RESEARCH ARTICLE

A Multiple Antigenic Peptide Mimicking Peptidoglycan Induced T Cell Responses to Protect Mice from Systemic Infection with *Staphylococcus aureus*

Xiang-Yu Wang¹, Zhao-Xia Huang¹, Yi-Guo Chen¹*, Xiao Lu¹, Ping Zhu¹, Kun Wen², Ning Fu¹,², Bei-Yi Liu¹*

¹ Department of Immunology, School of Basic Medical Sciences, Southern Medical University, Guangzhou, Guangdong, People’s Republic of China, ² Laboratory of Emerging Infectious Diseases and Division of Laboratory Medicine, Zhujiang Hospital, Southern Medical University, Guangzhou, Guangdong, People’s Republic of China

* Current Address: Medical Laboratory, Jiangxi Provincial People’s Hospital, Nanchang, Jiangxi, People’s Republic of China

* lbydodo@163.com

Abstract

Due to the enormous capacity of *Staphylococcus aureus* to acquire antibiotic resistance, it becomes imperative to develop vaccines for decreasing the risk of its life-threatening infections. Peptidoglycan (PGN) is a conserved and major component of *S. aureus* cell wall. However, it has not been used as a vaccine candidate since it is a thymus-independent antigen. In this study, we synthesized a multiple antigenic peptide, named MAP27, which comprised four copies of a peptide that mimics the epitope of PGN. After immunization with MAP27 five times and boosting with heat-inactivated bacterium one time, anti-MAP27 serum bound directly to *S. aureus* or PGN. Immunization with MAP27 decreased the bacterial burden in organs of BALB/c mice and significantly prolonged their survival time after *S. aureus* lethal-challenge. The percentage of IFN-γ⁺CD3⁺ T cells and IL-17⁺CD4⁺ T cells in spleen, as well as the levels of IFN-γ, IL-17A/F and CCL3 in spleen and lung, significantly increased in the MAP27-immunized mice after infection. Moreover, *in vitro* incubation of heat-inactivated *S. aureus* with splenocytes isolated from MAP27-immunized mice stimulated the production of IFN-γ and IL-17A/F. Our findings demonstrated that MAP27, as a thymus-dependent antigen, is efficient at eliciting T cell-mediated responses to protect mice from *S. aureus* infection. This study sheds light on a possible strategy to design vaccines against *S. aureus*.

Introduction

*Staphylococcus aureus* (*S. aureus*), one of the most common Gram-positive bacterium, causes a series of life-threatening diseases including sepsis, bacteremia and necrotizing pneumonia etc.
In recent years, the treatment has become more and more difficult for infections caused by methicillin-resistant *S. aureus* (MRSA), strains that are resistant to almost all the commonly used antibiotics [3]. This fact urges pharmaceutical companies to develop new and effective prophylactic vaccines and immunotherapies in the battle against the bacterium. However, attempts at making *S. aureus* vaccines have so far been unsuccessful [4, 5]. This failure has been attributed partly to the feature of the bacterium, which exhibits diverse arrays of virulence factors among individual clinical isolates, and can change its surface antigen repertoire potentially in different infection states [5]. Thus, it would be wise to select a conserved antigen that is expressed on bacterium persistently and steadily as a vaccine candidate. The second obstacle for development of *S. aureus* vaccine is that the relationship between the bacterium and the immune system remains ambiguous. *S. aureus* can evade the host immune system by inhibiting phagocytic uptake and its killing activity [6, 7]. Moreover, the effect of antibody-mediated opsonization is controversial [4, 5, 8]. Recently, more and more reports indicate that T cell-mediated immune responses, especially stimulating and secreting cytokines such as IL-17 or IFN-γ, might play an important role in prevention of *S. aureus* infection [9–17].

Peptidoglycan (PGN), accounting for about 50 percent weight of the bacterium wall, is made of polymeric meshwork of glycan strands crosslinked by short peptides [18]. In bacterium, PGN maintains the cell shape and osmotic pressure of cytoplasm. Therefore, it is a crucial and conservative component of *S. aureus*. However, as a thymus-independent antigen (TI antigen) with weak immunogenicity, PGN is not considered as a rational vaccine candidate. Mimetic peptides, acting as a thymus-dependent antigen (TD antigen) with relatively stronger immunogenicity, have been used as a promising surrogate for carbohydrate to generate vaccines. Immunization with a peptide, mimicking a carbohydrate antigen of tumor cells or polysaccharide in bacterium, could augment tumor-specific cellular responses or induce protective antibody responses to bacterium [19, 20]. Recently, we successfully obtained a series of PGN-mimicking peptides from phage peptide library by using an anti-PGN mAb as the target [21]. In this study, we synthesized a multiple antigenic peptide (MAP), named MAP27, which carried four copies of an identical peptide derived from the screening of the phage peptide library. Immunization with MAP27 decreased the bacterial burden in organs of the mice and significantly prolonged their survival time after *S. aureus* lethal-challenge. This protection is mainly correlated with the induction of IFN-γ- and IL-17A-producing T cells by MAP27 immunization.

## Materials and Methods

### Mice and Ethics Statement

BALB/c mice (4–6 weeks of age, female) were purchased from the Experimental Animal Center, Southern Medical University, Guangzhou, China. All of the animal experiments were approved by the Animal Ethical and Experimental Committee of the Southern Medical University (permit number: 2014–028). All mice were maintained under specific-pathogen-free conditions in a clean room at the Institute for Animal Experimentation in Southern Medical University. They were kept on a 12:12 h light-dark cycle with food and water provided *ad libitum*. Animal experiments were carried out in strict accordance with national guidelines for animal welfare. To alleviate pains, all mice were anesthetized with sodium pentobarbital (50 mg/kg) via abdominal cavity before *S. aureus* infection. According with the principle of animal ethics, the humane euthanasia was performed. During the bacterial lethal challenge, mice were observed every 2 hours for the first 48 hours, and the survival was monitored for at least 7 days. Mice that became moribund (such as hunched back, ruffled fur and lethargy), the survivors at
the endpoint of observation during \textit{S. aureus} lethal challenge, and the mice infected with \textit{S. aureus} for three days were sacrificed by CO\textsubscript{2} asphyxiation.

\textbf{Peptide Synthesis}

All peptides, including three linear peptides (SP27, SP27' and L2), a multiple antigenic peptide (named MAP27), and the attaching backbone of MAP (MAPctrl) were synthesized by Hybion Pharmaceutical (Shenzhen, China). Sequence accuracy of these peptides was confirmed by mass spectrometry and high performance liquid chromatography. Peptide purity was above 95% and the residual endotoxin was measured below 0.025 EU/ml. All of the peptides, provided as lyophilized powders, were dissolved in endotoxin-free water (Sigma) at 20mg/ml and stored at -70°C.

\textbf{The antigenicity of linear peptides and MAPs}

For examination of the antigenicity of linear peptides, enzyme linked immunosorbent assay (ELISA) was performed as described by Elshabrawy \textit{et al.} \cite{22, 23}. Briefly, 96-well plates were coated with 5 \(\mu\)g/ml monoclonal antibody (mAb) against PGN (AbD Serotec, MCA5792, clone No.11-232.3, isotype: mouse IgG3) or mAb against Lipoteichoic acid (LTA, Thermo scientific, MA1-7401, clone No. G35C, isotype: mouse IgG1) at 4°C for overnight. Biotin-labeled SP27, SP27' or L2 as a control peptide (sequence: Biotin-HSGHWDFRQWWQPSGG) was added to the wells, followed by detection with HRP-labeled streptavidin (Jackson ImmunoResearch, 1/10000 dilution). For the examination of MAP27, 5 \(\mu\)g/ml anti-PGN mAb or anti-\textit{S. aureus} polyclonal antibodies (stored at our laboratory, 1/800 dilution) were added to the wells coated with 80 \(\mu\)g/ml MAP27 or MAPctrl, and incubated at 37°C for 40 min. HRP-labeled goat anti-mouse and goat anti-rabbit IgG (Jackson ImmunoResearch, 1/10000 dilution) were then added (37°C for 40 min, respectively). Wells were washed three times with 0.1% PBS/Tween 20, followed by addition of the substrate solution containing TMB and H\textsubscript{2}O\textsubscript{2} for color reaction.

\textbf{Preparation of bacteria}

\textit{S. aureus} (ATCC 25923) were purchased from Wenzhou Kont Biology and Technology. The bacteria were grown in tryptic soy broth at 37°C with 250 rpm shaking for overnight. After centrifugation at 6000 rpm for 10 min, the pellet was resuspended and subsequently washed with sterile phosphate-buffer saline (PBS) for two times. The pellet was then diluted with PBS to an appropriate cell concentration as determined by spectrophotometry at 600 nm.

\textbf{Immunization and ELISA for specific antibodies}

The protocol of vaccination was based on our previous study with slight modifications \cite{21}. Briefly, female BALB/c mice were divided randomly into three groups. The first two groups were injected subcutaneously with 100 \(\mu\)g MAP27 and 100 \(\mu\)g MAPctrl for five times at 2-week intervals, respectively. The first immunization was performed in Freund’s complete adjuvant (Sigma) and the subsequent booster immunizations were administered in Freund’s incomplete adjuvant (Sigma). The third group of mice, without any peptide immunization, was bred at the same time and used as the blank control. After 14 days of the fifth MAP immunization, all of the three mice groups (MAP27-immunized, MAPctrl-immunized, and blank control mice) were finally immunized with heat-inactivated \textit{S. aureus} (2\times10\textsuperscript{7} CFU/mouse in sterile PBS without any adjuvant) via intraperitoneal injection. Serum samples were collected through retro-orbital bleeding 7 days after each immunization and frozen at -70°C for antibody evaluations.
Antibody titers and specificity were measured by ELISA as described previously [21]. Briefly, MAP27 (80 μg/ml), sonicated fragments of S. aureus (OD$_{600nm}$ = 0.5, 50 μl/well) and PGN (10 μg/ml) were coated in 96-well plates at 4°C for overnight, respectively. MAP27- and PGN-coated plates were blocked with 0.25% casein, whereas S. aureus fragments-coated plate were blocked with blocking buffer containing 1% guinea pig serum to avoid binding of staphylococcus protein A to IgG. The serial diluted antisera was subsequently added as the primary antibodies, followed by incubation with HRP-conjugated goat anti-mouse IgG as the secondary antibodies. The titer of antibody was defined as the highest dilution that gives more than twice the absorbance value of the blank control.

**Mice challenged with lethal live S. aureus**

Five days after the final boost, all of the mice were infected with S. aureus (ATCC 25923) at 5×10$^8$ CFU/mouse via tail vein injection. The mortality was monitored for 7 days (168 hours) post challenge.

**Measurement of bacterial burden in organs**

To determine the bacterial loads in organs, all of the three groups of mice were infected with S. aureus at 2×10$^7$ CFU/mouse via tail vein. Three days after infection, mice were sacrificed by CO$_2$ asphyxiation. The unilateral kidney and lung were aseptically removed and homogenized with 2 ml sterile PBS. The homogenates were then plated on agar media in a 10-fold serial dilution and incubated at 37°C for 18–24 h. The colony forming units (CFU) was determined.

**Levels of cytokines in organs post S. aureus systemic infection**

Mice were euthanized by CO$_2$ asphyxiation three days post infection. To measure the local inflammatory response, we centrifuged the homogenized lysates from spleen and lung. Supernatant were collected, added with protease inhibitors (Cell Signaling Technology) and analyzed by ELISA (Biolegend) according to the manufacturer’s instructions.

**Intracellular cytokine analysis**

Cytokines produced by CD$^+$ T cells were detected by flow cytometry based on a modified method as described by Chen et al. [24] and Bhattacharya et al. [25]. Briefly, the splenocytes were harvested three days post infection with S. aureus. 1–2×10$^6$ lymphocytes were incubated with cell stimulation cocktail (containing phorbol 12-myristate 13-acetate, PMA and ionomycin, eBioscience) and brefeldin A (BFA, eBioscience) for 6 h. Cells were then washed, stained with fluorescein-labeled antibodies (FITC labeled anti-CD3 mAb, Cat No. 11–0031, clone:145-2C11; APC labeled anti-CD4 mAb, Cat No.17-0042, clone:RM4-5; eBioscience). Following the fixation and permeabilization, PE-conjugated anti-mouse interferon gamma (IFN-γ) mAb (Cat No.12-7311, clone: XMG1.2, eBioscience) or interleukin 17A (IL-17A) mAb (Cat No. 12–7177, clone: eBio17B7, eBioscience) was used to measure the intracellular cytokine production in CD$^+$ T cells or CD$^+$ T cells, respectively. Data were acquired with a FACS Calibur (BD, Biosciences, San Jose, CA, USA) and analyzed using FCS Express software (De Novo Software, Los Angeles, CA USA). Isotype controls were included in each staining.

**In vitro stimulation of splenocytes**

Five days after the last heat-inactivated S. aureus boost, splenocytes from MAP27-immunized, MAPctrl-immunized and blank mice were obtained by squeezing followed by filtering through a stainless mesh (size, 70 μm) in RPMI1640 medium. Erythrocytes were lysed with RBC lysis
buffer (eBioscience). After being washed two times with RPMI1640, cells were resuspended with complete medium (RPMI1640 containing 10% fetal calf serum (Gibico), 100 U/ml of penicillin G and 100 μg/ml of streptomycin) in 96-well U-bottom culture plates. The splenocytes (2×10^5 cells/well) were incubated at 37°C with 100 μg/ml MAP27 for 24 h or 2×10^5 CFU/well heat-inactivated S. aureus for 72 h. The supernatants were collected after the incubation and stored at -70°C. The amount of interleukin 2 (IL-2), INF-γ, IL-17A/F and interleukin 4 (IL-4) was determined by ELISA (Biolegend) according to the manufacturer’s instructions. All samples were analyzed in triplicate.

**ELISPOT assays**

The IFN-γ and IL-17A spot ELISA assays were performed using a kit according to the manufacture’s instruction (eBioscience). Briefly, ELISPOT plates (Millipore) were coated with capture antibodies, and incubated at 4°C overnight. The plates were blocked with complete medium at room temperature for 1 h. Splenocytes were harvested and plated at 4×10^5/well, stimulated with 10 μg/ml MAP27 plus 1μg/ml anti-mouse CD28 antibody or 2×10^5 CFU/well heat-killed S. aureus at 37°C for 24 h. Cells and medium were decant from plates, and the plates were washed for three times with washing buffer. Biotinylated detection antibodies and HRP-conjugated avidin were added subsequently. The plates were extensively washed, and developed with substrate solution containing AEC (Sigma) and H2O2. The reaction was terminated with H2O. After drying, the plates were read using CTL analyzer (CTL S5 micro, Cellular Technology Ltd.).

**Statistical methods**

Results were expressed as means ± SEM. Statistical analyses of data to compare the different groups were performed using SPSS version 16.0 software. Data for specificity of antibody, cytokine production and ELISPOT were analyzed using either one-way ANOVA or Student’s t-test. Data for flow cytometry and bacterial load were analyzed by Mann-Whitney U of non-parametric Test. For comparison of survival in murine lethal challenge model, individual experiments were analyzed using log-rank test statistic (Mantel-Cox test) from the Prism software (Prism for Windows, version 5.01, GraphPad Software). Probability (P) values <0.05 were considered significant. 4 to 9 mice were used in each experiment and each experiment was repeated two or three times with consistent results.

**Results**

**Both linear peptides and tetra-branched, multiple antigenic peptide MAP27 bind to antibody specifically**

Both of the two linear peptides, SP27 (Biotin-SPHHHSRLRSESSAGG) and SP27’ (Biotin-SPHHHSRLRSESSAGG), contain the same core sequence SPHHHSRLRSE, flanked by 2–4 amino acids at N- and C- terminus (Fig 1A). The core sequence is derived from one of the positive phage clones we previously identified by using an anti-PGN mAb as the target [21]. As shown by ELISA, both SP27 and SP27’ can bind to anti-PGN in a dose-dependent manner. In contrast, a non-specific peptide L2 (Biotin-HSHGWDFRQWWQPSGG) did not bind to the anti-PGN mAb (Fig 1B). Moreover, neither SP27 nor SP27’ could bind to an unrelated anti-Lipoteichoic acid (LTA) mAb (Fig 1C). These results indicate that the core sequence (SPHHHSRLRSE), but not the flanking amino acids (SA, GG in SP27, or SAGG in SP27’) might mimic the epitope of PGN.
Compared with a linear peptide, a multiple antigenic peptide (MAP) has more advantages: it is more stable upon enzymatic degradation; it has bigger size that is sufficient for immunization without crosslinking with any carrier protein; moreover, MAP enhances molecular recognition by immune cells and induces stronger immune responses [26, 27]. Therefore, MAP has been used as an immunogen for developing experimental vaccines against various pathogens [28–32]. Based on this fact, we synthesized a MAP, named MAP27 using standard Fmoc chemistry. MAP27 contains four copies of SP27, with C-terminus of each peptide linked with the non-immunogenic, lysine-based dendritic scaffold (Fig 1D). The predicted molecular weight of MAP27 is 7137.7, big enough for immunization according to our previous work [21].

**Fig 1. Both SP27 and MAP27 bind to anti-PGN mAb specifically.** (A) The sequences of SP27 (Biotin-SASP HHHSRLRSE SGG) and SP27’ (Biotin-SASP HHHSRLRSE SAGG). Underlined letters represent nonspecific flanking amino acids. (B) Both SP27 and SP27’ bind to anti-PGN mAb in a dose-dependent manner. (C) Both SP27 and SP27’ (100 μg/ml) specifically bind to anti-PGN mAb, but not to anti-LTA mAb. For ELISA assays in panel B and C, anti-PGN mAb or anti-LTA mAb was used to coat the wells at a concentration of 5 μg/ml, SP27, SP27’ or non-specific peptide L2 (in panel B, sequence: Biotin-HSGHWDFRQWWQPSGG) was then added at indicated concentrations and incubated at 37°C for 40 min, followed by detection with HRP-labeled streptavidin. (D) The structure diagram of MAP27. MAP27 was synthesized in a tetra-branched form that contains four copies of a sequence (SASP HHHSRLRSE SGG) that mimics PGN epitope. (E) MAP27 binds to anti-PGN mAb specifically. (F) MAP27 binds to anti-S. aureus polyclonal antibodies specifically. For ELISA assays in panel E and F, MAP27 or MAPctrl was used to coat the wells of a microplate. Anti-PGN mAb or anti-S. aureus polyclonal antibodies was added, followed by detection with HRP-labeled antibodies. The absorbance was measured at OD450nm. The results are shown as means ±SEM. * P<0.05, ** P<0.01, *** P<0.001.

doi:10.1371/journal.pone.0136888.g001
Compared with the attaching backbone (MAPctrl), MAP27 specifically bound to anti-PGN mAb (Fig 1E) and anti-\textit{S. aureus} polyclonal antibodies (Fig 1F). These results indicate that a similar antibody-binding epitope as PGN was remained in MAP27.

**Last boost with heat-inactivated bacterium produced low titer antibody against \textit{S. aureus} in MAP27-immunized mice**

We then evaluated the immunogenicity of MAP27. Serum samples from mice immunized with MAP27 or MAPctrl were used for binding assays by indirect ELISA. The titer of antibodies against MAP27 in MAP27-immunized mice reached to $10^4$ after the third time of vaccination, whereas no detectable antibodies were produced in MAPctrl-immunized mice (Fig 2A). We also found that serum from MAP27-immunized mice weakly reacted with PGN after the fifth MAP immunization (Fig 2B, at the 1/200 dilution). In contrast, antibodies to \textit{S. aureus} were not detected in the same samples (data not shown). As shown in previous studies, boosting with natural antigens after immunization with peptide could significantly enhance the immune response [33, 34]. Therefore, we boosted all of the three groups of mice with heat-inactivated \textit{S. aureus} for only one time. Anti-\textit{S. aureus} IgG from MAP27-immunized mice could be detected with a dilution of 1/200. Conversely, the serum samples from either MAPctrl-immunized mice (P = 0.016) or blank control mice (P = 0.002) produced no detectable antibodies (Fig 2C). These results indicate that \textit{S. aureus} boosting specifically augmented the immune responses triggered by MAP27 immunization.

**Vaccination with MAP27 protected the mice against \textit{S. aureus} lethal-challenge and significantly reduced the bacterial burden in organs**

We then tested whether MAP27 immunization protects the mice against \textit{S. aureus} infection. All of the mice were challenged with live \textit{S. aureus} at a lethal dose on the fifth day post the last boost of heat-inactivated bacterium. The survival was then monitored. After seven days of lethal challenge, 50% of the MAP27-immunized mice were still alive, whereas, all of the control mice and MAPctrl-immunized mice died on day two (P = 0.000) and day five (P = 0.000), respectively (Fig 3A). This result indicates that MAP27 vaccination protects the mice against \textit{S. aureus} lethal challenge.

---

**Fig 2. BALB/c mice immunized with MAP27 produced anti-MAP27, anti-PGN and anti-\textit{S. aureus} antibodies.** (A) Titers of anti-MAP27 serum during the period of immunization. 96-well plates were coated with MAP27. Serum samples from mice immunized with MAP27, MAPctrl or blank were pooled and added in a 10-fold serial dilution, followed by incubation with HRP-conjunctive goat anti-mouse IgG. (B) Sera from MAP27-immunized mice bind to PGN after the fifth MAP immunization. (C) Sera from MAP27-immunized mice bind to \textit{S. aureus} after boosting with heat-killed \textit{S. aureus}. For ELISA assays in panel B and C, 96-well plates were coated with PGN or sonicated \textit{S. aureus} fragments. Serum samples were added in a 1/200 dilution as primary antibodies, followed by incubation with HRP-conjunctive goat anti-mouse IgG. The absorbance was measured at OD_{492nm}. The results are presented as means ±SEM. * P<0.05, ** P<0.01, n = 5–9 mice/group.

doi:10.1371/journal.pone.0136888.g002
To further determine the effect of MAP27 immunization on bacterial growth in animal organs, all of the mice were sacrificed three days after *S. aureus* infection. We found that the number of bacteria in the kidney of MAP27-immunized mice is significantly fewer than that of MAPctrl-immunized mice (P = 0.029) or blank control mice (P = 0.029) (Fig 3B). Similar results were also observed in lung (Fig 3C, MAP27 vs MAPctrl, P = 0.016; MAP27 vs blank control, P = 0.008). Taken together, these experiments indicate that vaccination with MAP27 reduced bacteria burden in animal organs.

IFN-γ, IL-17A/F and CCL3 were increased in organs of MAP27-immunized mice after *S. aureus* systemic infection

As we described above, although MAP27 immunization only induced the production of low titer antibodies against *S. aureus* or PGN, it protected the mice against *S. aureus* systemic infection. This fact led us to speculate that the protection effect is possibly through the in vivo T cell-mediated response. To test this hypothesis, we examined T cell-derived cytokines in spleen and lung from the mice three days post infection. As expected, the levels of IFN-γ and IL-17A/F, cytokines mainly secreted by T cells, were remarkably higher in MAP27-immunized mice than in MAPctrl-immunized mice and blank control mice (Fig 4). CCL3, a chemokine that activates macrophages and neutrophils [35, 36], as well as enhances IFN-γ production [37], was also increased in MAP27-immunized mice (Fig 4). These results indicate that T cell responses might mediate the protection of MAP27 immunization against the *S. aureus* systemic infection.

The IFN-γ⁺CD3⁺ and IL-17A⁺CD4⁺ T cells were increased in spleen of MAP27-immunized mice post *S. aureus* systemic infection

We further characterized cytokine expression profiles of infiltrated T cell population over the course of infection. To enhance the cytokine signals detected by flow cytometry, we conducted the PMA and inomycin treatment. As shown in Fig 5A and 5C, the percentage of IFN-γ⁺CD3⁺ CD4⁺ T cells from MAP27-immunized mice (about 7.18±0.96%) was significantly higher than that from MAPctrl-immunized mice (3.85±0.41%, P = 0.021) or blank control mice.
A Peptide Mimics Peptidoglycan As Vaccine Candidate for *S. aureus*

(Fig 4). Immunization with MAP27 promoted IFN-γ, IL-17A/F and CCL3 production in organs post infection with *S. aureus*. Five days after boosting with heat-inactivated *S. aureus*, all of the mice (n = 5 mice per group) were infected with *S. aureus* (2×10^7 CFU/mouse) for three days. Spleen and lung were aseptically taken and homogenized with sterile PBS. The supernatants were pooled and analyzed by ELISA. The concentrations of IFN-γ (panel A and D), IL-17A/F (panel B and E), and chemokine ligand 3 (CCL3, panel C and F) were measured in spleen and lung, respectively. * P<0.05, ** P<0.01, *** P<0.001.

doi:10.1371/journal.pone.0136888.g004

(3.57±0.73%, P = 0.03). Similar results were also observed for IFN-γ^+^CD3^+^CD4^+^ T cells (Fig 5B and 5C, MAP27 vs MAPctrl, P = 0.021; MAP27 vs blank control, P = 0.012). These results suggest that both CD4^+^ and CD4^−^ T cells were stimulated to produce IFN-γ in MAP27-immunized mice post *S. aureus* infection.

Th17 cells have been reported to play an important role in clearance of *S. aureus* [14, 38]. Here we also found the percentage of IL-17^+^CD3^−^CD4^−^ T cells from MAP27-immunized mice (about 2.4±0.38%) was higher than that from MAPctrl-immunized mice (about 1.34±0.13%, P = 0.021) and blank control mice (0.31±0.08%, P = 0.012) (Fig 5D and 5F). The percentage of IL-17^+^CD3^−^CD4^−^ T cells is not statistically different among the three groups (Fig 5E). Taken together, not only IFN-γ- but also IL-17-producing T cells of MAP27-immunized mice were stimulated effectively upon *S. aureus* infection.

**In vitro** MAP27 treatment stimulated the splenocytes from MAP27-immunized mice to produce IFN-γ

To further assess whether immunization with MAP27 was able to induce IFN-γ and IL-17 in *vitro*, we harvested splenocytes after five days of the last boost with heat-inactivated bacteria. Cells were incubated with 100 μg/ml MAP27 for 24 h. The supernatant was then collected and cytokine induction was analyzed by ELISA. The splenocytes from MAP27-immunized mice produced significantly more IL-2 and IFN-γ than cells from control mice upon stimulation by MAP27 (Fig 6A and 6B). In contrast, IL-4, which is mainly produced by Th2 cells, could only
be detected very weakly in all three groups (Fig 6C). This can be explained by the fact that high level of IFN-γ in MAP27-immunized mice might suppress Th2 cell differentiation [39]. Unexpectedly, we failed to detect IL-17A/F in supernatant of spleen cells from all the mice (data not shown).

We then used ELISPOT, a more sensitive assay than ELISA, to further determine whether the T cell-mediated response was induced by MAP27. As shown in Fig 6D and 6E, the number of IFN-γ-producing cells from MAP27-vaccinated mice was significantly higher than that from MAPctrl-immunized mice (P = 0.001) or blank control mice (P = 0.001). Again, we could not identify IL-17A-producing cells by ELISPOT assay. In light of this, we speculated that immunization with MAP27 might predominantly induce Th1 cell response.
Splenocytes from MAP27-immunized mice specifically recognized *S. aureus* and produced IFN-γ and IL-17

Our next question was whether *S. aureus* as a natural antigen could be recognized by splenocytes from MAP27-immunized mice. Splenocytes isolated from three groups of mice were incubated with heat-inactivated *S. aureus* for 72 h, and cytokines in the culture supernatant were analyzed by ELISA. Two classical cytokines produced by T cells, IL-2 and IFN-γ, were significantly produced in splenocytes from MAP27-immunized mice, but not MAPctrl-immunized mice or blank control mice (Fig 7A and 7B). Interestingly, IL-17A/F, a cytokine mainly secreted by Th17 cells, is also significantly induced in splenocytes from MAP27-immunized mice (Fig 7C). Similar to the *in vitro* assays with MAP27 stimulation (Fig 6C), IL-4 was not detectable in any group (Fig 7D). We next performed ELISPOT assay to quantitate IFN-γ- or IL-17A-producing cells after *S. aureus* stimulation. As shown in Fig 7E–7H, the number of IFN-γ- or IL-17A-producing cells from MAP27-immunized mice was significantly higher than that from the control groups (IFN-γ-producing cells: MAP27 vs MAPctrl, *P* = 0.0126; MAP27 vs blank control, *P* = 0.0041; IL-17-producing cells: MAP27 vs MAPctrl, *P* = 0.0034; MAP27 vs blank control, *P* = 0.0039). Collectively, these results indicate that T cells, especially IFN-γ- and IL-17-producing T cells in MAP27-immunized mice, recognized effectively the nature antigen *S. aureus in vitro*. 

---

**Fig 6. In vitro MAP27 treatment promoted the production of IFN-γ in splenocytes from MAP27-immunized mice.** Five days after the last boost immunization, splenocytes isolated from the mice (n = 5 mice per group) were cultured at 2×10⁵/well in U-bottom 96-well plates and stimulated with MAP27 (100 μg/ml) for 24 h. The concentrations of IL-2 (A), IFN-γ (B), and IL-4 (C) in supernatant were measured by ELISA. (D) Frequency of IFN-γ-producing cells induced by MAP27. Splenocytes isolated from the mice (n = 5 mice per group) were cultured at 4×10⁵/well in pre-coated plates, and stimulated with MAP27 (10 μg/ml) plus anti-mouse CD28 antibody (1 μg/ml) for 24 h. The number of IFN-γ-producing cells was then measured by ELISPOT and shown in box and whisker plots. (E) Representation of one of the ELISPOT assays. **P<0.01; ***P<0.001; ns: P>0.05. doi:10.1371/journal.pone.0136888.g006
Fig 7. Heat-inactivated S. aureus promoted Th1 and Th17 cell responses in the spleen of MAP27-immunized mice. Five days after the last booster with heat-inactivated bacteria, the splenocytes isolated from the mice (n = 5 mice per group) were cultured (2×10^5/well), and stimulated with 2×10^4 CFU/well heated-inactivated S. aureus for 72 h. The concentrations of IL-2 (A), IFN-γ (B), IL-17A/F (C) and IL-4 (D) were measured by ELISA. The isolated splenocytes were also cultured at 4×10^5/well in pre-coated plates and stimulated with heat-inactivated S. aureus for 24 h. Frequency of IFN-γ-producing (E) and IL-17A-producing cells (F) was determined by ELISPOT. Cell numbers were shown in box and Whisker plots. (G) Representation of one of the ELISPOT assays for IFN-γ-producing cells. (H) Representation of one of the ELISPOT assays for IL-17A-producing cells. * P<0.05; ** P<0.01; ns: P>0.05.

Discussion

To date, the failure to develop an effective vaccine against S. aureus is partly due to the high complexity of the numerous virulence factors that are expressed at different infection stages. Considering this, Lin and colleagues suggested that immunogens might not be restricted to microbial virulence factors, but any target antigen that effectively induces T cell immune responses could be selected [16]. Here, we used PGN, a conserved cell wall component of S. aureus as the target, and designed a de novo synthetic multiple antigenic peptide (MAP27) for mimicking a PGN epitope. The immunization outcome could be affected by various route of immunization. For example, vaccination of antigens via eyes [40, 41] or oral route [42, 43] has been shown to induce specific tolerance in BALB/c mice. In this study, we found that subcutaneous immunization, with peptides emulsified with Freund’s adjuvant, effectively induced immune response and protected the mice against S. aureus systemic infection. It would be very interesting to test whether other routes of immunization, such as via mucosal immunization, could further enhance the protective effect.

As shown in Fig 2B, immunization with MAP27 alone could only weakly induce IgG to bind to PGN. This can be explained by the fact that the peptide only mimics a single epitope of PGN, and possibly the peptide mimicking is not completely identical to the natural antigen. Moreover, as an artificial antigen, MAP27 probably has weaker immunogenicity compared to most of the protein immunogens. Since previous findings have showed that boosting with natural antigen after peptide immunization could induce a robust recall response [33, 34], we modified our immunization strategy by boosting the mice with heat-inactivated S. aureus after MAP immunization. Our results demonstrated that boosting with natural antigen improved the binding of the sera to the bacteria (Fig 2C). In contrast, the same boosting step had no effect on the control groups (Figs 2C and 3A). This result is consistent with the previous finding that vaccination with heat-killed S. aureus before infection does not affect bacterium outcome [44].

Although the titer of sera against PGN or S. aureus is low, MAP27-immunization could still protect the mice from S. aureus systemic infection (Fig 3). Previous studies have demonstrated that IL-17 and Th17 are critical for protection from S. aureus infection [9, 11, 14]. Moreover, IFN-γ is universally considered to improve the survival of staphylococcemia mice by activating macrophages and neutrophils to kill bacteria [45]. M1-polarized macrophages stimulated with IFN-γ could also inhibit the formation of S. aureus biofilm [46]. Based on these facts, we hypothesized that the protection is possibly through the activation of T cell-mediated responses. Several evidences to support this hypothesis were presented in this study: 1) Levels of IFN-γ and IL-17A/F were significantly increased in the early stage of bloodstream infection (Fig 4); 2) Both IFN-γ+CD3+CD4+ Th1 cells and IL-17+CD3+CD4+ Th17 cells significantly increased in MAP27-immunized mice after infection (Fig 5); 3) Our in vitro assay showed that splenocytes from MAP27-immunized mice specifically recognized S. aureus and produced IFN-γ and IL-17 (Fig 7). Interestingly, we found that IFN-γ+CD3+CD4+ T cells also increased in MAP27-immunized mice post infection (Fig 5B and 5C). We speculated that MAP27 immunization might induce different subsets of T cells to secrete IFN-γ post infection. For example, CD8+ T and γδ T cells have been reported to secrete IFN-γ and protect mice from S. aureus systemic infection [17, 47, 48].
Different from heat-inactivated *S. aureus* stimulation (Fig 7), MAP27 failed to induce IL-17A/F in splenocytes (data not shown), even though it induced high level of IFN-γ *in vitro* (Fig 6B). Again, this result can be explained by the fact of the inhibitory role of IFN-γ in Th17 differentiation [49]. We speculated that the artificial MAP antigen and the natural antigen (*S. aureus*) might trigger different responses. However, the exact mechanism is still unclear and needs to be elucidated. A previous study suggested that MAP can be internalized by antigen presenting cells (APCs) and presented as an epitope on cells [50]. It would be very interesting to investigate how APCs recognize this peptide to elicit T cell responses.

In summary, our results indicate that immunization with PGN mimicking peptide, MAP27, could protect mice against *S. aureus* systemic infection. This protection might be related with the stimulation of IFN-γ- and IL-17-producing T cells that specifically recognized *S. aureus*.

**Supporting Information**

S1 Fig. MAP27 immunization decreases the bacterial burden in organs. Mice were immunized with MAP27 or MAPctrl for five times at a two-week interval without bacterial boost (A, C), or with bacterial boost (B, D). All the mice were infected with *S. aureus* via tail vein post five days of the last immunization. The bacterial numbers in organs were measured after three days of infection. Bacterial burden in kidney (A, B) and in lung (C, D) were measured. (* P<0.05; ** P<0.01) (n = 4-11/group). (TIF)

**Acknowledgments**

We thank Da-Ming Zuo for his kind advice on design and technical assistance. We also thank Li-Chang Liu for handling of experimental animals.

**Author Contributions**

Conceived and designed the experiments: BYL NF. Performed the experiments: XYW ZXH YGC XL KW. Analyzed the data: XYW ZXH. Contributed reagents/materials/analysis tools: PZ. Wrote the paper: BYL NF.

**References**

1. Foster TJ. Colonization and infection of the human host by staphylococci: adhesion, survival and immune evasion. Vet Dermatol. 2009; 20(5–6): 456–470. doi: 10.1111/j.1365-3164.2009.00825.x PMID: 20178484
2. Lowy FD. Staphylococcus aureus infections. N Engl J Med. 1998; 339(8): 520–532. PMID: 9709046
3. DeLeo FR, Otto M, Kreiswirth BN, Chambers HF. Community-associated meticillin-resistant Staphylococcus aureus. Lancet. 2010; 375(9725): 1557–1568. doi: 10.1016/S0140-6736(09)61999-1 PMID: 20206987
4. Fowler VJ, Proctor RA. Where does a Staphylococcus aureus vaccine stand? Clin Microbiol Infect. 2014; 20 Suppl 5: 66–75. doi: 10.1111/1469-0691.12570 PMID: 24476315
5. Broughan J, Anderson R, Anderson AS. Strategies for and advances in the development of Staphylococcus aureus prophylactic vaccines. Expert Rev Vaccines. 2011; 10(5): 695–708. doi: 10.1586/erv.11.54 PMID: 21604989
6. Foster TJ, Geoghegan JA, Ganesh VK, Hook M. Adhesion, invasion and evasion: the many functions of the surface proteins of Staphylococcus aureus. Nat Rev Microbiol. 2014; 12(1): 49–62. doi: 10.1038/nrmicro3161 PMID: 24336184
7. Lowy FD. How Staphylococcus aureus adapts to its host. N Engl J Med. 2011; 364(21): 1987–1990. doi: 10.1056/NEJMmp1100251 PMID: 21612466
8. Broker BM, Holtfretter S, Bekeredian-Ding I. Immune control of Staphylococcus aureus—regulation and counter-regulation of the adaptive immune response. Int J Med Microbiol. 2014; 304(2): 204–214. doi: 10.1016/j.ijmm.2013.11.008 PMID: 24462009

9. Montgomery CP, Daniels M, Zhao F, Alegre ML, Chong AS, Daum RS. Protective immunity against recurrent Staphylococcus aureus skin infection requires antibody and interleukin-17A. Infect Immun. 2014; 82(5): 2125–2134. doi: 10.1128/IAI.01491-14 PMID: 24614564

10. Zuo QF, Yang LY, Feng Q, Lu DS, Dong YD, Cai CZ, et al. Evaluation of the protective immunity of a novel subunit fusion vaccine in a murine model of systemic MRSA infection. PLoS One. 2013; 8(12): e81212. doi: 10.1371/journal.pone.0081212 PMID: 24324681

11. Archer NK, Harro JM, Shirtliff ME. Clearance of Staphylococcus aureus nasal carriage is T cell dependent and mediated through interleukin-17A expression and neutrophil recruitment. Infect Immun. 2013; 81(6): 2070–2075. doi: 10.1128/IAI.00084-13 PMID: 23529621

12. Joshi A, Pancari G, Cope L, Bowman EP, Cua D, Proctor RA, et al. Immunization with Staphylococcus aureus iron regulated surface determinant B (IsdB) confers protection via Th17/IL17 pathway in a murine sepsis model. Hum Vaccin Immunother. 2012; 8(3): 336–346. doi: 10.4161/hv.18946 PMID: 22327491

13. Holley MM, Kielian T. Th1 and Th17 cells regulate innate immune responses and bacterial clearance during central nervous system infection. J Immunol. 2012; 188(3): 1360–1370. doi: 10.4049/jimmunol.1101660 PMID: 22190181

14. Cho JS, Pietras EM, Garcia NC, Ramos RI, Farzam DM, Monroe HR, et al. IL-17 is essential for host defense against cutaneous Staphylococcus aureus infection in mice. J Clin Invest. 2010; 120(5): 1762–1773. doi: 10.1172/JCI40891 PMID: 20364087

15. Narita K, Hu DL, Mori F, Wakabayashi K, Iwakura Y, Nakane A. Role of interleukin-17A in cell-mediated protection against Staphylococcus aureus infection in mice immunized with the fibrinogen-binding domain of clumping factor A. Infect Immun. 2010; 78(10): 4574–4580. doi: 10.1128/IAI.00700-08 PMID: 18644876

16. Lin L, Ibrahim AS, Xu X, Farber JM, Avanesian V, Baquir B, et al. Th1/Th17 cells mediate protective adaptive immunity against Staphylococcus aureus and Candida albicans infection in mice. PLoS Pathog. 2009; 5(12): e1000703. doi: 10.1371/journal.ppat.1000703 PMID: 20041174

17. Spellberg B, Ibrahim AS, Yeaman MR, Lin L, Fu Y, Avanesian V, et al. The antifungal vaccine derived from the recombinant N terminus of Als3p protects mice against the bacterium Staphylococcus aureus. Infect Immun. 2008; 76(10): 4574–4580. doi: 10.1128/IAI.00700-08 PMID: 18644876

18. Vollmer W, Seligman SJ. Architecture of peptidoglycan: more data and more models. Trends Microbiol. 2010; 18(2): 59–66. doi: 10.1016/j.trendsmicrobio.2009.12.004 PMID: 20060721

19. Monzavi-Karbassi B, Cunto-Amesty G, Luo P, Shamloo S, Blassczyk-Thurin M, Kieber-Emmons T. Immunization with a carbohydrate mimicking peptide augments tumor-specific cellular responses. Int Immunol. 2001; 13(1): 1361–1371. PMID: 11675368

20. Fleuridor R, Lees A, Pirofski L. A cryptococcal capsular polysaccharide mimotope prolongs the survival of mice with Cryptococcus neoformans infection. J Immunol. 2001; 166(2): 1087–1096. PMID: 11145609

21. Chen Y, Liu B, Yang D, Li X, Wen L, Zhu P, et al. Peptide mimics of peptidoglycan are vaccine candidates and protect mice from infection with Staphylococcus aureus. J Med Microbiol. 2011; 60(Pt 7): e81212. doi: 10.1099/jmm.0.028647-0 PMID: 21436375

22. Elshabrawy HA, Fan J, Haddad CS, Ratia K, Broder CC, Caffrey M, et al. Identification of a broad-spectrum antiviral small molecule against severe acute respiratory syndrome coronavirus and Ebola, Hendra, and Nipah viruses by using a novel high-throughput screening assay. J Virol. 2014; 88(8): 4353–4365. doi: 10.1128/JVI.00509-13 PMID: 24501399

23. Elshabrawy HA, Coughlin MM, Baker SC, Prabhakar BS. Human monoclonal antibodies against highly conserved HR1 and HR2 domains of the SARS-CoV spike protein are more broadly neutralizing. PLoS One. 2012; 7(11): e50366. doi: 10.1371/journal.pone.0050366 PMID: 23185609

24. Chen Z, Kim SJ, Chamberlain ND, Pickens SR, Volin MV, Volokh S, et al. The novel role of IL-7 ligation to IL-7 receptor in myeloid cells of rheumatoid arthritis and collagen-induced arthritis. J Immunol. 2013; 190(10): 5266–5266. doi: 10.4049/jimmunol.1201675 PMID: 23606539

25. Bhattacharya P, Fan J, Haddad C, Essani A, Gopisetty A, Elshabrawy HA, et al. A novel pancreatic beta-cell targeting bispecific-antibody (BsAb) can prevent the development of type 1 diabetes in NOD mice. Clin Immunol. 2014; 153(1): 187–198. doi: 10.1016/j.clim.2014.04.014 PMID: 24792135

26. Fujita Y, Taguchi H. Current status of multiple antigen-presenting peptide vaccine systems: Application of organic and inorganic nanoparticles. Chem Cent J. 2011; 5(1): 48. doi: 10.1186/1752-153X-5-48 PMID: 21861904
27. Tam JP. Synthetic peptide vaccine design: synthesis and properties of a high-density multiple antigenic peptide system. Proc Natl Acad Sci U S A. 1988; 85(15): 5409–5413. PMID: 3399498

28. Ma JH, Yang FR, Yu H, Zhou YJ, Li GX, Huang M, et al. An M2e-based synthetic peptide vaccine for influenza A virus confers heterosubtypic protection from lethal virus challenge. Virol J. 2013; 10: 227. doi: 10.1186/1743-422X-10-227 PMID: 23834899

29. Wang Y, Wang M, Wang G, Pang A, Fu B, Yin H, et al. Increased survival time in mice vaccinated with a branched lysine multiple antigenic peptide containing B- and T-cell epitopes from T. gondii antigens. Vaccine. 2011; 29(47): 8619–8623. doi: 10.1016/j.vaccine.2011.09.016 PMID: 21939715

30. Mahajan B, Berzofsky JA, Boykins RA, Majam V, Zheng H, Chattopadhyay R, et al. Multiple antigen peptide vaccines against Plasmodium falciparum malaria. Infect Immun. 2010; 78(11): 4613–4624. doi: 10.1128/IAI.00533-10 PMID: 20822310

31. Oscherwitz J, Yu F, Cease KB. A synthetic peptide vaccine directed against the 2ss2-2ss3 loop of Yersinia pestis coupled to palmitate in mice. Vaccine. 2011; 29(50): 9352–9360. doi: 10.1016/j.vaccine.2011.09.129 PMID: 2201881

32. Amexis G, Young NS. Multiple antigenic peptides as vaccine platform for the induction of humoral responses against dengue-2 virus. Viral Immunol. 2007; 20(4): 657–663. PMID: 18158738

33. Uppada SB, Bhat AA, Sah A, Donthamshetty RN. Enhanced humoral and mucosal immune responses after intranasal immunization with chimeric multiple antigen peptide of LcrV antigen epitopes of Yersinia pestis coupled to palmitate in mice. Vaccine. 2011; 29(50): 9352–9360. doi: 10.1016/j.vaccine.2011.09.129 PMID: 2201881

34. Bolesta E, Kowalczyk A, Wierzbiacki A, Rotkiewicz P, Bambach B, Tsao CY, et al. DNA vaccine expressing the mimotope of GD2 ganglioside induces protective GD2 cross-reactive antibody responses. Cancer Res. 2005; 65(8): 3410–3418. PMID: 15833876

35. Sato E, Simpson KL, Grisham MB, Koyama S, Robbins RA. Inhibition of MIP-1alpha-induced human neutrophil and monocyte chemotactic activity by reactive oxygen and nitrogen metabolites. J Lab Clin Med. 2000; 135(2): 161–169. PMID: 10695661

36. Wolpe SD, Cerami A. Macrophage inflammatory proteins 1 and 2: members of a novel superfamily of cytokines. FASEB J. 1989; 3(14): 2565–2573. PMID: 2687068

37. Karpus WJ, Lukacs NW, Kennedy KJ, Smith WS, Hurst SD, Barrett TA. Differential CC chemokine-induced enhancement of T helper cell cytokine production. J Immunol. 1997; 158(9): 4129–4136. PMID: 9126972

38. Ishigame H, Kakuta S, Nagai T, Kadoki M, Nambu A, Komiyama Y, et al. Differential roles of interleukin-17A and -17F in host defense against mucoprophthalial bacterial infection and allergic responses. Immunity. 2009; 30(1): 108–119. doi: 10.1016/j.immuni.2008.11.009 PMID: 19144317

39. Fitch FW, McKisic MD, Lanci DW, Gajewski TF. Differential regulation of murine T lymphocyte subsets. Annu Rev Immunol. 1993; 11: 29–48. PMID: 8475653

40. Farooq SM, Ashour HM. Type II collagen induces peripheral tolerance in BALB/c mice via the generation of CD8+ T regulatory cells. PLoS One. 2012; 7(11): e48635. doi: 10.1371/journal.pone.0048635 PMID: 23133648

41. Farooq SM, Ashour HM. Eye-mediated induction of specific immune tolerance to encephalitogenic antigens. CNS Neurosci Ther. 2013; 19(7): 503–510. doi: 10.1111/cns.12087 PMID: 23522052

42. Ishida W, Futkuda K, Harada Y, Sunti T, Taguchi O, Tsuda M, et al. Oral administration of Ag sup- presses Ag-induced allergic conjunctivitis in mice: critical timing and dose of Ag. Br J Ophthalmo. 2013; 97(4): 492–497. doi: 10.1136/bjophthalmol-2012-302639 PMID: 23343653

43. Miron N, Feldrihan V, Berindan-Neagoe I, Cristea V. The role of Staphylococcus enterotoxin A in achieving oral tolerance to myelin basic protein in adult mice. Immunol Inve. 2014; 43(3): 267–277. doi: 10.3109/08820139.2013.868474 PMID: 24354897

44. Schmaler M, Jann NJ, Ferracin F, Landmann R. T and B cells are not required for clearing Staphylococ- cus aureus in systemic infection despite a strong TLR2-MyD88-dependent T cell activation. J Immunol. 2011; 186(1): 443–452. doi: 10.4049/jimmunol.1001407 PMID: 21131426

45. Zhao YX, Nilsson IM, Tarkowski A. The dual role of interferon-gamma in experimental Staphylococcus aureus septicemia versus arthritis. Immunology. 1998; 93(1): 80–85. PMID: 9536122

46. Hanke ML, Heim CE, Angle A, Sanderson SD, Kielian T. Targeting macrophage activation for the preven- tion and treatment of Staphylococccus aureus biofilm infections. J Immunol. 2013; 190(5): 2159–2168. doi: 10.4049/jimmunol.1202348 PMID: 23365077

47. Murphy AG, O’Keeffe KM, Lalor SJ, Maher BM, Mills KH, McLoughlin RM. Staphylococcus aureus infection of mice expands a population of memory gammadelta T cells that are protective against sub- sequent infection. J Immunol. 2014; 192(8): 3697–3708. doi: 10.4049/jimmunol.1303420 PMID: 24623128
48. Cheng P, Liu T, Zhou WY, Zhuang Y, Peng LS, Zhang JY, et al. Role of gamma-delta T cells in host response against Staphylococcus aureus-induced pneumonia. BMC Immunol. 2012; 13: 38. doi:10.1186/1471-2172-13-38 PMID: 22776294

49. Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM, et al. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. Nat Immunol. 2005; 6(11): 1123–1132. PMID: 16200070

50. Ota S, Ono T, Morita A, Uenaka A, Harada M, Nakayama E. Cellular processing of a multibranched lysine core with tumor antigen peptides and presentation of peptide epitopes recognized by cytotoxic T lymphocytes on antigen-presenting cells. Cancer Res. 2002; 62(5): 1471–1476. PMID: 11888922