Immunogenicity of a New Enhanced Tetanus-reduced Dose Diphtheria-acellular Pertussis (Tdap) Vaccine Against *Bordetella Pertussis* in a Murine Model

Kyu Ri Kang
Catholic University of Korea
Dong Ho Huh
The Vaccine Bio Research Institute, Catholic University of Korea
Ji Ahn Kim
The Vaccine Bio Research Institute, The Catholic University of Korea
Jin Han Kang (kjhan@catholic.ac.kr)
Catholic University of Korea School of Medicine

Research article

Keywords: Tetanus-reduced dose diphtheria-acellular pertussis vaccine, immunogenicity, mouse study

Posted Date: December 16th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-127484/v1

Abstract

Background

The necessity of the tetanus-reduced dose diphtheria-acellular pertussis (Tdap) vaccine in adolescence and adults has been emphasized since the resurgence of small-scale pertussis in Korea and worldwide due to the waning effect of the vaccine and variant pathogenic stains in the late 1990s. GreenCross Pharma (GC Pharma), a Korean company, developed the Tdap vaccine GC3111 in 2010. Recently, they enhanced the former vaccine GC3111 to reinforce the antibody response against filamentous hemagglutinin (FHA). In this study, the immunogenicity and efficacy of the enhanced Tdap vaccine were compared and evaluated between the former Tdap vaccine GC3111 and the commercially available Tdap vaccine in a murine model.

Methods

Balb/C mice were primed with two doses of the diphtheria-tetanus-acellular pertussis (DTaP) vaccine followed by a single booster Tdap vaccine at 6 weeks using the commercially available Tdap vaccine or 2 Tdap vaccines from GC Pharma (GC3111, enhanced GC3111). Humoral response was assessed 1 week before and 2 and 4 weeks after Tdap booster vaccination. The INF-γ (Th1), IL-5 (Th2), and IL-17 (Th17) cytokines were assessed 4 weeks after booster vaccination by stimulation with three simulators: heat inactivated *Bordetella pertussis* (hBp), vaccine antigens, and hBp mixed with antigens. An intranasal challenge test was performed 4 weeks after booster vaccination.

Results

The enhanced GC3111 generated a humoral response to filamentous hemagglutinin (FHA) that was comparable to that of the commercial vaccine. Regarding cell-mediated immunity, cytokine secretion differed among the three simulators. However, no difference was found between the groups. All the vaccinated groups indicated a Th1/Th2 response. The mean value of INF-γ in the control and study groups (simulated with hBp mixed with antigens) was 12,551.69, and the mean value of IL-5 (simulated with antigens) was 1,782.47 pg/mL. On Day 5 post-intranasal challenge, *B. pertussis* colonies were absent in the lungs in all groups.

Conclusions

Our results confirmed the immunogenicity of GC Pharma's Tdap vaccine; enhanced GC3111 was equivalent to the presently used commercial vaccine in terms of humoral response as well as cell-mediated cytokine expression.

Background

Sporadic outbreaks of pertussis among adolescents and adults have continuously been reported worldwide, including in advanced countries such as Europe, Australia, the USA, and Japan, where the rate

of vaccination is above 90%, and yet, the disease is spreading steadily [1–3]. The reasons for the increased occurrence of pertussis include the following. The antibodies produced after pertussis vaccination last for approximately 5–6 years, and thus, the likelihood of reinfection increases during adolescence and adulthood, wherein the defence mechanism is lost due to the waning of the antibodies produced by the diphtheria-tetanus-pertussis (DTP) vaccine. Moreover, the present pertussis vaccine does not work against variant pathogenic strains, such as pertactin (PRN)-deficient variants [4–6], particularly in advanced countries, where the acellular pertussis vaccine (aP vaccine) is widely used. In addition, the pertussis vaccination rate is reported to be low in adolescents and adults. Furthermore, tolerance to macrolide antibiotics following pertussis outbreaks is a major challenge in some countries [7]. Therefore, to address the epidemiological changes, Tdap vaccination should be recommended to adolescents and adults, and simultaneously, new vaccines that protect against variant strains should be developed.

In Korea, the Korea National Institution of Health established the laboratory diagnostics of pertussis in 1999, and since then, only 18 incidences were observed annually until 2008. However, the numbers increased subsequently, with 66 cases in 2009, 27 in 2010, and 97 cases observed in 2011 [8]. In the first half of 2012, sudden small outbreaks were reported around the schools in certain regions. Since then, small sporadic outbreaks have continued to occur with a steady increasing trend. With epidemiological changes in Korea, immunization of adolescents and adults using the adult pertussis vaccine is necessary [9, 10]. Currently, no Tdap vaccine manufacturer exists in Korea, so the country relies on imported vaccines. Therefore, vaccination is limited, as the vaccine is not easily available. To resolve this issue, Green Cross Pharma (GC Pharma, Yongin, Korea) began developing a Tdap vaccine (GC3111) in 2010 and began Phase I and IIa clinical trials in 2017. During the trials, the antibody titre against pertussis toxin (PT), filamentous hemagglutinin antigen (FHA), and PRN antigens revealed positive seroconversion and seroprotection after vaccination; however, the vaccine induced a lower titre level of the antibody to FHA compared to the commercially available control vaccine Boostrix™ (GlaxoSmithKlein, Rixensart, Belgium). Based on this finding, an enhanced GC3111 Tdap vaccine with increased antigen volume was developed by improving FHA inactivation and purification. The present study aimed to investigate whether the enhanced vaccine (enhanced GC3111) had improved immunological outcomes and efficacy by comparing the former vaccine (GC3111) and the existing commercial vaccine using an animal-based model prior to conducting human trials.

Methods

Mice

During the animal research period, the mouse were housed in filter-top cages under semi-specific pathogenfree conditions and food and water are available freely. All animal research procedures were performed in accordance with the Laboratory Animals Welfare Act, the Guide for the Care and Use of Laboratory Animals and the Guidelines and Policies for Rodent Experiments provided by the IACUC (Institutional Animal Care and Use Committee) in the School of Medicine, The Catholic University of Korea. (Approval number: CUMS-2019-0100-01).

Immunization

Four-week-old BALB/c female mice from Orient-bio Co., Ltd. (Seongnam, Korea) were used. The mice were divided into 4 groups (30 mice per group) according to booster vaccine types: negative control boosted with saline, positive control with licensed Tdap vaccine (Boostrix^{TM}) from GSK (GlaxoSmithKline, Rixensart, Belgium) and two study groups; one study group with the previous GC Pharma Tdap vaccine GC3111 and the other study group with the enhanced FHA antigen of GC 3111 vaccine. All mice were vaccinated with one-fourth of the human dose (0.125 mL) via intramuscular (quadriceps muscle) injection and immunized with two doses of primary diphtheria-tetanus-acellular pertussis (DTaP) vaccine (provided by GC Pharma) at 3-week intervals, except for the negative control group, which was vaccinated with saline before booster vaccination. The study was conducted according to previous murine model studies at our laboratory at the Vaccine Bio Research Institute [11-12]. All Tdap vaccine components were equivalent to PT 8 μg, FHA 8 μg, and PRN 2.5 μg. The vaccination and assay schedule is described in Fig. 1.

Humoral immunity assessment

Blood samples from the retro-bulbar venous plexuses were collected in each group at 1 week before booster vaccination (n=6 per group) and 2, 4 week after booster vaccination (n=10). When sampling the blood, all mice were anesthetized with tiletamine, zolazepam and xylazine via intra peritoneal injection except last sampling. At 4 week after booster vaccination, mice were euthanized by 2% isoflurane inhalation while sampling and sacrificed via CO_2 inhalation. The humoral immunogenicity against pertussis antigens (anti-PT IgG, anti-FHA IgG and anti-PRN IgG) was evaluated by commercially available ELISA kits (Alpha Diagnostic International Inc., San Antonio, TX, USA). Additionally, anti-diphtheria toxoid (DT) IgG and antitetanus toxoid (TT) IgG titres were measured using commercially available ELISA kits (Alpha Diagnostic International Inc. San Antonio, TX, USA). All final results were analysed through optimal density using an Epoch ELISA plate reader (BioTek Instrumetns Inc., Winooski, VT, USA). Antibody titres of each tested antigen were compared between groups at each time point.

Cellular mediated immunity assessment

Four weeks after the booster vaccination, mouse spleen cells (n=5 per group) were prepared in RPMI-1640 (HyClone, GE Healthcare Life Sciences, SouthLogan, Utah, USA) medium containing penicillin, streptomycin, and 10% FBS. For cell-based experiments, 1 μg/mL pokeweed mitogen (PWM; Sigma-Aldrich, St. Louis, MO, USA) was used as a positive control, and the following three stimulators were tested: 1×10^6 CFU/mL of heat inactivated *Bordetella pertussis* (hBp), PT (8 μg/mL), FHA (8 μg/mL) and PRN (4 μg/mL) vaccine antigens, and the mixture of the two (hBp + antigens). Splenocytes (5×10^6 cells/mL) were treated with three simulators separately and cultured in 6-well plates for 3 days. Subsequently, the cytokine response was assessed by analysing the supernatant using ELISA kits (R&D Systems, Minneapolis, MN, USA).

Intranasal challenge test

The protective efficacy against *B. pertussis* infection was assessed with intranasal clearance tests. The challenge *B. pertussis* strain obtained from a Korean adult pertussis patient was supplied from the Korean Centers for Disease Control & Prevention (No. 13674) and was intranasally inoculated at 6×10^6 colony forming units (CFUs) 4 weeks after booster vaccination. Four mice in each group were euthanized by 2% isoflurane inhalation and their lungs were extracted 2 h, 2 days, 5 days and 8 days after infection. The extracted lungs were homogenized in 10 mL of PBS and diluted to concentrations of 10^{-1}, 10^{-3} and 10^{-5}. Each diluted homogenate was incubated on Bordet-Gengou agar media at 37 °C for 5 days. CFUs on each media were determined, and mean CFUs were compared between groups at each time point.

Statistical analysis

All results are expressed as the means ± standard errors of the means (SEM) and compared by two-way ANOVA with Tukey's multiple comparison test. Statistical analysis was performed using GraphPad Prism™ software v7.02 (GraphPad, San Diego, CA, USA), and statistical significance was defined as a p value ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$).

Results

Humoral response

The humoral immune response was examined 1 week before (n = 6 per group) and 2 and 4 weeks (n = 10 per group) after the booster vaccination. In the anti-DT IgG and anti-TT IgG humoral response, the enhanced GC3111 group led to a high titre among the groups (Fig. 2A). Humoral immunity against pertussis antigens was significantly elevated in the positive control group and the two study groups compared with the negative control group. The enhanced GC3111 group was not significantly different from the other groups; however, a statistically significant difference was noted in anti-FHA IgG titre between the GC3111 group and the positive control group. The mean anti-FHA IgG titre 2 weeks after booster vaccination was 73.10 U/mL in the GC3111 group and 117.70 U/mL in the positive control group ($p = 0.0109$, $p < 0.05$). Four weeks after booster vaccination, the anti-PRN IgG titre was higher in the GC3111 group than in the positive control group ($p = 0.0427$, $p < 0.05$; Table 1, Fig. 2B).

Table 1
Humoral responses against pertussis toxin (PT), filamentous hemagglutinin (FHA), and pertactin (PRN) (mean ± SEM)

	Negative control	Positive control	GC3111	Enhanced GC3111
Anti-PT IgG (U/mL)				
Naïve (N = 5)	0.86 ± 0.17	0.55 ± 0.16	0.66 ± 0.16	0.82 ± 0.31
1w before booster vaccination (N = 6)	0	9,804.25 ± 1,061.82	8,102.17 ± 1,002.83	10,608.92 ± 2,022.17
2w after booster vaccination (N = 10)	92.20 ± 63.98	15,457.80 ± 1,781.44	11,734.00 ± 886.59	16,580.25 ± 1,663.38
4w after booster vaccination (N = 10)	0	22,270.00 ± 3,286.30	19,936.00 ± 2,972.66	19,203.90 ± 3,494.35
Anti-FHA IgG (U/mL)				
Naïve (N = 5)	0.01	0	0	0.02 ± 0.01
1w before booster vaccination (N = 6)	0.88 ± 0.22	51.41 ± 9.98	48.39 ± 11.00	40.74 ± 7.85
2w after booster vaccination (N = 10)	0	117.70 ± 17.95	73.10 ± 15.04	93.90 ± 16.80
4w after booster vaccination (N = 10)	7.50 ± 3.84	78.00 ± 8.79	60.50 ± 3.45	62.00 ± 6.35
Anti-PRN IgG (U/mL)				
Naïve (N = 5)	0	0	0	0
1w before booster vaccination (N = 6)	2.88 ± 2.80	510.42 ± 42.25	436.08 ± 38.92	456.75 ± 52.19
2w after booster vaccination (N = 10)	96.40 ± 96.40	2,181.00 ± 225.35	2,629.10 ± 272.59	2,686.10 ± 320.84
4w after booster vaccination (N = 10)	18.90 ± 18.90	1,491.50 ± 127.52	2,101.40 ± 73.77	1,918.10 ± 237.37
(SEM = standard errors of the means)				

Cell mediated immune response

Cell-mediated immunity (CMI) was evaluated by stimulating mouse splenocytes (n=5 per group) to hBp, vaccine antigens, or hBp+ antigens, whereas the culture medium was used as a negative control, and 1 μg/mL PWM was used as a positive control. The results showed that the secretion of INF-γ, IL-17A, and

IL-5 did not differ significantly among the groups (except for the media negative control group and PWM control group). However, cytokine secretion was significantly different according to the stimulator used; the mean INF-γ expression levels of the three vaccinated groups, including the positive control and 2 study groups, were 12,551.69 pg/mL in the hBp+antigen stimulator and 2,289.63 pg/mL in the pertussis antigen stimulator. Even after excluding the saline-vaccinated negative control group results, the mean value of the vaccinated groups stimulated by hBp+antigen was three times higher than that of the antigen-stimulated group. Furthermore, IL-17A and IL-5 were significantly upregulated in the groups stimulated by the hBp and vaccine antigens, respectively (Fig. 3A). In this study, the CMI of the vaccinated groups confirmed Th1/Th2 immunity when the ratio was computed between the mean cytokine level of the vaccinated groups subtracted from the cytokine level of the negative control (saline vaccinated) group using the highest cytokine response regardless of the stimulators. IL-17A was overexpressed by the hBp stimulator in both the vaccinated groups and the negative control group. The mean titre of the three vaccinated groups was 1,062.67 pg/mL, and that of the negative control group was 1,095.92 pg/mL. When excluding the level of the negative control group from the vaccinated groups, limited IL-17A expression was observed. In contrast, when the level of the negative group was excluded, the mean cytokine response of the three vaccinated groups was 6,826.51 pg/mL and 1773.26 pg/mL for INF-γ and IL-5, respectively (Fig. 3B).

Intranasal lung clearance

The vaccine efficacy was evaluated against the clinical pertussis strain (n=4 per group). The results from the test using the clinically isolated strain showed that *B. pertussis* was removed quickly in the lungs and was almost eliminated after 5 days (Fig. 4). The results were the same in the two study groups and the positive control group. The CFUs of *B. pertussis* significantly decreased 2 days after intranasal challenge with the clinical strain in the study and positive control groups (Fig. 4). This result showed protective efficacy against *B. pertussis* in both the positive group and the two study groups.

Discussion

Since 2000, a serological study in Korea has confirmed incidents of pertussis infection with higher certainty than reported earlier, and small-scale pertussis outbreaks have occurred once every 3 years since 2009, leading to the requirement for Tdap vaccination. In 2010, GC Pharma, a national company, started developing Tdap and DTaP vaccines; and our laboratory, the Vaccine Bio Research Institute, conducted animal-based studies [11, 12] and performed clinical trials [13] using the Tdap booster vaccine. In animal studies, GC Pharma's new Tdap vaccine, GC3111, was compared with Boostrix™, a commercially available product in Korea. The humoral immunity was assessed after a single dose of DTaP vaccine followed by Tdap booster vaccine [12], and CMI was assessed after two doses of DTaP vaccine followed by Tdap booster vaccine [11]. After two animal studies and clinical trials, GC Pharma complemented GC3111 to improve the anti-FHA antibody response. The present study aimed to show the immunogenic response and efficacy of complemented GC3111 (enhanced GC3111) compared to Boostrix™ and the former GC3111 vaccine and to verify anti-FHA response reinforcement.

The protective effects of the humoral response to the aP vaccine were actively investigated soon after aP vaccine development, and the importance of the humoral response to PT, PRN, FHA, and fimbriae antigens was evaluated in different systems, including animal models [14–16]. Humoral immunity to these antigens of *B. pertussis* is known to protect the individual from pertussis infection by neutralizing the pathogenic antigens or by activating the complement system that activates CMI [14, 17, 18]. Among the immunogens present in the vaccine, PT is known as the most important immunogen and induces the generation of protective antibodies that provide direct protection from pertussis infection [19, 20]. Moreover, the humoral immunity generated by aP-vaccinated pregnant women can prevent infants from pertussis infection since the antibodies produced by the mother can deliver to the foetus [21–24]. Thus, evaluation of humoral response after pertussis vaccination is immensely significant. In this study, compared to the positive control, the GC3111 group showed a significantly lower anti-FHA antibody level 2 weeks after booster vaccination, which was in line with the observations of a previous study [13]; however, the antibody response was comparable between the enhanced GC3111 group and the positive control group with respect to all antigens and all time points (Fig. 2B). Hence, our results verified that the humoral immune response was improved with the enhanced GC3111 Tdap vaccine. With respect to the effect of two doses of DTaP vaccination, anti-PT, anti-FHA, and anti-PRN antibody titres were elevated even before the booster vaccination, and these levels were further enhanced after the booster vaccination and retained at a high level until 4 weeks after vaccination (Fig. 2B). Considering the humoral responses to tetanus and diphtheria, all groups except the negative control group revealed a titre of over 0.1 U/mL (the protective level) from 1 week before booster vaccination, and this is predicted by two doses of DTaP vaccination (Fig. 2A).

The fact that CMI plays crucial roles in preventing pertussis infection was first shown in a mouse model in 1993 [25] followed by clinical experiments [26]. The importance of both Th1 [27, 28] and Th17 [29–31] type CMI responses was confirmed in animal and clinical studies. INF-γ and IL-17 are the main cytokines that provide crucial protection. Recently, the resurgence of pertussis outbreaks [1–3] and the protective effects of the aP vaccine and whole cell pertussis (wP) vaccine [32] were compared frequently. This was based on the observations of some researchers who concentrated that the wP vaccine induces Th1/Th17 responses similar to natural infection [31, 33, 34], whereas the aP vaccine mainly induces the Th2 response, resulting in a weaker protective effect than that of the wP vaccine. Previous studies showed that the aP vaccine generated Th1/Th2 [35] or Th2/Th17 [34, 36] responses. This inconsistency in the findings may be attributed to the difference in animal models used in the studies [37] as well as the study design, such as vaccine schedule and stimulation condition. In general, the Th2 dominant response is the common CMI in aP vaccine-based studies. In this study, hBp was included as one of the simulators to indirectly examine the effects of natural exposure, while vaccine antigens were used as simulators to evaluate the response to the aP vaccine. After exposure to the stimulators, INF-γ, IL-17, and IL-5 showed significant differences between stimulators that were used but no differences between the positive control group and study groups. Notably, in the hBp + antigen stimulator group, the mean INF-γ level of the 3 vaccinated groups was 12,551.69 pg/mL, thereby revealing a difference of more than 5-fold when compared with the mean 2289.63 pg/mL in the antigen stimulator, suggesting that induction of INF-γ

may vary according to the type of simulator used for assay, and this can be applied to the other cytokines. Regardless of stimulators, the levels of INF-γ and IL-5 were significantly higher compared to the saline vaccinated negative control group, indicating that Th1 (INF-γ)/Th2 (IL-5) adaptive immunity was induced (Fig. 3B). This result is consistent with previous studies showing that the aP vaccine primarily induces the Th2 response but induces a dominant Th1 response when exposed to natural pertussis [38–41]. However, in this study, there are some limitations due to the in vitro system, and natural pertussis exposure could be substituted by hBp indirectly. In addition, the results of the intranasal challenge test using the clinical pertussis strain for real and reliable assessment showed that all vaccinated groups cleared the pathogen from Day 2 post-challenge, and by Day 5, the pathogen was hardly found in the lungs, thereby confirming the similar efficacy of the booster vaccines in the three vaccinated groups (Fig. 4).

Conclusions

GC Pharma's enhanced GC311 Tdap vaccine addresses the limitations of the previous GC311 Tdap vaccine, wherein a lower anti-FHA antibody response is observed compared to that of the commercially available product. Our study outcomes confirmed that after booster vaccination, the humoral as well as the CMI responses were comparable to those of the commercially available product with equivalent efficacy against the clinical strain. Our findings present strong evidence that similar findings may be obtained in the phase II clinical trial that is currently being carried out.

Abbreviations

aP: acellular *Bordetella pertussis*; CFU: colony forming unit; CMI: Cell mediated immunity; DT: diphtheria toxoid; DTaP: diphtheria-tetanus-acellular pertussis; FHA: filamentous hemagglutinin; PRN: pertactin; PT: pertussis toxin; PWM: pokeweed mitogen; SEM: standard errors of the means; Tdap: tetanus-reduced dose diphtheria-acellular pertussis; TT: tetanus toxoid; WP: whole cell pertussis

Declarations

Acknowledgements

Not applicable.

Authors' contributions

JHK and DHH designed the study. JAK, KRK and DHH conducted the experiments and analysed the data. KRK and JHK wrote the manuscript. All authors read and approved the final manuscript.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

Competing interests

There is no conflicts of interest in this study.

Ethics approval

All animal research procedures were performed in accordance with the Laboratory Animals Welfare Act, the Guide for the Care and Use of Laboratory Animals and the Guidelines and Policies for Rodent Experiments provided by the IACUC (Institutional Animal Care and Use Committee) in the School of Medicine, The Catholic University of Korea. (Approval number: CUMS-2019-0100-01).

Funding

This study was supported by Green Cross Pharma (Yongin, Korea, Grant No.5-2018-D0083-0001, 5-2019-D0083-00002).

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Figures

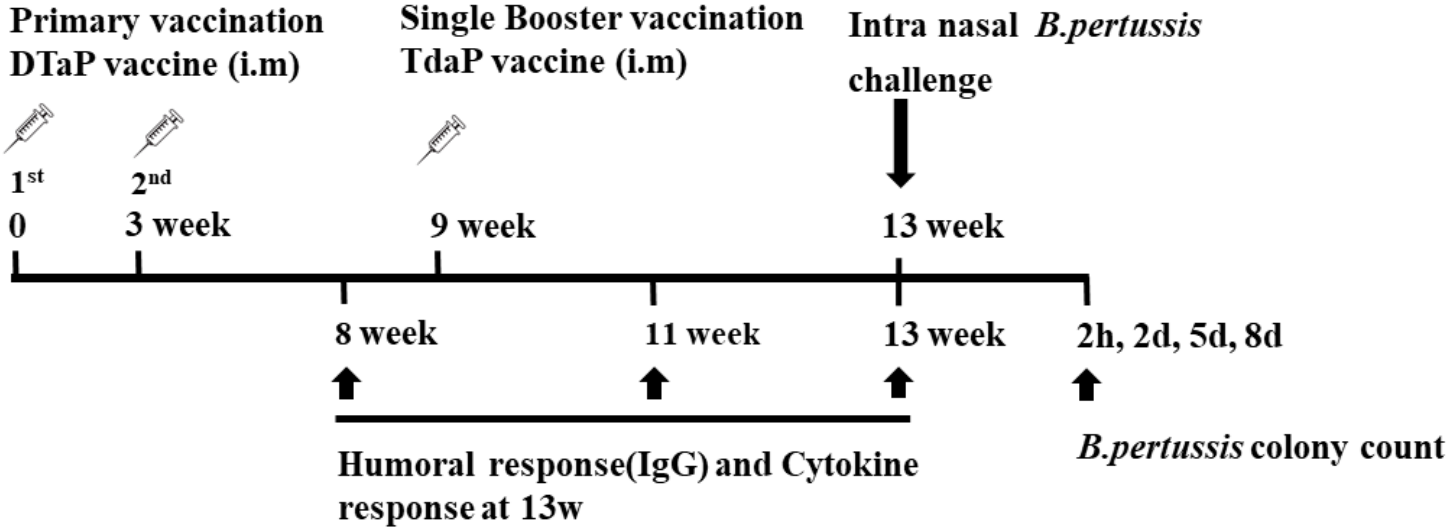
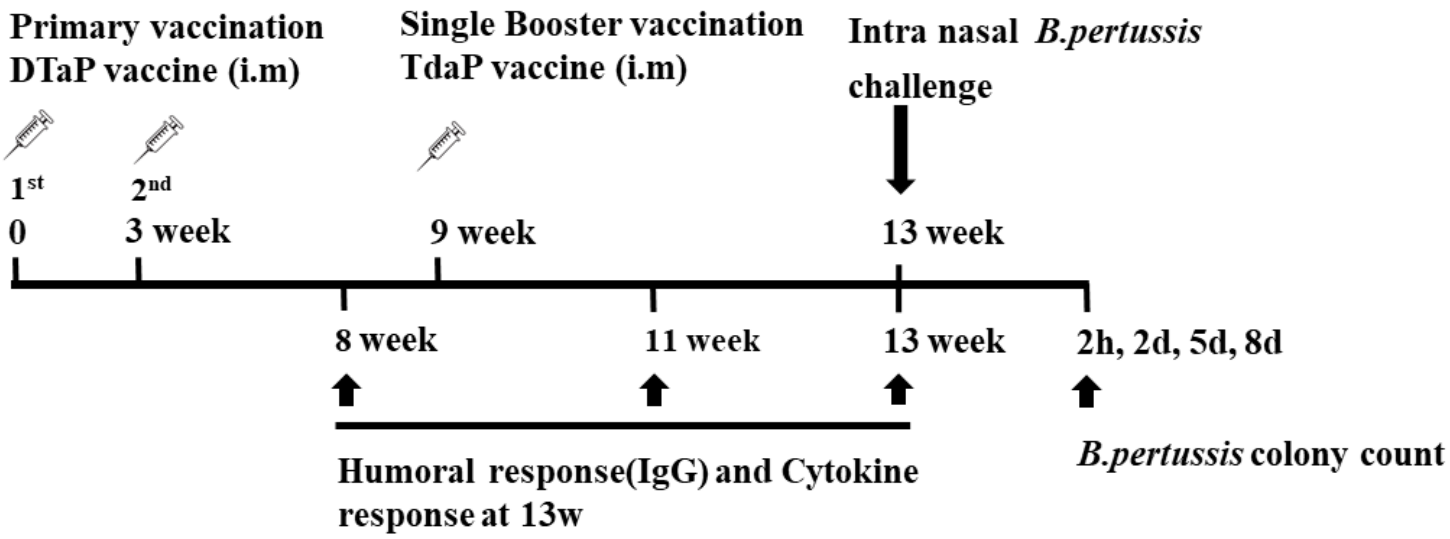


Figure 1

Schedule of the study



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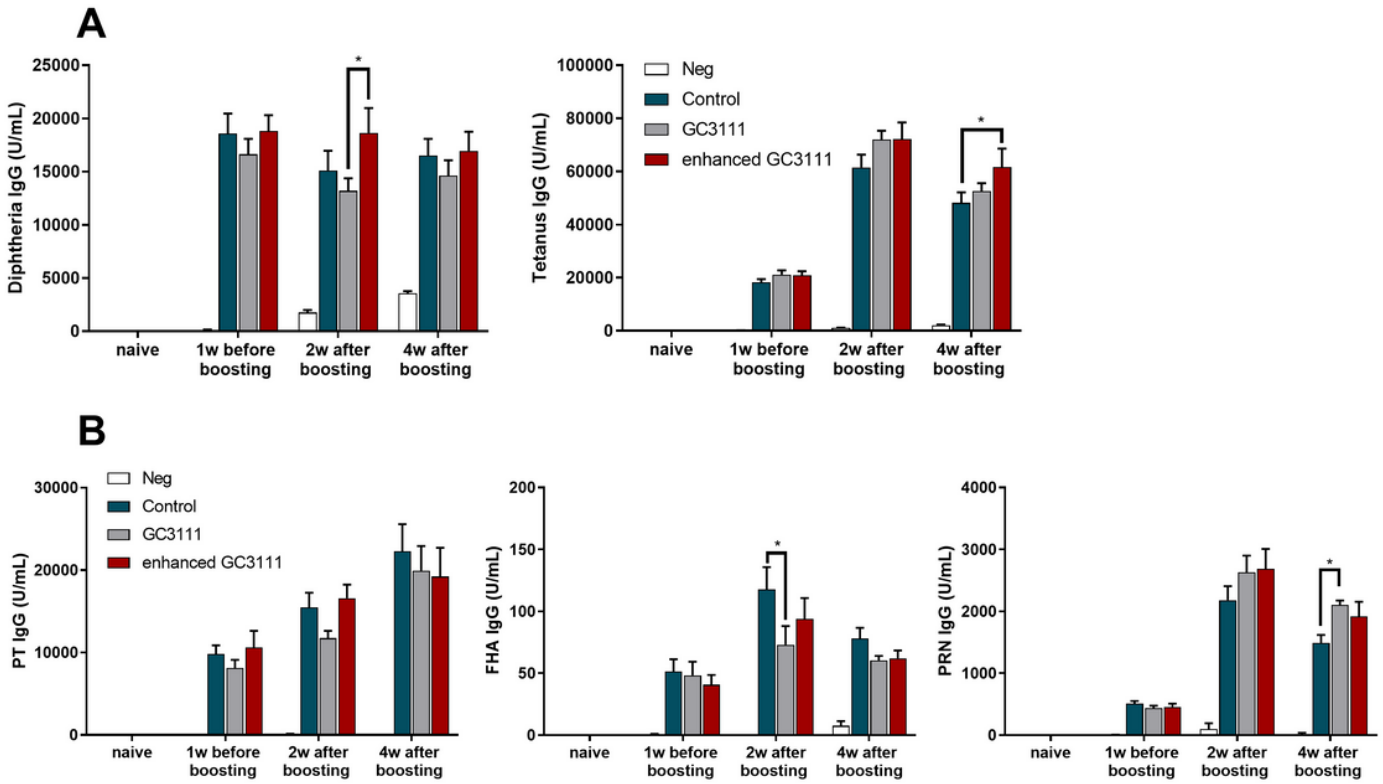
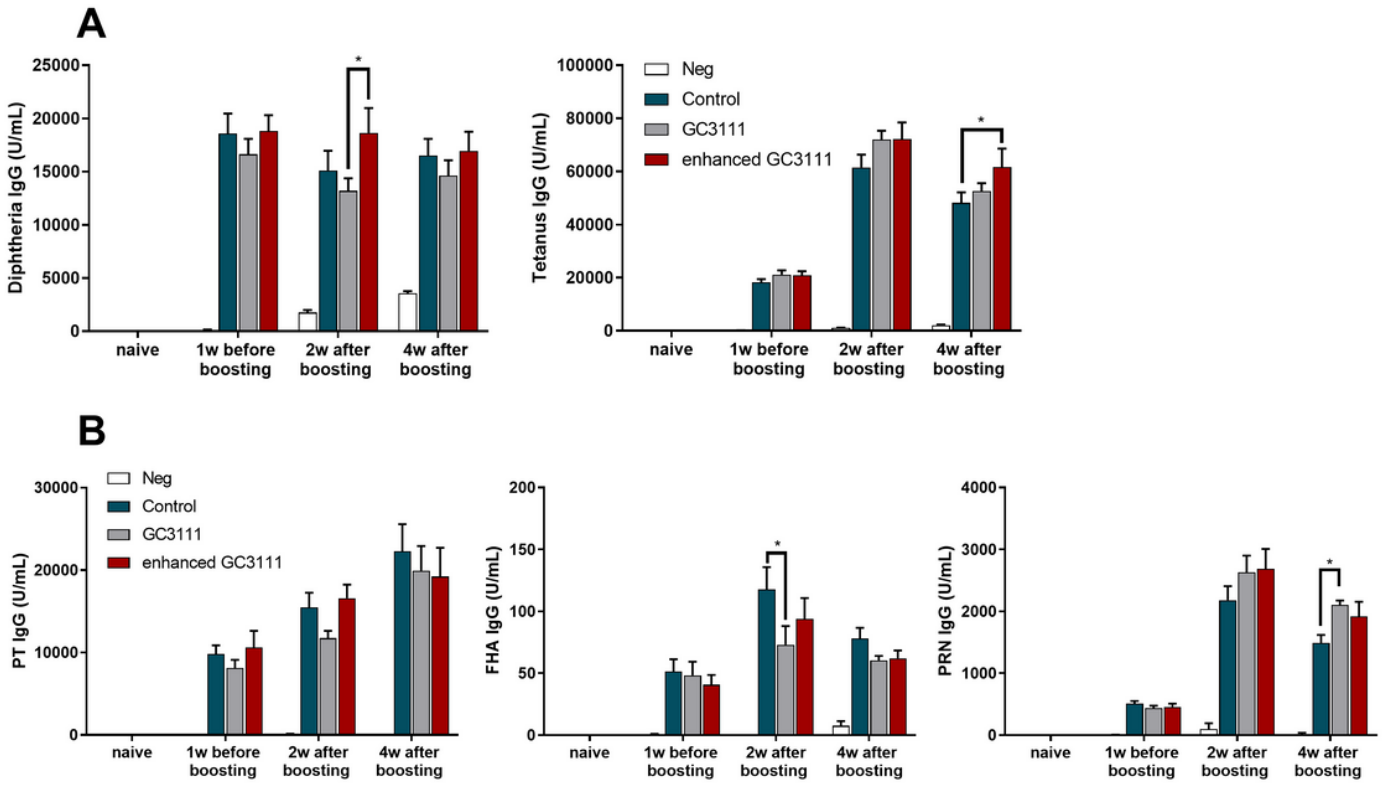


Figure 2

Humoral response. Humoral response was assessed 1 week before and 2 and 4 weeks after the booster vaccination. The results from commercially available ELISA kits are presented as the mean ± SEM (U/mL) in the graphs. The experiment was performed using five mice per group in the naïve groups and

six mice per group 1 week before vaccination. In all other conditions, experiments were performed on 10 mice per group. Statistical differences were tested with two-way ANOVA and Tukey's multiple comparison test. (A) IgG responses to diphtheria and tetanus were assessed by using commercially available ELISA kits. (B) Anti-PT, anti-FHA, and anti-PRN IgG titer levels were assessed by using commercially available kits. (SEM = standard errors of the means, PT = pertussis toxin, FHA = filamentous haemagglutinin, PRN = pertactin) (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).



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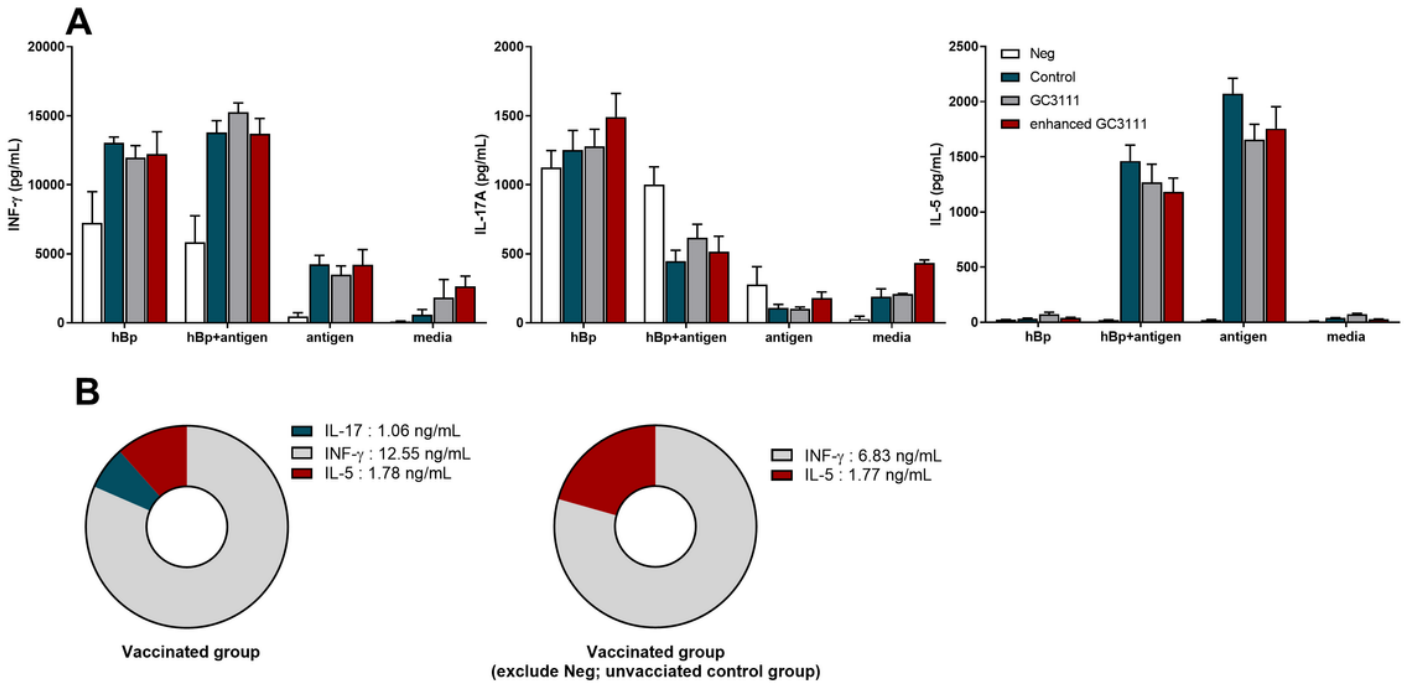
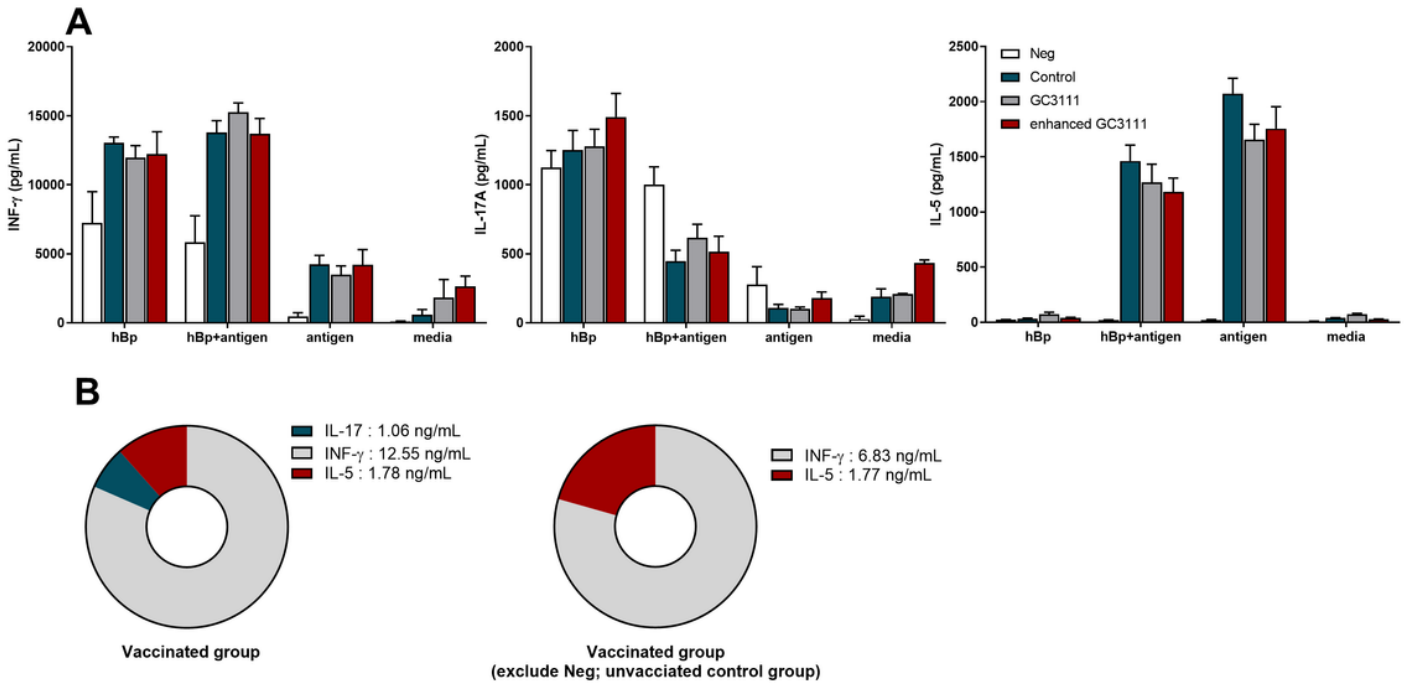


Figure 3

Cytokine production in each simulator Two and four weeks after booster vaccination, stimulation was carried out by three stimulators with heat-killed B. pertussis (hBp), PT, FHA, and PRN antigens or the mixture of the two (hBp+antigen) for 3 days (n = 5). The culture medium was used as a negative control, and 1 μg/mL PWM was used as a positive control. The results were obtained via commercially available cytokine ELISA kits and presented as the mean ± SEM (pg/mL). Statistical differences were tested with 2-way ANOVA and Tukey's multiple comparison test. (A) INF-γ, IL-17A, and IL-5 cytokine levels were assessed for each stimulator. Excluding the medium (used for the negative control), no statistically significant difference was observed between the groups; however, different cytokines revealed various response levels according to the stimulator type. (B) INF-γ had the highest expression level in the hBp+antigen simulator, IL-17A in the hBp simulator, and IL-5 in the antigen stimulator. Th1/Th2 immunity was confirmed by heightened cytokine responses. The ratio was calculated by subtracting the cytokine expression level of the negative control group that was vaccinated with saline (Neg) from the mean cytokine expression level of the three vaccinated groups, positive control group and two study groups. (SEM = standard errors of the means, PT = pertussis toxin, FHA = filamentous haemagglutinin, PRN = pertactin) (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).



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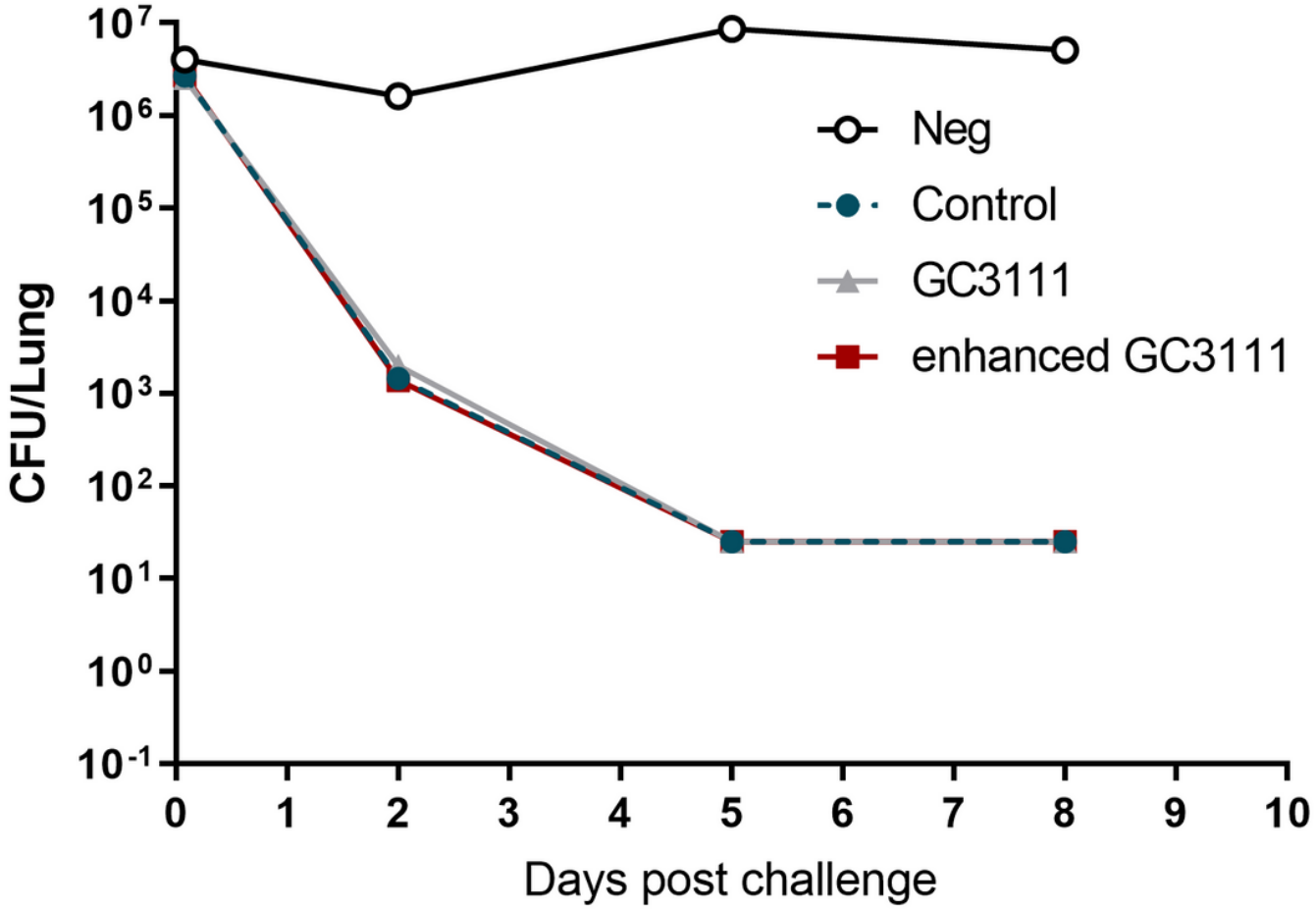
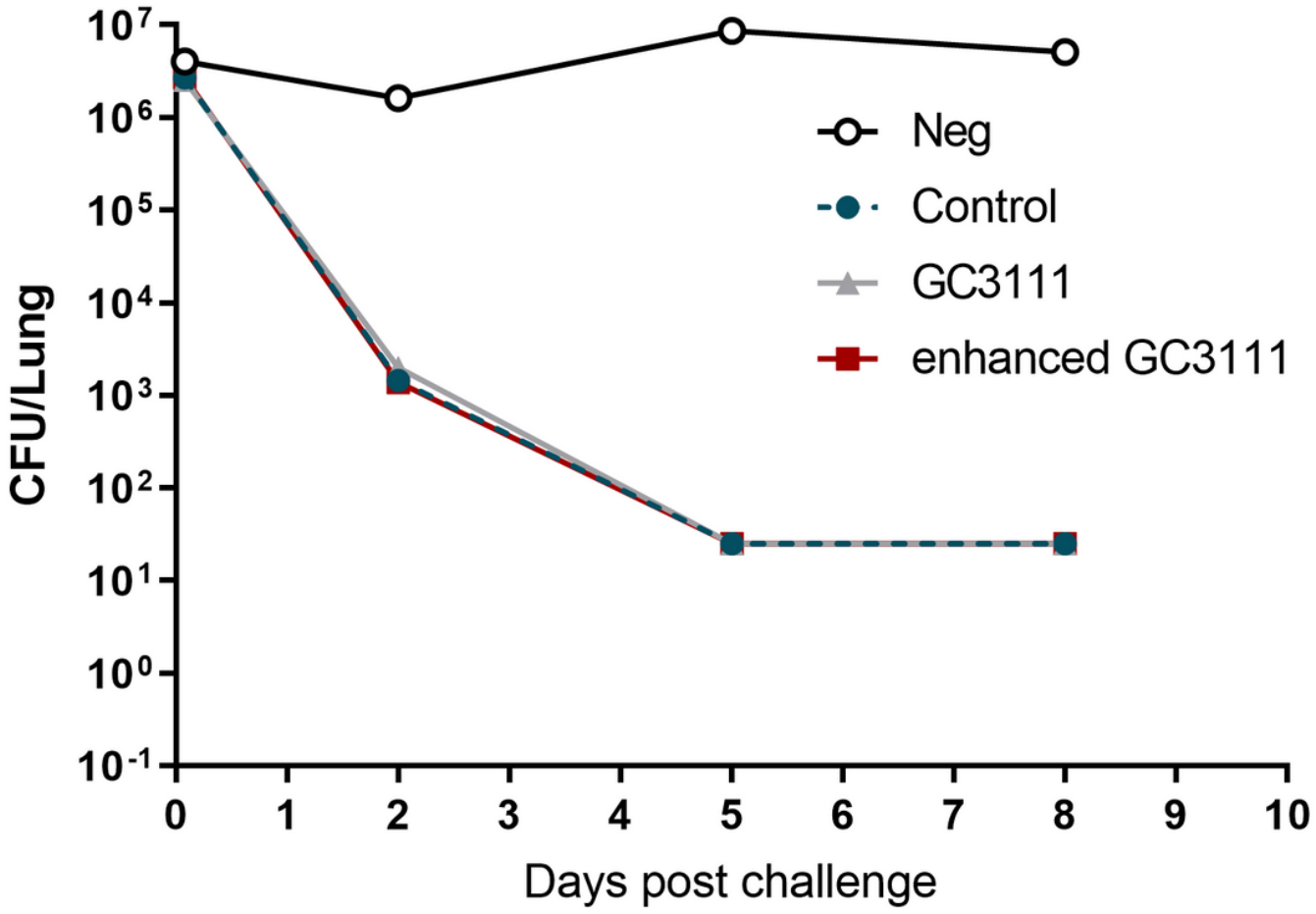


Figure 4

Lung clearance Lungs were extracted from the mice subjected to the challenge test, and the pertussis bacterial colonies were enumerated at 2 h and 2, 5, and 8 days after the challenge (n = 4). By Day 5 post-challenge, the bacterial colonies were hardly found in any of the groups, with the exception of the negative control group (Neg), which was injected with physiological saline solution.



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