Construction and immune effect of *Haemophilus parasuis* DNA vaccine encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in mice

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**A B S T R A C T**

*Haemophilus parasuis*, the causative agent of swine polyserositis, polyarthritis, and meningitis, is one of the most important bacterial diseases of pigs worldwide. The development of a vaccine against *H. parasuis* has been impeded due to the lack of induction of reliable cross-serotype protection. In this study the gapA gene that encodes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was shown to be present and highly conserved in various serotypes of *H. parasuis* and we constructed a novel DNA vaccine encoding GAPDH (pCgap) to evaluate the immune response and protective efficacy against infection with *H. parasuis* MD0322 serovar 4 or SH0165 serovar 5 in mice. A significant antibody response against GAPDH was generated following pCgap intramuscular immunization; moreover, antibodies to the pCgap DNA vaccine were bactericidal, suggesting that it was expressed in vivo. The gapA transcript was detected in muscle, liver, spleen, and kidney of the mice seven days post-vaccination. The IgG subclass (IgG1 and IgG2a) analysis indicated that the DNA vaccine induced both Th1 and Th2 immune responses, but the IgG1 response was greater than the IgG2a response. Moreover, the groups vaccinated with the pCgap vaccine exhibited 83.3% and 50% protective efficacy against the *H. parasuis* MD0322 serovar 4 or SH0165 serovar 5 challenges, respectively. The pCgap DNA vaccine provided significantly greater protective efficacy compared to the negative control groups or blank control groups ($P<0.05$ for both). Taken together, these findings indicate that the pCgap DNA vaccine provides a novel strategy against infection of *H. parasuis* and offer insight concerning the underlying immune mechanisms of a bacterial DNA vaccine.

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1. Introduction

*Haemophilus parasuis* (*H. parasuis*) is the causative agent of Glässer’s disease, which is characterized by fibrinous polyserositis, polyarthritis, and meningitis as well as other serious conditions [1]. Along with changes in world-wide production methods, cases of Glässer’s disease have increased significantly [2]. To date, 15 serovars of *H. parasuis* have been identified; serovars 4 and 5 have been most frequently found in many countries [2–4]. Because 15 serovars and non-typeable strains of *H. parasuis* exist and the pathogenesis of *H. parasuis* infection is poorly understood, prevention and control of this disease has become increasingly difficult.

Recently, researchers have constructed several inactivated vaccines and have discovered protective or immunogenic antigens that could potentially serve as vaccine candidates, such as PalA, Omp2, D15, HPS 06257 [5], OmpA [6], VtaA [7], and Tbp [8,9]. Although these candidates potentially afford partial protection against challenge by homologous serovars of *H. parasuis* by decreasing mortality but not morbidity, challenge by heterologous serovars has not been studied. Furthermore, inactivated vaccines or subunit vaccines have some disadvantages [10], and the cost and production technologies limit their use in a clinical setting. Therefore, development of a new vaccine to control Glässer’s disease has become a priority.

DNA vaccines have emerged as an exciting new approach to control and prevent bacteria [11,12], viruses [13], and parasites [14]. Compared to subunit vaccines and whole cell-based vaccines, DNA vaccines are safer, more stable, and easy to handle [12]. Furthermore, a DNA vaccine can induce both a humoral and cellular immune response or only one of them depending on the formulation, which is beneficial for targeting bacterial infections [15,16].
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a glycolytic enzyme that participates in the generation of bacterial energy, which is essential for growth in the absence of neoglycocogenic substrates [17]; it is a virulence-associated immunomodulatory protein in S. agalactiae [18]. To date, H. parasuis DNA vaccine has not been reported previously; thus, we constructed a DNA vaccine based on the gapA gene.

In this study, we constructed an experimental H. parasuis DNA vaccine pCGap and tested the immunoprotective efficacy of the vaccine using a lethal dose challenge of H. parasuis in a mouse model. To our knowledge, this is the first report of a DNA vaccine targeting H. parasuis capable of producing immunoprotective efficacy.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The H. parasuis SH0165 strain (serovar 5) and MD0322 (serovar 4) strain are highly virulent strains, isolated from the lung of a commercial pig with fibrinous polyserositis, arthritis, and meningitis (SH0165) and from the lung of a commercial pig with fibrinous polyserositis (MD0322). MD0322 and SH0165 were grown in TSB (Difco™, France) or TSA (Difco™, France) supplemented with 10 μg/ml NAD (Sigma, St Louis, MO) and 10% new-born calf serum (Gibco) at 37 °C.

2.2. PCR detection of gapA in the 15 serovars reference strains

The genomic DNA of H. parasuis MD0322 or SH0165 strain and 15 serovar reference strains was extracted using DNeasy Blood and Tissue Kits (QIAGEN) and was then used as a template with the following primers: gapF1: 5'-ATGGCAATTTAATGGTATG-3'; gapR1: 5'-TTAGCGCCGTTAGCTG-3' for PCR amplification of the target sequence. The master mixture contains in reaction 32 μl deionized water, 10 μl 5× PrimeSTAR Buffer (Mg2+ plus), 4 μl dNTP Mixture (2.5 mM each), 1 μl of each primer (10 μM), 1 μl PrimeSTAR HS DNA Polymerase (2.5 U/μl) (Takara, Dalian) and 1 μl template (100 ng/μl) and the PCR amplification was performed at 94 °C for 10 min and subjected to 30 cycles of 94 °C for 1 min, 57 °C for 45 s, and 72 °C for 1 min 30 s, followed by a final extension at 72 °C for 10 min. Amplified PCR products were analyzed by agarose-gel electrophoresis. By using the TIANgel Midi Purification Kit (TIANGEN, China) PCR products were purified and sequenced.

2.3. Plasmid construction and preparation

To construct pCGap, gapA was amplified by PCR using the following primers: gapF2 (5'-GGCCTTGTTAGCTGTACC-3'; underlined, BamHI site) and gapR2 (5'-GGCAATTTAATGGTATTGGGC-3'; underlined, EcoRI site). The PCR products were cloned into pDNA3.1 Directional TOPO vector (Invitrogen) and transformed into E. coli TOP10 and digested by BamH I and EcoRI, resulting in pCGap. An overview of the recombinant plasmid is shown in Fig. S1. The GAPDH protein was expressed as a His fusion.

The endotoxin-free plasmid DNA was extracted using QIAGEN endoFree plasmid maxi kit (QIAGEN, Valencia, USA) and were adjusted to 1 μg/μl in physiological saline and stored at −20 °C until use.

2.4. Expression of GAPDH by mammalian cells in vitro

Expression of GAPDH protein in MDBK cells was confirmed by indirect immunofluorescence assay (IFA) with some modifications [19]. The MDBK cells were transfected with 1 μg plasmid DNA using the transfecting reagent Lipofectamine TM200 (Invitrogen).

Twenty-four hours after transfection, the transfected cells were fixed with methanol. After washing three times with PBS/1% Triton X-100 (PBS/Triton) the cells were subsequently incubated with 5% bovine serum albumin (BSA) in PBS at 37 °C for 1 h. Then mouse anti-His antibody diluted 1:300 was added and were incubated at 37 °C for 30 min and 100 μl of 1:300 diluted fluorescein-conjugated goat anti-mouse IgG was added and samples were then incubated in the dark at 37 °C for 30 min and were examined with a fluorescence microscope (OLYMPUS, Japan).

The expression of the GAPDH protein in MDBK cells was also confirmed by Western blot as previously described [19]. Briefly, the total lysates of transfected cells were electrophoresed on 12% SDS-PAGE gels, transferred to nitrocellulose membrane and subsequently was blocked with 5% skim milk in TBST. Serially-diluted rabbit polyclonal antibody against GAPDH protein (initially 1:400) were added and incubated for 30 min at 37 °C. Anti-rabbit IgG-HRP (Southern Biotech, USA), diluted 1:5000 in washing buffer, was used as the secondary antibody and incubated for 30 min at 37 °C. The color was visualized with the DAB substrate.

2.5. Animals and vaccination

This study was carried out in strict accordance with the recommendations in the China Regulations for the Administration of Affairs Concerning Experimental Animals 1988 and the Hubei Regulations for the Administration of Affairs Concerning Experimental Animals 2005. The protocol was approved by China Hubei Province Science and Technology Department (Permit Number: SYXKER (2010-0029)). The animals were euthanized at the end of the experiments or when moribund during the experiment.

Four to five week old female BALB/c mice were divided randomly into three groups (30 mice per group): one group was injected intramuscularly in the hind thigh muscle with 100 μg of plasmid dissolved in 100 μl PBS; the other two groups were injected with pDNA 3.1 vector only as negative control or PBS as blank control. Mice received a vaccination boost of an equivalent dose on day 14 and 28 following the first immunization. Blood samples were obtained by tail bleeding before immunization, one day before each boost, and two weeks after the 2nd boost for immunologic assays.

2.6. PCR detection of plasmid DNA in vaccinated mice

Seven days after the first immunization, muscle, kidney, spleen and liver were taken from each group (three mice per group). DNA was obtained from the tissues using DNeasy Blood and Tissue Kit (QIAGEN, Germany) and used as template for PCR analysis with gapF1 and gapR1 primers.

2.7. Analysis of the expression of DNA vaccines by reverse transcriptase-PCR (RT-PCR)

To determine the gene gapA expression levels in the immunized mice, tissues were taken from each group (three mice per group) on the 7th day following the first immunization. Total tissue RNA was extracted using RNeasy Mini Kit (QIAGEN) and was treated with RNAase free DNAase and was then utilized for cDNA synthesis using the First Strand cDNA Synthesis Kit (TOYO-OBO, JAPAN) and then the cDNA product was used as template for PCR analysis. RT-PCR was performed using β-actin with the following primers actinF: 5'-GGCTTATCTCCCTCCATCG-3' and actinR: 5'-CCAGTTGGTACAACTGCGGTCAT-3' as the internal control. The PCR amplification reaction contains per reaction 38 μl deionized water, 5 μl 10× Ex Taq Buffer (Mg2+ plus), 4 μl dNTP Mixture (2.5 mM each), 1 μl of each primer (10 μM), 0.5 μl TaKaRa Ex Taq (5 U/μl)
(Takara, Dalian) and 0.5 μl cDNA (100 ng/μl). And the PCR amplification condition was performed at 94 °C for 10 min and subjected to 30 cycles of 94 °C for 1 min, 57 °C for 45 s, and 72 °C for 1 min 30 s, followed by a final extension at 72 °C for 10 min.

2.8. Determination of antibody titers

Serum for IgG titers against GAPDH protein was analyzed by ELISA, as described previously [12]. Briefly, 96-well microtiter plates were coated with purified rGAPDH and serially-diluted mouse serum was added and incubated for 30 min at 37 °C. Then anti-mouse IgG-HRP was added and the samples were incubated for 30 min at 37 °C. To measure IgG subclass, coated plates were incubated with dilutions of mice sera and added 100 μl of goat anti-mouse IgG1-HRP or IgG2a-HRP diluted 1:5000. The color was developed by adding activated substrate solution (sodium citrate buffer containing 1 mg/ml of 3, 3', 5, 5-tetramethylbenzidine and 0.03% H2O2) and reaction was stopped by adding 0.25% hydrochloric acid to each well. The plates were read at an absorbance of 630 nm.

2.9. Determination of cytokines by ELISA

Two weeks following the second vaccination boost, blood samples were collected. The levels of IL-2, primarily secreted by Th1 cells, and IL-4 and IL-10 levels, primarily secreted by Th2 cells, were determined in serum using an ELISA kit (R&D, USA) in accordance with the manufacturer’s instructions.

2.10. Whole blood killing bacteria assay

The whole blood killing bacteria assay was performed as previously described with minor modifications [12,20]. Three mice from each group were anesthetized and the blood was collected by retro orbital blood collection on the 14th day following the final immunization. The bacterial cultures were washed three times with sterilized PBS, and then diluted with PBS. A 10-μl dilution containing 106 bacteria cells was mixed with 190 μl of each inactivated serum sample, which had been diluted 50% with sterilized PBS; the control contained the PBS only. The dilution was incubated for 30 min at 37 °C and then placed on ice. Next, non-immune, heparinized (10 U/ml) mouse whole blood (100 μl) was added and the mixture was incubated at 37 °C with shaking for 1 h. The bacteria were plated on TSA containing 10% newborn calf serum and 1 μg/ml of NAD (Sigma). The bacteria colonies were determined after 24 h. The results were expressed as percent killing according to the following formula: (CFU after 1 h of growth with control serum) – (CFU after 1 h of growth with immune serum)/CFU after 1 h of growth with control serum × 100%. The results were obtained from three independent assays.

2.11. Challenge test

Two weeks after the second vaccination boost, mice immunized with pCgap were divided into groups 1 and 2 (12 mice/group), mice immunized with pcDNA 3.1 vector were divided into groups 3 and 4 (12 mice/group), and mice immunized with PBS were divided into groups 5 and 6 (12 mice/group), respectively. Next, the mice from groups 1, 3, and 5 were challenged intraperitoneally with a lethal dose of 3.0 × 109 CFU of H. parasuis SH0165 strain and the mice from groups 2, 4 and 6 were challenged intraperitoneally with a lethal dose of 3.0 × 109 CFU of H. parasuis MD0322 strain. Mice were monitored for seven days post-challenge, and morbidity and mortality were recorded.

2.12. Histopathology

The lungs from immunized and non-immunized mice that survived H. parasuis challenge were collected and fixed by immersion in 10% neutral-buffered formalin embedded in paraffin, and then 4-μm-wide tissue sections were stained with H&E according to a standard protocol and examined using light microscopy.

2.13. Passive immunization

Passive immunization was determined as previously described with some modifications [21]. Briefly, the newly purchased 32 female BALB/c mice were randomly divided into four groups which were groups 7–10. Groups 7 and 9 (eight mice per group) were intraperitoneally injected with 100 μl of serum from pCgap-immunized mice and groups 8 and 10 (eight mice per group) were intraperitoneally injected with 100 μl of serum from pcDNA3.1 vector-immunized mice. At 24 h post-immunization, groups 7 and 8 were challenged with a lethal dose 3.0 × 109 CFU of H. parasuis SH0165 strain and group 9 and 10 were challenged with a lethal dose 3.0 × 109 CFU of H. parasuis MD0322 strain. All mice were monitored as described above.

2.14. Statistical analysis

The experimental data were expressed as mean ± SD. The difference between two groups was analyzed using the Student’s t-test and the difference among three groups was analyzed using the ANOVA and survival analysis was used the Logrank test. A value of P < 0.05 was considered to indicate a statistically significant result.

3. Results

3.1. Detection of gene gapA in the H. parasuis strains

The results revealed that the gapA gene is widely present in all 15 serovar reference strains as well as the field isolate strains SH0165 and MD0322 (Fig. S2). Moreover, sequence analysis demonstrated that the gapA gene is highly conserved and similar in all 15 serovar reference strains (Fig. S3).

3.2. Gene expression in mammalian cells

IFA and Western blot were utilized to confirm whether the plasmid pCgap could be expressed in mammalian cells. IFA demonstrated that the GAPDH protein was highly expressed in the MDBK cells (Fig. 1A); additionally, no green fluorescence was detected in either cells transfected with vector only (Fig. 1B) or non-transfected MDBK cells (data not shown). Western blot analysis also showed that the GAPDH protein was expressed very well in the MDBK cells (Fig. 1C).

3.3. Antibody response to DNA vaccination in mice

The results indicated that both groups immunized with the pCgap DNA vaccine exhibited a significant antibody response (P<0.05) (Table 1). However, no detectable antibody production was induced in mice immunized with pcDNA 3.1 vector or PBS (Table 1). To further investigate the underlying immune response associated with the DNA vaccine, we performed IgG antibody isotyping. The results demonstrated that the levels of antibody iso- types (IgG1, IgG2a) were significantly higher in the DNA vaccine immunized groups compared to the blank control groups and negative control groups (IgG1, P = 0.0002; IgG2a, P = 0.0001) (Fig. 2A). Additionally, IgG1 titers predominated over IgG2a titers, suggesting a bias toward Th2 type immune response (P= 0.0195) (Fig. 2A).
Table 1
Antibody response of each group during three time immunization.

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Serum IgG titer*</th>
<th>2nd immunization</th>
<th>3rd immunization</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCgap</td>
<td>106.7 ± 46.2 (1:160, 1:80, 1:80)</td>
<td>1066.7 ± 369.5 (1:640, 1:1280, 1:1280)</td>
<td>6826.6 ± 2956.0 (1:5120, 1:10240, 1:5120)</td>
</tr>
<tr>
<td>Negative control</td>
<td>20 ± 0 (1:20, 1:20, 1:20)</td>
<td>26.7 ± 11.5 (1:20, 1:40, 1:20)</td>
<td>26.7 ± 11.5 (1:20, 1:40, 1:20)</td>
</tr>
<tr>
<td>Blank control</td>
<td>13.3 ± 11.5 (1:0, 1:20, 1:20)</td>
<td>20 ± 0 (1:20, 1:20, 1:20)</td>
<td>20 ± 0 (1:20, 1:20, 1:20)</td>
</tr>
</tbody>
</table>

* Serum antibody IgG titer was calculated as the reciprocal of the serum dilution which gave an OD630 of 0.2 above that of the preimmune sera.

3.4. Determination of cytokine production

The levels of cytokines of blood serum samples were analyzed by ELISA. Levels of IL-4 were significantly higher in serum from immunized animals compared to serum from mice that received negative control and blank control (P < 0.0001) (Fig. 2B). Levels of IL-10 production were also significantly higher in immunized animals (P = 0.0005) (Fig. 2B). However, there were no significant increases in IL-2 levels in vaccinated animals versus animals that received negative control and blank control (data not shown).

3.5. Mouse whole-blood killing bacteria assay

The results demonstrated that antiserum against the DNA vaccine significantly inhibited the growth of both strain MD0322 and strain SH0165 (Fig. 3) compared with the negative control groups and blank control groups (P < 0.05); this finding suggests that the antibodies induced by the DNA vaccine potentially confer a degree of immunoprotection against H. parasuis challenge.

3.6. Expression of gene gapA in mice tissues with vaccination

PCR and RT-PCR results demonstrated that both plasmid DNA pCgap and gapA transcripts were present in the kidney, spleen, liver, and muscle of pCgap-immunized mice on the seventh day following the first immunization (Fig. 4). However, no plasmid DNA and gapA transcripts were detected in pcDNA 3.1 vector or PBS-immunized mice (Fig. 4).

3.7. DNA vaccine induces protective efficacy against H. parasuis challenge

The results demonstrated that survival of mice immunized with the DNA vaccine challenged by MD0322 strain was significantly better compared to groups immunized with the DNA vaccine.
challenged by SH0165 strain, 83.3% and 50%, respectively (P < 0.05) (Fig. 5A). Moreover, the immunoprotective effects in groups immunized with the DNA vaccine were greater compared to the negative control groups and blank control groups (P < 0.05 for both), and no immunoprotective effect was observed in the negative control group and blank control group challenged with either MD0322 or SH0165 strain (Fig. 5A). Bacteriological analysis demonstrated that MD0322 or SH0165 strain was the only type of bacterial strain re-isolated from lung and brain of the moribund mice.

3.8. The significance of antibody response to immunoprotection efficacy

To determine whether the serum antibody production induced by pCgap contributed to immunoprotection efficacy, we performed passive immunization. We found that the percentages of surviving mice immunized with serum from pCgap challenged by the MD0322 or SH0165 strain were 62.5% and 37.5%, respectively (Fig. 5B).

3.9. Histopathological analysis

The results demonstrated that in the negative control group and in the blank control group challenged with either SH0165 or MD0322 strain, lung tissues exhibited extensive edema with massive proliferation of fibroblasts and formation of connective tissue (Fig. S4A1, A2, B4 and B5). Bronchioles were filled with cellular exudate containing a large number of neutrophils (Fig. S4A1, A2, B4 and B5). In contrast, only minor pathological changes were observed in lung tissues of animals immunized with the DNA vaccine (Fig. S4A3 and B6). These findings demonstrate that immunization with pCgap prevents pathological changes following challenge infection with H. parasuis, and confirm that the DNA vaccine induces robust immunoprotection against H. parasuis infection in mice.

4. Discussion

Great progress has been made in the development of H. parasuis vaccines, such as subunit vaccines [7,22] and use of attenuated live bacterium [23,24] to control the infection of H. parasuis. DNA vaccines offer certain advantages over conventional protein vaccines in terms of flexibility, potential for rapid manufacture, low cost, and ability to induce cellular immunity [25]. Research has shown that surface-displayed GAPDH of A. hydrophila effectively protects turbot from infection with A. hydrophila and Vibrio anguillarum [26]. In this study, we constructed pCgap and demonstrated that the DNA vaccine could provide 83.3% and 50% protective efficacy against lethal challenge of H. parasuis serovar 4 and 5, respectively. And the apparent homogeneity of response appeared either protected or non-protected among the animals. At the same time we found that there were no differences between protected and non-protected within the vaccinated group among the animals. However, there are currently no vaccines that exhibit a significant effect on infections with all pathogenic strains of H. parasuis. Gene gapA is highly conserved and is present in all 15 serovar reference strains; thus, there is the potential to confer immunoprotection against all pathogenic strains of H. parasuis.

Some previous studies have reported that specific serum antibodies were detected 3–4 weeks post vaccination in mice vaccinated with Actinobacillus pleuropneumoniae DNA vaccine [11,19]. In this study, we found that the DNA vaccine induced significant humoral immune responses, and specific serum antibodies were detected 2 weeks post vaccination. The blood killing bacteria assay showed that antiserum against the DNA vaccine significantly inhibited both MD0322 and SH0165 growth, and passive immunization exhibited a survival rate of 62.5% and 37.5% following challenge with MD0322 or SH0165 strains, respectively. These findings suggest that antibody-mediated immune responses are likely to play an important role in pCgap-induced immunoprotection. Furthermore, because the survival percentages with passive immunization were lower compared to pCgap immunization, alternatively the quantity of antibodies is lower or the distribution of the antibodies is different than in the active immunization.

The relevant IgG antibody subclass and Th type are critical for protection against a particular disease. The IgG1 and IgG2a subclasses have been further determined to be markers of Th helper 2 (Th2) and Th1 immune responses, respectively. Our findings indicated that, although IgG subclass analysis demonstrated production of both IgG1 and IgG2, the higher levels of IgG1 indicated the dominance of Th2 response over Th1 response. In order to further confirm this, cytokine production was determined and there was a significant increase in IL-4 and IL-10 levels in serum from
pCGap immunized mice and continued for some time, which may be related to the stimulation of the DNA vaccine, but long-term existence of cytokines could be prejudicial for the vaccinated individuals and the mechanism needs further study. However, there was no increase in IL-2 levels. These findings, taken together with passive immunization and blood killing bacteria assay results, suggest that the DNA vaccine pCGap is potentially associated with a cellular immune response, leading to greater immune protection efficacy.

In this study, the results demonstrated that pCGap and gene gapA transcripts are present in muscle, spleen, kidney, and liver of the immunized mice at 7 days post-vaccination. These data suggest that some pCGap plasmid enters directly into myocytes, but other plasmid is transported to tissues such as liver, spleen, and kidney in which the plasmid DNA is expressed following immunization. The wide distribution and presence of the plasmid pCGap in the immunized mice potentially leads to a high level of immunoprotection.

An important issue of using conserved antigens as vaccine candidates is that there is a possibility and concern which the antibody against the conserved antigens may interfere with related pathogens and naturally infected animals may produce antibodies against this protein. But there are a number of conserved genes as subunit vaccine in clinical practice such as Hsp70. On the other hand, the antibody against the GAPDH of the H. parasuis protein was not reaction with the GAPDH protein of the MDBK cell itself which proved its specificity and this may be due to different surface antigen recognition sites on the protein.

Taken together, our data suggests that the DNA vaccine pCGap is capable of inducing a significant humoral immune response and effective immunoprotection; it represents a novel approach that is effective against H. parasuis challenge. Therefore, this new vaccine, pCGap, could potentially be used to control and prevent H. parasuis infection. Certainly, the immunoprotection efficacy results in mice would not be the same as the results occurring in pigs. The murine model has been used to evaluate the immune responses and immunoprotection effect against H. parasuis challenges in several studies. Undoubtedly, there will be further research studying the potential in pigs using the pCGap DNA vaccine based on the present mice data.

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