The Combinations Chitosan-Pam₃CSK₄ and Chitosan-Monophosphoryl Lipid A: Promising Immune-Enhancing Adjuvants for Anticaries Vaccine PAC

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ABSTRACT We previously demonstrated that recombinant protein PAC could be administered as an anticaries vaccine. However, the relatively weak immunogenicity of PAC limits its application. In the present study, we investigated the effect of two adjuvant combinations of chitosan plus Pam₃CSK₄ (chitosan-Pam₃CSK₄) and of chitosan plus monophosphoryl lipid A (chitosan-MPL) in the immune responses to the PAC protein *in vivo* and *in vitro*. PAC-chitosan-Pam₃CSK₄ or PAC-chitosan-MPL promoted significantly higher PAC-specific antibody titers in serum and saliva, inhibited *Streptococcus mutans* colonization onto the tooth surfaces, and endowed better protection effect with significantly less caries activities than PAC alone. Chitosan-Pam₃CSK₄ and chitosan-MPL showed no statistically significant differences. In conclusion, our study demonstrated that the chitosan-Pam₃CSK₄ and chitosan-MPL combinations are promising for anticaries vaccine development.

KEYWORDS dental caries, PAC, adjuvant, chitosan, MPL, Pam₃CSK₄

PAC, a surface fibrillar protein of *Streptococcus mutans*, is a key virulence factor associated with the initial adherence of *S. mutans* to tooth surfaces (1, 2). Recombinant protein PAC includes two functional regions, namely, the alanine-rich region (A-region) and the proline-rich region (P-region) originating from amino acid residues 222 to 965 of the *pac* gene, which encodes the PAC protein of *S. mutans* MT8148 (3). It can induce salivary immunoglobulin A (IgA) antibodies to inhibit *S. mutans* adherence and colonization to teeth (4). By virtue of these traits, recombinant protein PAC (PAC) is utilized as an immunogen in various experimental systems to develop an anticaries vaccine (3, 5, 6). However, the aforementioned studies also indicated that PAC generates low immune responses that cannot induce and maintain adequate protection against dental caries without adjuvants (3, 5–8).

Adjuvants are therefore required to assist PAC to induce sufficiently effective and persistent immune responses for providing caries protection. Pattern-recognition receptors (PRRs) of the innate immune system, particularly Toll-like receptors (TLRs) and nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs), are ordinary targets of adjuvants that affect the type and strength of immune response to vaccination, primarily by activating the innate immune response, which in turn activates the acquired immune response (9–12). The effects of TLR and NLR stimulation have largely been investigated using Pam₃CSK₄ (a TLR2 agonist) (13, 14), muramyl

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dipeptide (MDP; a NOD2 agonist) (15), D-isoglu-meso-DAP (iE-DAP; a NOD1 agonist) (16), monophosphoryl lipid A (MPL; a TLR4 agonist) (17), and chitosan (an NLRP3 agonist) (18, 19).

PRR agonists can synergize and/or balance the immunomodulatory activity of one another (20) by combinations, such as TLR4-NLRP3 (21) and TLR4-TLR7 (9). Combinations of TLR and/or NLR adjuvants elicit effective immune responses and augment antibody responses to vaccines (20, 22–24).

In the present study, we selected two combinations from five TLR or NLR agonists as the immune-enhancing additives of PAC, which could boost antibody responses to PAC vaccines targeting dental caries. Therefore, we studied whether stimulation with the combination chitosan-Pam₃CSK₄ or the combination chitosan-MPL can enhance the antibody titers of mice and inhibit the colonization of *S. mutans* and whether this combination can augment caries protection in mice orally infected with *S. mutans*.

RESULTS

Adjuvant potential of five TLR or NLR agonists in mice. We first checked to determine whether a certain PRR agonist can serve as an adjuvant for immunizing antigens in an ovalbumin (OVA)-specific T cell receptor (TCR) transgenic mouse model. OVA-specific TCR transgenic mice were C57BL/6J mice that received naive OVA-specific T cells purified from CD45.1×OTII F₁ mice. OVA-specific TCR transgenic mice can produce a rapid immune response with a small dose of OVA. At 3 days after immunization, the average sizes of isolated lymph nodes in the phosphate-buffered saline (PBS), OVA, OVA-MDP, OVA-chitosan, OVA-Pam₃CSK₄, OVA-MPL, and OVA-iE-DAP groups were 1.83, 1.83, 1.86, 2.50, 4.00, 3.17, and 2.23 mm, respectively (Fig. 1A). The relative numbers of cells in the OVA-chitosan, OVA-Pam₃CSK₄, and OVA-MPL groups were 0.92×10^6 , 1.85×10^6 , and 1.84×10^6 , respectively. The relative numbers of OVA-specific T lymphocytes in the PBS, OVA, OVA-MDP, OVA-chitosan, OVA-Pam₃CSK₄, OVA-MPL, and OVA-iE-DAP groups were 0.90×10^2 , 1.80×10^2 , 3.16×10^2 , 3.10×10^2 , 8.88×10^2 , 10.89×10^2 , and 2.87×10^2 , respectively (Fig. 1B). The relative numbers of OVA-specific T lymphocytes in the OVA-Pam₃CSK₄ and OVA-MPL groups were significantly greater than for the OVA group ($P < 0.001$). Among other tested groups, chitosan was slightly more effective than MDP or iE-DAP ($P > 0.05$).

To understand further the impact of the five PRR agonists on the specific immunity of C57BL/6J mice, we analyzed lymphocytes in the submandibular lymph nodes on day 17. In the OVA-Pam₃CSK₄ and OVA-MPL groups, the average sizes of lymph nodes were 4.95 and 4.05 mm (Fig. 2A), which were much larger than those of other immunized groups. The relative numbers of cells in the OVA-Pam₃CSK₄ and OVA-MPL groups were 17.50×10^6 and 19.39×10^6 , respectively. In comparison to the PBS group, the numbers of cells in the OVA-Pam₃CSK₄ and OVA-MPL groups increased significantly as well ($P < 0.05$ or $P < 0.01$) (Fig. 2A and B). At day 17, the numbers of CD4⁺ T cells and CD8⁺ T cells in the OVA-MPL group were 4.34×10^6 and 3.85×10^6 , respectively, which were significantly higher ($P < 0.05$) than for the PBS group (Fig. 2C and D).

Compared to the OVA-immunized group, the serum antibody class immunoglobulin G1 (IgG1) and immunoglobulin G2b (IgG2b) titers increased in all tested groups (Fig. 2E). The antibody titers of IgG1 in the OVA-Pam₃CSK₄ group were the highest, followed by the OVA-chitosan and OVA-MPL groups. Notably, for IgG2b, the OVA-Pam₃CSK₄ or OVA-MPL group induced a stronger antibody response than did the other tested groups (Fig. 2E). The antibody assays implied that all PRR ligands tested enhanced both Th1- and Th2-type immune responses. On the basis of these results, chitosan, MPL, and Pam₃CSK₄ were chosen for the subsequent tests.

Enhanced antibody titers in mice immunized with OVA-chitosan-MPL or OVA-chitosan-Pam₃CSK₄. Immunization of mice with OVA-chitosan-MPL or OVA-chitosan-Pam₃CSK₄ enhanced serum antibody (IgG1 and IgG2b) responses compared to immunization with OVA-chitosan (Fig. 2F). With respect to IgG1, OVA-chitosan-Pam₃CSK₄ induced a stronger antibody response than OVA-chitosan-MPL. Notably, the antibody titers were enhanced in mice immunized with OVA-chitosan-MPL. Immunization with

FIG 1 Adjuvanticity of MDP, chitosan, Pam₃CSK₄, MPL, and iE-DAP to OVA in OVA-specific TCR transgenic mice. Naive OVA-specific T cells were purified from the spleens and inguinal lymph nodes of CD45.1 $\times$ OTII F₁ mice and transferred into female C57BL/6J mice. One day later, the recipients were immunized via buccal mucosal injection with one of seven compositions in 40 μ l, namely, 1 μ g of OVA, OVA plus 40 μ g of MDP, OVA plus 32 μ g of chitosan, OVA plus 25 μ g of Pam₃CSK₄, OVA plus 15 μ g of MPL, OVA plus 50 μ g of iE-DAP, or PBS control. Three days later, the mice were sacrificed. The draining submandibular lymph nodes were isolated, and the cells were counted (A). Single-cell suspensions were made for staining with CD45.1 plus CD4 and then run on a BD FACSaria II and analyzed for the number of OVA-specific T cells (B). *, Significantly different from the PBS group (*, $P < 0.05$; ***, $P < 0.001$). #, Significantly different from the OVA-immunized group (###, $P < 0.001$).

OVA-chitosan-MPL or OVA-chitosan-Pam₃CSK₄ generated higher serum IgG2b anti-OVA responses than immunization with OVA-chitosan at day 17 (Fig. 2F).

Anti-Pac antibody responses in mice. Mice were immunized twice with PAC, PAC-chitosan, PAC-MPL, PAC-Pam₃CSK₄, PAC-chitosan-Pam₃CSK₄, and PAC-chitosan-MPL by the buccal-mucosa route. Serum IgG and salivary IgA anti-PAC responses were induced in all mice of the experimental groups, and no serum IgG and salivary IgA responses were detected in the control groups (Fig. 3A to D).

The mean anti-PAC IgG levels in serum samples of mice vaccinated with PAC-chitosan-Pam₃CSK₄ or PAC-chitosan-MPL were 3-fold higher than in mice vaccinated with PAC at week 2 ($P < 0.01$) (Fig. 3C). In the PAC-chitosan-Pam₃CSK₄- or PAC-chitosan-MPL-immunized group, the level of serum anti-PAC IgG was significantly enhanced at week 2 ($P < 0.01$) and remained high throughout the experimental period compared to that in the PAC-immunized group. Immunization with PAC-chitosan-Pam₃CSK₄ significantly enhanced serum anti-PAC IgG antibody responses compared to PAC at week 6 ($P < 0.001$), weeks 2, 8, and 12 ($P < 0.01$), and weeks 4 and 10 ($P < 0.05$). Significant levels of serum anti-PAC IgG were detected in the PAC-chitosan-MPL group at weeks 6 and 8 ($P < 0.001$), week 2 ($P < 0.01$), and weeks 4, 10, and 12 ($P < 0.05$) compared to the PAC-immunized mice.

Similar enhancement of the serum PAC-specific IgG2b levels was detected in the PAC-chitosan-Pam₃CSK₄ or PAC-chitosan-MPL group (Fig. 3B). The IgG2b titers of the

FIG 2 Adjuvanticity of MDP, chitosan, Pam₃CSK₄, MPL and iE-DAP to OVA in C57BL/6J mice. The C57BL/6J mice were immunized on day 0 via buccal mucosal injection with 100 μ g of OVA, OVA plus 40 μ g of MDP, OVA plus 32 μ g of chitosan, OVA plus 25 μ g of Pam₃CSK₄, OVA plus 15 μ g of MPL, OVA plus 50 μ g of iE-DAP, or PBS and boosted on day 7. On day 14, the sera were collected for measuring OVA-specific IgG1 and IgG2b responses (see panel E). On day 15, the mice were administered with 100 μ g of OVA via buccal mucosal injection. On day 17, the mice were sacrificed; the draining submandibular lymph nodes were

(Continued on next page)

FIG 3 PAC-specific immune responses and the inhibition of colonization by *S. mutans* in mice. Mice were fed a cariogenic diet, infected orally with *S. mutans* MT8148 for 4 days (2×10^9 CFU/mouse/day), and immunized at weeks 0 and 2 with 50 μ g of PAC, PAC plus 32 μ g of chitosan, PAC plus 15 μ g of MPL, PAC plus 25 μ g of Pam₃CSK₄, PAC-chitosan-Pam₃CSK₄, PAC-chitosan-MPL, or PBS. Serum and saliva samples were collected at weeks 0, 1, 2, 4, 6, 8, 10, and 12 and analyzed for antibody responses by ELISA. (A to D) Serum PAC-specific IgG1 (A), serum PAC-specific IgG2b (B), serum PAC-specific IgG (C), and salivary PAC-specific IgA (D) of immunized mice. (E) Body weights of mice at weeks 2, 4, 6, 8, and 10. (F) Numbers of *S. mutans* CFU in oral cavities of mice, calculated by using oral swabs. The results are expressed as means $\pm$ the standard errors ($n = 6$). *, Significantly different from the PAC-immunized group (*, $P < 0.05$; **, $P < 0.01$). The statistical differences are not shown in graphs A, B, C, and D.

PAC-chitosan-Pam₃CSK₄ or PAC-chitosan-MPL group were obviously higher at week 2 ($P < 0.01$ or $P < 0.001$) and remained at high levels ($P < 0.001$) compared to the PBS group. Significant levels of PAC-specific serum IgG2b in the PAC-chitosan-Pam₃CSK₄ or PAC-chitosan-MPL group were detected at week 6 ($P < 0.001$), weeks 8, 10, and 12 ($P < 0.01$), week 4 ($P < 0.05$), and week 2 ($P < 0.05$ or $P < 0.01$) compared to the PAC-immunized mice.

The mice vaccinated with PAC-chitosan-Pam₃CSK₄ showed the highest anti-PAC serum IgG1 throughout the experimental period (Fig. 3A). Significant levels of PAC-

FIG 2 Legend (Continued)

isolated (A), and the cells were counted (B). (C and D) Single-cell suspensions were made for staining with CD4 plus CD8 and then run on a BD FACSaria II and analyzed for the numbers of CD4⁺ and CD8⁺ T cells. C57BL/6J mice were immunized with 100 μ g of OVA, OVA plus 32 μ g of chitosan, OVA plus chitosan plus 15 μ g of MPL, or OVA plus chitosan plus 25 μ g of Pam₃CSK₄ on day 0 and boosted on day 7, all via buccal mucosa injection. On day 14, the mice were sacrificed, and sera were harvested to measure the OVA-specific IgG1 and IgG2b responses (F). *, Significantly different from the PBS group (*, $P < 0.05$).

specific serum IgG1 in the PAC-chitosan-Pam₃CSK₄ group were detected at weeks 2, 6, 8, 10, and 12 ($P < 0.01$) and week 4 ($P < 0.001$) compared with the PAC-immunized mice. The peaks of serum PAC-specific IgG, IgG1, and IgG2b levels appeared at week 6 after the first immunization in all groups, but the peak of salivary PAC-specific IgA was detected at week 4 (Fig. 3A to D).

Further, the salivary anti-PAC IgA level in the PAC-chitosan-Pam₃CSK₄ group was the highest, followed by the PAC-chitosan-MPL group (Fig. 3D). Meanwhile, this level was significantly higher in the PAC-chitosan-Pam₃CSK₄ group than in the PAC group at weeks 6 and 8 ($P < 0.001$) and weeks 4, 10, and 12 ($P < 0.01$) (Fig. 3D). The salivary anti-PAC IgA level of the PAC-chitosan-MPL group was significantly higher than that of the sham-immunized group at weeks 8, 10, and 12 ($P < 0.01$) and weeks 4 and 6 ($P < 0.05$).

Inhibition of colonization on tooth surfaces by *S. mutans*. A colony-forming assay was used to detect the number of *S. mutans* CFU sustained on the fissures and occlusal surfaces of molars after immunization for 5 weeks (Fig. 3F). As we inferred, the numbers of *S. mutans* CFU in the PBS-immunized mice increased gradually and remained at high levels throughout the experimental 5 weeks. The numbers of *S. mutans* CFU were slightly reduced in the PAC-immunized group compared to the PBS group. When immunized with PAC-chitosan-Pam₃CSK₄, the numbers of *S. mutans* CFU were significantly lower than for the PAC group at weeks 4 ($P < 0.05$) and 5 ($P < 0.01$). Similarly, *S. mutans* exhibited a significant reduction at weeks 4 and 5 ($P < 0.05$) when the mice were immunized with PAC-chitosan-MPL. No significant difference was found between PAC-chitosan-Pam₃CSK₄-immunized mice and PAC-chitosan-MPL-immunized mice. Notably, significant inverse correlations were found between the CFU count of *S. mutans* and anti-PAC IgA in saliva ($r = -0.569$, $P < 0.01$) and anti-PAC IgG in serum ($r = -0.666$, $P < 0.01$). No significant difference in body weight was observed among all the tested groups ($P > 0.05$) (Fig. 3E).

Caries protection against *S. mutans* infection. The extension of lesions and the residual volumes of enamel and dentin in the mice can be regarded as the protective efficacies of the different treatments (Fig. 4). Figure 4A demonstrates the three-dimensional (3D) reconstructive images of molar caries scanned by micro-computed tomography (micro-CT). Dental damage caused by molar caries occurred in all mice. Mice immunized with PBS exhibited the most severe enamel and dentinal lesions of molars, with perforated enamel layers. The molar caries of mice in the PAC group were similar to but with less severe dentinal lesions than those in the sham-immunized group ($P < 0.05$). Mice immunized with PAC-chitosan-Pam₃CSK₄ or PAC-chitosan-MPL displayed significantly fewer enamel and dentinal lesions than mice immunized with PAC ($P < 0.01$) (Fig. 4B and C). The mice immunized with PAC-chitosan-Pam₃CSK₄ presented more residual enamel volumes and fewer dentinal volumes than the PAC-chitosan-MPL-immunized mice, but no significant difference was observed ($P > 0.05$).

Interestingly, significant positive correlations were found between dentinal volumes of mice and anti-PAC IgA in saliva ($r = 0.788$, $P < 0.01$) and anti-PAC IgG in serum ($r = 0.905$, $P < 0.01$).

DISCUSSION

The original intention of anticaries vaccine PAC was to provide adequate immune protection for decreasing caries activity. The antibody responses in mice that received immunization with PAC-chitosan-MPL or PAC-chitosan-Pam₃CSK₄ by submucosal injection were higher than in mice that received immunization with PAC. The level of distinct serum-specific IgG antibody response at week 2 postpriming in the PAC-chitosan-Pam₃CSK₄ group was 3-fold greater than in the PAC group. In addition, the PAC-chitosan-MPL or PAC-chitosan-Pam₃CSK₄ group maintained high antibody responses at week 10 after boost. Mouse model results showed that in the groups infected with *S. mutans*, the PAC-chitosan-MPL- and PAC-chitosan-Pam₃CSK₄-immunized mice had significantly fewer dental lesions than did PAC-immunized mice. These findings demonstrated that the new combinations of PAC-chitosan-Pam₃CSK₄ and PAC-chitosan-MPL

FIG 4 Caries protection against *S. mutans* infection in mice. On week 12, mice challenged with *S. mutans* were euthanized, and the maxillae and mandibles were harvested. The maxillae and mandibles of mice were scanned by using micro-CT at a resolution of 9 μm in all three spatial dimensions. (A) 3D reconstruction of mouse molars. Caries lesions are indicated by red arrows. (B and C) Mean volumes of enamel and dentinal tissues of mice. Values are expressed as means $\pm$ the standard errors. #, Significantly different from the PBS group (#, $P < 0.05$; ##, $P < 0.01$). *, Significantly different from the PAC-immunized group (*, $P < 0.05$; **, $P < 0.01$).

significantly enhanced the speed, magnitude, and longevity of the specific antibody response and increased the protective effect against *S. mutans* compared to immunization with PAc alone.

Caries protection efficacy is usually evaluated by caries scores of enamel and by slight dentinal and moderate dental caries of rats (25). In the present study, we estimated protection against caries caused by infection with *S. mutans* by examining the molar teeth of C57BL/6J mice. The molar teeth of mice are smaller than those of rats and are not suitable to hemisection. Therefore, micro-CT was used for accurate quantification of dental loss, allowing the assessment of residual volume of enamel and dentin without subjective bias. Using three-dimensional reconstruction, we can observe the decay degree of mouse molars intuitively and calculate the residual volumes of enamel and dentin in each mouse.

In adjuvant research, “one plus one” often equals more than two. Adjuvants can be composed of different components with homologous functions and activities instead of a single compound with intrinsic immunomodulatory properties (26). The current challenge facing anticaries recombinant protein vaccine PAc is to find an adjuvant that can enhance and eventually reach the desired caries protective immune response. The combination PAc-chitosan-Pam₃CSK₄ or PAc-chitosan-MPL is a mix that includes antigen, carrier, and immunostimulants. Our results showed that such combinations are more effective than PAc alone. They can also retain more dental tissue and enhance antibody titers in serum and salivary samples compared to PAc alone. Vaccine formulations containing MPL have been evaluated for their capacities to enhance both mucosal and systemic immune responses against influenza, tetanus toxoid, and hepatitis B surface antigens (12). Zom et al. exploited the potential of Pam₃CSK₄ as a vaccine adjuvant and discovered that Pam₃CSK₄ facilitates the maturation of antigen-presenting cells and enhances *in vivo* T cell priming. These authors demonstrated the superiority of Pam₃CSK₄ as a vaccine adjuvant in the enhancement of antitumor immunity induction (14).

Chitosan, a deacetylated form of chitin composed of D-glucosamine and N-acetyl-D-glucosamine, is a hydrophilic linear polysaccharide polymer with high potential in the delivery of various hydrophilic molecules (e.g., protein) and drug agents (e.g., nano- and microparticles) (27–29) and immunostimulatory abilities to promote immune responses (19, 30). In addition, chitosan is highly suitable for use in medical and pharmaceutical applications because it is biodegradable, biocompatible, nonallergenic, and nontoxic (31). Thus, we utilized chitosan as a constituent of a combined anticaries vaccine. The next step is to build biodegradable synthetic polymer nanoparticles containing PAc-chitosan-Pam₃CSK₄ or PAc-chitosan-MPL to achieve further caries prevention.

MATERIALS AND METHODS

Animals. Female C57BL/6J mice (15 to 20 g, 6 weeks old) were purchased from Vital River Laboratory Animal Technology Co. (Beijing, China). OT II mice (Jackson Laboratory, Bar Harbor, ME) were crossbred with CD45.1⁺ (Ly5.1) mice (Jackson Laboratory) to produce CD45.1×OT II F₁ mice in the Tang Lab at Taishan Medical University. All mice were maintained in specific-pathogen-free (SPF) conditions on a standard diet and water and were used when they were approximately 6 to 8 weeks old. Animal studies were performed according to the Regulations of the Administration of Affairs Concerning Experimental Animals in China, and the Laboratory Animal Care and Use Committee, School of Stomatology, Wuhan University, reviewed and approved the protocols (approval 2015C23).

Expression and purification of the recombinant PAc. The recombinant protein PAc was constructed and purified as previously described (32). PAc was expressed in *Escherichia coli* BL21(DE3)/plysS containing the recombinant plasmid pET20b(+)-AP with suitable IPTG (isopropyl-β-D-thiogalactopyranoside) and purified by using a Ni-NTA His bind resin with imidazole (GE Healthcare, Uppsala, Sweden). Residual endotoxin (lipopolysaccharide) was removed from the purified protein by using a DetoxiGel endotoxin removing gel (Thermo Scientific, Waltham, MA).

Adjuvanticity of PRR agonists on soluble antigen in mice. Spleens and inguinal lymph nodes were removed from CD45.1×OTII F₁ mice and minced through a 70-μm nylon mesh to obtain single cells. The cells were labeled with CD16/32 and a mixture of antibodies (CD45.1-FITC, CD4 Percp/Cy5.5, TCRα2-APC/Cy7, TCRβ5-APC, CD62L-PE, and CD25-BV650 [all from eBioscience, San Diego, CA]) sequentially before being sorted with CD4⁺ MACS beads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), and the recovered CD4⁺ cells were further sorted using a BD FACSAria II (BD Biosciences, San Jose, CA) for CD4⁺ CD45.1⁺ TCRα2⁺ TCRβ5⁺ CD62L⁺ CD25[−] fractions. The purified naive T cells were transferred

to female C57BL/6J mice at 10^6 cells/mouse via tail vein injection. After 24 h, the recipients were immunized via buccal mucosal injection with one of five compositions in 40 μ l, namely, 1 μ g of OVA (Sigma, St. Louis, MO), OVA plus 40 μ g of MDP (Invivogen, San Diego, CA), OVA plus 32 μ g of chitosan (Invivogen), OVA plus 25 μ g of Pam₃CSK₄ (ApexBio, Houston, TX), OVA plus 15 μ g of MPL (Invivogen), or OVA plus 50 μ g of iE-DAP (Invivogen), or PBS (pH 7.2) control. After 3 days, the mice were sacrificed, and the submandibular draining lymph nodes were isolated, imaged, and minced into a single-cell suspension for staining with CD45.1 and CD4 as described above. Cells were run on a BD FACSAria II.

Mouse immunization. Seven groups of C57BL/6J mice were immunized via the buccal mucosa with OVA (100 μ g), OVA plus 40 μ g of MDP, OVA plus 32 μ g of chitosan, OVA plus 25 μ g of Pam₃CSK₄, OVA plus 15 μ g of MPL, or OVA plus 50 μ g of iE-DAP on day 0 and boosted on day 7. A sham-immunized group received only PBS. On day 14, sera were collected for antibody assays. On day 15, mice were injected via the buccal mucosa with 100 μ g of OVA and sacrificed for submandibular lymph node isolation on day 17.

C57BL/6J mice were immunized with 100 μ g of OVA, OVA plus 32 μ g of chitosan, OVA plus chitosan plus 15 μ g of MPL, or OVA plus chitosan plus 25 μ g of Pam₃CSK₄ on day 0 and boosted on day 7, all via subbuccal mucosa injection. On day 14, the mice were sacrificed for harvested serum.

Flow cytometric analysis. Submandibular lymph nodes were removed from immunized mice and minced through a 70- μ m nylon mesh to obtain single cells. The cells were labeled with CD16/32 and a mixture of antibodies (CD3-biotin, CD8-APC/Cy7, and CD4-eFluor 450 [all from eBioscience]) and SA-BV650 (eBioscience) and Alexa 430-succinimidyl ester sequentially before being run on a BD FACSAria II.

Antibody assays. Specific anti-PAC serum IgG, IgG isotypes (IgG1 and IgG2b) and salivary IgA antibodies in mice were determined by using enzyme-linked immunosorbent assay (ELISA) as reported previously (33). Sera were diluted serially or diluted 400 times; saliva samples were not diluted. Rat anti-mouse IgG, IgG1, IgG2b, and IgA-biotin antibodies were diluted to 0.25 μ g/ml for incubation before being labeled with streptavidin-horseradish peroxidase. TMB (3,3',5,5'-tetramethylbenzidine) solution was then added sequentially to each well, and the plates were read with a microplate reader at 450 nm.

Experimental mouse caries model. SPF female C57BL/6J mice were fed a cariogenic diet (Keyes 2000) (34) until the experiment was terminated. Antibiotics (ampicillin, chloramphenicol, and carbenicillin, 1.0 g/kg diet) were added from days 5 to 7 before the first immunization to temporarily suppress the oral flora in order to facilitate the cariogenic bacterial colonization. The mice were then challenged orally with 2×10^9 CFU of *S. mutans* MT8148 by applying swabs presoaked with bacterial solution for four consecutive days.

On week 0, seven groups of six animals each were immunized via buccal mucosa injection with 50 μ g of PAC, PAC plus 32 μ g of chitosan, PAC plus 15 μ g of MPL, PAC plus 25 μ g of Pam₃CSK₄, PAC-chitosan-Pam₃CSK₄, PAC-chitosan-MPL, or PBS and boosted on week 2. Cariogenic bacterial levels were tested from weeks 1 to 5 (35). We used swabs presoaked in PBS to collect *S. mutans* on fissures and smooth surfaces of molar teeth and transferred the samples to 500 μ l of PBS by vortexing. The *S. mutans* CFU were assayed by culture of serial 10-fold dilutions of samples on mitis salivarius agar medium (Hopebio, Qingdao, China).

Collection of saliva and blood was performed at weeks 0, 1, 2, 4, 6, 8, 10, and 12, as previously described (33). We stimulated the salivary flow by intraperitoneal injection of each mouse with 0.2% pilocarpine (0.15 mg/20 g [weight]), followed by centrifugation to acquire the supernatants. Blood samples were harvested from retro-orbital plexus, and then sera were collected by centrifugation.

At week 12, the mice were euthanized, and the maxillae and mandibles were harvested.

Micro-CT assay. Maxillae and mandibles of mice were scanned by using micro-CT (Skyscan 1172; Bruker, Germany) (36, 37). 3D dental loss was quantified. The specimens were scanned at a resolution of 9 μ m in all three spatial dimensions. The scans were Gaussian filtered and segmented using a multilevel global thresholding procedure for the segmentation of enamel and dentin. Reconstruction into cross-section slices was performed using the Skyscan NRecon v.1.6.6 software interface. Other settings included the input of optimal contrast limits for the specific material scanned. After reconstructing molars, the dental crown and dental root were separated at the cemento-enamel junction. The enamel and dentin in the crown were differentiated according to the density difference, and the enamel and dentin volumes were calculated. The residual enamel or dentinal volume was determined separately.

Statistical analysis. Data are presented as the means $\pm$ the standard errors and were assessed by using one-way analysis of variance or a Student *t* test with SPSS software. A *P* value of <0.05 was considered statistically significant.

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M.F. and Y.L. conceived the study. Y.B., J.X., Z.L., and Y.Y. performed the experiments. Y.B., H.T., L.S., and Q.X. analyzed the data. Y.B., L.S., and Q.X. wrote the manuscript.

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