Peptide mimics of peptidoglycan are vaccine candidates and protect mice from infection with *Staphylococcus aureus*

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Staphylococcus aureus drug resistance to antibiotics is a serious situation that has drawn greater attention to immunotherapy and prophylaxis. Peptidoglycan (PGN) is a common and conserved component of the cell wall of Gram-positive bacteria such as *S. aureus*. However, PGN, as a thymus-independent antigen, cannot be considered a vaccine candidate because of its very weak immunogenicity. In this study we have attempted to enhance the immunogenicity of PGN by identifying a PGN peptide mimic sequence that would act as a thymus-dependent antigen. Several peptide sequences were obtained from a phage display peptide library using a mAb against *S. aureus* PGN, and a 12-mer linear single peptide (Sp-31) and a four-branch multiple antigen peptide (MAP) (MAP-P31) were synthesized. Both Sp-31 and MAP-P31 were shown to bind directly to anti-PGN mAb and a polyclonal antibody against *S. aureus*. These peptides could also inhibit the binding of PGN to a mAb against PGN. Furthermore, MAP-P31 was able to provoke an effective secondary antibody response in mice to PGN and to cell-wall fragments isolated from *S. aureus*, *Escherichia coli*, *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* by sonication. In addition, the MAP-P31 antiserum showed a potent bactericidal or bacteriostatic activity against *S. aureus* in the presence and absence of complement *in vitro*. Importantly, immunization with MAP-P31 significantly prolonged the survival and enhanced bacterial clearance in BALB/c mice challenged with live *S. aureus*. In addition, the serum IgG-type antibodies against PGN persisted in mice, demonstrating that MAP-P31, as a peptide mimicking epitopes on PGN, provokes an effective secondary or memory antibody response, which can only be induced by a thymus-dependent antigen and which protects against infection with *S. aureus*. These results suggest that MAP-31 may be a novel vaccine candidate against *S. aureus*.

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INTRODUCTION

Staphylococcus aureus is a leading cause of community-associated and nosocomial infections. Since the appearance of methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *S. aureus* in the 1960s, immunotherapy and prevention of infection have been explored. At present, there are five therapeutic antibodies, and two out of ten potential vaccines have been authorized for clinical trials, although none has been approved for clinical application (Baker, 2006; Schaffer & Lee, 2008). Previous attempts to produce *S. aureus* vaccines, including those using attenuated *S. aureus* strains, polysaccharide–protein conjugates, protein, glucosamine and DNA, have been unsuccessful or

remain to be clarified (Buzzola *et al.*, 2006; Schaffer & Lee, 2009). Recent studies have indicated that *S. aureus* by itself cannot provoke an efficient specific protective immunity, as B and T cells exhibit anergy in response to infection with *S. aureus*, and B and T cells activated by vaccination with heat-killed *S. aureus* could not improve clearance from organs (Schmaler *et al.*, 2011; Spellberg & Daum, 2010). As most vaccine candidates that have been reported are prepared with intact *S. aureus* or its components derived or modified from conjugation, isolation or recombinant expression, it may be necessary to explore new vaccine candidates with distinct immunogen characteristics unlike the natural components of *S. aureus*. In this work, we tried to create peptide mimics of peptidoglycan (PGN) as a vaccine candidate.

PGN is a vital component of the cell wall of many kinds of bacteria, especially Gram-positive bacteria such as *S. aureus* (Wergeland *et al.*, 1989), and can be detected in the blood

Abbreviations: FCA, Freund's complete adjuvant; HRP, horseradish peroxidase; IL-6, interleukin 6; LTA, lipoteichoic acid; MAP, multiple antigen peptide; MRSA, methicillin-resistant *Staphylococcus aureus*; PGN, peptidoglycan; pMØ, mouse peritoneal macrophages; TNF- α , tumour necrosis factor alpha.

of >80% of patients with serious bacterial infections (Merkel & Scofield, 2001). However, PGN, a thymus-independent antigen, is a poor immunogen as it does not induce the production of memory B cells or an effective secondary antibody response. Our strategy was to enhance the immunogenicity of PGN by identifying a PGN peptide mimic sequence that would instead act as a thymus-dependent antigen. We screened PGN peptide mimics from a random phage display peptide library using an anti-PGN mAb and synthesized a single-chain (Sp-31) and a four-branch multiple antigenic peptide (MAP) (MAP-P31) that mimicked epitopes on PGN. Our results indicate that MAP-P31 may be a vaccine candidate that can induce an effective secondary antibody response and a protective effect against infections with *S. aureus*.

METHODS

Reagents, bacteria and animals. A PhD-12 phage display peptide library kit was purchased from New England Biolabs. PGN and polymyxin B sulphate were from Sigma. mAb (IgG3) against *S. aureus* PGN was purchased from ABD Serotec, mAb (IgG1) to lipoteichoic acid (LTA) was from LifeSpan Biosciences, and rabbit anti-*S. aureus* antiserum and normal rabbit serum were prepared in our laboratory. Iscove's modified Dulbecco's medium and RPMI 1640 were purchased from Gibco, and fetal bovine serum was from Sijiqing Biological Engineering Materials. The tumour necrosis factor alpha (TNF- α) and interleukin-6 (IL-6) ELISA kits were from eBioscience. *S. aureus* (ATCC 25923) and *Staphylococcus epidermidis* (ATCC 12228) were purchased from Wenzhou Kont Biology and Technology, and *Escherichia coli* and *Pseudomonas aeruginosa* were isolated from clinical samples. BALB/c mice were purchased from the Experimental Animal Center, Southern Medical University, Guangzhou, China. The animal experiments were approved by the Ethics Committee for Experimental Animals at Southern Medical University and were carried out in accordance with national guidelines for animal welfare.

Screening and characterizing of phage clones that mimic PGN

Panning of phage display peptides. Phage clones that mimicked PGN were screened from a PhD-12 phage display peptide library using a commercial anti-PGN mAb as a target. Briefly, 1.0×10^{11} phage particles were added to one well coated with anti-PGN mAb ($20 \mu\text{g ml}^{-1}$) in 0.1 M NaHCO_3 buffer, and this was followed by ten rapid washes with Tris-buffered saline (50 mmol Tris, 150 mmol NaCl in 1 l deionized water, pH adjusted with HCl) plus 0.1% Tween 20 to remove unbound phages. Bound phages were eluted with 50 μl 0.2 M glycine/HCl (pH 2.2) containing 1 mg BSA ml^{-1} , followed immediately by neutralization with 1 M Tris/HCl buffer (pH 9.1). The eluted phages were titrated, amplified and purified for the next round. After three rounds of screening, single colonies were picked randomly from agar plates and analysed by phage ELISA.

Phage ELISA. Phage particles (1.0×10^{11} p.f.u. ml^{-1} , 50 μl per well) from each individual phage clone were added to microplates coated with anti-PGN mAb or left uncoated as a control, and this was followed by blocking with 1% casein. Finally, horseradish peroxidase (HRP)-labelled anti-M13 (MEK13 phage vector) antibody was added, and then thorough washing was carried out. A colour reaction was developed using tetramethylbenzidine with hydrogen peroxide and stopped with 2 M sulphuric acid. The absorbance values were read at 450 nm (A_{450}) using a microplate reader (Elx800; BioTek).

Sequencing of phage clones. Fourteen positive phage clones identified by phage ELISA were amplified, and the single-stranded DNA was isolated for DNA sequencing by Invitrogen.

Synthesis and characterization of peptide mimics of PGN

Peptide synthesis. A single-chain peptide (Sp-31) and an MAP form comprising a four-branch peptide (MAP-P31) that were deduced from phage clone number 31 were synthesized by Hybio Pharmaceutical. The accuracy and purity of the final products were confirmed by MS and HPLC, which demonstrated the correct sequence and 96.32% purity.

Determination of antigenicity by indirect and competitive ELISAs. Biotin-labelled Sp-31 was added to microplates coated with different antibodies against PGN or LTA, and detected by HRP-streptavidin. For the identification of MAP-P31, anti-PGN mAb or anti-*S. aureus* polyclonal antibody was added to microplates coated with MAP-P31 and detected by HRP-labelled goat anti-mouse or goat anti-rabbit antibody, respectively. The colour reaction and evaluation of results were performed as described above. The specificity of Sp-31 and MAP-P31 were also confirmed by competitive ELISA. Briefly, Sp-31 or MAP-P31 as a competitor was pre-incubated with anti-PGN mAb before the mixture was added to microplates coated with PGN, and HRP-labelled goat anti-mouse IgG was then added. PGN was also used to demonstrate inhibition of the binding between biotin-labelled Sp-31 and anti-PGN mAb coated on microplates.

Induction and measurement of cytokines. Mouse peritoneal macrophages (pMØ) were collected from the peritoneal cavities of BALB/c mice by washing three times with 5 ml RPMI 1640. Cell suspensions were centrifuged at 800 g at 4 °C and resuspended in fresh RPMI 1640 containing 10% fetal bovine serum, followed by culturing in 24-well plates at 1×10^6 cells per well for 24 h. Adherent cells (pMØ) were stimulated with MAP-P31 to produce cytokines. Finally, the supernatant from cultured cells was collected, and the levels of TNF- α and IL-6 were measured using commercially available ELISA kits (eBioscience) according to the manufacturer's instructions.

Identification of antiserum following immunization with MAP-P31.

Six- to eight-week-old BALB/c mice were divided randomly into four groups to be immunized as follows: (i) MAP-P31 emulsified with Freund's complete adjuvant (FCA) for the first immunization, followed by a boost with Freund's incomplete adjuvant; (ii) FCA for 1 week prior to MAP-P31; (iii) MAP-P31 alone; (iv) an unrelated MAP and PBS as controls. Immunizations in all groups were performed by subcutaneous injection of 100 μg five times, each with a 2 week interval. Antisera were collected for evaluation of antibodies to MAP-P31, PGN and *S. aureus* by indirect ELISA. Briefly, microplates were coated with 80 μg MAP-P31 ml^{-1} , 10 μg PGN ml^{-1} , 10 μg LPS ml^{-1} , 10 μg LTA ml^{-1} or 5×10^8 c.f.u. sonicated fragments of *S. aureus*, *S. epidermidis*, *E. coli* or *P. aeruginosa* at 4 °C overnight, followed by blocking with 1% casein, except for the microplates coated with sonicated fragments of *S. aureus*, which were blocked with normal rabbit serum to avoid the binding of staphylococcus protein A to IgG. The diluted antiserum or control serum was added, and the microplates were incubated with HRP-labelled goat anti-mouse IgG. The colour reaction was developed as described above.

Assay of bactericidal or bacteriostatic activity. The bactericidal activity of anti-MAP-P31 serum against *S. aureus* was performed as described by Beninati *et al.* (2004) with slight modifications. Briefly, the sera from six mice were pooled. *S. aureus* (1×10^7 c.f.u.) was incubated with diluted heat-inactivated antiserum or control serum with or without mouse complement at 37 °C for 30 min. The mixture was vortexed and the diluted antisera were plated onto tryptic soy

agar plates for 18–24 h at 37 °C and the number of bacterial c.f.u. on the plates was calculated.

Evaluation of MAP-P31-induced protection

Bacterial clearance *in vivo*. Immunized mice ($n=3$) were challenged with 2×10^7 c.f.u. *S. aureus* by intravenous tail injection and sacrificed 6 h later, as described by Murphey *et al.* (2008). Spleen, liver and kidney samples were aseptically isolated, weighed and homogenized in sterile saline (0.9% NaCl) using sterile grinders. The tissue homogenates were diluted, plated on nutrient agar and cultured at 37 °C for 18–24 h. The number of bacterial c.f.u. was calculated to estimate the bacterial burden of the tissues.

Evaluation of survival of mice infected with *S. aureus*. On day 10 after the fifth immunization with MAP-P31, BALB/c mice were inoculated with 6×10^8 c.f.u. live *S. aureus* by intraperitoneal injection. All mice were observed at 2 h intervals for the first 48 h and survival was monitored for at least 14 days. Evaluation of the statistics of survival was carried out using a log-rank test.

Statistical analysis. Results were expressed as means $\pm$ SD. Student's *t*-test and one-way ANOVA were performed using SPSS version 13.0 software to compare data between different groups; survival curves were analysed by a log-rank test. $P < 0.05$ was considered statistically significant. All figures were produced using GraphPad Prism (version 5.01 for Windows; GraphPad Software).

RESULTS

Characteristics of phage clones with peptide mimics of PGN

The screening of phage clones showed an enrichment process from the first to the third round. A total of 14 of 50 phage clones were positive clones that bound anti-PGN mAb identified by indirect ELISA (Fig. 1), suggesting that these phages displayed a mimotope of PGN.

Fig. 1. Binding of phage clones to anti-PGN mAb. Each phage clone was added to a well coated with anti-PGN mAb at $2 \mu\text{g ml}^{-1}$, or with PBS as a control to exclude non-specific absorption of phages to the plate or MEK13 (vector) as a negative control. One representative result of three experiments is presented. A_{450} values were calculated as $A_{450 \text{ coating}} - A_{450 \text{ PBS control}}$.

Analyses of sequences

The amino acid sequences of the 14 positive phage clones deduced from DNA sequences were analysed and are shown in Table 1. Three phage clones, numbers 28, 31 and 43, had the consensus sequence ATWxHxLxSAGL, which also showed higher reactivity with anti-PGN mAb than other clones (Fig. 1). Phage clone numbers 1, 27 and 39 shared the conserved sequence xHx or xH found in clone number 31.

Antigenicity of peptides Sp-31 and MAP-P31 that mimic PGN

The sequence of clone number 31, termed Sp-31, was synthesized with the addition of the amino acids SA and GG at the N and C termini, respectively. To enhance the immunogenicity, a four-branch MAP based on the sequence of Sp-31 was synthesized (MAP-P31). The results in Fig. 2 showed that both Sp-31 and MAP-P31 bound to anti-PGN mAb and a polyclonal antibody against *S. aureus* in a dose-dependent manner. Moreover, both Sp-31 and MAP-P31 inhibited the binding of PGN to anti-PGN mAb in a dose-dependent manner (Fig. 3a); conversely, PGN could inhibit the binding of Sp-31 to the anti-PGN mAb (Fig. 3b). The data in Figs 2 and 3 indicated that both Sp-31 and MAP-P31 mimicked epitopes on PGN.

Production of TNF- α and IL-6 by pMØ stimulated with MAP-P31 *in vitro*

As PGN can stimulate the production of pro-inflammatory cytokines, the levels of TNF- α and IL-6 produced by pMØ after induction by MAP-P31 were measured. Polymyxin B ($20 \mu\text{g ml}^{-1}$), which does not interfere with the production of cytokines, was used to neutralize the influence of LPS if present in the supernatant. The results showed that TNF- α and IL-6 were produced by pMØ stimulated with MAP-P31 in dose-dependent manner (Fig. 4; $P < 0.01$), suggesting that MAP-P31 can also mimic the biological activity of PGN, despite the weaker activity of MAP-P31 in stimulating cytokine production than that of PGN.

Table 1. Amino acid sequences of phages binding to anti-PGN mAb

Phage clone no.	Amino acid sequence*
39	GRW <u>xHxVxW</u> AGL
28, 31, 43	ATW <u>xHxLxS</u> AGL
27	SPH <u>xHS</u> RLRSES
1	<u>xHx</u> VVLLSLTAH
7, 11	FSTNWMNQHRPS
41	LAALTRTPVVSP
46	DPLLTGNYKEAS
5, 23, 38, 44	GWWPYAALRLAS

*Areas underlined and in bold represent areas of homology.

Fig. 2. Binding of Sp-31 and MAP-P31 to antibodies against PGN and *S. aureus*. (a) Binding of Sp-31 to antibodies. Anti-PGN mAb (●), anti-*S. aureus* polyclonal antibody (▲) and anti-LTA mAb (■) as a control were used to coat the wells of a microplate at a concentration of $2 \mu\text{g ml}^{-1}$; biotin-labelled Sp-31 was added at the indicated concentrations and incubated at 37°C for 30 min, followed by detection with HRP-labelled streptavidin. (b) Binding of MAP-P31 to antibodies. MAP-P31 was used to coat the wells of a microplate, and anti-PGN mAb (●) or anti-*S. aureus* polyclonal antibody (□) was added at a concentration of $2 \mu\text{g ml}^{-1}$, followed by detection with HRP-labelled antibodies. The results are presented as A_{450} . Experiments were performed three times and results are shown as means $\pm$ SD.

Binding of antiserum to MAP-P31 and different bacteria or bacterial components

Antisera from mice immunized with MAP-P31 were evaluated for binding activities by indirect ELISA. The results in Fig. 5(a) indicated that the best antibody response was found in mice immunized with MAP-P31 mixed with FCA. Therefore, the antiserum of mice immunized with MAP-P31 plus FCA was used to observe the binding of different antigens and bactericidal or bacteriostatic activity. The results in Fig. 5(b) showed that

Fig. 3. Inhibition effect of peptide mimics of PGN on the binding of anti-PGN mAb to PGN or peptide mimics. (a) Sp-31 and MAP-P31 inhibition of the binding of PGN to anti-PGN mAb. Mixtures of Sp-31 (○) or MAP-P31 (□) at the indicated concentration and anti-PGN mAb were pre-incubated and added to microplates that had been coated with PGN. HRP-labelled goat anti-mouse IgG was added, and the A_{450} was determined. Inhibition was calculated as the percentage of the absorbance of mAb without Sp-31 or MAP-P31 added. (b) PGN inhibition of the binding of Sp-31 to anti-PGN mAb. Mixtures of PGN and biotin-labelled Sp-31 were added to microplates that had been coated with anti-PGN mAb. HRP-streptavidin was added and A_{450} was determined. Inhibition was calculated as the percentage of the absorbance of Sp-31 without PGN added. Experiments were performed three times and results are shown as means $\pm$ SD.

the antiserum could bind effectively to PGN but only weakly to LTA and LPS. The antiserum could also bind to sonicated fragments of *S. aureus*, *S. epidermidis*, *E. coli* and *P. aeruginosa* (Fig. 5c). Consistent with our expectations, the MAP-P31 antiserum reacted with PGN and *S. aureus*; this reaction could be considered a secondary antibody response or memory response, as shown in Fig. 5(d).

Fig. 4. Production of TNF- α (a) and IL-6 (b) in mouse pMØ stimulated with MAP-P31. pMØ from BALB/c mice were cultured with or without MAP-P31 at the indicated concentrations for 12 h. TNF- α and IL-6 in the culture supernatants were measured by ELISA. Polymyxin B (PMB) was used as an inhibitor of endotoxin (LPS), and PGN (20 $\mu\text{g ml}^{-1}$) was used as a positive control. Experiments were performed three times in duplicate wells and the results are shown as means $\pm$ SD. ***, $P < 0.001$.

Bactericidal or bacteriostatic activity of MAP-P31 antiserum

In the presence or absence of complement, by adding fresh or inactivated murine serum, anti-MAP-P31 antiserum or control unrelated MAP antiserum was incubated with live *S. aureus* (ATCC 25923), and the number of bacterial c.f.u. on agar plates was calculated. The results in Fig. 6 showed that anti-MAP-P31 antiserum had a more potent bactericidal or bacteriostatic activity than that of an unrelated MAP antiserum and normal serum in a dose-dependent manner, with or without complement.

Bacterial clearance and protection against infection mediated by immunization with MAP-P31

Bacterial clearance and protection against infection with lethal live *S. aureus* were determined in 6–8-week-old BALB/c mice immunized with MAP-P31 or an unrelated MAP and PBS as controls. Bacterial clearance in spleen, liver and kidney tissues was enhanced in mice immunized with MAP-P31 compared with that in mice immunized

with an unrelated MAP (Fig. 7a). Moreover, when mice were challenged with 6×10^8 live *S. aureus* by intraperitoneal injection on day 10 after the fifth immunization, the results showed that survival of mice immunized with MAP-P31 was notably longer than that of mice immunized with an unrelated MAP or with the PBS control (Fig. 7b), and after 1 week no mice died. Consequently, we concluded that MAP-P31 can induce protection against a lethal infection with *S. aureus*, and this may be associated with enhanced bacterial clearance *in vivo*.

DISCUSSION

In this work, we successfully identified a PGN peptide mimic that acts as a thymus-dependent antigen, to improve the immunogenicity of PGN, which is a thymus-independent antigen. These results may contribute to the development of a new vaccine candidate for *S. aureus*. The use of MAP-P31 as an immunogen provoked an effective protection against infection with *S. aureus in vivo* and antibody-mediated bactericidal or bacteriostatic activity *in vitro*. Additionally, we demonstrated that MAP-P31 not only mimicked the antigen specificity of PGN to induce the production of antibodies that bound to PGN as well as to sonicated fragments of *S. aureus*, *S. epidermidis*, *E. coli* and *P. aeruginosa*, but also induced the production of the pro-inflammatory cytokines TNF- α and IL-6, even when used at a lower level than that of PGN as a stimulator. Compared with the *S. aureus* vaccine candidates that have been reported so far, MAP-P31 is not a natural component of *S. aureus* that would be expected to overcome the anergy of T and B cells in response to *S. aureus* infection (Schmalzer *et al.*, 2011; Spellberg & Daum, 2010).

Concerning the significance and application of PGN antibodies, several reports have described the use of antibodies against PGN in human or animals for diagnosis (Jacob *et al.*, 1987; Minors *et al.*, 1988; Nozawa, 1985; Verbrugh *et al.*, 1981, 1983; Wheat *et al.*, 1983). However, only a few studies have focused on the opsonic activities of anti-PGN antibodies against *S. aureus* (Schuman *et al.*, 2007; Verbrugh *et al.*, 1980). It is worth noting that the MAP-P31 antiserum showed a potent complement-independent bactericidal or bacteriostatic activity *in vitro*; most notably, MAP-P31 immunization significantly prolonged survival and enhanced bacterial clearance in BALB/c mice challenged with live *S. aureus* ATCC 25923, a methicillin-sensitive *S. aureus*. Moreover, the IgG-type antibodies against PGN endured for 8 weeks after the last immunization, which indicates that MAP-P31 as a thymus-dependent antigen induces a secondary antibody response to *S. aureus*, as only thymus-dependent antigens can induce secondary antibody responses and persist in the body for this length of time. We also found that MAP-P31 stimulated pMØ to release IL-6 and TNF- α independently of LPS, which suggests that MAP-P31 can also mimic the biological activities of PGN, as PGN plays a vital role in inflammation by inducing excessive amounts of inflammatory factors (Lin

Fig. 5. Reactivities of MAP-P31 antiserum determined by ELISA. (a) Binding of MAP-P31 to antiserum from mice immunized with: MAP-P31 emulsified with FCA (FCA + MAP-P31), FCA injection 1 week prior to MAP-P31 injection (FCA prior to MAP-P31), MAP-P31 alone, and an unrelated MAP or PBS. (b) Specificity of MAP-P31 antiserum or antiserum to an unrelated MAP (both diluted 1 : 200) to PGN, or to LPS, LTA or PBS as controls. Grey bars, coated with PGN; white bars, coated with LPS; hatched bars, coated with LTA; black bars, coated with PBS. (c) Antiserum from mice immunized with MAP-P31 emulsified with FCA bound to sonicated fragments of *S. aureus*, *S. epidermidis*, *E. coli* and *P. aeruginosa*. Black bars, anti-MAP-P31; white bars, anti-unrelated MAP. Experiments in (a)–(c) were carried out three times and results are shown as means $\pm$ SD. (d) Titres of MAP-P31 antiserum binding to MAP-P31, PGN and *S. aureus*. MAP-P31 ($\circ$), PGN ($\square$), *S. aureus* ($\blacksquare$) and an unrelated MAP ($\diamond$) were used as coating antigens. Antiserum to MAP-P31 was collected and diluted with PBS. The experiment was then performed as a routine indirect ELISA. An A_{450} value that was more than twice that of the control was used as the positive end-point titre. The results shown represent one of three independent experiments, with the titre being the reciprocal of the dilution of antiserum. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

et al., 2010; MacKenzie *et al.*, 2010; Visser *et al.*, 2005; Xie *et al.*, 2010).

Studies of peptide mimics of PGN have not been reported previously; however, this strategy has been used in research on non-protein vaccines, such as peptide mimics to induce the production of protective antibodies against *Cryptococcus neoformans* (Datta *et al.*, 2008), *Vibrio cholerae* (Falkind-Jerkrus *et al.*, 2005) and *Entamoeba histolytica* (Melzer *et al.*, 2003), as well as the production of cytotoxic antibodies against tumour cells (Monzavi-Karbassi *et al.*, 2001; Pashov *et al.*, 2009). In addition, we synthesized a cyclic peptide mimic of LPS that provoked an

IgG-type antibody response and protective immunity against endotoxic shock in mice challenged with LPS, and prolonged the survival of mice infected with live *Salmonella typhimurium* (Jiang *et al.*, 2009; Luo *et al.*, 2008).

Nevertheless, there are important problems that remain to be resolved for peptide mimic vaccines. For instance, an ideal vaccine candidate for *S. aureus* should induce a specific humoral and T-cell-mediated immune response, and should protect patients or experimental animals from different strains of *S. aureus*. However, to date, there is no evidence to show that *S. aureus* or its components can

Fig. 6. Bactericidal or bacteriostatic activity of MAP-P31 antiserum. The sera from six immunized mice were pooled. (a) *S. aureus* (1×10^7 c.f.u.) was incubated with MAP-P31 antiserum, control unrelated MAP antiserum or normal mouse serum at the indicated dilutions at 37 °C for 30 min. The mixture was vortexed, diluted and then plated onto tryptic soy agar plates at 37 °C for 18–24 h. The number of bacterial c.f.u. on the plates was determined. (b) *S. aureus* (1×10^7 c.f.u.) was incubated with untreated or heat-inactivated MAP-P31 antiserum or normal mouse serum at 37 °C for 30 min. The mixture was treated as in (a) and the number of c.f.u. was determined. Experiments were carried out three times and results are shown as means $\pm$ SD. *, $P < 0.05$; **, $P < 0.01$; NS, not significant.

provoke a protective specific T-cell response. We supposed that MAP-P31 might induce a specific CD4⁺ T-cell-mediated cellular immunity against *S. aureus*. However, neither MAP-P31 nor Sp-31 specifically stimulated T-cell proliferation or the release of gamma interferon and IL-2, which indicated that MAP-P31 and Sp-31 do not contain T-cell epitopes. Another problem with our results is that MAP-P31 only induced a protective response against methicillin-sensitive *S. aureus*, and not against MRSA, which is increasing in clinical specimens (Styers *et al.*, 2006). In subsequent work, we aim to redesign the sequence of MAP-P31 by adding a T-cell recognition epitope into the sequence, and to optimize more sequences binding to

Fig. 7. MAP-P31 enhances clearance of bacteria and survival of immunized mice challenged with *S. aureus*. (a) Clearance of bacteria. Mice immunized with MAP-P31 (black bars) or an unrelated MAP (control) (white bars) were infected with 2×10^7 live *S. aureus* by intravenous tail injection and sacrificed after 6 h. The number of bacterial c.f.u. was calculated to estimate the bacterial burden of each tissue. *, $P < 0.05$ ($n = 3$ per group). (b) Survival of mice. Mice immunized with MAP-P31 or with an unrelated MAP or PBS (controls) were challenged with 6×10^8 live *S. aureus* by intraperitoneal injection and monitored post-challenge. **, $P < 0.01$ ($n = 10$ per group). Δ , MAP-P31 + FCA ($n = 10$); $\diamond$, unrelated MAP + FCA ($n = 10$); $\circ$, PBS ($n = 10$).

anti-PGN antibodies that would be expected to induce a more efficient protective immune response to MRSA.

In conclusion, our results indicate that MAP-P31 may be used as a vaccine candidate to induce a protective immune response to *S. aureus*. This protection may be associated with protective antibodies to increase bacterial clearance in the major organs of infected animals challenged with live *S. aureus*. Our results provide a novel approach in the fight against *S. aureus*, although there remains much work to be done before MAP-P31 can be used as a clinical vaccine against *S. aureus*.

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