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Dual Immunization with Lipoprotein Tp0663 and Flagellin FlaB3 Offers Augmented Protection against *Treponema pallidum* in Mice

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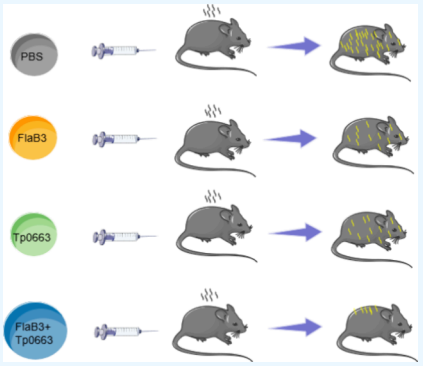
ABSTRACT: Syphilis is a significant multistage sexually transmitted infection caused by the bacterium *Treponema pallidum*. The pathogenesis of this pathogen remains inadequately understood, impeding the progress of syphilis vaccine development. Our prior study has demonstrated the potential of the Tp0663 protein as a viable candidate for a vaccine against *T. pallidum*. In the present study, we sought to explore the protective response of dual immunization using two different antigenic entities (i.e., flagellin FlaB3 and lipoprotein Tp0663) against *T. pallidum* in a murine model. Our investigation revealed that FlaB3 + Tp0663 can elicit robust humoral and cellular immune responses. In addition, the FlaB3 + Tp0663 vaccine demonstrated a notable reduction in the Treponemal burden within various anatomical sites of infected mice, including the blood, brain, liver, lymph nodes, spleen, and testicles. It is worth noting that the FlaB3 + Tp0663 vaccine suppressed the dissemination of *T. pallidum* in C57BL/6 mice. The findings demonstrate that *T. pallidum* flagellin FlaB3 may augment the immunoprotection of Tp0663. This represents a valuable practical perspective and offers insights into developing a syphilis vaccine.



1. INTRODUCTION

Syphilis is a detrimental multistage sexually transmitted infection, attributed to *Treponema pallidum* subsp. *pallidum* (*T. pallidum*) and is known to result in multiple organ failure. It is primarily transmitted through sexual contact. Additionally, it can be vertically transmitted from mother to fetus during pregnancy and delivery. This mode of transmission may result in miscarriage, stillbirth, premature delivery, teratosis, or fetal syphilis infection. Despite understanding syphilis transmission and its curability with antibiotics, it remains common in low and middle-income countries and has resurged among men who have sex with men. There has also been an increase in syphilis cases among women, leading to more congenital infections.[1] Historically, over 90% of syphilis infections were concentrated in low- and middle-income countries. However, there is now a notable increase in syphilis incidence in regions including Europe, the UK, the US, Canada, and China.[1−4] Congenital syphilis is one of the important reasons for the increase in stillbirths worldwide. The prevalence of congenital syphilis was 0.69% in 2016, with a rate of 473 cases per 100,000 live births.[5] Syphilis is regarded as the second most prevalent cause of stillbirths after malaria.[6] Approximately 7.7% of the worldwide stillbirth rate is associated with congenital syphilis infection.[5−7] Although syphilis is readily diagnosable and treatable, current methods pose challenges in discerning an early-stage infection. Furthermore, there is a challenge of macrolide antibiotic resistance in syphilis replacement therapy. Hence, the eradication of syphilis through syphilis screening and treatment alone presents considerable challenges. Consequently, the development of effective syphilis vaccines is imperative for the prevention and control of this disease.

Syphilis vaccines have progressed through several developmental stages, including whole-cell vaccines, live attenuated vaccines, recombinant subunit vaccines, and nucleic acid vaccines.[8] Although whole-cell vaccines can provide complete protection, the application of them to humans is impractical. Whole-cell and live attenuated vaccines can induce strong immune protection effects because of their natural adjuvant components, but they also exhibit some side effects. Many other types of vaccines do not offer complete protection. Recombinant subunit vaccines have emerged as an important area of focus for developing syphilis vaccines. These vaccines provide safety benefits and minimal side effects. However, they have been found to have poor immunogenicity, meaning that they cannot induce long-lasting and effective protective immunity on their own. If the antigen type of the combined immunization is similar or if certain antigens can be used as carrier proteins, it would enhance the immune effect produced. Furthermore, vaccine composition has a significant impact on the immune response produced; however, there is little

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research respecting multiple antigen combinations on immune responses, especially on *T. pallidum* infection.

Flagellin is a potent immunogen capable of activating various immune cells and eliciting innate and adaptive immune responses by interacting with TLR5. This interaction enhances the immunogenicity of antigens and facilitates local and systemic immune protection.[9] Furthermore, flagellin predominantly elicits an effect similar to that of vaccine adjuvants through the combination or fusion with exogenous antigens. Research has demonstrated that the coadministration of flagellin and exogenous antigens via intranasal drops significantly elevates exogenous antigen-specific IgA levels in both mucosal secretions and serum of the subjects under investigation.[10] In addition, the combined vaccine provided full protection against a lethal challenge posed by the homologous virus.[11] Several vaccines incorporating flagellin have advanced to the clinical trial stage.[12]

Previous research on *T. pallidum* has employed various animal models, with the rabbit model being the most extensively utilized.[13,14] The rabbit model has the advantage of being easily infected with *T. pallidum*,[15] and it has partly similar serological reactions, skin lesions, and tissue immunopathological changes to humans.[14,16,17] However, considering the New Zealand rabbit's outbred nature, genetic manipulation presents notable challenges and is subject to substantial limitations. Moreover, the scarcity of specialized research reagents hinders comprehensive investigations of rabbit-associated studies. Hence, identifying novel animal models can advance *T. pallidum* research significantly. During the middle of the 20th century, it was documented that mice could be infected with *T. pallidum* and the bacterium could persist in mice for a prolonged duration. However, no discernible skin lesions were evident.[18] In the 1980s, Klein et al. observed that specific strains of mice were susceptible to developing cutaneous lesions after infection with *T. pallidum*.[19] Inbred strain mice provide the advantage of a well-defined genetic background and are amenable to genetic manipulation. Furthermore, there is a wealth of related research reagents available. Accordingly, C57BL/6 mice were adopted as animal models for syphilis vaccine research in this investigation.

Our previous results have confirmed that the *T. pallidum* outer membrane protein Tp0663 exhibits a good immune protection effect.[20] Since the selected Freund's adjuvant cannot be used in the human body and the combined immunization with flagellin and other antigens can enhance the immune protection effect, the safe and effective *T. pallidum* flagellar core protein FlaB3 was chosen to further study the immunoprotective effect of recombinant protein Tp0663 in C57BL/6 mice in this study.

2. MATERIALS AND METHODS

2.1. Preparation of Recombinant Proteins FlaB3 and Tp0663. The purification of recombinant FlaB3 and Tp0663 proteins was performed as previously described[21,22] and then treated with a Detoxi-Gel Endotoxin Removing Gel. Limulus amebocyte lysate (Chinese Horseshoe Crab Reagent Manufactory, Ltd., Xiamen, China) was used to detect the endotoxin in the purified proteins, which was found to be less than 0.03 endotoxin unit (EU)/mL.

2.2. *T. pallidum* Propagation. Propagation of *T. pallidum* (Nichols strain) was performed as previously described.[23]

2.3. Immunization Procedure. C57BL/6 mice were randomly divided into four groups: PBS control group, FlaB3-immunized group, Tp0663-immunized group, and FlaB3 + Tp0663-immunized group, with six animals in each group. Each animal was intraperitoneally injected three times at 2-week intervals (0, 2, and 4 weeks) with 30 μg of test antigen or PBS at each immunization. The antiserum was collected to estimate the serum antibody titers. 2 weeks following the final immunization, three mice were randomly selected from each group. Spleens from these mice were harvested for splenocyte proliferation experiments and the detection of related cytokines, while the remaining mice were allocated for subsequent *T. pallidum* infection experiments.

2.4. ELISA Analysis of Antibody Levels. Tp0663 recombinant protein was diluted to 100 μg/mL with PBS, and 100 μL was added to each well and coated overnight in a 4 °C refrigerator. The next day, the liquid in the well was discarded and the plate soaked and washed three times in PBST, 2∼3 min each time. The washing solution was dried in the ELISA plate, 250 μL of blocking solution (prepared with 5% skim milk powder) added to each well, and then incubated at 37 °C for 2 h. The blocking solution was discarded and the plate washed five times in PBST, 2∼3 min each time. 100 μL of 5% skimmed milk was added to each well in advance; 100 μL of 1000-fold diluted immune mouse serum was taken from the first well and serially diluted (PBS control mouse serum as a control); the dilution residue was discarded in the last well and the ELISA plate placed in a 37 °C incubator and then incubated for 2 h. The liquid in the well was discarded, the plate soaked and washed five times in PBST, 2∼3 min each time. Goat antimouse IgG secondary antibody was diluted at 1:5000, 100 μL added to each well of the ELISA plate, and incubated at 37 °C for 1 h. The liquid was discarded in the well and the plate soaked and washed for five times in PBST, 2∼3 min each time. 100 μL of chromogenic substrate was added dropwise to each well and reacted for 15 min at 37 °C in the dark. Then, 100 μL of 1 M sulfuric acid was added to stop the color reaction. Each experiment was repeated three times, and the absorbance was measured at a wavelength of 450 nm (BioTek Instruments, Winooski, VT, USA).

2.5. Splenic Cell Proliferation Assay. Three spleens from each group were harvested, and a part of the splenic cells from each mouse was resuspended in RPMI-1640 (1% antibiotics) to evaluate the Tp0663-specific cellular immune response. Cells were diluted to 6×10^6/mL and seeded onto culture plates. Each well received 10 μg/mL of purified Tp0663 recombinant protein (add the same volume of PBS as a control) for restimulation, and the plate was incubated at 37 °C with 5% CO_2 for 44 h. Lymphocyte proliferative capacity was estimated using a Cell Counting Kit-8 (Dojindo, Japan).

2.6. Cytokine Determinations from the Spleen by ELISA. The spleen cell suspension was taken, the cell density adjusted to 6×10^6/mL, and 800 μL added to each well of a 24-well cell culture plate. Then, 10 μg of Tp0663 recombinant protein was added to each well (the same volume of PBS was added as a control), gently blown, and mixed. The cell culture plate was placed in a 5% CO_2 cell culture incubator, cultured at 37 °C for 72 h, centrifuged at 5000 rpm for 20 min, and the splenocyte supernatant collected. The production of cytokines IL-4 and IFN-γ was determined using commercially available ELISA kits (eBioscience, San Diego, CA, USA), separately, following the instructions provided by the manufacturer.

2.7. Flow Cytometry. 800 μL 6×10^6/mL spleen cell suspension was added to each well of a 24-well cell culture plate, then 0.5 μL of BD GolgiStop added, and gently blown

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Table 1. Primer Sequences Used in qRT-PCR

Gene	Organism	Sequence (5' to 3')	Annealing Temp
FlaA	*T. pallidum*	F: AACGCAAACGCAATGATAAA	55°C
		R: CCAGGAGTCGAACAGGAGATAC	
β-actin	Mouse	F: CCTTCCTTCTTGGGTATGGA	55°C
		R: ACGGATGTCAACGTCACACT	

and mixed; the cell culture plate was put into a 5% CO_2 cell culture incubator and incubated at 37 °C for 5 h. Collecting cell pellets by centrifugation, 1 mL of precooled PBS was added to each tube to wash the cells, centrifuged at 300g, 4 °C for 5 min, and repeated twice. 50 μL of the staining buffer was added, the cells gently blown and resuspend, the appropriate amount of anti-CD3 and CD4 fluorescent monoclonal antibodies was added, the cells were incubated at 4 °C for 30 min, and 1 mL of the staining buffer was added to each tube to wash the cells twice at 250g and then centrifuged at 4 °C for 5 min. 250 μL of the permeabilization solution was added to the above cells, the cells blown and mixed well, and the membrane permeated at 4 °C for 20 min, then 1 × BD perm/wash buffer 1 mL was added to each tube to wash the cells and repeated two times. An appropriate amount of IL-4 and INF-γ fluorescent antibody or negative control with BD perm/wash buffer was diluted to 50 μL; the diluted antibody solution was added to the fixed and ruptured cells and the cells fully resuspended. It was stained at 4 °C in the dark for 30 min. To collect cell aggregates, it was centrifuged, 1 mL of 1 × BD perm/wash buffer added to each tube to wash the cells twice, and then resuspend with 500 μL of staining buffer. Cells were acquired on a FACS Calibur instrument (BD Bioscience). Data were analyzed with the FlowJo.

2.8. Intradermal Challenge. Two weeks after the final immunization, C57BL/6 mice were infected by intragastric injection, intraperitoneal injection, intradermal injection, and cavernous injection with 2.5×10^6 freshly isolated *T. pallidum* (Nichols strain), and the total amount of *T. pallidum* infection per mouse was 1×10^7. During *T. pallidum* infection, it was observed whether there were obvious skin lesions on the back of the infected C57BL/6 mice and whether their testes were swollen and inflamed. Thirty days after *T. pallidum* infection, all the infected mice were killed by anesthesia, and the lymph nodes of the mice were isolated for subsequent lymphatic suspension transfer experiments; the testicle, liver, and spleen tissues of the mice were isolated and soaked in 10% neutral formaldehyde. The specimen was fixed in the solution for subsequent immunohistochemical analysis; blood was taken from the eyeball and part of the testicle, liver, spleen, rectum, lymph node, and brain tissue of the mouse taken out in a 1.5 mL clean EP tube and stored at −80 °C for subsequent tissue extraction. Genomic DNA analysis of the *T. pallidum* load was conducted in each organ.

2.9. Mouse Lymphatic Suspension Transfer Experiment. All of the mice were sacrificed under anesthesia after being infected for 30 days, and three lymph nodes in the groin, humerus, and axillary of the mice were isolated and transferred to be suspended. Mouse lymph solution was injected into the rabbit testicle according to the subculture method of *T. pallidum*. The rabbit testicle was observed every day to determine whether there was inflammation. Blood was drawn from the veins of the rabbit's ears every week, and then seroconversion was detected by TPPA and RPR. Nine weeks after the mouse lymphatic transfer to New Zealand rabbits, the rabbits were anesthetized and air embolized, the testicular parenchyma solution was collected, and the *T. pallidum* content was detected by qRT-PCR and dark field.

2.10. Extraction of *T. pallidum* DNA from Tissues and qPCR. The genomic DNA (gDNA) was extracted from *T. pallidum*-challenged C57BL/6 mice tissues, including blood, brain, liver, lymph node, spleen, and testicle, by using a DNeasy Blood and Tissue Kit (Qiagen, Shanghai, China) according to the protocol recommended by the manufacturer. Three tissue samples from each C57BL/6 mouse organ were analyzed for reproducibility. The gDNA was used as the template in the qPCR mixture according to the manufacturer's standard protocol for QuantiFast SYBR one-step qPCR (Qiagen, Shanghai, China) and performed using a LightCycle 96 apparatus (Roche, Basel, Switzerland). Quantification of *T. pallidum* gDNA was determined using primers for the endoflagellar sheath protein (*flaA*) gene of *T. pallidum* and the *β-actin* gene of C57BL/6 mice (Table 1). A standard curve was created for *flaA* using a 10-fold serial dilution range from 10^7 to 10^1 copies of *T. pallidum* gDNA. A standard curve was created for *β-actin* using a twofold serial dilution from 100 to 0.0488 ng/mL of mice gDNA. The original gDNA concentration of C57BL/6 mice tissue used to create the standard curve was obtained from spectrophotometric measurements. PCR conditions for flaA and *β-actin* were as follows: initial denaturation at 95 °C for 7 min, followed by 40 cycles of 95 °C for 10 s, 56 °C for 20 s, and 72 °C for 20 s. A melt-curve analysis was performed with the following program: 95 °C for 10 s, 65 °C for 260 s, and 97 °C for 1 s. Each assay was performed in triplicate. In addition, each assay was run with a no-template control.

2.11. Histopathology. Tissues taken from the testicles, livers, and spleens of each mouse were fixed in formalin and embedded in paraffin. Tissues were then stained with S−P immunohistochemistry 30 d after infection with *T. pallidum*. The UltraSensitiveSP IHC Kit, with a mouse anti-*T. pallidum* was used as the first antibody to detect *T. pallidum*. Mice immunized with PBS were used as controls.

2.12. Ethics Statement. C57BL/6 mice were obtained from Hunan SJA Laboratory Animal Co. Ltd. (Changsha, China, Animal Production License No. SYXK 2021-0002) and kept in the Animal Experiment Research Center at the University of South China. Mice were maintained under standard conditions and treated by following the institutional guidelines of laboratory animals. All animal experiments were approved by the Experimental Animal Ethical Committee of the University of South China.

2.13. Statistical Analysis. The GraphPad Prism 6.0 software (GraphPad Software, Inc., La Jolla, CA) was used to analyze the data statistically. For specific statistical methods, refer to the corresponding legend. Results are reported as means ± SD. All comparisons of differences between the test

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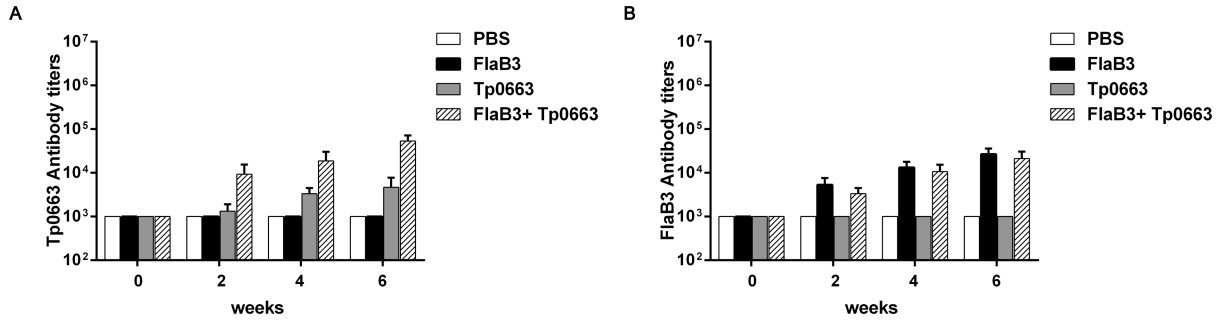

Figure 1. Detection of serum IgG in mice immunized with recombinant Tp0663 protein or flagellin FlaB3. C57BL/6 mice were immunized with 100 μg of recombinant Tp0663, FlaB3, or 100 μg of recombinant Tp0663 mixed with 100 μg of recombinant FlaB3. The control mice were immunized with PBS. Serum samples were collected from the mice's tail veins at 0, 2, 4, and 6 weeks, and the IgG antibody levels were determined by indirect ELISA. Each set of data is based on measurements derived from three mice.

and control groups were assessed via the Student's *t* test. $P < 0.05$ indicates a statistically significant difference in the results.

3. RESULTS

3.1. Analysis of Specific Antibody Levels in the Serum of Immune Mice. The levels of Tp0663-specific IgG antibodies in the serum of mice within each group were assessed using indirect ELISA. The findings are depicted in Figure 1A. Upon challenge to the recombinant protein Tp0663 for 2 weeks, C57BL/6 mice demonstrated the production of IgG antibodies. Notably, the titer of Tp0663-specific IgG antibodies exhibited an increase in correlation with the number of immunizations compared to both the PBS control group and the FlaB3-immunized group. Furthermore, the concentration of Tp0663-specific IgG antibody generated from coimmunizing C57BL/6 mice with recombinant protein Tp0663 and FlaB3 exceeded that of the group solely immunized with Tp0663. This observation indicates the potential of *T. pallidum* flagellar core protein FlaB3 in augmenting the immunogenic properties of Tp0663, while Tp0663 showed no enhanced effect for FlaB3 immunogenicity (Figure 1B).

3.2. Intracellular Cytokine Detection of $CD4^+$ T Cells in Mouse Splenic Lymphocytes. A multicolor staining method was employed for flow cytometry analysis to evaluate the expression of intracellular cytokines in $CD4^+$ T cells within mouse splenic lymphocytes after the combined immunization of C57BL/6 mice with recombinant proteins FlaB3 and Tp0663. The findings revealed that the $CD4^+$ T cells in the splenocytes of the mice in each group mainly secreted IFN-γ and secreted only a small amount of IL-4 (Figure 2). Moreover, the proportion of $CD4^+$ T cells secreting IFN-γ in the combined immunization group FlaB3 + Tp0633 was notably higher when compared with that immunized with the antigen alone.

3.3. Detection of Cytokines in Mouse Spleen Lymphocyte Supernatant. Spleen cells from C57BL/6 mice were cultured *in vitro* with the recombinant protein Tp0663. Subsequently, the levels of IL-4 and INF-γ secreted by the spleen lymphocytes were quantified using ELISA (Figure 3). The findings highlight the importance of using two antigens in combination, which elicits a significantly higher level of INF-γ secretion. Moreover, while the FlaB3 + Tp0663 combined immunization regimen effectively stimulated splenocytes to produce higher levels of IL-4 compared with the control group receiving PBS, the quantity of IL-4 secretion was notably lower than that of INF-γ. Consequently, the combined immunization primarily elicited INF-γ secretion.

3.4. Proliferative Response of Mouse Splenic Lymphocytes. The recombinant protein Tp0663 elicited a proliferation response in C57BL/6 mouse splenocytes when tested *in vitro*. Cell proliferation was assessed by using CCK8. The results are illustrated in Figure 4. The cell stimulation index (SI) of the FlaB3 + Tp0663 combined immunization group demonstrated a significant increase compared to both the PBS control group ($P < 0.01$) and the Tp0663 immunization group ($P < 0.05$).

3.5. Combined Immunization of C57BL/6 Mice with FlaB3 and Tp0663 Inhibits *T. pallidum* Proliferation. Following *T. pallidum* infection for 30 days, qRT-PCR analysis revealed detectable *T. pallidum* loads in the rectum, brain, lymph node, blood, liver, spleen, and testicle tissue of the mice in each immunization group (Figure 5). The results indicated that the *T. pallidum* load in the blood of the mice was most pronounced in the PBS control group. In contrast, the FlaB3 + Tp0663 combined immunization group exhibited a significantly lower *T. pallidum* load compared to the single immunization group ($P < 0.05$). The *T. pallidum* load in the rectum of the FlaB3 + Tp0663 combined immunization group exhibited levels similar to those observed in both the PBS control group and the Tp0663 immunization group. These indicated that combined immunization with FlaB3 and Tp0663 could inhibit the dissemination of *T. pallidum* in C57BL/6 mice.

3.6. *T. pallidum* Load in Infected Mouse Tissues Detected by Immunohistochemistry. Immunohistochemistry was employed to qualitatively detect *T. pallidum* in the liver, spleen, and testicle tissues of infected mice (Figure 6A,B,C) to further investigate the potential of combined immunization with FlaB3 and Tp0663 to impede the dissemination of *T. pallidum* in C57BL/6 mice. The findings demonstrated a notable reduction in the presence of *T. pallidum* brown filaments in the interstitial space of the testes of mice in the FlaB3 + Tp0663 combined immunization group compared with both the PBS control group and the Tp0663 immunization group. Furthermore, the brown-yellow filaments in the liver and spleen of the FlaB3 + Tp0663 combined immunization group were significantly lower than those in the PBS control group and the Tp0663-immunized group. These observations further revealed that the combination of FlaB3 + Tp0663 immunization inhibits the dissemination of *T. pallidum* in C57BL/6 mice.

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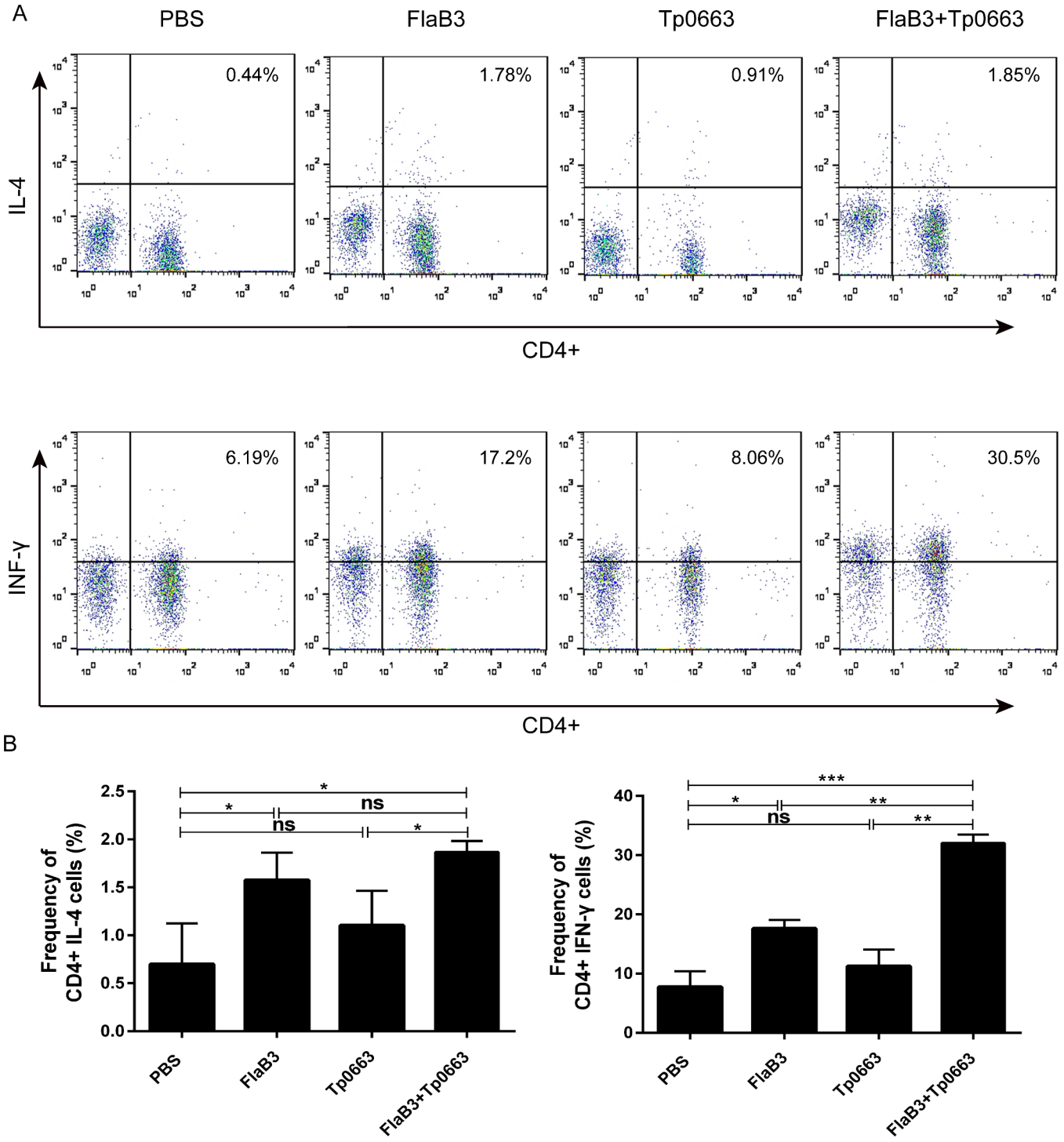

Figure 2. FlaB3 + Tp0663-induced specific T cell responses in the spleen. Splenocytes were isolated from three mice of each group at day 30 postimmunization and assayed for Tp0663-specific $CD4^+$ T cell response. (A) Tp0663-specific $CD4^+$ T cell response was determined by intracellular IL-4 and IFN-γ staining. (B) Statistical analysis of Tp0663-specific $CD4^+$ T cell response of three mice in each group. Data are presented as the means ± SD. Statistical significance was determined using Student's t test ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

3.7. Mouse Lymphocyte Suspension Transfer Experiment. To further verify the potential immune protection offered by the combined immunization of FlaB3 + Tp0663, lymph nodes were extracted from C57BL/6 mice of each immunized group following a 30-day challenge with *T. pallidum* (three mice per group). Subsequently, the lymph nodes were transplanted into the testes of New Zealand rabbits. Weekly blood samples were obtained from the rabbits' ear veins and analyzed for serum reactivity using RPR and TPPA. Nine weeks later, the New Zealand rabbits underwent anesthesia and were subsequently euthanized. The activity of *T. pallidum* was observed using dark-field microscopy, and the *T. pallidum* load in the testicular tissue was quantified using qRT-PCR. The findings are detailed in Table 2. Following 20 days post inoculation of the PBS control group mouse lymph suspension into New Zealand rabbits, the rabbits demonstrated

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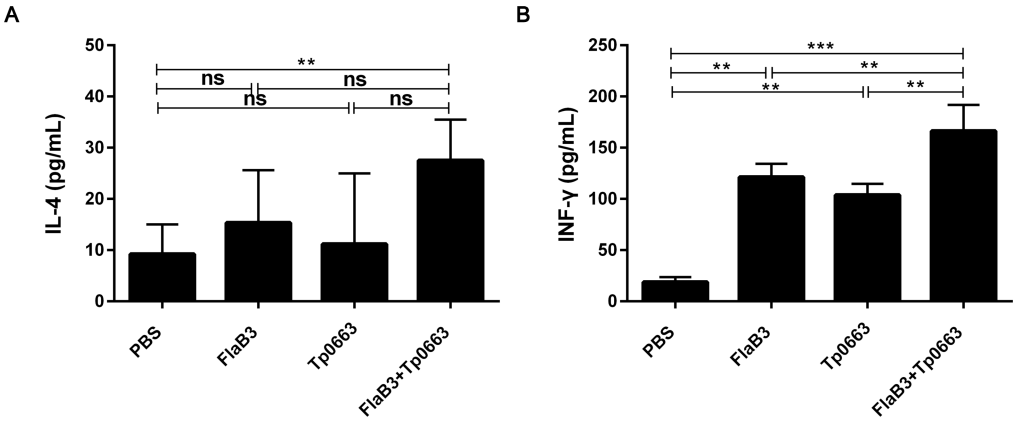

Figure 3. Cytokine productions by splenocytes isolated from immunized mice after restimulation with Tp0663. Splenocytes collected from immunized and nonimmunized mice were detected using mice IL-4 (A) and IFN-γ (B) ELISA kit according to the manufacturer's instructions. The results are expressed as the mean ± SD from three individual mice in each group. Each experiment was performed in three independent experiments (ns = not significant, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

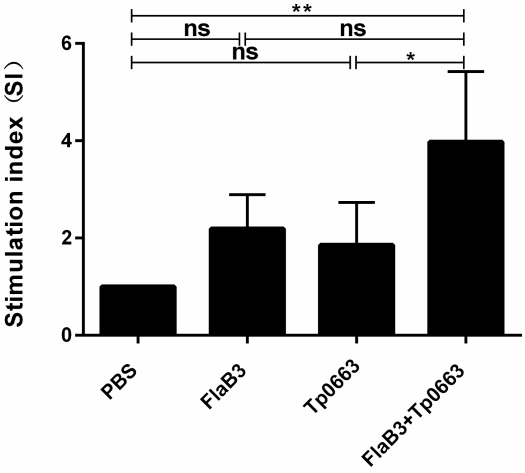

Figure 4. SI of Tp0663-specific splenic T lymphocyte proliferation. Spleen cells collected from immunized and nonimmunized mice were restimulated with 10 μg of Tp0663. After culturing for 44 h, T lymphocyte proliferations were detected using a CCK8 kit according to the manufacturer's instructions. SI is presented as the *A450* value of the stimulation group/control group. The results are expressed as the mean ± SD from three individual mice in each group. Each experiment was performed in three independent experiments ($*P < 0.05$, $**P < 0.01$).

seropositivity, registering an RPR titer of 1:1. The New Zealand rabbits demonstrated seroconversion 32 days following the inoculation of the lymph suspension from mice in the FlaB3 and Tp0663-immunized groups. Seroconversion in the FlaB3 + Tp0663-immunized group of New Zealand rabbits occurred 52 days after the inoculation of the lymphatic suspension from mice. In addition, dark-field microscopy findings demonstrated the presence of viable *T. pallidum* exclusively in the testes of New Zealand rabbits that had been inoculated with the lymph suspension of PBS control mice (Table 2). At the same time, the qRT-PCR results indicated that the *T. pallidum* load in the testes of New Zealand rabbits inoculated with the lymph suspension of PBS control mice was higher compared to the other three groups (Table 2).

4. DISCUSSION

Currently, *T. pallidum* still lacks an effective vaccine. Its outer membrane proteins are widely regarded as the most promising syphilis vaccine candidate and are extensively utilized in syphilis vaccine research.[8,24,25] Since the pathogenesis of pathogens such as *T. pallidum* is a multifactorial process mediated by multiple virulence factors, modern vaccine development strategies focus on the rational design of vaccines to target these antigens to trigger more targeted and effective immune responses. For example, Vij et al. have reported substantially enhanced protective immunity against enteric fever after immunizing the mice with a combination of CdtB protein and flagellin epitope.[26] Considering the augmented protective potential offered by a combination of antigens, this study also exploits flagellar core protein FlaB3 and outer membrane protein Tp0663 in combination with *T. pallidum*.

The adjuvant effect of flagellin has been extensively researched in various viral and bacterial vaccines.[27−30] However, its adjuvant effect in a syphilis vaccine has not yet been reported. The adjuvant effect of flagellin predominantly relies on its capacity to activate innate and adaptive immunity, thereby augmenting the production of specific antibodies.[31] The interaction between flagellin and TLR5 on the surface of innate or nonimmune cells can prompt the expression of a wide array of proinflammatory cytokines and chemokines. Consequently, this process activates the antigen-specific adaptive immune response.[31,32] Studies have demonstrated that the inclusion of flagellin in vaccines results in a significant increase in its concentration within the draining lymph nodes. Consequently, this activates dendritic cells and other nonimmune cells within the draining lymph nodes, producing cytokines and chemokines. Consequently, T and B lymphocytes are recruited to the draining lymph nodes, ultimately optimizing the recognition of cognate antigens by antigen-specific lymphocytes.[33,34] In our prior research, it has been demonstrated that the *T. pallidum* flagellar core protein effectively binds to TLR5 located on the surface of monocytes, thereby eliciting the secretion of proinflammatory cytokines.[35] This investigation sought to assess the potential immune effect when coimmunizing mice with *T. pallidum* flagellar core protein FlaB3 and outer membrane protein Tp0663. The findings demonstrate that augment immunogenicity was observed in the mice group immunized with FlaB3 and

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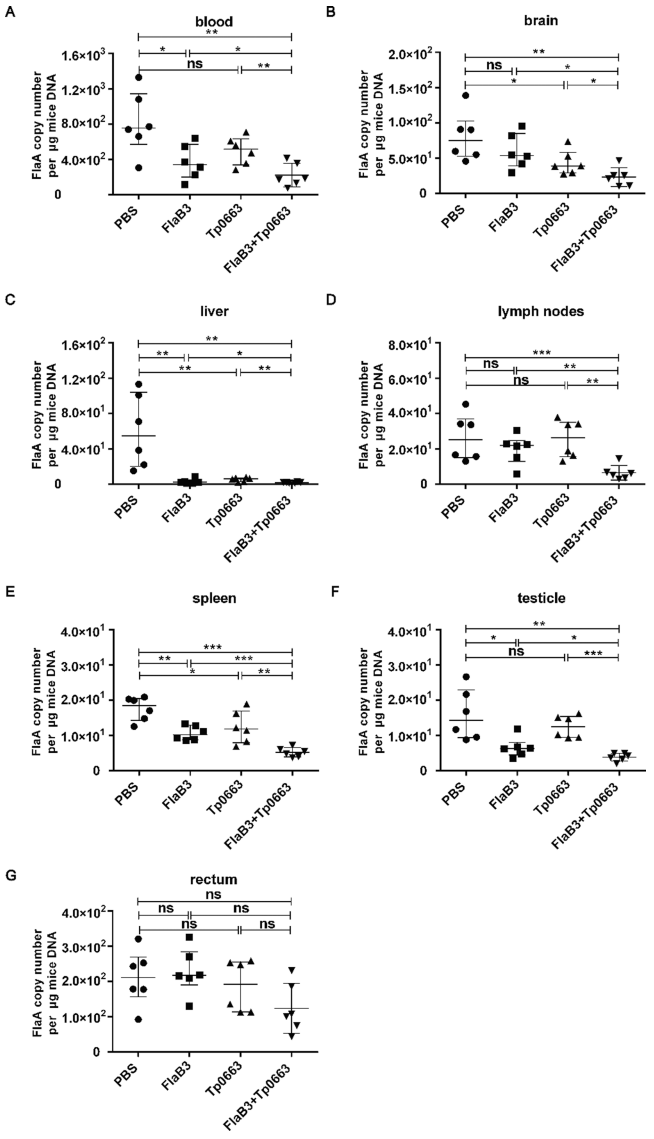

Figure 5. Immunization with FlaB3 + Tp0663 inhibited *T. pallidum* dissemination. Spirochete numbers were evaluated in PBS, FlaB3, Tp0663, or FlaB3 + Tp0663-immunized animals using qRT-PCR to measure *flaA* DNA concentrations in lesion biopsies. Bacterial burdens in the blood (A), brain (B), liver (C), lymph node (D), spleen (E), testicle (F), and rectum (G) at day 30 postinfection. Results were normalized within each tissue type based on the concentration of mouse gDNA and presented as the median ± interquartile range. Significance was assessed using Student's *t* test (ns = not significant; $P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). Points corresponding to two separately extracted tissue samples from each mouse organ (six total points in each group) were analyzed for reproducibility.

Tp0663 when compared to the mice immunized with the antigen alone at the same concentration, implying its promising role in enhancing immunoprotection.

The effective clearance of *T. pallidum* within the body primarily relies on the activation of macrophages by Th1 cytokines produced by $CD4^+$ T cells and on *T. pallidum*-specific antiserum to enhance the opsonophagocytosis of macrophages.[36,37] Previous research has indicated that the heightened expression of Th1 cytokine INF-γ has been observed in the serum of individuals with syphilis. This particular cytokine is closely associated with eradicating *T. pallidum* at the site of infection.[38,39] Furthermore, prior research has validated that administering the outer membrane protein Tp0663 increased serum INF-γ content in New Zealand rabbits.[20] The present research further explored whether Tp0663 can induce a Th1 immune response in mice with the assistance of *T. pallidum* flagellar core protein FlaB3. The enumeration of INF-γ-secreting $CD4^+$ T cells and IL-4-secreting $CD4^+$ T cells in the splenocytes of *T. pallidum*-infected mice was conducted through flow cytometry. The results showed that the proportion of INF-γ-secreting $CD4^+$ T cells was significantly higher than IL-4-secreting $CD4^+$ T cells following coimmunization with FlaB3 and Tp0663 in mice, indicating that the cellular immune response mediated by $CD4^+$ Th1 cells was dominant after FlaB3 and Tp0663 coimmunized C57BL/6 mice. Following stimulation of *T. pallidum*-infected mouse splenocytes with Tp0663 *in vitro*, ELISA was employed to quantify the levels of INF-γ and IL-4 in the splenocyte supernatant. The results demonstrated a significant elevation in INF-γ levels compared to IL-4, further indicating that Th1-type immune response was dominant in mice coimmunized with FlaB3 and Tp0663. Moreover, when C57BL/6 mice were immunized with either Tp0663 or FlaB3 alone in this study, there was a predominant presence of $CD4^+$ T cells secreting IFN-γ in the splenocytes. However, the levels of these cells were significantly lower than those that received combined immunization with FlaB3 and Tp0663. This indicates that FlaB3 may have the potential to enhance the immunoprotective effects of specific antigens.

Vascular diffusion is one of the important factors in the pathogenesis of *T. pallidum*. Given that *T. pallidum* can penetrate the endothelium, placenta, and blood−brain barrier during the initial stages of infection and can invade various organs of the body,[13,40,41] inhibiting the spread of *T. pallidum* could serve as a crucial criterion for evaluating potential syphilis vaccines. In the current investigation, qRT-PCR was employed to ascertain the *T. pallidum* load in the blood, rectum, liver, spleen, lymph node, testicle, and brain tissue of mice within each specific immune group after *T. pallidum* infection. The quantification of *T. pallidum* in the blood and brain tissue of the control group demonstrated the highest levels, suggesting a potential correlation with the transmission pattern of *T. pallidum* within the body. Previous studies have indicated that *T. pallidum* can invade the central nervous system in approximately 40% of patients with early syphilis.[13,42] This phenomenon may help explain the higher presence of *T. pallidum* in the brain tissue of the control mice. However, after coimmunizing mice with FlaB3 and Tp0663, the *T. pallidum* loads in the blood, liver, spleen, lymph nodes, testicle, and brain tissues of the mice were significantly lower than those in the Tp0663-immunized group and the PBS control group. This indicates that combined immunization with FlaB3 and Tp0663 can inhibit the spread of *T. pallidum* in mice. Furthermore, this suggests that FlaB3 can enhance the immune protection of Tp0663. Previous research has demonstrated that Tp0751 can impede the dissemination of *T. pallidum* following the immunization of New Zealand rabbits because the Tp0751-specific antibody can inhibit the interaction of *T. pallidum* with extracellular matrix components and endothelium, thereby inhibiting its spread.[43] Our previous research demonstrated that FlaB3 can induce the expression of MMP-9 and MMP-13 in skin keratinocytes, thereby facilitating *T. pallidum* invasion.[44] Additionally, FlaB3 can impede *T. pallidum* proliferation following the immunization of New

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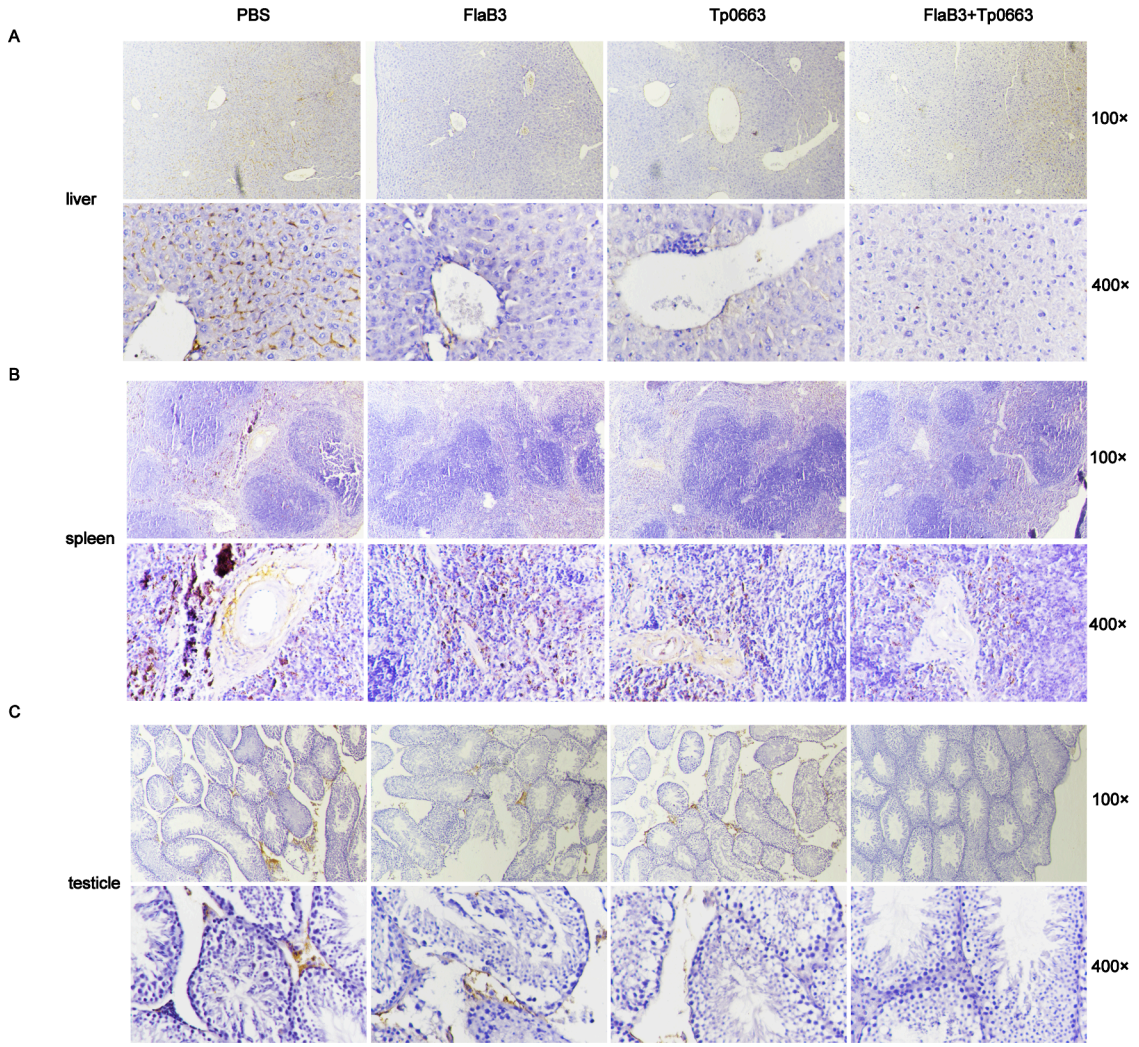

Figure 6. Detection of *T. pallidum* by immunohistochemistry in tissues of FlaB3 + Tp0663-immunized mice. Liver (A), spleen (B), and testicle tissues (C) of PBS, FlaB3, Tp0663, or FlaB3 + Tp0663-immunized mice were sectioned and stained with S−P immunohistochemistry 30 days after infection with *T. pallidum*. The UltraSensitiveSP IHC Kit, with a rabbit anti-*T. pallidum* was used as the first antibody to detect *T. pallidum*. Mice immunized with PBS were used as controls. Those with brown granules contain *T. pallidum*.

Table 2. Serological and Physical Markers of Testicular Infection in RIT Rabbits

rabbit testicles inoculated with lymph nodes from mice	seroconversion[a]	dark-field analysis	qRT-PCR[b]
PBS (n = 3)	+ (day 20)	+	108.88 ± 27.61
FlaB3 (n = 3)	+ (day 32)		68.33 ± 32.01
Tp0663 (n = 3)	+ (day 32)		65.85 ± 30.63
FlaB3+Tp0663 (n = 3)	+ (day 52)		69.37 ± 33.54

[a]The "+" indicates positive seroconversion (reactive RPR 1:1 and TPPA). [b]The differences in spirochete burden were observed by qRT-PCR from rabbit testicles (n = 3).

Zealand rabbits.[45] In the present investigation, it was observed that the combined administration of FlaB3 and Tp0663 in mice resulted in suppression of *T. pallidum* proliferation. This inhibitory impact was found to be markedly more robust than that observed in either the Tp0663 or FlaB3-immunized groups. There may be two mechanisms: (1) Tp0663-specific antibody directly assists macrophages in clearing pathogens at the initial site of infection; (2) Tp0663 can indirectly assist *T. pallidum* in invading host cells, similar to FlaB3. Thus, its specific antibody can immobilize the pathogen at the original infection site and prevent its further diffusion and development.

Unlike New Zealand rabbits, no obvious clinical symptoms were observed after C57BL/6 mice were infected with *T. pallidum* in this investigation. These findings align with prior research outcomes,[18] intimating the incongruity of the mouse model for discerning clinical symptoms of *T. pallidum* infection. However, the findings of the study demonstrate that *T. pallidum* can spread in mice, suggesting that the mouse model is suitable for assessing the effectiveness of the syphilis vaccine in inhibiting the spread of *T. pallidum*. In addition, the mouse model can also detect a diverse array of immune indicators. Therefore, the effectiveness of syphilis vaccines can be thoroughly assessed in combination with those of rabbit and mouse models in the future. This will significantly advance the development of syphilis vaccines.

In summary, the findings of this research suggest that the dual immunization with *T. pallidum* flagellar core protein FlaB3 and Tp0663 exhibits stronger immune protection against *T. pallidum*. At the same time, Tp0663 demonstrated inhibitory effects on the dissemination of *T. pallidum* in

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C57BL/6 mice. As a result, Tp0663 may serve as a promising candidate for a syphilis vaccine, warranting further extensive investigation.

■ ASSOCIATED CONTENT

Data Availability Statement

Data used to support the findings of this study are included in the article.

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Yafeng Xie and Man Xu designed the experiments and wrote the paper. Yafeng Xie and Man Xu revised the manuscript. Yiming Zhou and Jing Yang played important roles in data analysis. All authors have read and agreed to the published version of the manuscript.

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Notes

The animal study protocol was approved by the Ethics Committee of the Animal Experiment Research Center at the University of South China (Animal Production License No. SYXK 2020-0002). The mice operations conformed to National Laboratory Animal Care and Use Guidelines.

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