Live-attenuated Pru:Δ*cdpk2* strain of *Toxoplasma gondii* protects against acute, chronic and congenital toxoplasmosis

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Running title: Pru:Δ*cdpk2*-based vaccine for *Toxoplasma gondii*.

Short summary: We have demonstrated the development of a balanced Th1/Th2 immune response in mice following a vaccination regime using *Toxoplasma gondii* Pru:Δ*cdpk2* mutant. Vaccinated mice survived the challenge with homologous and heterologous strains, and were protected against different forms of toxoplasmosis.

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Abstract

Background:

The threat of *Toxoplasma gondii* infection in immunocompromised individuals and pregnant women necessitates the development of a safe and effective vaccine. Here, we examined the immune protection conferred by live-attenuated strain of *T. gondii*.

Methods:

We tested the efficacy of intraperitoneal vaccination using 500 Ca^{2+}-dependent protein kinase 2 (*cdpk2*)-deficient tachyzoites of *T. gondii* Pru strain against acute, chronic and congenital toxoplasmosis in mice. The kinetics of antibody response, cytokines, and other quantifiable correlates of protection against *T. gondii* infection were determined.

Results:

Vaccination with Pru:Δ*cdpk2* induced a high level of anti-*T. gondii* IgG titer, Th1 response at 28 days post-vaccination (dpv), and mixed Th1/Th2 response at 70 dpv. All vaccinated mice survived a heterologous challenge with 1000 tachyzoites of RH or ToxoDB#9 (PYS or TgC7) strains. Also, vaccination protected against homologous infection with 20 *T. gondii* Pru cysts, and improved pregnancy outcome by reducing parasite cyst load in the brain, maintaining litter size and body weight of pups born to vaccinated dams challenged with 10 Pru cysts compared to pups born to unvaccinated dams.

Conclusion:

The use of *T. gondii* Pru:Δ*cdpk2* mutant strain represents a promising approach to protection against acute, chronic and congenital toxoplasmosis in mice.

Keywords: *Toxoplasma gondii*, vaccination, Ca^{2+}-dependent protein kinase 2, attenuated vaccine, protective immunity, toxoplasmosis

INTRODUCTION

Toxoplasma gondii is an obligate intracellular protozoan parasite and the causative agent of toxoplasmosis, a prevalent disease that can affect all warm-blooded animals and humans [1-3]. Infections in immunocompromised individuals, such as organ transplant patients and AIDS patients, can cause severe or even a fatal outcome [1-3]. Primary infection during pregnancy can cause neonatal malformations, miscarriage, chorioretinitis, blindness, mental retardation and hydrocephalus in the infected fetus [3-6]. Current treatments of toxoplasmosis are challenged by side effects of current medications [7] and the occurrence of drug-resistant strains [8]. New approaches are therefore needed to develop more effective interventions for better prevention and control of toxoplasmosis.

Vaccination using live-attenuated strains is promising because it can trigger a protective humoral and cellular immunity through simulating natural infection without leading to the disease [9,10]. A commercially available vaccine, Toxovax®, developed from *T. gondii* S48 strain, is licensed for use in sheep and goats to prevent abortion in the UK, New Zealand, France, and Ireland [11]. Attenuation of the virulence of *T. gondii* through deletion of certain genes has been achieved in several studies [12-18]. The Mic1-3KO RH strain, lacking *mic1* and *mic3* micronemal proteins protected mice against chronic and congenital toxoplasmosis [12] and reduced *T. gondii*-induced abortion and tissue cyst burden in lambs born to vaccinated ewes [13]. Recently, we have shown that immunization of Kunming mice with RH:Δ*GRA17* conferred significant protection against acute, chronic and congenital *T. gondii* infection [14]. Other groups have shown that uracil auxotrophic mutants elicited a protective immunity against acute and chronic *T. gondii* infection in mice [15-18].

Toxoplasma gondii possesses 14 Ca^{2+}-dependent protein kinases (CDPKs), which play important roles in the parasite's motility, colonization, replication and egress [19-23]. Recently, *cdpk2* has been shown to play a role in the regulation of parasite amylopectin synthesis and degradation [23]. Disruption of *cdpk2* induced abnormal accumulation of massive amylopectin granules in *T. gondii* tachyzoites and in the vacuolar space of the residual bodies, together with complete ablation of cyst formation [23]. Because of its inability to establish chronic infection in mice [23], Pru:Δ*cdpk2* mutant has emerged as an attractive candidate to produce a safe and efficacious vaccine [24].

In this study, we determined the protective efficacy of a live attenuated *T. gondii* Pru mutant with a target deletion of *cdpk2* (designated Pru:Δ*cdpk2*), delivered intraperitoneally. Kunming mice vaccinated with 500 Pru:Δ*cdpk2* tachyzoites developed a protective immune response against acute, chronic and congenital toxoplasmosis. We also explored the possibility that if disruption of *cdpk2* in RH strain impairs its virulence in mice, then a RH:Δ*cdpk2* mutant might be a potential vaccine candidate.

MATERIALS AND METHODS

Mice

Eight-week-old Kunming mice were obtained from Laboratory Animal Center of Lanzhou University. All procedures were approved by the Animal Ethics Committee of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences. Kunming mice were used in this study because of their susceptibility to acute and chronic *T. gondii* infection [14, 25-27] and because of their ability to produce more pups per litter, to better assess protection against congenital infection, compared with BALB/c and C57BL/6 mice [28].

Parasites

Tachyzoites of *T. gondii* type I (RH, PYS and TgC7), type II (Pru), and mutant strains RH:Δ*cdpk2* and Pru:Δ*cdpk2* were maintained in human foreskin fibroblast cultures, as previously described

[22, 29]. Cysts of *T. gondii* Pru strain were isolated from brain homogenates of Kunming mice as previously described [25-27].

Disruption of CDPK2 by CRISPR-Cas9 system

CRISPR-Cas9 system was used to knockout *cdpk2* gene as previously described [30]. Guide RNA and primers used in the study to generate mutant Δ*cdpk2* strains are listed in Supplementary Table S1. The pattern of *in vitro* growth of tachyzoites of *T. gondii* Δ*cdpk2* deletion mutant was compared to wild type (WT) strain as previously described [14].

Virulence of Mutants *Versus* Wild-type Strains in Mice

Freshly harvested 200 tachyzoites of RH strain or RH:Δ*cdpk2* mutant, or 1×10^5 tachyzoites of Pru strain or Pru:Δ*cdpk2* mutant were injected intraperitoneally (i.p.) into mice (10 mice/group/parasite strain). These mice were monitored daily for the development of clinical signs of acute illness and mortality was recorded until all mice died.

Vaccination of Mice

In these experiments, the efficacy of Pru:Δ*cdpk2* mutant strain in conferring protective immunity was evaluated against acute, chronic, and congenital *T. gondii* infections (Supplementary Figure

S1). Mice were either vaccinated once with 500 freshly harvested Pru:Δ*cdpk2* tachyzoites or mock-vaccinated in a total of 200 µl PBS i.p. We used i.p. vaccination route because it can trigger both systemic and mucosal immune responses.

Antibody Measurement Post-vaccination

Serum samples were obtained at day 28 and 70 post-vaccination. Total IgG content and subclasses of IgG antibodies, IgG2a and IgG1 as indicators of Th1 and Th2 responses, respectively were tested by ELISA as described [14, 25-27].

Vaccine-Induced Cytokine Production

Mice were sacrificed seventy days post-vaccination (dpv) and single splenocyte suspensions were obtained as previously described [14, 25-27]. Splenocyte cultures were incubated *in vitro* with 10 µg/ml *T. gondii* soluble tachyzoite antigen (STAg) and supernatants were tested by ELISA for the presence of secreted cytokines IL-2 (24-h post-incubation), IL-10 (72-h post-incubation), IL-12 and IFN-γ (96-h post-incubation) in accordance with the manufacturer's instructions (eBioscience® Bender MedSystems GmbH, Austria).

Protection against Acute and Chronic Infection

Seventy dpv, both vaccinated and age-matched naïve mice were challenged i.p. with 200 µl PBS containing 1,000 tachyzoites of RH, ToxoDB#9 (PYS or TgC7) strain to test efficacy against acute infection; or were inoculated orally with 20 Pru cysts to test efficacy against chronic infection. Strains of ToxoDB#9, the predominant genotype in China, have a comparable virulence to type I RH strain [31, 32]. Thus, it was sensible to determine whether immune responses induce by the Pru:Δ*cdpk2* mutant strain protects against infection with the local strains (TgC7 and PYS) of ToxoDB#9 genotype. It was also important to evaluate the ability of Pru:Δ*cdpk2* to confer protection against challenge with Pru strain, the predominant genotype during congenital infection in humans and sheep [33, 34]. The degree of illness and survival of all mice were observed daily for 35 days.

Seven days post-infection (dpi), peritoneal fluid and serum samples collected from Pru:Δ*cdpk2*-vaccinated mice infected with RH, non-vaccinated + RH infected mice, and non-vaccinated + uninfected mice were tested for Th1 cytokines (IFN-γ and IL-12) during acute infection by ELISA. For chronic infection, mice were euthanized at 35 dpi, and their brains were removed and individually homogenized in 1 ml of PBS. Brain cyst's burden was assessed by examining dilutions of DBL-stained brain homogenates using a Zeiss wide-field epifluorescence microscope with 10×objective, as described [14].

Protection Against Congenital Transmission

Female mice vaccinated with 500 Pru:Δ*cdpk2* tachyzoites were mated with male mice 70 dpv. Two female mice were housed in a cage with one male and every 12 h female mice were

inspected for the presence of vaginal plugs. The day of presence of vaginal plug was designated day 1 of gestation. On day 12 of gestation, mice were infected orally with 10 *T. gondii* Pru cysts. Control mouse groups included: non-vaccinated uninfected mice (negative control), and non-vaccinated mice infected orally infected with 10 Pru cysts. The level of protection against congenital *T. gondii* infection was evaluated by analyzing litter size and survival rate of the naturally delivered pups at birth and 35-day old. The body weight of pups at 35-day old were also used to evaluate the protective efficacy. The level of maternal protection in Pru cysts-challenged dams and their pups was determined by quantifying the brain tissue cyst burden in surviving pups at 35 days of age, and in their dams at 30 days after delivery. Maternal splenocytes from pregnant mice challenged with 10 Pru cysts on day 12 of gestation were collected six days later. Splenocyte culture was stimulated with 10 μg/ml STAg and the level of Th1 (IFN-γ, IL-2, IL-12), and Th2 (IL-10) cytokines in vaccinated + infected mice, non-vaccinated + infected mice, and non-vaccinated + uninfected mice was evaluated using ELISA, as described above.

Statistical Analysis

The differences in the level of cytokines, anti-*T. gondii* antibodies and parasite cysts' burdens were compared using two-tailed, unpaired Student's t-test (for comparing means between two groups) or one-way ANOVA analysis (for comparing means between ≥ three groups). The SD was derived from three independently performed experiments with three replicates per

experiment for the *in vitro* assays. Values of $P < 0.05$ were considered statistically significant. Mortality was determined by plotting survival curves of the different mouse groups stratified by *T. gondii* infection and vaccination status by Mantel–Cox log-rank test.

RESULTS

Deletion of *cdpk2* gene in *T. gondii* RH and Pru Strains

The *cdpk2* gene was successfully knocked out in RH and Pru strains using CRISPR-Cas9 system. In both strains, the DHFR* was inserted into the gRNA-targeted coding sequence region through nonhomologous end joining (NHEJ) (Supplementary Figure S2A). Single, stable pyrimethamine-resistant clones were generated and verified by specific PCR method (Supplementary Figure S2B). Phenotypic characterization of RH:Δ*cdpk2* or Pru:Δ*cdpk2* tachyzoites revealed abnormal morphology and excessive accumulation of granules at both the tachyzoites basal end and within the tachyzoites residual body, whereas tachyzoites of the WT RH and Pru strains appeared normal (Supplementary Figure S3). These results demonstrate that *cdpk2* gene was successfully knocked out in the mutant RH and Pru strains.

Disruption of *cdpk2* does not affect virulence in mice

Deletion of *cdpk2* gene did not result in attenuation of the virulence of mutant RH or Pru strains, as indicated by the comparable survival rates of mice infected with the parental WT or mutant

strains (Supplementary Figure S4). In an effort to achieve the required level of immunogenicity of the attenuated Pru:Δ*cdpk2* strain, without inducing an excessive immune response or death in the vaccinated mice, we reduced the number of type II Pru:Δ*cdpk2* tachyzoites used in the vaccination experiments from 10^5 to 500 tachyzoites. Mice challenged with 500 Pru:Δ*cdpk2* mutant tachyzoites survived and consistent with previous work [23], tissue cysts were not detected in the brain of mice challenged with 500 Pru:Δ*cdpk2* tachyzoites, but brain tissue cysts were detected in mice challenged with 500 WT Pru tachyzoites (data not shown).

Humoral immune responses induced by vaccination

The immunogenicity of Pru:Δ*cdpk2* was assessed by determination of specific anti-*T. gondii* IgG antibody titers and IgG isotypes in the serum of vaccinated mice at 28 and 70 dpv by quantitative ELISA. At day 28 post-vaccination, all vaccinated mice had seroconverted with a higher level of anti-*T. gondii* IgG antibodies compared with non-vaccinated mice. This level of IgG titer remained high at 70 dpv (Figure 1). These results suggest that Pru:Δ*cdpk2* induced a strong humoral response. We then tested whether a Th1 and Th2 response was elicited in the vaccinated mice by evaluating the levels of STAg-specific IgG subclasses, IgG2a and IgG1 isotypes, respectively. Compared to non-vaccinated mice, the level of IgG2a was significantly higher in vaccinated mice at 28 and 70 dpv. The level of IgG1 was only increased in the vaccinated mice at 70 dpv, compared with non-vaccinated mice (Figure 1). These results suggest

that vaccination with Pru:Δ*cdpk2* in mice elicits a Th1-type immune response at 28 dpv and a mixed Th1/Th2 immune response at 70 dpv.

Cytokine production after vaccination

Cytokine production in splenocyte culture supernatants stimulated with STAg was assessed by ELISA at 70 dpv. The levels of Th1 type cytokines (IFN-γ, IL-2 and IL-12) were significantly higher than those in non-vaccinated mice. Also, the level of Th2 type cytokine (IL-10) was significantly higher than that of non-vaccinated mice (Figure 2).

Pru:Δ*cdpk2* immunogenicity and potency for protection against acute infection

Kunming mice were vaccinated with 500 Pru:Δ*cdpk2* tachyzoites, and 70 days later, challenged with 1,000 tachyzoites of *T. gondii* type I RH strain, or ToxoDB#9 (PYS or TgC7) strain. As expected, all non-vaccinated mice infected with 1,000 RH strain or ToxoDB#9 (PYS or TgC7) strains died within 10 days after infection, whereas all mice vaccinated survived regardless of the challenging strains (Figure 3). We further analyzed Th1 cytokines (IL-12 and IFN-γ) in the serum and peritoneal fluids at 7 days after infection. Significantly elevated levels of IL-12 and IFN-γ were found in the non-vaccinated + RH infected mice, while only modestly elevated levels of the same cytokines was observed in Pru:Δ*cdpk2* vaccinated + RH infected mice (Figure 4).

Protection against chronic infection

The protective efficacy of vaccination with Pru:Δ*cdpk2* against chronic infection was investigated in Kunming mice at 70 dpv. All Pru:Δ*cdpk2*-vaccinated mice survived infection with 20 *T. gondii* Pru parasite cysts, whereas only 40% of non-vaccinated infected mice survived (Figure 5A). At 35 days post-challenge, parasite cyst burden in the brain of survived Pru:Δ*cdpk2*-vaccinated compared with non-vaccinated mice was determined. Non-vaccinated mice challenged with 20 Pru cysts had 4296 ± 687 cysts per brain, whereas Pru:Δ*cdpk2*-vaccinated mice challenged with the same number of Pru cysts had significantly fewer cysts per brain (78 ± 48 cysts/brain) ($P < 0.001$; Figure 5B).

Protection Against Congenital Toxoplasmosis

Pregnant mice were orally challenged with 10 *T. gondii* Pru cysts on day 12 of gestation, the litter size and body weight of the neonates were determined. The cyst burden in the brain of neonates and dams was evaluated. The litter size and survival of pups of Pru:Δ*cdpk2*-vaccinated + Pru cysts infected dams, was similar to that of non-vaccinated uninfected mice **(**Figure 6A). Body weight of neonates born to Pru:Δ*cdpk2*-vaccinated + Pru cysts infected dams was similar to neonates born to non-vaccinated uninfected dams (Figure 6B). In contrast, the litter size and body weight of pups of non-vaccinated + Pru cysts infected mice were significantly lower when compared to that of non-vaccinated uninfected mice, or Pru:Δ*cdpk2*-vaccinated + Pru cysts

infected mice. The brain cyst burden in the neonates was determined at day 35 post-partum. The average brain cyst number in all neonates (n = 19) born to non-vaccinated + Pru cysts infected dams was 919 ± 339. In contrast, 41.4% (24 out of 58) of neonates born to Pru:Δ*cdpk2*-vaccinated + Pru cysts infected dams had an average brain cyst number of (60 ± 33). Interestingly, examination of the brain of 58.6% (34 of 58) of neonates born to vaccinated + infected dams revealed no cysts. The brain cyst burden in dams was also determined at day 30 post-partum. The average brain cyst number was significantly higher in non-vaccinated + Pru cysts infected dams (3287 ± 569 cysts/brain) than in that of Pru:Δ*cdpk2*-vaccinated + Pru cysts infected dams (77 ± 58 cysts/ brain).

Immune Responses to Infection During Pregnancy

The levels of *T. gondii*-specific Th1 (IFN-γ, IL-12, IL-2) and Th2 (IL-10) cytokines from Pru:Δ*cdpk2*-vaccinated + Pru cysts infected mice were significantly higher compared with those in non-vaccinated + Pru cysts infected mice, and non-vaccinated uninfected mice ($P < 0.001$; Figure 7). However, the difference in the cytokines levels between non-vaccinated + Pru cysts infected, and non-vaccinated uninfected mice were not statistically significant ($P > 0.05$).

DISCUSSION

A highly effective vaccine against toxoplasmosis is urgently required, with leading vaccination strategies targeting both horizontal transmission and vertical transmission from pregnant dams to their offspring. Previous study has shown that *cdpk2* is essential for the development of viable *T. gondii* tissue cysts, as *cdpk2*-deficient parasite strains failed to form cysts in the brain of mice [23]. Based on this observation, we inferred that *cdpk2*-deficient *T. gondii* might be a promising live-attenuated vaccine to prevent toxoplasmosis. In the present study, *cdpk2* was successfully disrupted in *T. gondii* RH and Pru strains using CRISPR-Cas9 method, and the mutant strains exhibited the anticipated morphological abnormalities, and no parasite cyst was detected in the brain of mice challenged with Pru:Δ*cdpk2* tachyzoites, which was consistent with previous study [23].

Our results showed that deletion of *cdpk2* gene did not result in the attenuation of the virulence of RH or Pru strains, as indicated by the comparable survival rates of mice infected with the parental WT (RH or Pru) or mutant (RH:Δ*cdpk2* or Pru:Δ*cdpk2*) strains. A high dose (10^5) of Pru:Δ*cdpk2* tachyzoites caused mice death in 11 days, however all mice infected with the same strain, but with a smaller dose (500) survived and no parasite cyst was detected in their brain. Therefore, 500 Pru:Δ*cdpk2* tachyzoites were used in all subsequent experiments as the vaccination dose because it exhibited the type of balance between safety and high immunogenicity that should be expected of a promising live-attenuated vaccine strain.

Consistent with other attenuated *T. gondii* vaccines, vaccination with Pru:Δ*cdpk2* induced a high level of anti-*T. gondii* IgG antibodies in mice [12-18]. These specific IgG antibodies can neutralize the attachment of *T. gondii* and limit its ability to establish infection [35-38].

Pru:Δ*cdpk2*-vaccinated mice also developed a sequential Th1 and mixed Th1/Th2-type immune response, as indicated by the high level of Th1 immunity (IgG2a) at day 28 post-vaccination; and the elevation of Th2 protective (IgG1) level at day 70 dpv when compared to that in non-vaccinated mice. The high level of IgG2a and IgG1 in Pru:Δ*cdpk2*-vaccinated mice was substantiated by high levels of Th1 cytokines (IFN-γ, IL-2 and IL-12) and Th2 cytokines (IL-10) at 70 dpv. Indeed, these immune response induced by Pru:Δ*cdpk2* vaccination completely protected mice (i.e. 100% survival rate) post-challenge with RH, or ToxoDB#9 (PYS or TgC7) strain, whereas non-vaccinated mice died within 10 dpi. In addition, Pru:Δ*cdpk2* significantly reduced the development of chronic toxoplasmosis. The parasite cysts were significantly reduced in the brain of vaccinated mice after infection with 20 cysts of *T. gondii* Pru strain compared with non-vaccinated + Pru cysts infected mice. In our study, Th1 cytokines from the non-vaccinated + RH-infected mice was significant higher than that from the Pru:Δ*cdpk2* vaccinated + RH-infected mice, indicating that vaccination with Pru:Δ*cdpk2* may have down-regulated Th1 response and reduced the severe inflammatory response that accompanies acute *T. gondii* infection [39-42].

Th1-type cytokine response with high level of IFN-γ, IL-2 and IL-12, are needed to limit congenital transmission and to protect pregnant mice against *T. gondii* infection [43-46]. However, Th1-type immune response can be detrimental to pregnancy and potentially deleterious for the conceptus [43, 44]. Thus, a balanced Th1/Th2 immune response detected at day 70 post-vaccination seems to contribute to the observed successful pregnancy outcomes; because unsuccessful pregnancies were observed at day 28 post-vaccination (data not shown)

where Th1 response was dominating (Figure 1). Therefore, the high level of Th2-related anti-inflammatory cytokine, IL-10, which suppresses the production of Th1 cytokines (IFN-γ and IL-12) detected in Pru:Δ*cdpk2*-vaccinated pregnant mice infected with Pru cysts on day 12 of gestation (Figure 7) may have contributed to better pregnancy outcomes when compared to non-vaccinated + Pru-infected pregnant mice. Higher IL-10 levels appear to play a role in protection against infection, because IL-10-deficient mice died during acute *T. gondii* infection due to a strong Th1 inflammatory response [47]. Previous studies showed that pregnancy outcomes can be improved in *T. gondii*-infected mice by administration of recombinant IL-10 and can be worsened in IL-10-deficient mice [48]. Despite the inhibitory effect of maternal and fetal Th2 polarized immune response that occurs during pregnancy on Th1 cytokine production, vaccination seems to elicit a protective immune response that enabled the pregnant mice to control the infection. These results show that vaccination of mice with Pru:Δ*cdpk2* achieved a critical balance between Th1 and Th2 responses for optimal control of infection, while minimizing overt inflammation and severe pathology.

Although the immune responses induced by Pru:Δ*cdpk2* vaccination cannot completely block the vertical transmission, a statistically lower brain cyst burden was observed in pups from Pru:Δ*cdpk2*-vaccinated + Pru-infected dams, compared to pups of non-vaccinated + Pru-infected dams. In addition, there were significantly less numbers of brain cysts in Pru:Δ*cdpk2*-vaccinated + Pru-infected dams than in non-vaccinated + Pru-infected dams. Also, neonates born to Pru:Δ*cdpk2*-vaccinated + Pru-infected dams had similar body weights to neonates born to non-vaccinated + uninfected mice. These results indicate that vaccination with

Pru:Δ*cdpk2* can confer significant protection against subsequent oral infection with Pru cysts during the second trimester of gestation. Unexpectedly, infection with *T. gondii* Pru strain at 12 days of gestation failed to elicit more cytokines in non-vaccinated + Pru-infected mice, compared with non-vaccinated + uninfected mice (Figure 7). At present, the reason for this observation is unknown. However, it is possible that the time elapsed after infection was not long enough to elicit a detectable level of cytokines nor to induce potent CD8+ T cell responses without prior antigenic stimulation.

In conclusion, data reported in this study demonstrate that a single i.p. vaccination of 500 Pru:Δ*cdpk2* tachyzoites provides cross-protection against homologous and heterologous challenge with multiple *T. gondii* strains of the same and different genotypes in mice. Pru:Δ*cdpk2* strain generated an immune response, which improved the survival rate and reduced parasite cyst burden in the brain of the vaccinated mice. Strong protection against congenital toxoplasmosis was demonstrated by a significant reduction in the brain tissue cyst burden in the pups and their dams, along with improvement in the body weight and survival rate of pups born to vaccinated dams compared with non-vaccinated dams. Given the potential efficacy of Pru:Δ*cdpk2* live-attenuated vaccine, the data generated in Kunming mice merit further exploration and should be evaluated in larger animals, such as sheep.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

NOTES

***Authors contributions*.** XQZ, SYH and HME conceived the project, designed the experiments, and critically revised the manuscript. JLW, TTL and KC performed the experiments and analyzed the data. JLW drafted the manuscript. WC, WBY and MJB helped in the implementation of the study. All authors reviewed and approved the final version of the manuscript.

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Potential conflicts of interest. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REFERENCES

1. Montoya JG, Liesenfeld O. Toxoplasmosis. Lancet **2004**; 363:1965-76.

2. Robert-Gangneux F, Belaz S. Molecular diagnosis of toxoplasmosis in immunocompromised patients. Curr Opin Infect Dis **2016**; 29:330-9.

3. Petersen E, Pollak A, Reiter-Owona I. Recent trends in research on congenital toxoplasmosis. Int J Parasitol **2001**; 31:115-44.

4. Robert-Gangneux F. It is not only the cat that did it: how to prevent and treat congenital toxoplasmosis. J Infect **2014**; 68 Suppl 1:S125-33.

5. Pomares C, Montoya JG. Laboratory diagnosis of congenital toxoplasmosis. J Clin Microbiol **2016**; 54:2448-54.

6. Elbez-Rubinstein A, Ajzenberg D, Dardé ML, et al. Congenital toxoplasmosis and reinfection during pregnancy: case report, strain characterization, experimental model of reinfection, and review. J Infect Dis **2009**; 199:280-5.

7. Alday PH, Doggett JS. Drugs in development for toxoplasmosis: advances, challenges, and current status. Drug Des Devel Ther **2017**; 11:273-293.

8. Doliwa C, Escotte-Binet S, Aubert D, et al. Sulfadiazine resistance in *Toxoplasma gondii*: no involvement of overexpression or polymorphisms in genes of therapeutic targets and ABC transporters. Parasite **2013**; 20:19.10.

9. Hiszczyńska-Sawicka E, Gatkowska JM, Grzybowski MM, et al. Veterinary vaccines against toxoplasmosis. Parasitology **2014**; 141:1365-78.

10. Zhang NZ, Wang M, Xu Y, et al. Recent advances in developing vaccines against *Toxoplasma gondii*: an update. Expert Rev Vaccines **2015**; 14:1609-21.

11. Buxton D, Thomson K, Maley S, et al. Vaccination of sheep with a live incomplete strain (S48) of *Toxoplasma gondii* and their immunity to challenge when pregnant. Vet Rec **1991**; 129:89-93.

12. Ismael AB, Dimier-Poisson I, Lebrun M, et al. Mic1-3 knockout of *Toxoplasma gondii* is a successful vaccine against chronic and congenital toxoplasmosis in mice. J Infect Dis **2006**; 194:1176-83.

13. Mévélec MN, Ducournau C, Bassuny Ismael A, et al. Mic1-3 Knockout *Toxoplasma gondii* is a good candidate for a vaccine against *T. gondii*-induced abortion in sheep. Vet Res **2010**; 41:49.

14. Wang JL, Elsheikha HM, Zhu WN, et al. Vaccination with *Toxoplasma gondii* GRA17 deletion mutant induces partial protection and survival in challenged mice. Front Immunol **2017**; 8:730.

15. Fox BA, Bzik DJ. De novo pyrimidine biosynthesis is required for virulence of *Toxoplasma gondii*. Nature **2002**; 415:926-9.

16. Gigley JP, Fox BA, Bzik DJ. Long-term immunity to lethal acute or chronic type II *Toxoplasma gondii* infection is effectively induced in genetically susceptible C57BL/6 mice by vaccination with an attenuated type I vaccine strain. Infect Immun **2009**; 77:5 380-8.

17. Fox BA, Bzik DJ. Avirulent uracil auxotrophs based on disruption of orotidine-5'-monophosphate decarboxylase elicit protective immunity to *Toxoplasma gondii*. Infect Immun **2010**; 78:3744-52.

18. Fox BA, Bzik DJ. Nonreplicating, cyst-defective type II *Toxoplasma gondii* vaccine strains stimulate protective immunity against acute and chronic infection. Infect Immun **2015**; 83:2148-55.

19. Long S, Wang Q, Sibley LD. Analysis of noncanonical calcium-dependent protein kinases in *Toxoplasma gondii* by targeted gene deletion using CRISPR/Cas9. Infect Immun **2016**; 84:1262-73.

20. Billker O, Lourido S, Sibley LD. Calcium-dependent signaling and kinases in apicomplexan parasites. Cell Host Microbe **2009**; 5:612-22.

21. Lourido S, Shuman J, Zhang C, et al. Calcium-dependent protein kinase 1 is an essential regulator of exocytosis in *Toxoplasma*. Nature **2010**; 465:359-62.

22. Wang JL, Huang SY, Li TT, et al. Evaluation of the basic functions of six calcium-dependent protein kinases in *Toxoplasma gondii* using CRISPR-Cas9 system. Parasitol Res **2016**; 115:697-702.

23. Uboldi AD, McCoy JM, Blume M, et al. Regulation of starch stores by a Ca(2+)-dependent protein kinase is essential for viable cyst development in *Toxoplasma gondii*. Cell Host Microbe **2015**; 18:670-81.

24. Tomavo S. Too much sugar puts a parasite in jeopardy. Cell Host Microbe **2015**; 18:641-3.

25. Chen J, Li ZY, Petersen E, et al. DNA vaccination with genes encoding *Toxoplasma gondii* antigens ROP5 and GRA15 induces protective immunity against toxoplasmosis in Kunming mice. Expert Rev Vaccines **2015**; 14:617-24.

26. Zhang NZ, Xu Y, Wang M, et al. Protective efficacy of two novel DNA vaccines expressing *Toxoplasma gondii* rhomboid 4 and rhomboid 5 proteins against acute and chronic toxoplasmosis in mice. Expert Rev Vaccines **2015**; 14:1289-97.

27. Li ZY, Chen J, Petersen E, et al. Synergy of mIL-21 and mIL-15 in enhancing DNA vaccine efficacy against acute and chronic *Toxoplasma gondii* infection in mice. Vaccine **2014**; 32:3058-65.

28. Liu X, Liu K, Yang L, et al. Measurement and analysis of the reproductive performance in six commonly used SPF mice and rats. Chin J Comp Med **2016**; 10: 9-13. (Translated from Chinese).

29. Wang JL, Li TT, Elsheikha HM, et al. Functional characterization of rhoptry kinome in the virulent *Toxoplasma gondii* RH Strain. Front Microbiol **2017**; 8:84.

30. Shen B, Brown KM, Lee TD, et al. Efficient gene disruption in diverse strains of *Toxoplasma gondii* using CRISPR/CAS9. MBio **2014**; 5:e01114-14.

31. Gao JM, Xie YT, Xu ZS, et al. Genetic analyses of Chinese isolates of *Toxoplasma gondii* reveal a new genotype with high virulence to murine hosts. Vet Parasitol **2017**; 241:52-60.

32. Shwab EK, Zhu XQ, Majumdar D, et al. Geographical patterns of *Toxoplasma gondii* genetic diversity revealed by multilocus PCR-RFLP genotyping. Parasitology **2014**; 141:453-61.

33. Ajzenberg D, Conge´ N, Paris L, et al. Genotype of 86 *Toxoplasma gondii* isolates associated with human congenital toxoplasmosis, and correlation with clinical findings. J Infect Dis **2002**; 186:684-9.

34. Owen MR, Trees AJ. Genotyping of *Toxoplasma gondii* associated with abortion in sheep. J Parasitol **1999**; 85:382-4.

35. Sayles PC, Gibson GW, Johnson LL. B cells are essential for vaccination-induced resistance to virulent *Toxoplasma gondii*. Infect Immun **2000**; 68:1026-33.

36. Hunter CA, Sibley LD. Modulation of innate immunity by *Toxoplasma gondii* virulence effectors. Nat Rev Microbiol **2012**; 10:766-78.

37. Pifer R, Yarovinsky F. Innate responses to *Toxoplasma gondii* in mice and humans. Trends Parasitol **2011**; 27:388-93.

38. Spellberg B, Edwards JE Jr: Type 1/Type 2 immunity in infectious diseases. Clin Infect Dis **2001**; 32:76-102.

39. Oldenhove G, Bouladoux N, Wohlfert EA, et al. Decrease of Foxp3+ Treg cell number and acquisition of effector cell phenotype during lethal infection. Immunity **2009**; 31:772-86.

40. Tenorio EP, Fernández J, Castellanos C, et al. CD4+ Foxp3+ regulatory T cells mediate *Toxoplasma gondii*-induced T-cell suppression through an IL-2-related mechanism but independently of IL-10. Eur J Immunol **2011**; 41:3529-41.

41. Mordue DG, Monroy F, La Regina M, et al. Acute toxoplasmosis leads to lethal overproduction of Th1 cytokines. J Immunol **2001**; 167:4574-84.

42. Gazzinelli RT, Wysocka M, Hieny S, et al. In the absence of endogenous IL-10, mice acutely infected with *Toxoplasma gondii* succumb to a lethal immune response dependent on CD4+ T cells and accompanied by overproduction of IL-12, IFN-gamma and TNF-alpha. J Immunol **1996**; 157:798–805.

43. Menzies FM, Henriquez FL, Roberts CW. Immunological control of congenital toxoplasmosis in the murine model. Immunol Lett **2008**; 115:83-9.

44. Zhang YH, He M, Wang Y, Liao AH. Modulators of the balance between M1 and M2 macrophages during pregnancy. Front Immunol **2017**; 8:120.

45. Shirahata T, Muroya N, Ohta C, et al. Enhancement by recombinant human interleukin 2 of host resistance to *Toxoplasma gondii* infection in pregnant mice. Microbiol Immunol **1993**; 37:583-90.

46. Abou-Bacar A, Pfaff AW, Letscher-Bru V, et al. Role of gamma interferon and T cells in congenital *Toxoplasma* transmission. Parasite Immunol **2004**; 26:315-8.

47. Nguyen TD, Bigaignon G, Markine-Goriaynoff D, et al. Virulent *Toxoplasma gondii* strain RH promotes T-cell-independent overproduction of proinflammatory cytokines IL12 and gamma-interferon. J Med Microbiol **2003**; 52: 869-76.

48. Lao K, Zhao M, Li Z, et al. IL-10 regulate decidual Tregs apoptosis contributing to the abnormal pregnancy with *Toxoplasma gondii* infection. Microb Pathog **2015**; 89:210-6.

Figures legends

Figure 1: Humoral response and antibody isotype profile in the serum of mice vaccinated with Pru:Δ*cdpk2* by intraperitoneal route. Levels of IgG and IgG subclass (IgG2a and IgG1) antibodies were evaluated in the sera of mice at 28 and 70 days after Pru:Δ*cdpk2*-vaccination compared to non-vaccinated mice. The patterns of IgG2a to IgG1 show the induction of a Th1 immune response at day 28 post-vaccination, followed by a mixed Th1/Th2 immune response at day 70 post-vaccination. Results are expressed as mean of OD450 ± SD. Significance compared with control (uninfected + non-vaccinated) mice: ***$P < 0.001$.

Figure 2: Levels of Th1 and Th2 cytokines produced by splenocytes culture of Pru:Δ*cdpk2*-vaccinated mice. Spleen cells from six mice were obtained 70 days post-vaccination and stimulated *in vitro* with 10 µg/ml soluble *T. gondii* tachyzoite antigen (STAg). Cell-free supernatants were harvested and evaluated for Th1 (IFN-γ, IL-2 and IL-12) and Th2 (IL-10) cytokines using ELISA. Significance compared with control (uninfected + non-vaccinated) mice: $^{**}P < 0.01$, $^{***}P < 0.001$.

Figure 3: Protection of mice against acute *Toxoplasma gondii* infection. Survival curves of Pru:Δ*cdpk2*-vaccinated mice, challenged intraperitoneally with 10^3 tachyzoites of RH, PYS, or TgC7 strains 70 days post-vaccination. The survival of mice was monitored for 35 days. A Log-rank (Mantel–Cox) test showed significant difference in the survival rates between vaccinated + infected groups compared to non-vaccinated+ infected groups ($P = 0.003$). All mice in the vaccinated groups remained alive at day 35 after infection, but all mice in the non-vaccinated groups died between day 7 and 10 after infection. In addition to the separate curves that show the difference in the survival rate between vaccinated and non-vaccinated mouse groups infected with RH, PYS, or TgC7 strains (A–C), the overall survival rates in all vaccinated mouse groups compared with the non-vaccinated mouse groups were combined in one graph (D).

Figure 4: Pro-inflammatory cytokines produced by Pru:Δ*cdpk2*-vaccinated mice after infection with *Toxoplasma gondii* RH. Mice were challenged with 10^3 RH tachyzoites 70 days post-vaccination and levels of IFN-γ and IL-12 in the serum and peritoneal washes were assessed by ELISA at 7 days post infection. Highest levels of pro-inflammatory cytokines were found in the non-vaccinated infected mice. Significance compared with uninfected and non-vaccinated control mice: $^{**}P < 0.01$, $^{***}P < 0.001$.

Figure 5: Vaccination with Pru:Δ*cdpk2* promoted survival and reduced brain cyst's burden after infection. (A) Survival curves following challenge of Pru:Δ*cdpk2*-vaccinated mice with 20 cysts of *T. gondii* Pru strain, compared with non-vaccinated mice, 70 days post-vaccination. The survival of mice was monitored for 35 days. A Log-rank (Mantel–Cox) test demonstrated a significant difference between the vaccinated and non-vaccinated groups ($P = 0.0001$). **(B)** Cyst burden in the brain of mice that survived to 35 days after challenge (Pru:Δ*cdpk2*-vaccinated + Pru-infected mice *vs* non-vaccinated + Pru-infected mice). Data points indicate means ± SD. $^{***}P < 0.001$

Figure 6: Protection of mice against type II Pru cyst infection on day 12 of gestation. (A) The litter size and survival of pups from non-vaccinated uninfected mice, non-vaccinated + Pru-infected mice, and Pru:Δ*cdpk2*-vaccinated + Pru-infected mice was assessed at birth and 35 days after birth. **(B)** The average body weight of 35-day-old pups. Data

points indicate means ± SD. Significance compared with control (uninfected + non-vaccinated) mice: ***$P < 0.001$.

Figure 7: Profile of *Toxoplasma gondii*-specific Th1 and Th2 cytokines in Pru:Δ*cdpk2*-vaccinated mice after infection with Pru cysts. Vaccinated, pregnant mice were infected with *T. gondii* Pru cysts 12 days of gestation and spleen cells were collected 6 days after infection. Splenocyte culture was stimulated with 10 µg/ml *T. gondii* soluble tachyzoite antigen. Cell-free supernatants were evaluated for Th1 (INF-γ, IL-2 and IL-12) and Th2 (IL-10). Cytokine concentrations represent mean ± SD after subtraction of background control values with medium only. Significance compared with control (uninfected + non-vaccinated) mice: ***$P < 0.001$.

Supplementary material

Supplementary TABLE S1: General information of guide RNA (gRNA) and primers used in this study

Supplementary Figure S1: Schematic illustration of the vaccination regimens. Experimental overview of the present investigation from vaccination of Kunming mice with 500 Pru:Δ*cdpk2* tachyzoites **(A)**, assessment of immune response in the serum of vaccinated mice prior to

infection **(B)** to the evaluation of the immunogenicity and protection of vaccination against acute infection **(C)**, chronic infection **(D)** and congenital infection **(E)**. For simplicity, only experiments that involved the vaccinated and infected groups are shown. More details about other experimental mouse groups and controls can be found in "Materials and Methods." Abbreviations: i.p., intraperitoneal; dpi, days post infection.

Supplementary Figure S2: Generation of *cdpk2* mutant strains using CRISPR-Cas9. A. Schematic illustration of gene disruption by insertion of a pyrimethamine-resistant DHFR (DHFR*) cassette into the coding sequence of *cdpk2* gene. **B.** KO-*cdpk2*-F and KO-*cdpk2*-R primers were used to amplify the small fragment around the gRNA. Small fragment was lost due to insertion of the larger fragment created by insertion of DHFR* under short extension times.

Supplementary Figure S3: Morphological characteristics of *Toxoplasma gondii* Δ*cdpk2* mutant strains growing in human foreskin fibroblast cells. Parasite morphology was examined by indirect fluorescence staining using anti-SAG1 antibody that labels the extracellular coat of the parasite. The arrows indicate the site of amylopectin accumulation in RH:Δ*cdpk2* and Pru:Δ*cdpk2* strains. Tachyzoites of wild-type RH and Pru strains appeared normal.

Supplementary Figure S4: Comparative virulence between *Toxoplasma gondii* wild-type and *cdpk2* mutant RH and Pru strains in mice. The survival rate of Kunming mice infected

with wild-type (WT) RH and Pru strains were compared to their respective mutant strains. Mice were infected intraperitoneally (i.p.) with 200 tachyzoites of WT RH or RH:Δ*cdpk2* strain, or with 10^5 tachyzoites of WT Pru or Pru:Δ*cdpk2* strain. In addition to separate curves that show the difference in the survival rate between WT RH and Pru strains and their corresponding mutant strains (A–D), the overall survival rates in all infected mouse groups were combined in one graph (E).

Figure 1.

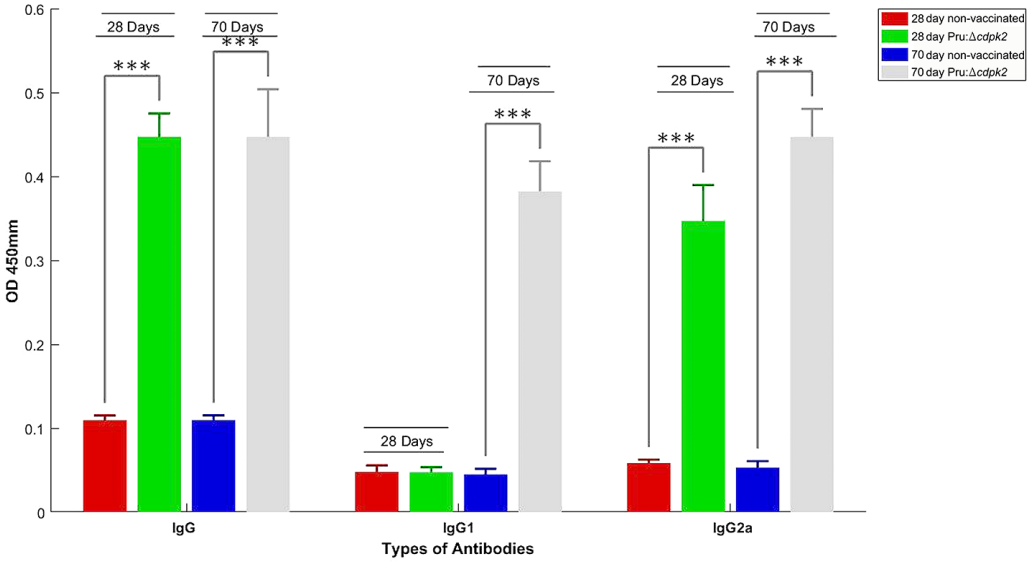
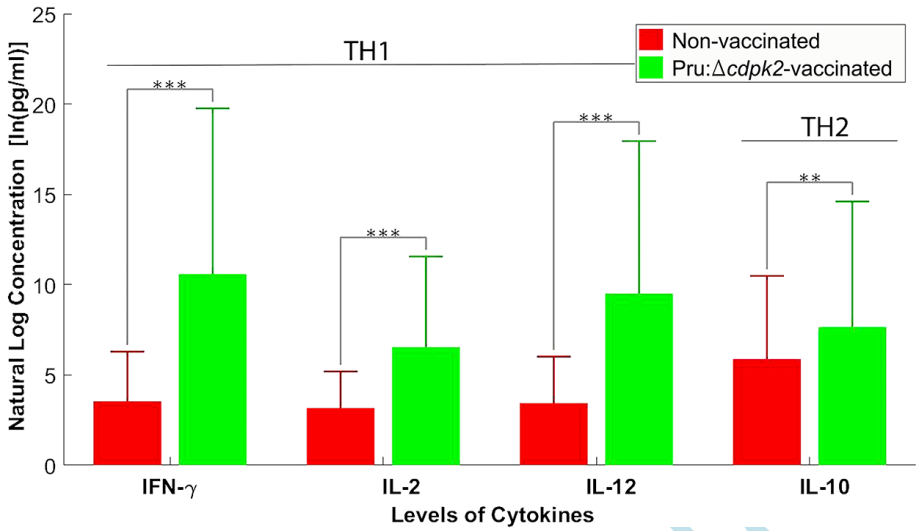


Figure 2.



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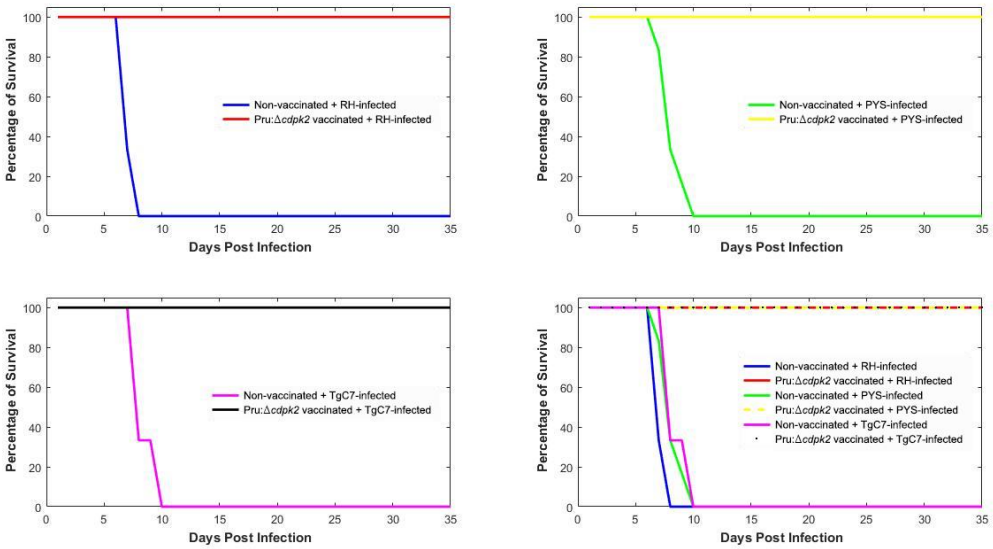

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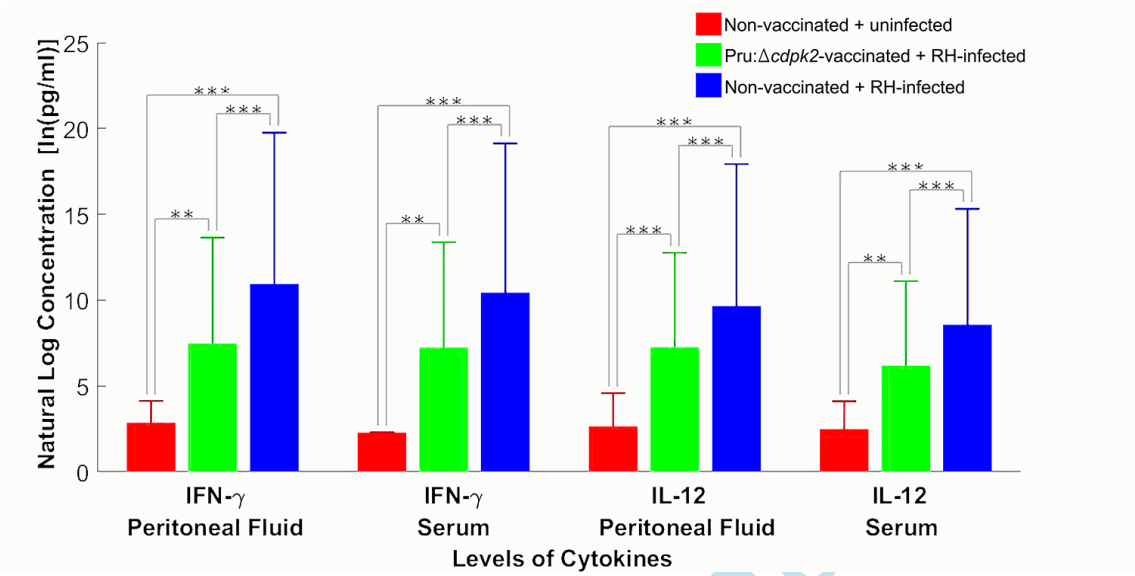

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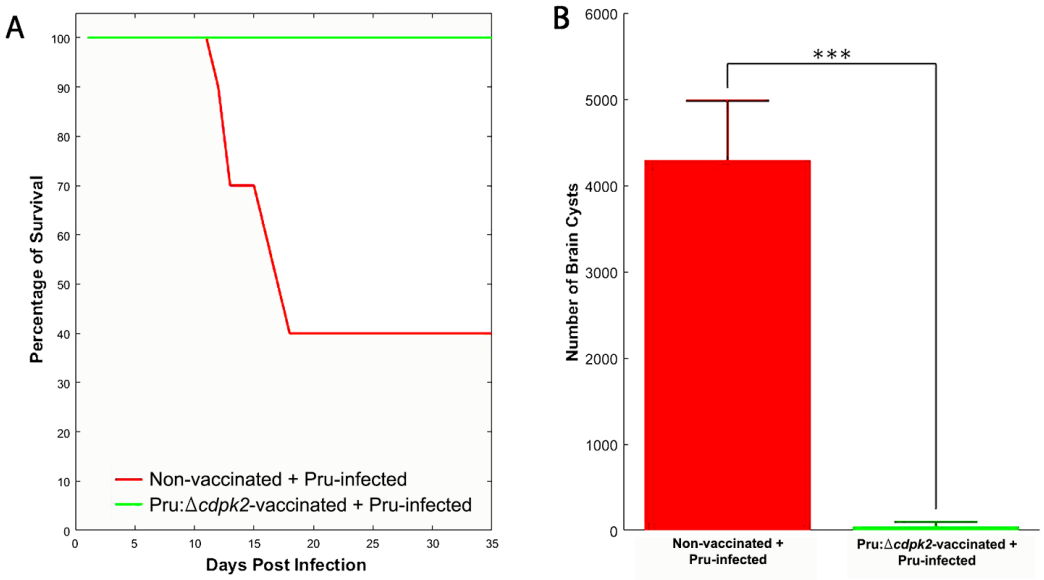

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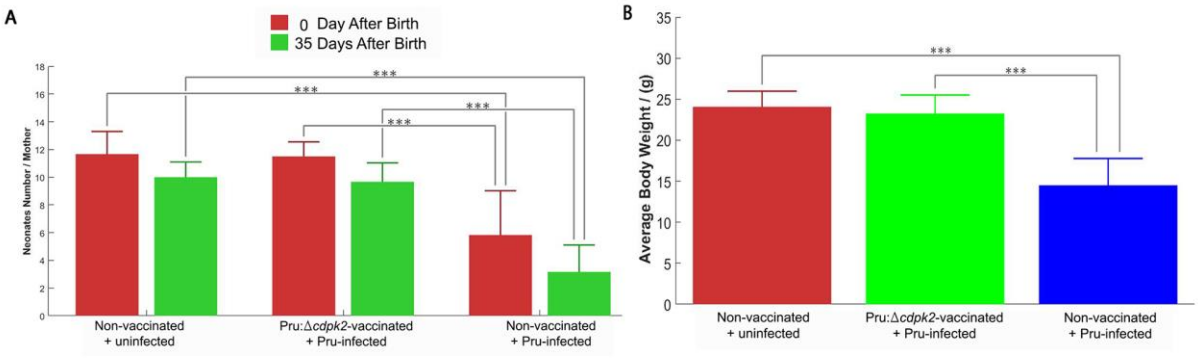

Figure 7.