Als3-Th-cell-epitope plus the combined adjuvant of MDP, CpG and FIA synergistically enhanced the immune responses triggered with recombinant TRAP proteins in mice

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Abstract

Here, Als3-Th-cell-epitope （Als3 epitope） was connected to the N-terminal of TRAP by flexible linker, and the Als3-Th-cell-epitope-TRAP (ATT) proteins were prepared, then, the ATT proteins plus freund's adjuvant were inoculated in mice to evaluate Als3 epitope to increase the immunogenicity of TRAP. To strengthen the immunogenicity of ATT protein, the proteins plus the novel combined adjuvants of MDP, CpG and FIA were immunized in mice. After the booster immunization, the results showed that the mice immunized with ATT protein plus Freund's adjuvant exhibited significantly higher level for IFN-γ, IL-4, IL-10 and IL-17A, and displayed the stronger humoral immune response against TRAP than the control groups, importantly, the survival rate of these mice was significantly higher than the control groups. In addition, the mice immunized with ATT protein plus CpG+MDP+FIA adjuvants exhibited significantly higher level for IFN-γ and IL-17A than other groups, and the level of IgG antibody against TRAP was higher than other groups, moreover, the survival rate of these mice was obviously higher than other groups. These data

suggested that the immune protection triggered with ATT was significantly stronger than TRAP or TRAP+Als3 epitope did, which indicted Als3 epitope significantly enhanced the immune responses triggered with TRAP through their fused forms of expression. Additionally, these data manifested that ATT plus the novel combined adjuvant, MDP, CpG and FIA, induced the strongest immune response and protection against *S.aureus* among all the groups, revealing the synergistic effect on different adjuvant. This study provides an important reference for the further development of a new effective vaccine against *S.aureus*.

Key words: Als3 epitope, ATT protein, MDP, CpG, FIA, Combined adjuvant

Introduction

Staphylococcus aureus (*S.aureus*) is a gram-positive opportunistic pathogen, which is distributed in water, dust and other natural environments, and also exists in human and animal skin, excreta and cavities (Mah et al., 2014; Piewngam & Otto, 2020; Sakr, Bregeon, Mege, Rolain, & Blin, 2018). *S.aureus* infection is the most common cause of human-related pneumonia, endocarditis, medical devices in hospitals (Galar, Weil, Dudzinski, Munoz, & Siedner, 2019; Projan, Nesin, & Dunman, 2006; Self et al., 2016), it also causes a variety of infections, such as mastitis in sheep and bovine, and canine pyoderma (Loeffler & Lloyd, 2018; Pu et al., 2014; Vasileiou et al., 2019). Over the years, the increasing emergence of resistant strains, such as Methicillin-resistant *Staphylococcus aureus* (MRSA) and Vancomycin-resistant *Staphylococcus aureus*, was attributed to the excessive use of antibiotics in many countries and regions (Dadashi, Hajikhani, Darban-Sarokhalil, van Belkum, & Goudarzi, 2020; Durai, Ng, & Hoque, 2010; Howden, Davies, Johnson, Stinear, & Grayson, 2010; Mehraj et al., 2014). Clinical studies have shown that the vaccine is effective in preventing *S.aureus* infection, therefore, developing an effective vaccine against *S.aureus* is urgently needed (Miller, Fowler, Shukla, Rose, & Proctor, 2020; Proctor, 2012; Schaffer & Lee, 2009).

TRAP（Target of RNAIII Activating Protein）is a membrane-bound protein composed of 167 amino acid residues, which is relatively conservative and is consistently expressed from *S. aureus*. TRAP activates downstream target proteins by binding RAP, which in turn can activate and promote the synthesis of RNAIII, and ultimately increase the expression level of toxin. Researches have shown that TRAP can protect DNA from natural mutations, adaptive mutations and oxidative damage during the process of *S. aureus* stress response (Kiran & Balaban, 2009). The mice immunized with TA21 peptide from TRAP generated immune protective response against *S.aureus*, and the research results in our laboratory showed that TRAP triggered the stronger immune protection and the higher level of IFN-γ, IL-4, and IL-17 (Song et al., 2019) in mice, an epitope of TRAP can induce Th17 cell

differentiation and improves production of IL-17. Therefore, TRAP displayed the strong immunogenicity, however, its immunogenicity was still needed to be further increased to effectively prevent *S. aureus* infection.

Als3（Agglutinin-like sequence 3）, a critical adhesion factor, plays a crucial role for improving *Candida albicans* (*C. albicans*) to adhere to host cell surface (Lin et al., 2009; Mayahara et al., 2014; Spellberg et al., 2008), its three-dimensional structures are similar to clumping factor A (ClfA) of *S. aureus* (Yeaman et al., 2014) . Preclinical studies demonstrated that Als3p can protect mice from intravenous challenge with *C. albicans* and *S. aureus*, and promotes the secretion of IFN-γ and IL-17A from Th1/Th17 cells (Lin et al., 2009; Schmidt et al., 2012; Spellberg et al., 2008), indicating immune cross-reaction against *S. aureus* and *Candida* infection. Bar et al. found that an Als3-Th-cell-epitope (Als3 epitope) derived from Als3 proteins acted as an efficient vaccine when used in combination with an adjuvant improving IL-17A secretion from peptide-specific T cells (Bar et al., 2012).

Un-methylated cytosine-phosphate-guanosine oligodeoxynucleotides (CpG-ODN (CpG)), as short synthetic agonists, binds Toll-like receptor 9 (TLR9), promoting B cells and dendritic cells activated, and inducing Th1-mediated immune response (Hayen et al., 2018; Klinman, 2004; Kulis, Gorentla, Burks, & Zhong, 2013; Majewska-Szczepanik et al., 2016; Sepulveda-Toepfer et al., 2019). In addition, muramyl dipeptide (MDP) induces the secretion of pro-inflammatory factors through binding NOD2 receptor, then, improves cellular immune response, which mainly induces Th1- mediated cellular immune response (Behr & Divangahi, 2015; Laman et al., 2016; Poecheim, Barnier-Quer, Collin, & Borchard, 2016; Tukhvatulin et al., 2016). Freund's incomplete adjuvant（FIA）is an oil-water-emulsion emulsified with oil (paraffin oil or vegetable oil) and emulsifier (lanolin or Twin-80), as the most commonly used adjuvant in animal experiments. FIA sustains slow release of antigen and enhances the immunogenicity induced with antigen by up-regulating Th2-mediated immune response (Hekmat et al., 2019; Rivera & Espino, 2016). However, an adjuvant often triggers a weak immune response for antigen, the combined adjuvants exert synergy to maximize immune effect. Hence, we predicted

that the combinations of CPG, MDP and FIA adjuvant could generate a synergistic function to enlarge immune response for desired antigen, which was responsible for driving the polarization of naïve CD4 + T cells toward Th1, Th2, Th17 cells.

In this study, *Als3* epitope and *trap* genes were tandemly expressed to prepare ATT protein, furthermore, Als3 epitope acted as a synergistic effect in combination with CPG, MDP and FIA adjuvant to enlarge the immune response triggered with TRAP.

Materials and Methods

Mice and Bacterial strains

Female C57/B6 mice (6-8 weeks) were ordered from the Changchun Institute of Biological Products (Changchun, China). Animal experiment was performed in accordance with animal ethics guidelines approved by the Animal Ethics Committee of Heilongjiang BaYi Agricultural University. *Staphylococcus aureus* strain Newman and *S. aureus* strain Wood46 were grown in tryptic soy agar (TSA), and *Escherichia coli* strain BL21 was grown in Luria-Bertani (LB) broth at 37°C overnight.

Construction of recombinant plasmids

The *trap* gene was obtained by Polymerase Chain Reaction (PCR) with forward primer 5′- GGATCCAAGAAACTATATACATCTT-3′ (*Bam*H I site underlined) and reverse primer AAGCTTTTCTTTTATTGGGTAT (*Hin*d III site underlined) from the pET-32a (+)-*trap* plasmid, its structural cassette of gene was shown in figure. 1 A (Fig. 1 A). The PCR conditions were as follows: denaturation at 94 °C for 5 min; followed by 30 cycles of 94 °C, 45s; 56 °C, 40 s; 72 °C, 40 s ; extension at 72 °C for 8 min. Finally, The *trap* fragments were linked into pET-28a (+) vectors.

The *Als3-Th-cell-epitope* (*Als3-epitope*)-*trap* (*att*) was acquired from the pET-28a (+)-*trap* plasmid by PCR with forward primer 5′- *GGATCCTGGAATTATCCGGTTTCATCTGAATCA*GGTAGTGGTAGTGGTAGTGG TAGTAAGAAACTATATACATCTT-3′ (*Bam*H I site with italicized and underlined, *Als3-epitope* sequence with italicized, linker sequence underlined) and reverse primer 5′- AAGCTTTTCTTTTATTGGGTAT-3′ (*Hin*d III site underlined),

the structural cassette of *att* was exhibited in figure. 1 B (Fig. 1 B). The PCR conditions were as follows: denaturation at 94 °C for 5 min; followed by 30 cycles of 94 °C, 45s; 58 °C, 40 s; 72 °C, 40 s ; extension at 72 °C for 8 min. The *att* fragments were cloned into pET-28a (+) vectors.

eAls (epitope *Als*) gene includes the DNA sequence that *Als3-epitope* (TGGAATTATCCGGTTTCATCTGAATCA) connected with flexible linker (GGTGGTAGCGGTGGCGGTTCTGGTGGCGGCTCTGGT) was repeated for 6 times, and the same linker was added to the 5 ' end of the first *Als3-epitope* and the 3' end of the last *Als3-epitope*, and *Bam*H I, *Hin*d III restriction endonuclease sites were added at 5 ' end and 3' end of the whole sequence, respectively, and its structural cassette of gene was shown in figure. 1 C (Fig. 1 C). This sequence was synthesized by Sangon Biological Engineering Technology Service Co., LTD, and inserted into pET-28a (+) plasmid.

Expression, purification and analysis of protein

The recombinant plasmids, pET-28a (+)-*att*, pET-28a (+)-*trap* and pET-28a (+)-*eAls,* were transformed into in *E. coli* BL21 (DE3) (Tiangen, Beijing, China) and were expressed the ATT, TRAP and eAls proteins with 0.1 mM isopropyl-β-D -1-thiogalactopyranoside (IPTG, Biosharp, Hefei, China) induction at 37 °C for 4 h, respectively. Then, the bacterial cells were obtained by centrifugation and were ultrasonicated, and the suspension was acquired. The His-tagged ATT, TRAP and eAls proteins were purified by using His-Binding-resin (Novagen, Germany) according to the manufacturer's instructions. These proteins were confirmed with SDS-PAGE and Western blot. For Western blot, anti-His tag monoclonal antibodies (mAbs) (Sigma) and HRP-conjugated goat anti-mouse IgG antibodies (Sigma) were used as the primary antibodies, the secondary antibodies, respectively.

Mice immunization

After ATT proteins were prepared, we next assessed their immunogenicity. 100 female C57/B6 mice（6-8 weeks）were randomly divided into 5 groups，including ATT, TRAP, eAls group, TRAP+eAls and Phosphate Buffer Solution (PBS) group, there were 20 mice in each group. C57/B6 mice were immunized intramuscularly with the

dosage of 100 μg ATT, TRAP, eAls and TRAP+eAls or PBS mixed with equal volume Freund's incomplete or complete adjuvant (Sigma-Aldrich (St. Louis, MO)) to a final volume of 0.2 mL on days 0 and 21, respectively. All the animals were fed in a special pathogen-free environment.

In addition, 160 female C57/B6 mice were randomly divided into 8 groups, including ATT+CpG+MDP+FIA, ATT+MDP+FIA,ATT+CpG+FIA, ATT+MDP+CpG, ATT+CpG, ATT+MDP, ATT+FIA, PBS+FIA groups. ATT+CpG+MDP+FIA group was immunized with the dosage of 100 μg ATT, 10 ng CpG and 10 ng MDP plus FIA at a volume ratio of 1:1, and ATT+ MDP+FIA group vaccinated with the dosage of 100 μg ATT and 10 ng MDP plus FIA at a volume ratio of 1:1, ATT+CpG+FIA group vaccinated with the dosage of 100 μg ATT and 10 ng CpG plus FIA at a volume ratio of 1:1, ATT+MDP+CpG group vaccinated with the dosage of 100 μg ATT and 10 ng MDP plus 10 ng CpG, ATT+CpG group vaccinated with the dosage of 100 μg ATT and 10 ng CpG, ATT+MDP group vaccinated with the dosage of 100 μg ATT and 10 ng MDP, ATT+FIA group vaccinated with the dosage of 100 μg ATT plus FIA at a volume ratio of 1:1, PBS+FIA group vaccinated with PBS plus FIA at a volume ratio of 1:1. Each mouse was immunized intramuscularly at a dose of 200 μl in the muscle of the lateral thigh. Booster immunization was performed on 21 days after the first immunization, the specific immunization method, dose and location were the same as that of the first immunization.

Cytokine profile analysis

The amounts of cytokines were detected using enzyme-linked immunospot (ELISpot) assay or enzyme-linked immuno sorbent assay (ELISA), respectively. ELISpot assay was performed according to the kit instructions. Briefly, lymphocytes were separated from spleens of mice from different groups by using lymphocyte separation fluid. To stimulate lymphocytes, cells (1×10^6 cells/well) were seeded into 96-well culture plates and cultured for 24 h at 37℃ in 1640 medium supplemented with 10% foetal bovine serum, either alone (unstimulated) as negative control or with phorbol myristate acetate (PMA, 50 ng/ml) as positive control or with the desired proteins (TRAP, 10 μg/mL). The data were expressed as the number of spot-forming cells (SFCs)/10^6 splenoytes. For cytokine profile analysis, the treated cells were

cultured at 37℃ for 48 h, then, the supernatant were collected and analyzed by ELISA.

ELISA for specific antibodies and antibody subclasses

The serum was separated from blood samples in mice on 14 days after booster immunization. IgG antibodies in sera were detected by ELISA as described previously (Ma, Luo, Huang, Song, & Liu, 2012). Briefly, TRAP proteins were used to coat the 96-well plates at a concentration of 10 μg/ml and incubated overnight at 4 °C. After washing three times with PBST, the plates were blocked with 3% BSA for 2 h at 4 °C, then, a twofold serial dilution of samples were added into the wells and incubated for 2 h at room temperature. After washing with PBST, HRP-conjugated goat anti-mouse IgG or IgG1, IgG2a, IgG2b, IgG3 mAbs were added and incubated for 1h at room temperature. After washing, 3, 3', 5, 5'-tetramethylbenzidine (TMB) solution was incubated with the plate for 15 min. To stop the reaction, 2N sulfuric acid was added, then, (Optical Densities) OD_{450nm} value was measured with an automated ELISA plate reader.

Challenge assay

To assess the immune-protective effect of the desired proteins plus the corresponding adjuvant, the challenge assay was performed. Two weeks after booster immunization, the C57/B6 mice in the challenge groups were infected by intraperitoneal injection with the lethal dose of 8×10^8 colony-forming units (CFU) of *Staphylococcus aureus* strain Newman, and 6×10^8 CFU of *Staphylococcus aureus* strain Wood46, respectively. The mice were detected for mortality and recorded every day after infection. Finally, the survival rate of mice from each group was determined after 14 days.

Statistical analysis

The results were analyzed by using an unpaired Student's t-test of the software SPSS 13.0. The data were presented as the means plus standard deviations. *P* values < 0.05 or *P* values < 0.01 were considered as significant difference.

Results

Confirmation of ATT, TRAP and eAls expression

To obtain recombinant pET-28a (+)-*att* plasmids, the *att* fragments were amplified from pET-32a(+)-*trap* with a pair of specific primers carrying the nucleotide sequence of *Als3 epitope*, and a nucleotide sequence encoding a GSGSGSGS linker was introduced between *Als3 epitope* and *trap* fragments to maintain the native conformation of Als3-TRAP protein. The segments of 558 bp were obtained by PCR and linked into pET-28a (+) plasmids (Fig. 2 A), and the analytical results showed that 558 bp segments were exhibited (Fig. 2 B), indicating the recombinant pET-28a (+)-*att* plasmids were correctly constructed. The segments of 498 bp were acquired using PCR (Fig. 2 A), and after pET-28a (+)- *trap* plasmids were treated with the *Bam*H I and *Hin*d III restriction endonuclease and PCR method, 498 bp segments were displayed (Fig. 2 C). In addition, the results of pET-28a (+)-*eAls* plasmids digested with restriction endonuclease revealed 426 bp segments production (Fig. 2 D). Therefore, these data demonstrated that the desired recombinant plasmids were correctly constructed.

The SDS-PAGE results indicated that ATT (24kDa) (Fig. 3 A), TRAP **(**22 kDa**)** (Fig. 3 B), eAls **(**18kDa**)** (Fig. 3 C) proteins were expressed by the recombinant *E. coli* BL21 (DE3) strains with pET-28a (+)-*att*, pET-28a (+)-*trap* and pET-28a (+)-*eAls* plasmids, respectively, and these proteins were successfully purified (Fig. 3 A, 3 B, 3 C). Western blot results also confirmed that the size of bands obtained by exposure was consistent with the expected molecular weight of the desired proteins, ATT (Fig. 3 D), TRAP (Fig. 3 E) and eAls (Fig. 3 F), indicating these proteins were successfully expressed.

Als3 epitope enhanced TRAP immunogenicity

I. Als3 epitope increased IFN-γ, IL-4, IL-10 and IL-17A production from lymphocytes

In order to assess Th1 cell immune response elicited by the desired antigens, the IFN-γ level of spleen lymphocytes in each group was determined by ELISpot assay. The representative images of TRAP-specific spot forming cells are shown in figure 4 A, the results showed that IFN-γ level in ATT group was higher than that in TRAP, TRAP+eAls group ($P < 0.05$), and IFN-γ level in ATT group was significantly

different from that in eAls group (P <0.01) (Fig. 4 A, 4 B). In addition, ELISA assay was performed to detect the levels of IL-4, IL-10 and IL-17A in supernatant of the spleen lymphocytes stimulated by desired stimulus. The results indicated the levels of IL-4 and IL-10 in ATT group were significantly different from those in TRAP and eAls group (P <0.01), and the levels of IL-10 in the supernatant of spleen lymphocytes from ATT group were significantly different from those in TRAP + eAls group (P <0.01), while the levels of IL-4 in ATT group were not significantly different from those in TRAP + eAls group (Fig. 4 C, 4 D), in addition, the secretion of IL-17A in ATT group was stronger than that of TRAP group (P <0.05), and was significantly different from that of eAls group (P < 0.01), but slightly lower than that of TRAP + eAls group (Fig. 4 E).

II. **Als3 epitope increased humoral immune response of TRAP**

To evaluate the level of antibody against TRAP in serum from immunize mice, ELISA assay was performed. As shown in figure 5 A, the level of IgG antibody against TRAP from TAA group was higher than that from TRAP and eAls groups (P <0.01), but slightly low compared with TRAP+eAls group (Fig. 5 A). In addition, ELISA was used to detect the level of IgG antibody subclasses in each group. As shown in figure 5 B, the results displayed IgG1 level was the highest among IgG1, IgG2a, IgG2b and IgG3 subclasses, and the level of IgG1 from ATT group was higher than that in Trap and eAls groups (P <0.01), but low compared with Trap + eAls group (Fig. 5 B).

III. **Als3 epitope boosted protective immune response of TRAP**

The immunized mice were challenged with *S.aureus* Newman strain and *S.aureus* Wood46 strain, respectively. After 3 days challenged with *S.aureus* Newman strain, the challenged results showed that the immune survival rate of PBS group was 20%, that of ATT group was 80%, that of TRAP group was 60%, that of eAls group was 50%, and that of TRAP+eAls group was 40% (Fig. 6 A). After 3 days challenged with *S.aureus* Wood46 strain, all the mice in PBS group died, and the immune survival rate of ATT group was 80%, that of TRAP group was 60%, that of eAls group was 40%, and that of TRAP+eAls group was 30% (Fig. 6 B). These data

299 indicated that Als3 epitope obviously boosted the protective immune response of
300 TRAP.

301 **The combined adjuvant improved important cytokine production from spleen**
302 **lymphocytes**

303 To detect Th1 and Th2 cell immune responses induced with antigen, the
304 secretion of IFN-γ and IL-4 from spleen lymphocytes of mice in each group was
305 determined by ELISpot assay. The representative images of TRAP-specific spot
306 forming cells for IFN-γ secretion were shown in figure 7 A, the statistic results
307 showed the production of IFN-γ in ATT+MDP+CpG+FIA group was highest in all
308 the groups ($P < 0.01$) (Fig. 7 A,7 B). Furthermore, the representative images of
309 TRAP-specific spot forming cells for IL-4 secretion were exhibited in figure 7 C, the
310 statistic results manifested that the production of IL-4 in ATT+MDP+CpG+FIA
311 group was significantly higher than that in other groups, but was slightly low
312 compared with that in ATT+CpG+FIA group (Fig. 7 C, 7 D).

313 In addition, IL-10 and IL-17A level in the supernatant of spleen lymphocytes
314 from mice in each group was analyzed by ELISA assay. As shown in figure 8 A,
315 IL-10 level in the supernatant of spleen lymphocytes in ATT+MDP+CpG+FIA group
316 was significantly different from that in ATT+CpG, ATT+FIA and ATT+CpG+MDP
317 groups ($P < 0.01$, $P < 0.05$), while no significant difference was exhibited in
318 comparation with other groups (Fig. 8 A). By contrast, the results showed that IL-17A
319 level in the supernatant of spleen lymphocytes in ATT+MDP+CpG+FIA group was
320 significantly higher than that in other groups ($P < 0.01$) (Fig. 8 B), indicating that the
321 combinations of MDP+CpG+FIA markedly promoted IL-17A production.

322 In general, IL-4 and IL-10 are characteristic of the Th2 immune response and
323 mediates the immune activity of B cell. By contrast, Th1 immune response is
324 characterized by increasing the levels of IFN-γ. IL-17 is mostly produced by Th17
325 cells and promotes neutrophil recruitment, enhances inflammation. In consequence,
326 from these above results, the combined adjuvants of MDP+CpG+FIA can exert a
327 coaction to induce the immune activation of Th1, Th2 and Th17 cells, as a result, to
328 further enlarge T-cell-mediated immune response.

The combined adjuvant increased humoral immune response

To evaluate if Als3 epitope plus the combinations of CpG, MDP and FIA adjuvant act a synergistic effect to enlarge the immune response triggered by TRAP. The level of IgG antibody against TRAP in the serum from immunized mice was detect with ELISA. As shown in figure 9 A, the serum from mice immunized with ATT+CpG+MDP+FIA group exhibited the highest IgG level among all groups, and obviously higher than that of ATT+CpG, ATT+MDP, ATT+FIA, ATT+CpG+MDP groups, displayed the high level of IgG compared with ATT+CpG+FIA and ATT+MDP+FIA group. Furthermore, the data of IgG antibody subclasses showed that IgG1 level was the highest level among IgG1, IgG2a, IgG2b and IgG3 subclasses, and the serum from mice immunized with ATT+CpG+MDP+FIA generated the highest IgG1 level among all the groups, and obviously higher IgG1 level than ATT+CpG, ATT+MDP, ATT+FIA, ATT+CpG+MDP groups (P <0.01) (Fig. 9 B). These results showed that Als3 epitope plus CpG, MDP and FIA adjuvant obviously increased the humoral immune response triggered with TRAP.

The combined adjuvants enhanced protective immune response

On 14 days after the boost immunization, challenge assay was performed by using *S. aureus* strain Newman and Wood46 strain, respectively. As shown in figure. 10 A, after 3 days challenge with *S. aureus* Newman strain, the survival rate of mice in PBS+FIA group was 20%, while that in ATT+CpG+MDP+FIA group was 80%, that in ATT+CpG+FIA group was 80%, that in ATT+MDP+FIA group was 60%, that in ATT+CpG+MDP and ATT+CpG groups was 50%, that in ATT+ MDP group was 40%, that in ATT+FIA group was 30% (Fig. 10 A). After 3 days challenge with *S. aureus* Wood46 strain, ATT+CpG+MDP+FIA group exhibited the highest the survival rate of 80% in all the groups, the survival rate of ATT+CpG+FIA group was 70%, that of the ATT+MDP+FIA was 60%, that of ATT+CpG was 50%, that of ATT+CpG+MDP was 40%，that of ATT+MDP was 30%， that of ATT+FIA was 30%，and that of PBS + FIA group was 10% (Fig. 10 B). These data indicated that the combined adjuvants plus Als3 epitope obviously enhanced the protective immune response triggered with TRAP.

Discussion

In this study, ATT proteins were prepared when *Als*3 epitope and *trap* were expressed by fusion, and Als3 epitopes obviously increased TRAP immunogenicity. With the synergistic effect of Als3 epitope, the combined adjuvants of CpG+MDP+FIA strengthened the immune response triggered with TRAP. These data might provide a novel strategy for enhancing the immuno-protection of vaccine candidates.

Recently, TRAP proteins, one of the important surface proteins from *S. aureus*, have exhibited the great potential as a new vaccine candidate. Balaban N et al. found that the antibodies against TRAP significantly reduced the secretion of *S. aureus* exotoxin. TRAP, as an immunogen, can effectively inhibit the toxins generation from *S. aureus*, and displays a protective effect on preventing *S. aureus* infection (Balaban et al., 2001). However, the TRAP immunogenicity is not up to people's ideal requirements for preventing *S. aureus* infection, therefore, how to further enhance the immunogenicity of TRAP protein has been performed in this study.

Owing to Als3, similar to three-dimensional structures of *S. aureus* ClfA, and Als3 epitope to trigger immune response against *S. aureus* and *C. albicans* infection (Bar et al., 2012; Schmidt et al., 2012; Yeaman et al., 2014), we prepared the ATT proteins to confirmed if Als3 epitopes promote TRAP immunogenicity. Firstly, ELISA and ELISpot data revealed that the levels of IL-4, IL-10, IL-17 and IFN-γ secreted from the spleen cells of ATT group were significantly higher than those of control groups. Secondly, the results of antibody detection showed that the level of IgG against TRAP from ATT group was higher than that of TRAP group, moreover, IgG1 generation was significantly different between ATT group and TRAP group, indicating that ATT protein could elicit the strong humoral immune response in mice. Finally, the challenged results showed that the immune protection effect of ATT was higher than TRAP, indicating that Als3 epitopes were able to enhance the immune-protective effect of TRAP. Therefore, our data demonstrated that Als3 epitopes obviously enhanced the TRAP immunogenicity by their fusion expression.

CpG, as the agonist of TLR9, MDP, as an activator of NOD2, both can improve the development of cellular immune response towards Th1/Th17 (Inohara et al., 2003; van Heel et al., 2005). The liquid paraffin and surfactant components in FIA can adsorb antigens and active innate immunity, moreover, they slow antigen to release into the microenvironment and extend antigen-induced immune responses in vivo. Recent studies reveal that the combined adjuvant can cooperate the synergic effect and obviously enhances the protective immune responses induced with antigens (Mount et al., 2013). Therefore, the combined adjuvant is a new trend in the vaccine development. Presently, coactions of different adjuvant have been gradually utilized, for example, the combined utilization of CpG ODN and nanoemulsion adjuvant (NE02), CpG ODN and Poly(I:C) (Polyinosinic-polycytidylic acid), streptavidin-4-1BBL (SA-4-1BBL) and monophosphoryl lipid A (MPLA), aluminum salts and CpG ODN plus innate defense regulator peptide HH2, which effectively attains the desired immune response and improves generation of multiple cytokines, then, boosting the immune effect of antigens (Pirahmadi et al., 2019; Srivastava, Yolcu, Dinc, Sharma, & Shirwan, 2016; Tian et al., 2017; Wang et al., 2020). In recent years, IL-17, IFN-γ cytokine plays a crucial role for killing *S. aureus.* IL-17 can recruit neutrophils to eliminate *S. aureus*, IFN-γ activates and makes macrophages chemotactic to engulf and kill *S. aureus* (Sathiyaseelan et al., 2006). IL-17 and IFN-γ were mainly secreted from Th1 cells, Th17 cells, respectively. Thus it can be indicated that Th1/Th17-mediated immune response plays a key role in killing *S. aureus*. Hence, on the development of vaccines against *S.aureus* infection, it is particularly important for alliance of different adjuvants to active Th1 cells and Th17 cells.

Therefore, in this study, we prepared the combine adjuvant of CPG, MDP and FIA to enlarge the immune protective effect of ATT against *S. aureus* infection. In the challenge experiment, the immune results shown that ATT+CpG+MDP+FIA group exhibited 80% survival rate against *S. aureus* strain Newman or *S. aureus* strain Wood46, which was obviously higher than the control groups. In addition, ELISA and ELISPOT data revealed that the secretion levels of IL-17 and IFN-γ from

ATT+CpG+MDP+FIA group were significantly higher compared with other experimental groups, which indicated that Th1 and Th17 cells might have been activated, increasing the immune response against *S. aureus* infection. However, it will be further performed to evaluate Th1 /Th17 cell-mediated immune response triggered with ATT plus CpG+MDP+FIA adjuvant in future.

By detecting IgG antibody level, it was found that there was no significant difference on antibody level when ATT+CpG+MDP+FIA group compared with ATT+CpG+FIA and ATT+MDP+FIA groups, but there was significant difference compared with other experimental groups. In addition, IgG1 level was the highest in all groups, indicating that ATT plus CpG, MDP and FIA adjuvant could stimulate the immunized mice to generate the stronger humoral immune response. Therefore, the Als3 epitopes plus the combinations of CpG, MDP and FIA adjuvant were able to generate the synergistic effect to enhance the immune response triggered with TRAP. However, to further increase the immunogenicity of TRAP, other TLR agonists, for example, Poly (I:C), MPLA and so on, will be considered in conjugation with ATT in our future research (Poteet et al., 2015; Renu et al., 2020; Temizoz, Kuroda, & Ishii, 2016). In addition, in this study, we only selected *S. aureus* strain Newman and *S. aureus* strain Wood46 as the challenge strains, and other *S. aureus* strains should be used to further evaluate the immuno-protection effect triggered with the ATT plus CpG+MDP+FIA complex in future.

Conclusions

Taken together, our data showed that ATT successfully obtained and Als3 epitopes significantly strengthen the immune response triggered with TRAP by their fused expression, the combined adjuvants of CpG+MDP+FIA exhibited the stronger immunomodulatory function and significantly heightened TRAP-induced immune responses in conjunction with Als3 epitopes, which may provide an important basis for the novel vaccines development against *S. aureus* infection.

Author contributions

Jinzhu Ma contributed design of the study, and analyzed the data. Wei Liu

performed the experiments, and revised the article. Beiyan Wang, Simiao Yu, Liquan Yu, Baifen Song, Yongzhong Yu and Zhanbo Zhu contributed to data collection.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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List of non-standard abbreviations

Als3: Agglutinin-like sequence 3, Als3 epitope: Als3-Th-cell-epitope, ATT: Als3-Th-cell-epitope-TRAP, *eAls*: epitope *Als*, TRAP: Target of RNAIII-activating Protein, ClfA: Clumping factor A, TLR9: Toll-like Receptor 9, TMB: 3, 3', 5, 5'-tetramethylbenzidine, mAbs: Monoclonal Antibodies, OD: Optical Densities, *S. aureus*: *Staphylococcus aureus*, ELISpot: enzyme-linked immunospot, ELISA: Enzyme-linked immuno sorbent assay, CpG ODN (CpG): Cytosine-phosphate-guanosine oligodeoxynucleotides, MDP: Muramyl Dipeptide, FIA: Freund's incomplete adjuvant, PBS: Phosphate Buffer Solution, IPTG: isopropyl-β-D -1-thiogalactopyranoside, PMA: Phorbol Myristate Acetate, MPLA: Monophosphoryl Lipid A.

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