/kg of AdC7-ZGP was sufficient to fully protect Guinea pigs from a lethal challenge with ZEBOV. Importantly, pre-existing immunity to AdHu5 had only a slight impact on T and B cell responses generated by AdC7-ZGP in mice and none on survival following challenge with mouse-adapted ZEBOV. The next step is formal evaluation of this approach in nonhuman primates. Pilot studies of rhesus macaques vaccinated with AdC7-EboGP showed detectable antigen specific T cell responses and levels of ZEBOV NAB comparable to that seen in mice. This study was performed using mouse- or Guinea pig-adapted strains which exhibit some mutations leading to amino acid changes compared to human ZEBOV. Stocks of chimpanzee adenovirus Ebola vaccine vector were recently produced for evaluation in nonhuman primates against human ZEBOV to assess efficacy in a more relevant model of Ebola hemorrhagic fever.

Materials and methods

Construction and production of adenoviral vectors

Molecular clones of E1-deleted and/or E1/E3-deleted human and chimpanzee adenovirus vectors expressing ZEBOV glycoprotein were created using a green-white selection method as described previously (Gao et al., 2003a). Correct molecular clones were confirmed by sequencing. The viral vectors were rescued by transfecting the linearized molecular clones into 293 cells, propagated to large-scale infections (1 × 10⁹ cells) and purified by the standard CsCl gradient sedimentation process. Genome structures of vectors were analyzed by restriction digestions of viral DNA in comparison with those of the molecular clones. Infectivity of vectors was determined by the standard plaque assay on 293 cells.

Animal models

BALB/c mice were used to evaluate protection lethal doses of mouse-adapted Zaire Ebola virus since it is the preferred strain of mice for BSL-4 related experiments based on a number of technical and safety issues. Antibody responses to ZGP were measured in BALB/c and B10.BR as indicated. The
T cell response was analyzed by evaluating the frequency of CD8+ T cells positive for INF-γ production upon peptide stimulation by flow cytometry (FACS) in B10.BR (MHC H-2^k) where a dominant CD8 epitope has been mapped. Analysis for CD8+ T cell activation in response to vaccination in BALB/c was not possible because screening of a peptide library encompassing the Ebola GP failed to identify a restricted dominant epitope in this strain of mice (MHC H-2^b). However, experiments in C57BL/6 mice revealed that the amount of specific cell lysis monitored by Cr51 release (CTL assays) following incubation of target cells with activated cytotoxic lymphocytes (effector cells) was comparable to data accumulated in B10.BR and correlated with protection efficacy observed in BALB/c mice (data not shown). Hartley guinea pigs were between 300 and 400 g at the time of vaccination.

**Immunization of guinea pigs and mice with adenoviral vectors and challenge**

Guinea pigs, BALB/c, and B10.BR mice were immunized by intramuscular injection in the right limb with 100 μl of recombinant adenoviral vector diluted in PBS. Guinea pigs and BALB/c mice were challenged by intraperitoneal injection with indicated doses of the Guinea pigs-adapted or mouse-adapted Zaire Ebola virus strain Mayinga. The mouse-adapted strain of ZEBOV was isolated following nine serial passages of the human strain in progressively older mice (Bray et al., 1998). The guinea pig-adapted strain was isolated after 4 passages of the human strain in guinea pigs (Connolly et al., 1999). After challenge, the animals were weighed every day for 11 days and monitored for clinical signs of infection for 30 days after the challenge.

**Passive transfer of pooled human immune globulin (Casimiro et al.)**

BALB/c and B10.BR mice were injected intravenously with 500 μl containing 120 mg of pooled human Ig (Panglobulin NF; American Red Cross, Washington, DC) 24 and 2 h before vaccination. At the time of vaccination, serum showed NAB titers for AdHu5 at 1:1280. BALB/c mice were challenged as indicated above.

**Neutralizing antibody assay**

Sera collected from immunized mice were inactivated at 56 °C for 45 min. Serial dilutions of each sample (1:10, 1:20, 1:40, etc., in 50 μl of DMEM) was mixed with equal volume of Zaire Ebola GP (ZGP)- pseudotyped HIV-based vector (see below for details) encoding the LacZ reporter gene (15–30 transducing units/well) and incubated at 37 °C for 60 min. The mixture was then transferred onto subconfluent HeLa cells in 96-well flat-bottomed plates and incubated for 90 min at 37 °C in 5% CO2. Control wells were infected with equal amount of viral vector without addition of serum or with non-immune serum. 100 μl of DMEM supplemented with 20% FBS was then added to each well and plates were incubated at 37 °C in 5% CO2 for 48 h. Cells were subsequently stained with X-gal and examined under a microscope. Sample dilutions showed >50% reduction in the number of blue cells compared to controls that scored positive for neutralizing antibody. ZGP-pseudotyped HIV vector was produced as previously described (Kobinger et al., 2001).

Briefly, 180 μg of endotoxin-free DNA mixture, containing ZEBOV/GP expression vector, HIV packaging plasmid, and transfer vector encoding for LacZ in a 3:1:2 molar ratio, was transfected into each 150 mm plate of 293T cells using CalPhos Mammalian Transfection Kit (BD Biosciences Clontech). At 44 h after transfection, medium was added to each plate for 16 h before collection of vector. Cell-free supernatant containing virus was further concentrated by ultracentrifugation at 28,000 rpm for 2 h at 4 °C using a SW28 rotor (Beckman, Fullerton, CA). Concentrated virus was then resuspended into complete DMEM and stored at −80 °C in small aliquots.

**Frequency of INF-γ positive CD8 T cells**

Splenocytes (1 × 10^6/sample) were culture for 5 h at 37 °C in 96-well round bottom microtiter plate wells in DMEM supplemented with 10% FBS and 10^-6 M 2-ME. GolgiStop (BD PharMingen, San Diego, CA) was added at 1 μl/ml. The TELRTFSI peptide was used for stimulation at a concentration of 2 μg/ml. Control cells were incubated with an unrelated peptide or without peptide. After washing, cells were stained with 100 μl of 1/100 dilution of a FITC-anti mouse CD8a (BD PharMingen) at 4 °C for 30 min. Cells were washed again and permeabilized in 1 × Cytofix/Cytoperm (BD PharMingen) for 20 min at 4 °C, washed with 1 × Perm/Wash (BD PharMingen), and stained with 100 μl of 1/100 dilution of a PE-anti mouse IFN-γ (BD PharMingen) in the same buffer at 4 °C for 30 min. After washing, cells were examined by two-color flow cytometry using an EPICS Elite XL (Beckman Coulter, Miami, FL), and data were analyzed by WinMDi software. The TELRTFSI (Rao et al., 1999) peptide which carries the immunodominant MHC class I epitope of ZEBOV GP for mice of the H-2k haplotype was synthesized by Mimotopes (Clayton-Victoria, Australia). Peptide was diluted in DMSO to a concentration of 5 mg/ml and stored at −80 °C.

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