Specific Humoral Immune Response Induced by *Propionibacterium acnes* Can Prevent *Actinobacillus pleuropneumoniae* Infection in Mice

Feng Yang,[a] Qiuyue Ma,[a] Liancheng Lei,[a]* Jing Huang,[b] Qun Ji,[a] Ruidong Zhai,[a] Lei Wang,[a] Yu Wang,[a] Linxi Li,[a] Changjiang Sun,[a] Xin Feng,[a] Wenyu Han[a]

College of Veterinary Medicine, Jinlin University, Changchun, People's Republic of China[a]; Clinical Laboratory, The First Hospital of Jilin University, Changchun, People's Republic of China[b]

Porcine contagious pleuropneumonia, caused by *Actinobacillus pleuropneumoniae*, has a major impact on economics, ecology, and animal welfare in the pig-rearing industry. *Propionibacterium acnes*, a facultative anaerobic Gram-positive corynebacterium, exists widely in normal healthy adult animals. We have shown previously that *P. acnes* can prevent *A. pleuropneumoniae* infections in mice and pigs. To elucidate the mechanism of this effect and to identify novel *A. pleuropneumoniae* vaccines, the role of anti-*P. acnes* antibodies in preventing infection was analyzed by indirect immunofluorescence and opsonophagocytosis assays *in vitro*. The role of the specific humoral immune response induced by *P. acnes* was confirmed in a B cell depletion mouse model. The survival rates of mice challenged with *A. pleuropneumoniae* exhibited a highly significant positive rank correlation with the levels of anti-*P. acnes* antibodies. The specific antibodies induced by *P. acnes* had the ability to combine with *A. pleuropneumoniae* and increase opsonization of *A. pleuropneumoniae* for phagocytosis. Furthermore, analysis in the murine B cell depletion model confirmed that the humoral immune response induced by *P. acnes* played an important role in resistance to *A. pleuropneumoniae* infection. In this study, we further elucidated the reasons that *P. acnes* can prevent *A. pleuropneumoniae* infection, which provides useful evidence for the development of heterologous vaccines for the control of porcine contagious pleuropneumonia.

Porcine contagious pleuropneumonia is a highly contagious and fatal respiratory infectious disease, and the mortality of the most acute cases often reaches 80 to 100% (1). It was first reported in Britain in 1957 and has become widespread in many countries around the world since the 1980s. This disease, which is caused by *Actinobacillus pleuropneumoniae*, affects swine of all ages and has a major impact on economics, ecology, and animal welfare in the pig-rearing industry (2, 3). However, *A. pleuropneumoniae* has been divided into 15 serotypes (4), with the lack of cross-protection between the main serotypes (5, 6) resulting in slow progress in the development of vaccines. To date, many studies have been reported and several vaccines have been commercialized, but complete satisfaction has not been obtained in the protection of pigs against *A. pleuropneumoniae* infection (7, 8). Therefore, the development of new vaccines is urgently required.

In 2007, during an analysis of the genomic differences between *A. pleuropneumoniae* serotypes 1 and 5, we made the serendipitous discovery of a strong cross-reaction between the antisera against *Propionibacterium acnes* and *A. pleuropneumoniae* (9). Furthermore, through immunization and challenge studies in mice and pigs, we found that *P. acnes* can prevent *A. pleuropneumoniae* infection (9).

P. acnes bacteria, which are relatively avirulent organisms, are Gram-positive pleomorphic rods that grow under anaerobic conditions. They are the most frequent inhabitants of the sebaceous glands of normal skin, hair follicles, the mouth, and the upper respiratory tract and a frequent contaminant in laboratory cultures (10). Moreover, *P. acnes* is rarely identified as a cause of significant infection (11). In the present study, *P. acnes* induced biological effects that modulate the innate and acquired immune responses, causing increased phagocytic and tumoricidal activities in macrophages (12–14), increased antibody responses (15, 16), and increased resistance to different pathogens (15, 17). There-

fore, the use of *P. acnes* and its components as immune enhancers and antineoplastic agents has become a focus in clinical and basic research.

The use of *P. acnes* to prevent *A. pleuropneumoniae* infection represents a novel and effective strategy for the prevention and control of porcine contagious pleuropneumonia and potentially other infections in the future. However, the effective applications for *P. acnes* are limited by the lack of information regarding the responses induced by *P. acnes* and how this organism can prevent *A. pleuropneumoniae* infection. In this study, we aimed to confirm that the humoral immune response induced by *P. acnes* plays an important role in resistance to *A. pleuropneumoniae* infection.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. *A. pleuropneumoniae* reference serotype 1 strain Shope 4074 (*A. pleuropneumoniae* S 4074), donated by the Shanghai Entry-Exit Inspection and Quarantine Bureau (Shanghai, China), was cultured in brain heart infusion (BHI; Difco Laboratories, Detroit, MI, USA) supplemented with NAD (10 μg/ml; Sigma) at 37°C for 6 h, while shaking at 150 rpm. *Propionibacterium acnes* strain 14 (*P. acnes* 14), which was separated and identified by our laboratory, was

TABLE 1 Frequencies of CD3, CD4, CD8, and CD19 cells determined by flow cytometry

Group	Immunogen	Immunization dose (CFU/ml in 0.2-ml suspension)	Frequency (%)				Ratio	
			$CD3^+$	$CD3^+ CD4^+$	$CD3^+ CD8^+$	$CD3^- CD19^+$	$CD3^+/CD19^+$	$CD4^+/CD8^+$
1	PBS	0.2 ml	39.41 ± 0.66	25.36 ± 0.33	11.71 ± 0.38	45.38 ± 0.85	0.87 ± 0.01	2.17 ± 0.06
2	Viable *P. acnes*	10^8	39.95 ± 0.33	27.27 ± 0.21^a	9.86 ± 0.16^a	48.57 ± 0.67^a	0.82 ± 0.01^a	2.77 ± 0.07^a
3	Viable *P. acnes*	10^9	40.76 ± 0.40^a	28.12 ± 0.23^a	9.81 ± 0.20^a	49.33 ± 0.43^a	0.83 ± 0.01^a	2.87 ± 0.03^a
4	Viable *P. acnes*	10^{10}	32.5 ± 0.65^a	22.63 ± 0.48^a	6.95 ± 0.63^a	55.07 ± 0.50^a	0.59 ± 0.01^a	3.27 ± 0.23^a
5	Inactivated *P. acnes*	10^8	49.76 ± 0.71^a	35.29 ± 0.36^a	14.28 ± 0.33^a	42.46 ± 0.91^a	1.17 ± 0.03^a	2.47 ± 0.04^a
6	Inactivated *P. acnes*	10^9	51.28 ± 0.71^a	39.01 ± 0.31^a	12.49 ± 0.44^a	45.04 ± 0.49	1.14 ± 0.01^a	3.13 ± 0.10^a
7	Inactivated *P. acnes*	10^{10}	52.74 ± 0.59^a	38.46 ± 0.38^a	9.64 ± 0.20^a	40.35 ± 0.75^a	1.31 ± 0.01^a	4.02 ± 0.05^a

[a] The mean difference between the immunization group and the control group is significant at the 0.05 level.

streaked on brucella broth agar (BD, Sparks, MD, USA), supplemented with 5% (vol/vol) defibrinated sheep blood (Lampire Biological Laboratories, Pipersville, PA, USA), vitamin K (5 μg/ml; Remel, Lenexa, KS, USA), and hemin (50 μg/ml; Remel), under anaerobic conditions using an anaerobic chamber (Mac500; Down Whitley Scientific, West Yorkshire, United Kingdom; 10% carbon dioxide, 10% hydrogen, 80% nitrogen) at 37°C. A single colony was inoculated in reinforced clostridium medium (Oxford, Hampshire, England) at 37°C under anaerobic conditions.

Immunogen preparation. The preparation of viable *P. acnes* 14 antigen was performed as follows: *P. acnes* 14 was cultivated for 72 h to an optical density at 600 nm (OD_{600}) of 1.0 to 3.0 (logarithmic growth phase). Bacteria were harvested by centrifugation at $5,000 \times g$ for 10 min, washed with phosphate-buffered saline (PBS) three times, finally suspended in PBS, and adjusted to an OD_{600} of 1.0. The concentration of this suspension was approximately 1×10^9 CFU/ml, according to the relationship between OD_{600} and the *P. acnes* colony count. The suspension was concentrated or diluted 10 times to give suspensions of viable *P. acnes* 14 antigen of 1×10^8 CFU/ml, 1×10^9 CFU/ml, and 1×10^{10} CFU/ml for use in immunizations.

Inactivated *P. acnes* 14 antigen was prepared by heat inactivation of viable *P. acnes* 14 at 60°C for 30 min. After inactivation, *P. acnes* was unable to grow on an agar plate (data not shown). Inactivated *P. acnes* was harvested by centrifugation at $5,000 \times g$ for 5 min and resuspended to an appropriate concentration in PBS.

P. acnes 14 antigen in adjuvant was prepared as follows: 10 g astragalus polysaccharide powder (adjuvant) was dissolved in distilled water (4% aqueous solution) and filtered (0.22 μm) to remove residual bacteria. Equal amounts of astragalus polysaccharide aqueous solution were mixed with viable or inactivated *P. acnes* 14 antigen under aseptic conditions. The resulting emulsions were vortexed and stored at 2 to 8°C for later use.

Animal immunizations and challenge. Female BALB/c mice (aged 6 to 8 weeks; 18 to 22 g) were purchased from the Animal Experiment Center of Jilin University. Female Japanese rabbits (obtained from the same source) were used for generation of rabbit hyperimmune antiserum. BALB/c mice were randomly assigned to seven groups: the experimental groups (2 to 7) are shown in Table 1. Each group comprised 15 mice (10 mice for toxicity evaluation and 5 for T cell detection), and mice were immunized subcutaneously (s.c.) with viable *P. acnes* 14 (2×10^7 CFU, 2×10^8 CFU, and 2×10^9 CFU) and inactivated *P. acnes* 14 (2×10^7 CFU, 2×10^8 CFU, and 2×10^9 CFU). The control group (group 1) received 0.2 ml PBS only. One week later, all mice received the same dose of the respective immunogens. Thirty-five days after the first immunization, blood was drawn from the tail to collect serum for enzyme-linked immunosorbent assays (ELISAs). For the generation of antiserum to *P. acnes* or *A. pleuropneumoniae* serotype 1, rabbits were immunized s.c. on days 0, 14, and 35 with 1×10^9 CFU of the viable *P. acnes* 14 or *A. pleuropneumoniae* serotype 1, respectively. All animal experiments and procedures were approved by the Institutional Animal Care and Use Committee, and all animals were cared for in accordance with the standards of the Associ-

ation for Assessment and Accreditation of Laboratory Animal Care International.

A. pleuropneumoniae serotype 1 was cultured in BHI medium for 6 h at 37°C. The bacteria were washed three times with PBS and then resuspended. At 35 days after the first immunization, the mice in all groups were challenged intraperitoneally (i.p.) with 0.2 ml of *A. pleuropneumoniae* bacterial suspension containing 4.0×10^7 CFU (equivalent to $5\times$ 50% lethal dose [LD_{50}]). The survival rates of all groups were calculated at day 7 postchallenge.

For evaluating the dynamic relationship between levels of anti-*P. acnes* antibodies and prevention of *A. pleuropneumoniae* infection, BALB/c mice were immunized with the optimal immunization condition of *P. acnes*. Then, mice were divided into various groups based on the different IgG titers at different time points. The control groups were the PBS control group and the astragalus polysaccharide control group. The mice in each group were challenged with *A. pleuropneumoniae* as soon as they were selected on different IgG titers.

ELISAs. Serum antibody responses to *P. acnes* or *A. pleuropneumoniae* were assessed by ELISA as previously described (18). Wells of microtiter plates (Thermo Labsystems) were coated overnight at 4°C with *P. acnes* 14 or *A. pleuropneumoniae* serotype 1 bacteria (1×10^6 CFU per well) lysed by ultrasonic treatment in 0.05 M carbonate buffer (pH 9.5). The wells were then washed three times with PBS containing 0.05% Tween 20 (PBST). Blocking for nonspecific interactions was carried out by the addition of 200 μl of 5% skim milk in PBST per well, and plates were incubated at 37°C for 1 h. Serial 2-fold dilutions of individual mouse or rabbit sera were analyzed using horseradish peroxidase (HRP)-conjugated secondary antibodies specific for either rabbit IgG or mouse IgG (Tianjin Sungene Biotech Co., Ltd., Tianjin, China) at a 1:4,000 dilution. Plates were washed five times with PBST, and 3,3′,5,5′-tetramethylbenzidine (Sigma) was used as the substrate. Absorbance (OD_{450}) was determined using the Labsystems Multiskan Plus plate reader. Absorbance was plotted against dilution, and the lower limit of detection of the ELISA was the reciprocal dilution of 100. The titer obtained was the reciprocal of maximum dilution when the OD_{450} value of serum was greater than 2.1 times that of the preimmune serum.

The concentrations of IgA and IgM at different IgG levels of mouse serum were determined using a mouse enzyme-linked immunosorbent assay kit for immunoglobulin A (IgA) (Uscn Life Science Inc.) and mouse enzyme-linked immunosorbent assay kit for immunoglobulin M (IgM) (Uscn Life Science Inc.), respectively, according to the manufacturer's instructions.

Flow cytometry. Erythrocyte-depleted single-cell suspensions of individual mouse spleens were stained on ice using optimal concentrations (0.5 μg per million cells) of rat anti-mouse CD16/CD32 (Fc block; BD Biosciences, San Diego, CA, USA). For detection of lymphocytes (including $CD3^+ CD4^+$ T cells, $CD3^+ CD8^+$ T cells, and $CD3^- CD19^+$ B cells) in mouse spleens, the single-cell suspensions were stained with optimal concentrations of antibodies specific for mouse CD3 (1.0 μg fluorescein isothiocyanate [FITC] anti-mouse CD3 per million cells in a 100-μl vol-

ume), CD4 (0.25 μg phycoerythrin [PE] anti-mouse CD4 per million cells in a 100-μl volume), CD8 (0.25 μg PE anti-mouse CD8a per million cells in a 100-μl volume), and CD19 (0.25 μg PE anti-mouse CD19 per million cells in a 100-μl volume) (Biolegend, Inc., San Diego, CA, USA). For depletion experiments, the single-cell suspensions were incubated with directly conjugated antibodies specific for mouse CD19 and mouse CD3.

Cells with light scattering properties of lymphocytes were then assessed by immunofluorescence on a FACScan cytometer using Cellquest software (BD Biosciences, San Diego, CA, USA). A total of 10,000 gated events were acquired for each sample.

Indirect immunofluorescence. For indirect immunofluorescence, *A. pleuropneumoniae* serotype 1 bacteria were cultured, washed, and resuspended in PBS. Then, bacteria were fixed with 1% paraformaldehyde for 20 min at room temperature. Following a 5-min centrifugation at 8,000 × *g*, bacteria were washed three times with PBS and resuspended in PBS containing 10% fetal bovine serum (FBS) and 1% NaN$_3$. *P. acnes* or *A. pleuropneumoniae* hyperimmune rabbit serum was added, and tubes were incubated at 37°C for 1 h. There was no *P. acnes* or *A. pleuropneumoniae* hyperimmune rabbit serum in the negative control and blank control, and the blank control also did not include *A. pleuropneumoniae* serotype 1 bacteria. Bacteria were washed three times with PBS, and bound antibodies were detected using FITC-labeled goat anti-rabbit IgG (Tianjin Sungene Biotech Co., Ltd.) antibodies. Then, bacteria were washed three times with PBS and resuspended in PBS containing 3% bovine serum albumin (BSA), 1% NaN$_3$ in darkness. The samples were observed by fluorescence microscopy (Lavision Biotec, Germany), and photographs were taken as soon as possible.

Opsonophagocytosis assay. The murine macrophage cell line J774A.1 was cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% FBS (HyClone) and 4 mM L-glutamine (Invitrogen). Approximately 2×10^5 cells were seeded into a 24-well cell culture plate (Corning) and incubated overnight at 37°C under 5% CO$_2$. For the opsonophagocytosis assay [19], *P. acnes* hyperimmune rabbit serum, *A. pleuropneumoniae* hyperimmune rabbit serum, preimmune rabbit serum, and unrelated glutathione *S*-transferase(GST) hyperimmune rabbit serum were heat inactivated by incubation at 55°C for 15 min. *A. pleuropneumoniae* serotype 1 was grown to an OD$_{600}$ of 1, and approximately 2×10^6 cells were incubated with heat-inactivated *P. acnes* hyperimmune rabbit serum (at 1% or 10%), *A. pleuropneumoniae* hyperimmune rabbit serum (10%), preimmune rabbit serum (10%), unrelated (GST) hyperimmune rabbit serum (10%), or PBS in 100 μl at 37°C for 30 min. The assay was performed by incubating macrophage cells with the previously mentioned mixture of serum or PBS containing bacteria for 1 h. This was followed by gentamicin (100 μg/ml) (Invitrogen) treatment for 1 h to kill the extracellular bacteria, washing twice with PBS to remove adherent bacteria, and lysing with 0.1% Triton X-100 (Invitrogen). Different dilutions were plated to enumerate the number of phagocytosed bacteria. The fold change in CFU for each treatment was calculated by dividing the intracellular CFU obtained from different serum groups by the CFU obtained from the PBS control. The assay was performed in triplicate and repeated two to three times.

Humoral immune response analysis in B cell depletion model mice vaccinated with *P. acnes*. Referring to studies of Oscherwitz et al. [20], we constructed and expressed a recombinant immunogen which contains six copies of the 22-amino-acid peptide sequence from the C terminus of the murine CD20 extracellular domain (ECD), covalently linked to the C terminus of maltose-binding protein (MBP) (henceforth referred to as CD20ECD-6). Figure 1 is a schematic representation of CD20ECD-6. The recombinant immunogen is capable of eliciting antibodies that recognize the folded CD20 ECD sequence and effectively binds cell surface CD20, leading to the pronounced depletion of splenic B cells to generate a murine B cell depletion model. In brief, the process is as follows: six copies of the DNA sequence (5′-CGGCTAC GTGGACATCTACGACTGCGAACCGTCCAACTCCTCTGAGAAG AACTCTCCGTCCACCCAGTA-3′) which encodes a 22-amino-acid
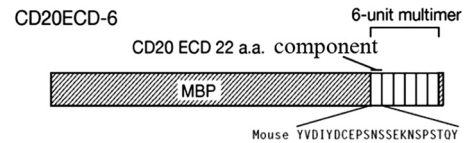
FIG 1 Schematic representation of CD20ECD-6. Each of the six CD20 components represents amino acids 155 to 176 of the murine CD20 ECD. A single glycine spacer is interposed between the CD20 component sequences, and the repeats are covalently linked to the C terminus of maltose-binding protein. Shown below the diagram is the single-letter amino acid sequence for mouse CD20 in the ECD region. The diagram is not to scale.

peptide sequence (amino acids 155 to 176; GYVDIYDCEPSNSSEKNS PSTQY) from the C terminus of the murine CD20 ECD (GenBank accession no. P19437) were synthesized by ShengGong Biological Engineering (Shanghai, China). The construction, expression in *Escherichia coli*, and purification of the fusion proteins using amylose agarose were as described previously [21]. Two groups of BALB/c mice (13/group) were immunized s.c. five times at 2-week intervals with 40 μg CD20ECD-6 immunogen in complete Freund's adjuvant (CFA) for priming immunizations and incomplete Freund's adjuvant (IFA) for boosting immunizations. Mice in the normal control group were immunized with the same dose of adjuvant alone by the same protocol. Ten days after the final immunization, erythrocyte-depleted single-cell suspensions of individual mouse spleens were assessed using flow cytometry to enumerate CD19$^+$ B cells as previously described.

Mice in one B cell depletion model group and one normal control group were immunized s.c. with viable *P. acnes* (2×10^8 CFU) on two occasions at 1-week intervals. Mice in the other B cell depletion model group were immunized with PBS. At day 35 after the first immunization, blood was drawn from the tail to collect serum for ELISAs. The mice in all groups were challenged i.p. with 0.2 ml of *A. pleuropneumoniae* serotype 1 bacterial suspension containing 4.0×10^7 CFU (equivalent to 5× LD$_{50}$). The survival rates of all groups were calculated on day 7 postchallenge.

Statistical analysis. Student's *t* test was used for comparison of the differences between different groups. Spearman rank correlation analysis was used for analysis of the dynamic relationship between levels of anti-*P. acnes* antibody and prevention of *A. pleuropneumoniae* infection. The survival rates of mice challenged with *A. pleuropneumoniae* were analyzed by the Kaplan-Meier method. All statistical analysis was performed using SPSS software (IBM Corporation, USA).

RESULTS

Analysis of humoral immune responses induced by *P. acnes*. At day 35 after immunization with *P. acnes*, IgG antibodies specific for *P. acnes* and *A. pleuropneumoniae* serotype 1 were detected in the peripheral blood in all groups by ELISA (Fig. 2). The antibody titers of mice vaccinated with viable *P. acnes* were significantly higher than those of mice vaccinated with inactivated *P. acnes* ($P < 0.05$). The serum antibody titers were found to increase in a dose-dependent manner, especially in the viable *P. acnes* groups. However, in the inactivated *P. acnes* groups, high immunization doses were required to induce high levels of antibodies (Fig. 2). It was worth noting that the serum antibody titers of mice vaccinated with *P. acnes* were also high when detected for *A. pleuropneumoniae* serotype 1 (Fig. 2B).

The types and proportions of lymphocytes in mouse spleen cell suspensions prepared from all groups on day 35 postimmunization were analyzed by flow cytometry (Table 1). The percentage of CD3$^+$ and CD4$^+$ T cells in the inactivated *P. acnes* immunization groups increased significantly compared with that of the control group ($P < 0.05$); the ratio increased in a dose-dependent man-
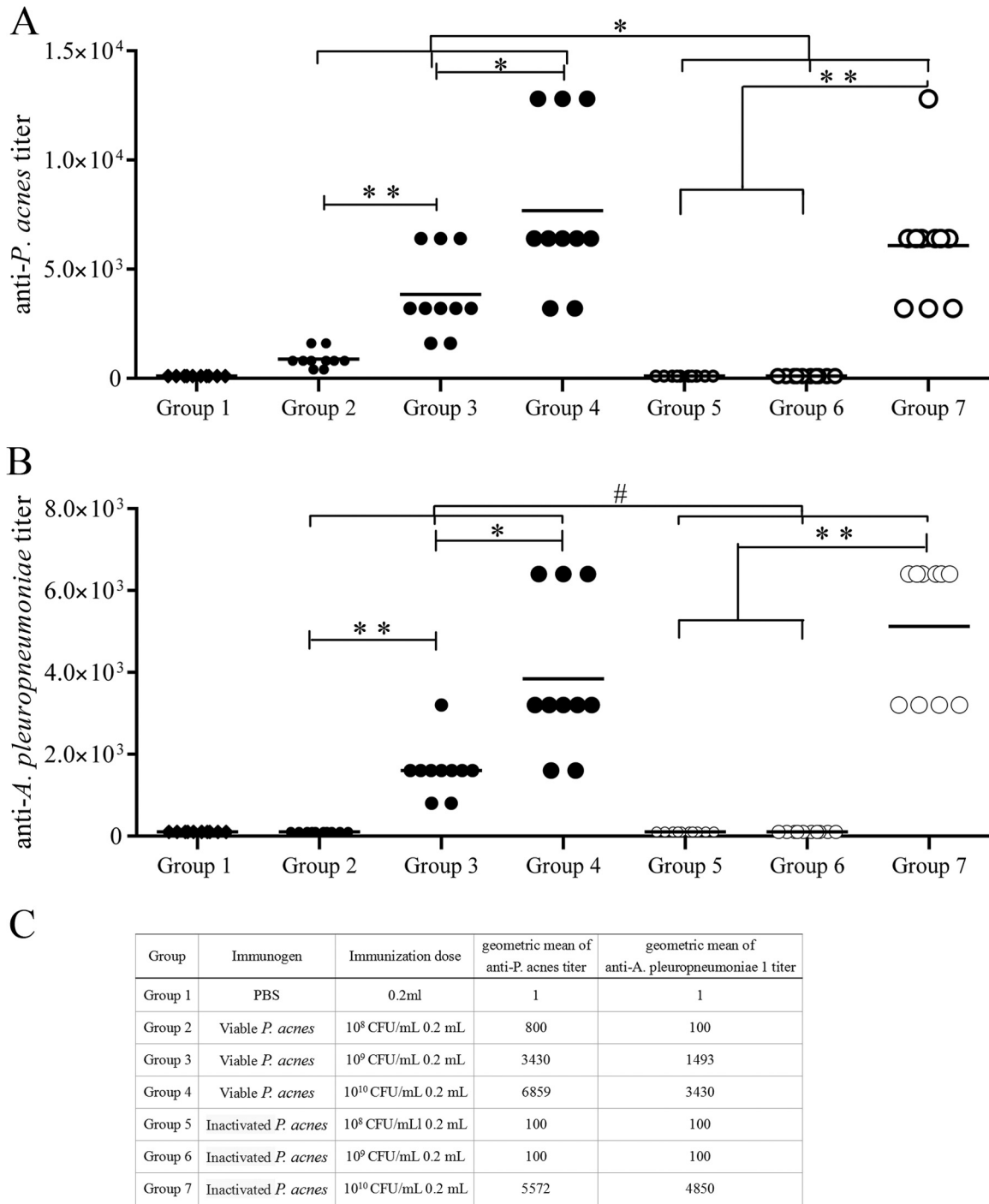
FIG 2 Specific antibody responses in mice elicited through immunization with *P. acnes*. BALB/c mice were randomly assigned to seven groups (*n* = 15 per group). Mice in groups 2 to 7 were immunized subcutaneously (s.c.) with viable *P. acnes* 14 (2×10^7 CFU, 2×10^8 CFU, and 2×10^9 CFU) and inactivated *P. acnes* 14 (2×10^7 CFU, 2×10^8 CFU, and 2×10^9 CFU). The control group (group 1) received 0.2 ml PBS only. One week later, all mice received the same dose of the respective immunogens. Thirty-five days after the first immunization, blood was drawn from the tail to collect serum for enzyme-linked immunosorbent assays (ELISAs). (A) Serum anti-*P. acnes* antibody titers. (B) Serum anti-*A. pleuropneumoniae* antibody titers. (C) Geometric mean of anti-*P. acnes* titers and anti-*A. pleuropneumoniae* serotype 1 titers. *, $P < 0.05$; **, $P < 0.01$; #, $P > 0.05$.

ner. Also, the ratio of CD4$^+$/CD8$^+$ T cells in all immunization groups increased significantly compared with that of the control group ($P < 0.05$), which showed that the CD4$^+$ T cell population was predominant after vaccination with *P. acnes*.

On day 35 postimmunization, all groups were challenged with *A. pleuropneumoniae* serotype 1 (5 times the LD). The survival rates varied with the immunization dose (Fig. 3). The survival rates of groups vaccinated with viable *P. acnes* were higher than
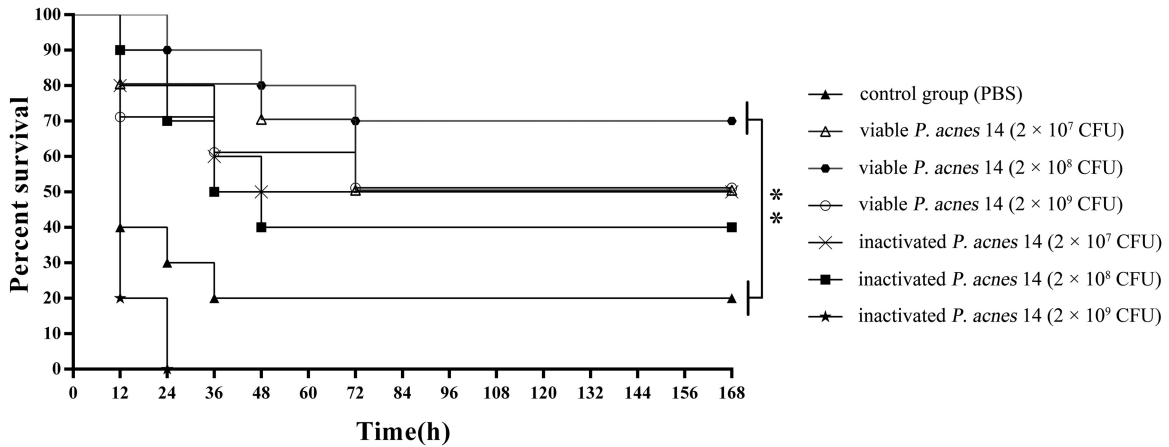
FIG 3 Survival rates of mice challenged with *A. pleuropneumoniae* after immunization with *P. acnes*. BALB/c mice were randomly assigned to seven groups ($n = 10$ per group). Mice in groups 2 to 7 were immunized subcutaneously (s.c.) with viable *P. acnes* 14 (2×10^7 CFU, 2×10^8 CFU, and 2×10^9 CFU) and inactivated *P. acnes* 14 (2×10^7 CFU, 2×10^8 CFU, and 2×10^9 CFU). The control group (group 1) received 0.2 ml PBS only. At 35 days after the first immunization, the mice in all groups were challenged intraperitoneally (i.p.) with 0.2 ml of *A. pleuropneumoniae* bacterial suspension containing 4.0×10^7 CFU (equivalent to $5\times$ LD$_{50}$). The survival rates of all groups were calculated at day 7 postchallenge. Group 1, control group, solid triangles; group 2, viable *P. acnes* 14 (2×10^7 CFU), open triangles; group 3, viable *P. acnes* 14 (2×10^8 CFU), hexagons; group 4, viable *P. acnes* 14 (2×10^9 CFU), circles; group 5, inactivated *P. acnes* 14 (2×10^7 CFU), crosses; group 6, inactivated *P. acnes* 14 (2×10^8 CFU), squares; group 7, inactivated *P. acnes* 14 (2×10^9 CFU), stars. **, $P < 0.01$.

those of groups vaccinated with inactivated *P. acnes* and the control group. Also, the protective effects in group 3, vaccinated with viable *P. acnes* (2×10^8 CFU/mouse), were significantly greater than those in the control group ($P < 0.01$). It is important to note that the mice of group 7 all died. It accorded with the literature reporting that injection with high doses of heat–killed *P. acnes* and then challenge with lipopolysaccharide (also existing in *A. pleuropneumoniae*) can induce fulminant hepatitis, which causes acute death, in many kinds of animals, including mice (22–24).

Based on the results of these experiments, 2×10^8 CFU/mouse was selected as the optimal immunization dose of viable *P. acnes*. This dose was used in all subsequent experiments.

Dynamic relationship between levels of anti-*P. acnes* antibodies and prevention of *A. pleuropneumoniae* infection. After immunization with *P. acnes* emulsified with astragalus polysaccharide adjuvant, *P. acnes*-specific IgG antibodies were detected by ELISA in the peripheral blood of all mice at the indicated time points. Based on the different IgG titers, all mice were divided into eight groups (groups 1 to 8: PBS control group, astragalus polysaccharide control group, group with titer of <100 at day 35, and groups with titers of 400, 800, 1,600, 3,200, and 6,400, respectively). At the same time the concentrations of IgA and IgM in

each group were examined (Table 2). The concentrations of IgA in groups 7 and 8 (IgG titers of approximately 3,200 and 6,400, respectively) were significantly higher than those in the two control groups ($P < 0.01$). However, there were no significant differences in the concentrations of IgM between the groups with different IgG titers.
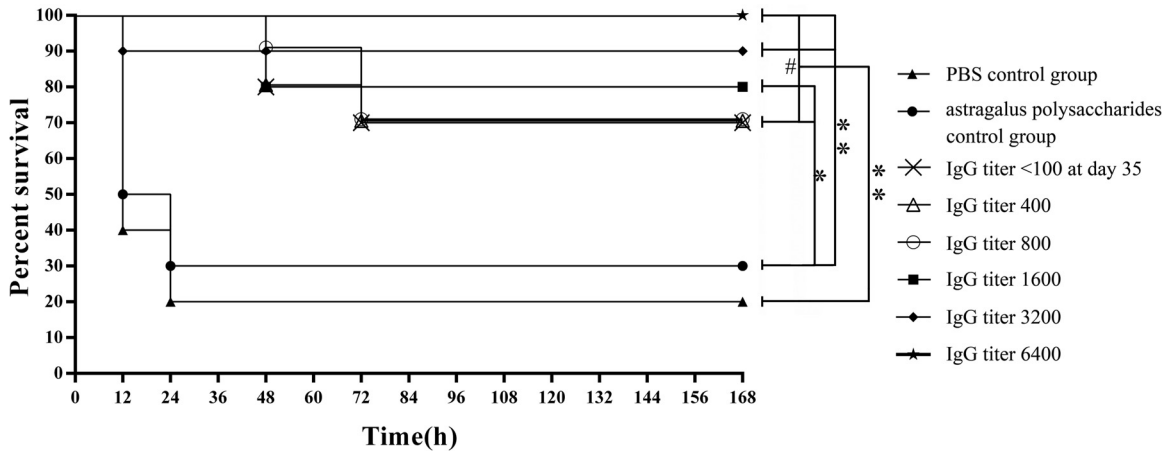
Selected mice in each group were challenged with *A. pleuropneumoniae* serotype 1 ($5\times$ LD$_{50}$). The survival rates of all groups are shown in Fig. 4. The presence of anti-*P. acnes* antibodies had protective effects against *A. pleuropneumoniae* infection. Spearman rank correlation analysis demonstrated a significantly positive rank correlation between the survival rates of mice challenged with *A. pleuropneumoniae* serotype 1 and the levels of anti-*P. acnes* antibodies ($P < 0.01$). Partial protection against *A. pleuropneumoniae* infection was observed in mice with low levels of anti-*P. acnes* antibodies (<100, 400, or 800) at day 35 after immunization with *P. acnes*.

Cross-reaction between anti-*P. acnes* serum and *A. pleuropneumoniae*. Rabbits were immunized with *P. acnes*, and anti-*P. acnes* hyperimmune serum was prepared (titer of ≈40,000). No immunofluorescence was detected in the absence of anti-*P. acnes* hyperimmune serum (Fig. 5A and B). Positive indirect immuno-

TABLE 2 IgA and IgM titers at different IgG titers

Group	Immunogen	Anti-*P. acnes* IgG titer	Mean concn (µg/ml)	
			IgA	IgM
1	PBS	0	139.142 ± 2.558	6.434 ± 0.150
2	Astragalus polysaccharides	0	140.482 ± 1.258	6.276 ± 0.492
3	*P. acnes* plus astragalus polysaccharides	<100 at 35 days	141.887 ± 4.104	6.205 ± 0.260
4	*P. acnes* plus astragalus polysaccharides	400	135.798 ± 0.547	5.761 ± 0.126
5	*P. acnes* plus astragalus polysaccharides	800	139.091 ± 1.531	5.671 ± 0.233
6	*P. acnes* plus astragalus polysaccharides	1,600	142.240 ± 2.912	6.391 ± 0.458
7	*P. acnes* plus astragalus polysaccharides	3,200	146.418 ± 2.900^a	6.350 ± 0.138
8	*P. acnes* plus astragalus polysaccharides	6,400	146.597 ± 0.785^a	6.338 ± 0.443

^{a} The concentrations of IgA in groups 7 and 8 were significantly higher than those in the two control groups ($P < 0.01$).

FIG 4 Dynamic relationship between level of anti-*P. acnes* antibody and survival rates. Based on the different IgG titers at the indicated time points, all mice immunized with *P. acnes* with astragalus polysaccharide adjuvant were divided into eight groups (group 1, PBS control group, solid triangles; group 2, astragalus polysaccharide control group, solid circles; group 3, IgG titer of <100 at day 35, crosses; group 4, IgG titer of 400, open triangles; group 5, IgG titer of 800, open circles; group 6, IgG titer of 1,600, squares; group 7, IgG titer of 3,200, diamonds; group 8, IgG titer of 6,400, stars). Selected mice in each group were challenged with *A. pleuropneumoniae* serotype 1 ($5\times$ LD_{50}). The survival rates of all groups were calculated at day 7 postchallenge. There were no significant differences among groups 3 to 8 ($P > 0.05$). Also, the survival rates of groups 3 to 8 were significantly higher than those of control groups 1 and 2. *, $P < 0.05$; **, $P < 0.01$; #, $P > 0.05$.
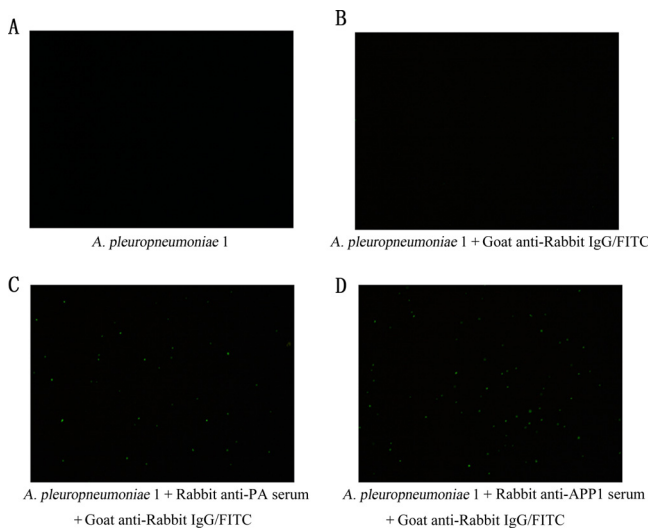
fluorescence signals indicated binding of anti-*P. acnes* hyperimmune serum with *A. pleuropneumoniae* serotype 1 (Fig. 5C). This was consistent with the positive control (Fig. 5D). These data suggest that the antibodies induced by *P. acnes* had the ability to combine with *A. pleuropneumoniae*, which was consistent with the results of ELISA shown in Fig. 2B. These was strong cross-reaction between anti-*P. acnes* serum and *A. pleuropneumoniae*.

Anti-*P. acnes* sera were opsonic. The presence of IgG in the immune serum has been correlated with high opsonic activity (25). We examined the efficiency of *P. acnes* hyperimmune rabbit serum in promoting opsonization and phagocytosis of *A. pleuro-*

FIG 5 Indirect immunofluorescence. Rabbit anti-*P. acnes* hyperimmune serum was prepared (titer, ≈1:40,000). No immunofluorescence was detected in the absence of anti-*P. acnes* hyperimmune serum (A and B). Positive indirect immunofluorescence signals indicated binding of anti-*P. acnes* hyperimmune serum with *A. pleuropneumoniae* serotype 1 (C). This was consistent with the positive control (D).
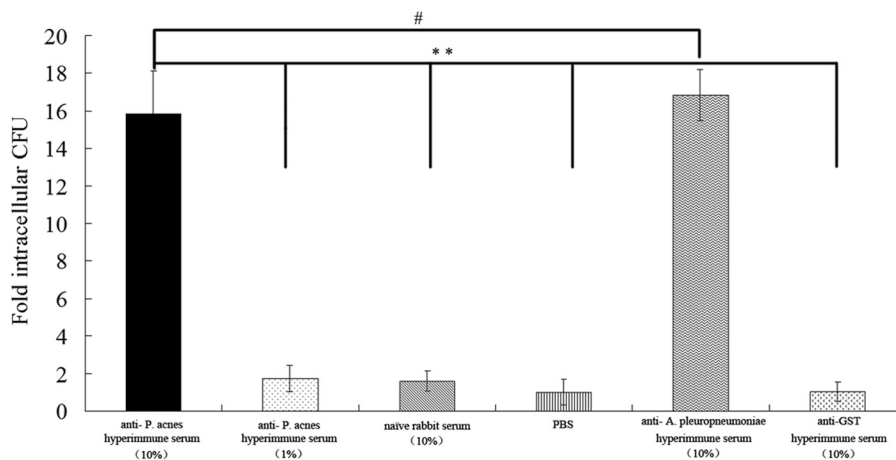
pneumoniae by J774A.1 murine macrophage cells (Fig. 6). These cells are frequently utilized to study pathogenic bacteria and for phagocytic assays (26, 27). Preimmune rabbit serum, PBS, and unrelated (GST) hyperimmune rabbit serum were used as negative controls, and titers of all the hyperimmune rabbit sera were approximately 40,000. Opsonization with *P. acnes* hyperimmune rabbit serum increased the efficiency of uptake of *A. pleuropneumoniae* by J774A.1 macrophages compared to incubation with preimmune rabbit serum, PBS, and unrelated (GST) hyperimmune rabbit serum (Fig. 6). In contrast, there were no significant differences in the phagocytosis of *A. pleuropneumoniae* following opsonization with either *P. acnes* hyperimmune rabbit serum or *A. pleuropneumoniae* hyperimmune rabbit serum. These observations confirm the specificity of anti-*P. acnes* antibody-mediated opsonization. These results therefore suggest that one of the mechanisms for the observed *P. acnes*-mediated protection is increased opsonization and phagocytosis of *A. pleuropneumoniae*.

Humoral immune response analysis in B cell depletion model mice vaccinated with *P. acnes*. BALB/c mice were immunized five times at 2-week intervals with the CD20ECD-6 immunogen using CFA for priming immunizations and IFA for boosting immunizations. At the conclusion of the immunization protocol, individual mouse spleens were assessed for determination of B cell numbers by flow cytometry as described in Materials and Methods. Immunization with CD20ECD-6 led to significant levels of B cell depletion compared to mice in the control group, with a 40% reduction in splenic B cells (Fig. 7). This was consistent with previous reports (20) which showed that a murine B cell depletion model was successfully established.

At day 35 after vaccination of B cell depletion model mice with *P. acnes*, *P. acnes*-specific IgG antibodies were detected by ELISA in the peripheral blood in all groups (Fig. 8). However, the serum antibody titer of B cell depletion model mice vaccinated with *P. acnes* was significantly lower than that of control mice (800 versus >6,400) ($P < 0.01$). This suggested that the titer of mice vacci-

FIG 6 Opsonization with anti-*P. acnes* serum enhances the phagocytosis of *A. pleuropneumoniae* by J774 murine macrophages. Opsonization with *P. acnes* hyperimmune rabbit serum increased the efficiency of uptake of *A. pleuropneumoniae* by J774 macrophages compared to control serum. In contrast, the fold intracellular CFU of group anti-*P. acnes* hyperimmune rabbit serum (10%) was significantly more than that of negative-control groups, including the preimmune rabbit serum, PBS, and unrelated (GST) hyperimmune rabbit serum groups ($P < 0.01$). Also, there were no significant differences in the uptake of *A. pleuropneumoniae* following opsonization with either *P. acnes* hyperimmune rabbit serum or *A. pleuropneumoniae* hyperimmune rabbit serum. Results are expressed as fold CFU of intracellular bacteria over the PBS treatment and are representative of three independent experiments performed in triplicate. Bars represent means ± standard deviations. Statistical analysis was carried out using the unpaired two-tailed Student *t* test. **, $P < 0.01$; #, $P > 0.05$.

nated with *P. acnes* is markedly reduced due to the depletion of B cells.

At day 35 postimmunization, the mice in all groups were challenged with *A. pleuropneumoniae* serotype 1 ($5 \times LD_{50}$) (Fig. 9). The survival rate of B cell depletion model mice vaccinated with *P. acnes* was significantly lower than that of the normal control group, and no difference was observed from the rate observed in B cell depletion model mice vaccinated with PBS. This implied that there were significant reductions both in the level of antibodies

FIG 7 Splenic B cell depletion in BALB/c mice. Two groups of BALB/c mice (13/group) were immunized s.c. five times at 2-week intervals with 40 μg CD20ECD-6 immunogen in complete Freund's adjuvant (CFA) for priming immunizations and incomplete Freund's adjuvant (IFA) for boosting immunizations. Mice in the normal control group were immunized with the same dose of adjuvant alone by the same protocol. Ten days after the final immunization, erythrocyte-depleted single-cell suspensions of individual mouse spleens were assessed using flow cytometry to enumerate CD19+ B cell numbers as described in Materials and Methods. Bar graphs represent group means ± standard deviations of B cell numbers (%). **, $P < 0.01$.
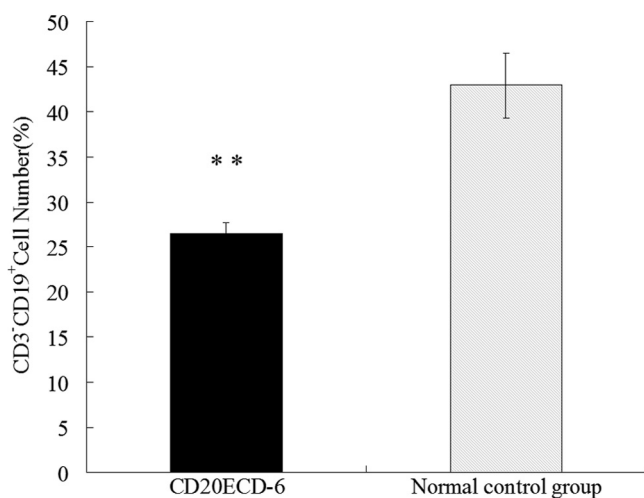
induced by *P. acnes* and in the ability to resist *A. pleuropneumoniae* infection.
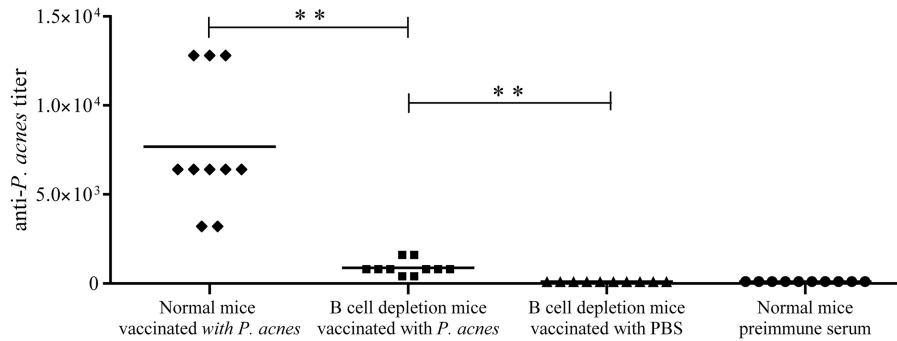
DISCUSSION

Despite all the research and trials in *A. pleuropneumoniae* vaccination conducted in the past, a safe vaccine that offers complete protection against all serotypes has not yet reached the market (5). Our previous studies have demonstrated that vaccination with *P. acnes* provides protection against *A. pleuropneumoniae* infection in mice and pigs. In these models, *P. acnes* vaccination inhibits the proliferation of the pathogen upon infection with *A. pleuropneumoniae* and the animals usually remain asymptomatic. Furthermore, over time, complete clearance of *A. pleuropneumoniae* by the immune system can be achieved in vaccinated animals (9).

The innate immune system is the first line of defense against infection. In the absence of complete pathogen clearance, the adaptive immune response is stimulated, in which specific antibodies, including IgA and IgG, play a vital role in clearing extracellular bacteria such as *A. pleuropneumoniae*.

The predominance of the CD4+ T cell population after vaccination with *P. acnes* implied the importance of humoral immunity in the response to *A. pleuropneumoniae*. Anti-*P. acnes* polyclonal antibodies can bind to and promote phagocytosis of *A. pleuropneumoniae* by phagocytes through specific opsonization. Its effect is the same as that of the anti-*A. pleuropneumoniae* antibody. Mice were immunized with the immunogen CD20ECD-6, resulting in significant elimination of B cells in the spleen and successful establishment of the murine B cell depletion model. The main effect of the B cell response is to produce specific antibodies to clear pathogens through neutralizing toxins, activating complement, opsonization, and antibody-dependent cellular cytotoxicity (28). The production of specific antibodies is hindered by B cell depletion. Thereby, the significant reduction both in the level of antibodies induced by *P. acnes* and in the ability to resist *A. pleuropneumoniae* infection confirmed that the humoral immune re-
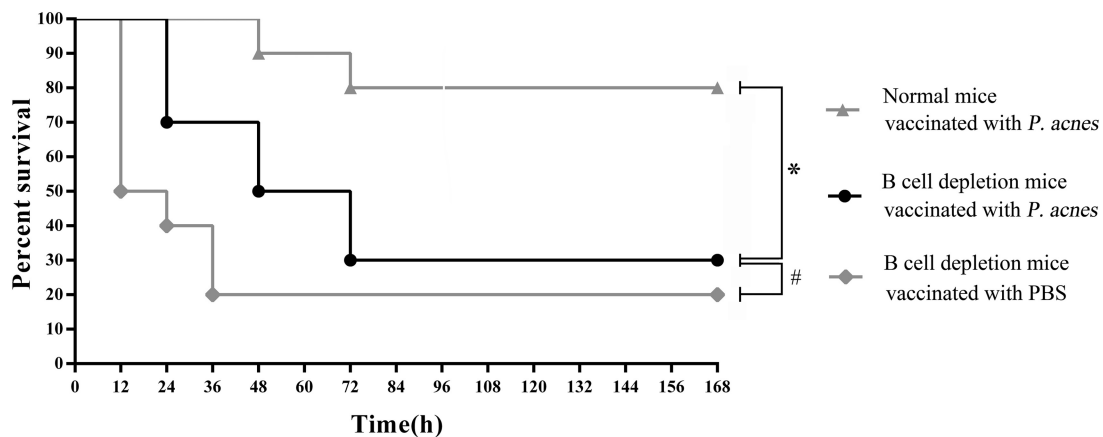
FIG 8 Antibody responses in B cell depletion mice elicited through immunization with *P. acnes*. Mice in one B cell depletion model group and one normal control group ($n = 10$ per group) were immunized s.c. with viable *P. acnes* (2×10^8 CFU) on two occasions at 1-week intervals. Mice in the other B cell depletion model group were immunized with PBS. At day 35 after the first immunization, blood was drawn from the tail to collect serum for ELISAs. Immune serum reactivity was compared with that of preimmune serum of normal mice. The anti-*P. acnes* serum titers of B cell depletion mice vaccinated with *P. acnes* were significantly lower than those of normal mice vaccinated with *P. acnes* ($P < 0.01$). **, $P < 0.01$.

sponse induced by *P. acnes* plays an important role in resisting infection by *A. pleuropneumoniae*.

The survival rates of mice challenged with *A. pleuropneumoniae* serotype 1 exhibited a significantly positive rank correlation with the antibody levels of mice immunized with *P. acnes*. It was noticeable that partial protection against *A. pleuropneumoniae* infection was observed in mice with low levels of anti-*P. acnes* antibodies (<100, 400, or 800) at day 35 after immunization with *P. acnes*. It is possible that the cytokines stimulated by *P. acnes* also play an important role in resistance to *A. pleuropneumoniae* infection. This speculation is consistent with our previous whole-genome screening of mice immunized with *P. acnes* for differentially expressed genes by high-throughput microarray. Significant differentially expressed cytokines included Epor, Retnlg, Selplg, Xcl1, interleukin 33 (IL-33), Pik3ip1, IL-17ra, IL-2, IL-7, IL-11, IL-15, and gamma interferon (IFN-γ). These cytokines are known to (i) regulate immune recognition (for instance, IFN-γ induces the expression of major histocompatibility complex II molecules by antigen-presenting cells to promote antigen presentation), (ii) participate in immune cell proliferation of immune cells (for example, IL-2, IL-4, IL-5, and IL-6 induced by *P. acnes* [29] promote

T cell and B cell activation, proliferation, and differentiation), (iii) participate in immune effects (for example, IFN-γ released by CD8^{+} effector T lymphocytes has the ability to inhibit viral replication and resolve intracellular infection without killing cells; *P. acnes* enhances Th17 cells to secrete IL-17, IL-6, tumor necrosis factor [TNF], and CXCL1, which recruit neutrophils to infected sites at the onset of the infection [30]; *P. acnes* also has the ability to promote Th1 cells to produce cytokines such as TNF-α, IFN-γ, and granulocyte-macrophage colony-stimulating factor [GM-CSF], which may enhance phagocytosis and cytotoxicity mediated by macrophages [30]; and IL-3 and GM-CSF secreted by Th1 and Th2 cells stimulate the bone marrow to generate macrophages, granulocytes, and dendritic cells), and (iv) participate in immune regulation. Immune cells are capable of stimulating and inhibiting each other by secreting cytokines during the course of immune response, thereby mediating effective regulation.

P. acnes and *A. pleuropneumoniae* are Gram-positive and Gram-negative bacteria, respectively. Why could the Gram-positive bacteria provide cross protection against Gram-negative bacterial infection? From our experimental results, we speculated that the possible reasons are as follows: (i) there may be some Forss-

FIG 9 Survival rates of B cell depletion mice challenged with *A. pleuropneumoniae* after immunization with *P. acnes*. Mice in one B cell depletion model group and one normal control group were immunized s.c. with viable *P. acnes* (2×10^8 CFU) on two occasions at 1-week intervals. Mice in the other B cell depletion model group were immunized with PBS. At day 35 after the first immunization, the mice in all groups were challenged i.p. with 0.2 ml of *A. pleuropneumoniae* bacterial suspension containing 4.0×10^7 CFU (equivalent to $5 \times$ LD$_{50}$). The survival rates of all groups were calculated on day 7 postchallenge. *, $P < 0.05$; #, $P > 0.05$.

man antigens shared between *P. acnes* and *A. pleuropneumoniae*, which are unrelated to the species and are the common antigens among humans, animals, and microorganisms; (ii) Forssman antigen is a glycosphingolipid with antigenic specificity determined by extramembrane haptenic sugars similar to blood group antigens and antigens that are the main barriers to xenogeneic organ transplantation (31); and (iii) there may be some common B cell epitopes between *P. acnes* and *A. pleuropneumoniae*, which we have published elsewhere (32). It is possible that the cytokines stimulated by *P. acnes* also play an important role in resistance to *A. pleuropneumoniae* infection. However, these hypotheses need to be studied further.

In this study, we further elucidate the mechanism by which *P. acnes* prevents porcine contagious pleuropneumonia, which provides the basis of further investigations into the development of heterologous vaccines for the control of porcine pleuropneumonia.

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