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Immunogenicity of the Non-toxic Form of Staphylococcus *Aureus* α-Hemolysin Using a Chimeric Fusion in Mice

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ABSTRACT

Background: *Staphylococcus aureus* is an opportunistic pathogen responsible for various infections with diverse clinical presentation and severity. The α-hemolysin is a major virulence factor in the pathogenesis of *S. aureus* infections.

Objective: To produce a chimeric fusion protein for hemolytic detection of the *S. aureus* isolates and as a component of a multi-antigen vaccine.

Methods: The fused strategy employed a flexible linker to incorporate the possible B cell and T cell determinants into one chimera (HlaD). The humoral and cellular response to the HlaD in mice was assessed to reveal a non-significant difference compared with the full-length α-hemolysin mutant (Hla_{H35L}).

Results: The results of the protective effect, the mimetic lung cell injury, and bacterial clearness demonstrated that the mice vaccinated with the HlaD alleviated the severity of the infection of the *S. aureus*, and the HlaD could similarly function with Hla_{H35L}.

Conclusion: The chimeric fusion (HlaD) provided a diagnostic antigen for hemolysis of the *S. aureus* strains and a potential vaccine component.

Keywords: α-hemolysin, Fusion, Immunogenicity, *Staphylococcus aureus*

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INTRODUCTION

Staphylococcus aureus (*S. aureus*) is a potentially morbid and mortal pathogen. It can cause a variety of disease states ranging from minor skin infections to systemic diseases such as septicemia, necrotizing pneumonia, toxic shock syndrome, meningitis, and septic arthritis [1]. An array of virulence factors secreted by the *S. aureus* is associated with various disease states, of which the example is α-hemolysin, acting as an essential factor in the pathogenesis of pneumonia [2]. Methicillin-resistant *S. aureus* strain USA300 with high α-hemolysin production plays a crucial role in the occurrence of clinically relevant diseases. This study has found that USA300 is a key pathogen causing sepsis and necrotizing pneumonia, and the production of α-hemolysin is closely related

to the virulence of USA300 [3]. Though antibiotics are critical to the therapy of disease, expensive cost and high occurrence of antibiotic- resistant strains such as USA300 are challenging in the treatment of the *S. aureus* infection. It has been currently demonstrated that the preexisting antibodies against the *S. aureus* α-hemolysin in the serum of human individuals play a critical role in protecting against the *S. aureus* pneumonia [4]. Therefore, according to the notion that vaccination could ameliorate or prevent disease, developing an effective *S. aureus* vaccine may provide a useful alternative approach to combating illness.

The α-hemolysin is a secreted protein containing 293AA residues, which can disrupt the innate and adaptive immune responses in animals [5]. One, it could induce pro-inflammatory mediators [6] and promote a breach of the epithelial barrier at least in part by binding and activating ADAM10 at low concentrations [7]. Second, the α-hemolysin can form functional transmembrane pores at high concentrations and has cytolytic activity toward susceptible host cells such as epithelial cells [8]. The importance of α-hemolysin has been demonstrated in the mouse models of the *S. aureus* pneumonia [9]. Active or passive immunization studies in mouse or rabbit model have shown that the neutralization of antibody against α-hemolysin lead to protection against skin infection, pneumonia, and endocarditis [8, 10, 11]. Huseby et al. showed that the β-hemolysin facilitated infective endocarditis progression [12]. Numerous other factors of the *S. aureus* such as superantigens or cytolysins may also have contributed to the reduced ability to cause pneumonia or endocarditis. A multi-target vaccine may result in superior efficacy in the development of a vaccine against the *S. aureus* illness. An alternative approach to developing a multi-target vaccine is to incorporate an immunodominant fragment of positive antigens into a single protein [13].

In this study, an attempt was made to conduct a non-toxic fusion encompassing two truncated regions of α-hemolysin. The immunogenicity of the fused protein was evaluated in the mice by examining humoral and cellular immunity. The fused protein will be potentially useful in constructing a multi-target vaccine or detecting wild-type toxins.

MATERIALS AND METHODS

Bacterial Strain, Growth Conditions, and Gene Cloning

Escherichia coli DH5α (Invitrogen, Carlsbad, CA) and BL21 (DE3) (Novengen Inc. Madison, WI, USA) were used as hosts for the expression of recombinant plasmids. *Escherichia coli* strains transformed with plasmids were cultured in Luria-Bertani broth (Difco, Becton-Dickinson. Sparks, MD. USA) at 37 ℃, in the presence of antibiotic (100 μg of ampicillin or 50 μg of kanamycin per ml) when necessary. The *S. aureus* strain Newman, a capsular type 5 strain, was obtained from the Eijkman Winkler Laboratory of University Medical Center Utrecht, Netherlands. Newman was aerobically cultured in tryptic soy broth (TSB) or agar (Difco, Becton-Dickinson. Sparks, MD. USA) at 37 ℃.

For gene cloning, the primers (Table 1) used in this study were custom synthesized from Sangon Biotech (Shanghai, China), and the gene sequence of α-hemolysin was retrieved from the NCBI database. The DNA of the *S. aureus* Newman was isolated following Marmur [14]. The inserted gene in the yielded plasmids, pET28a-hla harboring the full α-hemolysin (Hla), pET28a-Hla_{H35L} harboring the mutant α-hemolysin (Hla_{H35L}), and pET28a-HlaD harboring the fused α-hemolysin (HlaD), were similarly inserted into expression vector pET28a after digested with *Nco* I and *Xho* I.

Construction of Fusion

First, the Hla was amplified by PCR from Newman chromosomal DNA using the F1/R2 pairs.

Table 1. Primers used in this study.

Name	Sequence (5′→3′) [a]	Restriction site
F1	CATGCCATGGCAGATTCTGATATTAATATT	*Nco* I
R1	AACTATAAAATACTTTTTTGAGCATGCCATTTTCTTTAT	
F2	CAAAAAAGTATTTTATAGTT	
R2	CCGCTCGAGATTTGTCATTTCTTCTT	*Xho* I
LF	CCGCCATGGATTAATATTAAAACCGGTAC	*Nco* I
R3	GGTGGCGGTGGAAGCGGCGGTGGCGGAAGCGGCGGTGGCGGCA GCACTTTTGTTAGCACCTT	
F4	GCTGCCGCCACCGCCGCTTCCGCCACCGCCGCTTCCACCGCCACCA GAGCCCAACTGATAAAA	
LR	CATGCTCGAGAGAACTTGCTTTGTTAGGAT	*Xho* I

[a]The restriction sites are underlined. Mutation from H_{35} to L_{35} in α-hemolysin was introduced and the corresponding nucleotides in primer were shown with a gray background. A 45 bp linker sequence of $(G_4S)_3$ flexible bridge (shown by boxing) was added to the overlapping primer for HlaD

The purified PCR products were cloned into pET28a, yielding pET28a-Hla. For the construction of fusion, the hlaH35L was amplified by the overlap extension PCR from the positive pET28a-Hla using the F1/R1 or F2/R2 pairs, respectively. The following PCR was carried out using the above PCR mixed products as PCR templates with F1 and R2. The purified PCR products were then inserted into pET28a, yielding pET28a-Hla_{H35L}. Before the HlaD was cloned, the cell epitope of the *S. aureus* α-hemolysin was predicted using Protean (DNAStar, Madison, WI, USA) and two online software programs (http://www.imtech.res.in/raghava/bcepred/ and http://www.iedb.org/). According to a comparative analysis, the prediction results of the Hla, the structure characteristics of the Hla, and the construction method of fusion protein reported in the literature as a reference [13-16], two truncated fragments forming the HlaD were designed. One fragment of amino acid residues from I_1 to S_{72} of α-hemolysin contained a major B cell epitope. Another fragment of amino acid residues from E_{153} to S_{213} of α-hemolysin contained a major T cell epitope. So the HlaD was amplified through a flexible glycine linker by overlap extension PCR from the positive pET28a-Hla_{H35L}. It was mentioned that the 45 bp linker sequence of $(G_4S)_3$ flexible bridge (shown by boxing in Table 1) was added to the overlapping primer for the HlaD. It should be also noted that the glycine linker was flexible and would not affect the spatial structure of the two proteins in the fusion protein. Similarly, the PCR was carried out with LF/R3 or F4/LR pairs, respectively. The following PCR was carried out using the above PCR mixed products as templates with LF and LR. The purified PCR products were then inserted into pET28a, yielding pET28a-HlaD. The yielded plasmids were verified by DNA sequencing, and the service was provided by the Sangon Biotech (Shanghai, China).

Protein Production, Purification, and Western Blot

In the experiment, we designed the chimeric fusion HlaD (I_1-S_{72}-linker-E_{153}-S_{213}) to be convenient for multi-antigen vaccine generation or pathogen confirmation. In the present study, the target genes were cloned into the pET28a vector respectively and overexpressed in *E. coli* BL21 (DE3). The three plasmids, pET28a-Hla, pET28a-Hla_{H35L}, and pET28a-HlaD, were extracted and then transformed into *E. coli* BL21 (DE3). Recombinant *E. coli* BL21 was enriched in LB medium containing 50 μg/mL kanamycin. The temperature was 37 °C and the shaker operated at 170 rpm. When the OD600 of the bacterial culture reached 0.4-0.6, IPTG with a final concentration of 1 mmol/L was added,

and the induction was carried out for 4 hrs. The harvested bacterial cells were broken by ultrasonication. The ultrasonic conditions are as follows: 40% power ultrasonic crushing, the total ultrasonic time is 2 min, 30 each time, a total of 4 times, and the sample is cooled in an ice bath between cycles.

The purification of recombinant proteins was carried out under denaturation conditions by Ni-NAT affinity. His-tagged proteins were fractionated with Ni-NTA His·Bind^{R} Resin according to the manufacturer's recommendations (Novagen, Switzerland). Fractions containing his-tagged proteins were pooled, and endotoxin was removed by filtration through a Zeta-Plus Biofilter (CUNO, Ultrafilter Scandinavia, Denmark). Protein quantification was performed by the Bradford method using a protein assay kit (Bio-Rad, USA). Induced and purified proteins were sampled for SDS-PAGE. Purified proteins separated by SDS-PAGE were blotted onto nitrocellulose membranes (Membrane Solutions, USA). The membranes were probed with anti-His-tag antibody and goat anti-mouse IgG conjugated to horseradish peroxidase (HRP) (Sigma Chemical, St. Louis, MO, USA) with DAB staining according to the manufacturer's instructions.

Immunization and Challenge Tests

Specific-pathogen-free female ICR mice, Six-week-old, weighing 16-18 g, procured from the Experimental Animal Center of Changchun Institute of Biological Products, CO. Ltd. (Changchun, China). The mice were fed according to the Regulations for the Administration of Affairs Concerning Experimental Animals. A total of 48 ICR mice were randomly divided into 3 groups (16 per group), and each group was named the HlaD, $Hla_{H35L,}$ and PBS. The mice were inoculated intramuscularly with 100 μg of antigen mixed with Freund's complete adjuvant at a dose of 0.2 ml. On day 21 after the primary immunization, the mice were inoculated with 100 μg of antigen mixed with Freund's incomplete adjuvant at a dose of i.m. The control mice were inoculated with an equal volume of PBS, which served as antigen. Three mice per group were bled from the caudal vein sera (about 50 μl once) on days 0, 7, 14, 21, 28, and 35 after immunization for antibody detection and run individually. On day 7 after the immunization, three mice per group were dislocated neck to death and used for the lymphocyte cytokine response analysis (IL-4 and IFN-γ). The sera samples obtained on day 35 were also used for lactic dehydrogenase (LDH) and cytokines (TNF-α and IL-17) ELISA assay. On day 35 after the immunization, 13 animals from each vaccinated group were challenged by intranasal administration (i.n.) with the *S. aureus* Newman. In challenging, 10 mice per group received a lethal dose of bacteria (3×10^8 CFU) for testing the effect of immune protection. The residual 3 mice per group received a sublethal dose of bacteria (2.5×10^8 CFU) for ensuring the survival of mice within a certain period and bacterium propagates, and were dislocated neck to death for enumeration of bacterial load. The mortality of the mice was recorded daily and for 7 successive days.

Antibodies Titers and Isotyping

The serum IgG titer was measured using 2 fold serial dilutions of anti-HlaD sera by ELISA as described [13]. Endpoint titers were determined as the maximum antibody dilution whose mean O.D. was twice more than or equal to the mean O.D. value of the negative sample. It should be mentioned that the serum samples from the mice immunized only with PBS were taken as negative samples. Antibody titers were expressed as the reciprocal of the highest positive dilution, and final titers were expressed as the mean±SD of triplicate determinations. For isotyping, serum samples (1:1000 dilutions of the 35th-day sera) from 3 mice were pooled and assayed by mouse sub-typing kit (Thermo Scientific, Thermo Fisher Scientific, Inc. USA) following the manufacturer's instructions.

LDH Release Assay

The A549 cells (lung epithelial cell line) from the American Type Culture Collection were cultured in a 37 ℃, 5 % CO_2 incubator in RPMI-1640 supplemented with 10% fetal bovine serum and 2 mM L-glutamine. The A549 cells were seeded at 2×10^4 cells/well in a microplate. On the following day, washed A549 were cultured with 100 µl of immune sera (1:1000 dilutions) or PBS per well in culture medium in a triplicate well. After 2 hrs, pre-incubation, 100 µl of 20 nM Hla was added for incubation for an additional 4 hrs. Then, the lactate dehydrogenase activity was determined using a cytotoxicity detection kit (LDH; Roche, Switzerland) according to the manufacturer's recommendations, and measured on a microplate reader (Tecan, Austria). The results were representative of a minimum of two independent experiments and expressed as the percentage of maximal lysis obtained after detergent treatment of the A549 cells.

Enumeration of Lung Bacterial Burden

The mice were euthanized 24 hrs after the challenge and the lungs were aseptically removed to determine bacterial load. The lung was homogenized with a mortar and taken up in a total volume of 2 mL PBS. The samples were smeared in different dilutions on TSB agar plates and CFU was enumerated after incubation at 37 ℃. It should be noted here that the statistical value of CFU is the number of bacteria per gram of mouse lung.

Cytokines Assay

The amounts of TNF-α and IL-17A in the immune sera were determined by ELISAs using commercially available mouse cytokine ELISA kits (Dakewe Biotech, Beijing, China). In each assay, the recommended standard curve were generated, and the sample concentrations were transformed from the standard curve. The ELISA assay process was as follows: the process was: A 100 µL of the sample or standard was added into each well and incubated at 37 ℃ for 2 hrs. The supernatant was removed after incubation, and the well was washed with washing solution for 4 times. A 100 µL of a biotinylated antibody was added, and then incubated at 37 ℃ for 1 hr. The plates were washed 3 times after incubation, and the 100 µL of Streptavidin-HRP was added and incubated at 37 ℃ for 30 min. The plates were washed 5 times after the incubation. A 100 µL of the stabilized chromogen was added to each well, followed by incubation at 37 ℃ for 30 min in the dark. Finally, a 100 µL of a stop solution was added to each well, and the dates were measured within 10 min using a microplate reader at a wavelength of 450 nm. The splenic lymphocyte cytokine response (IL-4 and IFN-γ) was analyzed using Elispot kits (Dakewe Biotech, Beijing, China) according to the manufacturer's instructions. Briefly, fresh prepared mice splenocytes (5×10^5 cells/well) were added into the pre-coated Elispot plates immediately after the addition of 10µg/ml of the HlaD, HlaH35L, or 10 ng/ml of PMA, respectively. An additional 1 hr incubation was conducted with 2 µg/ml biotinylated detection antibody against mouse IFN-γ or IL-4 after being incubated and washed. ELISPOT development was performed with avidin-HRP complex after 1 hr incubation, followed by incubating with peroxidase substrate AEC for 30 min. The data of Elispot was provided by Dakewe Biotech.

Statistics

Antibody titer ($\log_{10}$ transformed) was determined using a one-way ANOVA after confirming residuals were normally distributed and variances were equal for data sets. The independent Student's t-test was used to assess the other experimental data. A P-value <0.05 was considered to be statistically significant.

RESULTS

HlaD on Mutant α-hemolysin by Chimeric Fusion

According to the design scheme (Figure 1A),

the target gene was cloned and induced to express in vitro. To confirm that the amino acid mutation at position 35 of the Hla did not affect the molecular size of the protein, the Hla was also used as a control for gene cloning and protein production in vitro (Figure 1B). The presence of a 17.8 kDa (molecular weight of HlaD) chimeric protein or 33.4 kDa (molecular weight of Hla_{H35L}, being a mutant Hla) recombinant protein (Figure 1B) band on the SDS-PAGE gel was shown. The purified recombinant protein was also confirmed by SDS-PAGE (Figure 1C). The expression of recombinant protein was confirmed by Western blot using an anti-His-tag antibody again (Figure 1D). These results showed that the correct recombinant protein was generated.

Response to Immunization with HlaD in Mice

The serum IgG responses to HlaD were determined in ICR mice immunized intramuscularly with the purified protein emulsified in complete or incomplete Freund's adjuvant (CFA/IFA). The mice immunized with recombinant protein in the presence of CFA/IFA developed significantly higher HlaD-specific IgG titers than the ones immunized with PBS ($P<0.01$) (Figure 2A). The level of IgG against HlaD was similar to that against Hla_{H35L}, and both showed a consistent antibody tendency (Figure 2A). It showed that IgG1 was predominantly immunoglobulin elicited by HlaD. But the significance of IgG1 elicited by HlaD was not found in the comparison with IgG1 elicited by Hla_{H35L} (Figure 2B). To evaluate the cellular immune response elicited by HlaD, the level of the important pro-inflammatory cytokine was assessed. As shown in Figure 3, a significant difference in the production of detected cytokine was not found compared with the HlaD group and Hla_{H35L} group. This data demonstrated that HlaD similarly functioned with full-length Hla_{H35L} in the humoral immune response and cellular immune response.

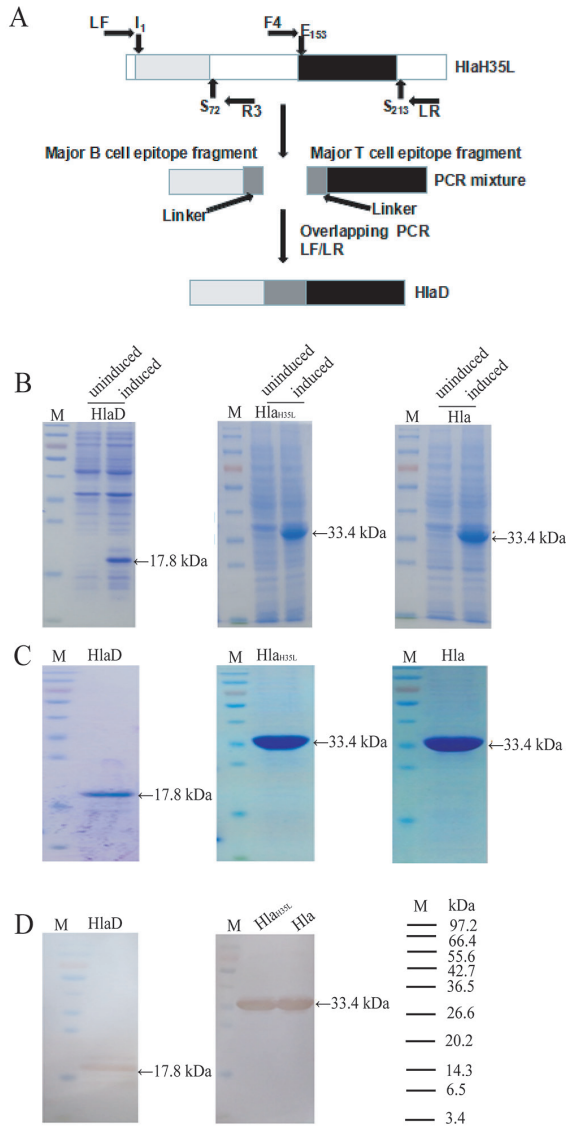

Figure 1. Production and validation of recombinant proteins. Construction schema of HlaD. (B) Expression of the proteins induced by 1 mM IPTG for 4 h. (C) purification of recombinant protein. The lysic bacterial cells or purified fractions were analyzed on a 10% SDS-PAGE followed by Coomassie brilliant blue staining. (D) Western blot assay of recombinant protein with anti-His tag antibody. Pre-stained protein standard was used as molecular mass markers (kDa) and schematic marked.

Protective Effect of HlaD Against S. aureus Pneumonia

The protective effect of HlaD against the *S. aureus* pneumonia was assessed by nose inoculation in the mice. From the data recorded after infection, mortality from pneumonia in the HlaD mice group is not significant in comparison with Hla_{H35L} mice group (Figure 4).

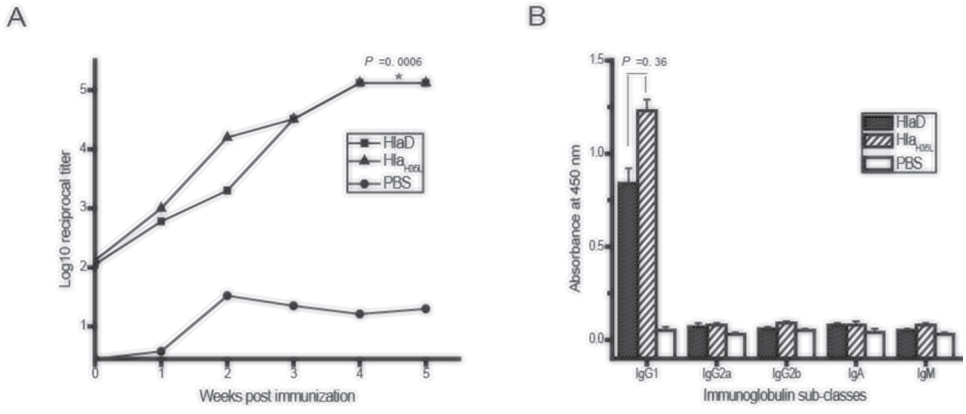

Figure 2. Induction of total IgG response to HlaD and immunoglobulin subclass determination in serum samples of mice immunized with recombinant protein. (A) HlaD-specific total IgG curve after the first and second dose. All mice immunized with HlaD had consistent in IgG dynamic curve with Hla_{H35L}. (B) Immunoglobulin subclass response in mice submitted to the different immunization regiments. No significant difference was found in primary IgG1 level in HlaD and HlaH35L group (P=0.36).

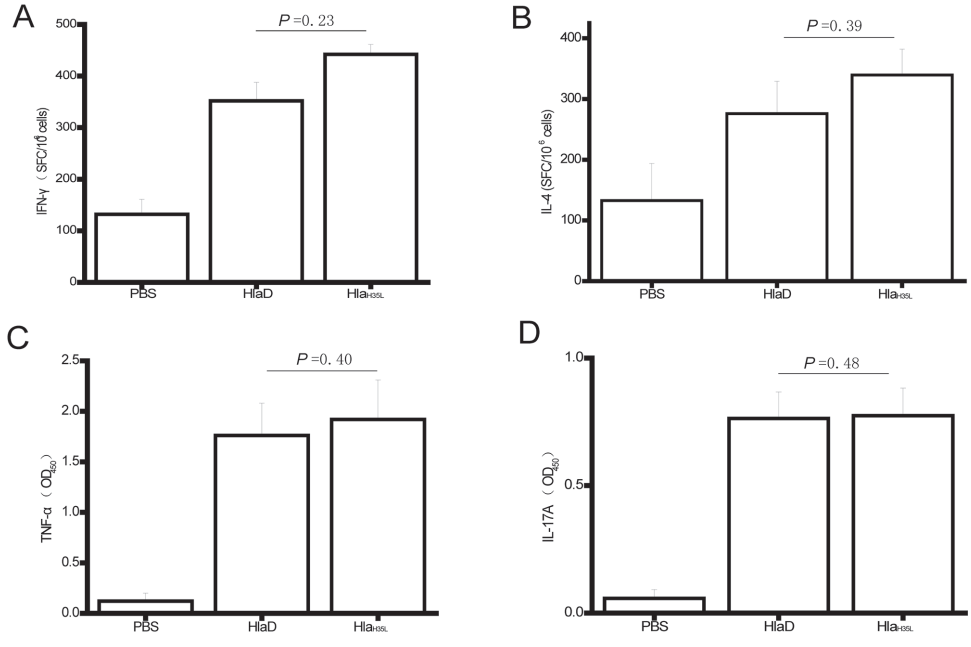

Figure 3. Specific cytokine level in mice immunized with HlaD. IFN-γ (A) or IL-4 (B) Elispot assay was done 1 week after the first immunization. The results are given as IFN-γ SFC/10^6 cells or IL-4 SFC/10^6 cells, and the data represent the mean ± SD of three mice per group. TNF-α (C) or IL-17A (D) level was determined using indirect ELISA. The corresponding P value was indicated.

To further demonstrate the protection correlation of bacterial number and pneumonia-induced mortality in gross, CFU recovered from the lung of HlaD-immunized animals were observed. A non-significant difference between HlaD and Hla_{H35L} groups was found in the bacterial burden of infected mice lungs (Figure 5). The results were consistent with the data revealed by mice survival.

Contribution of HlaD to the Injury of Lung Tissue

Co-incubation of A549 cells with α-hemolysin in the presence of PBS revealed cell death. It should be noted that the sera sample was collected on days 7, 14, 28, or 35 after the immunization. It had been found that the addition of mice anti-serum to Hla_{H35L} (the sera sample was collected on day 35 after the first immunization) led to a marked reduction in A549 injury.

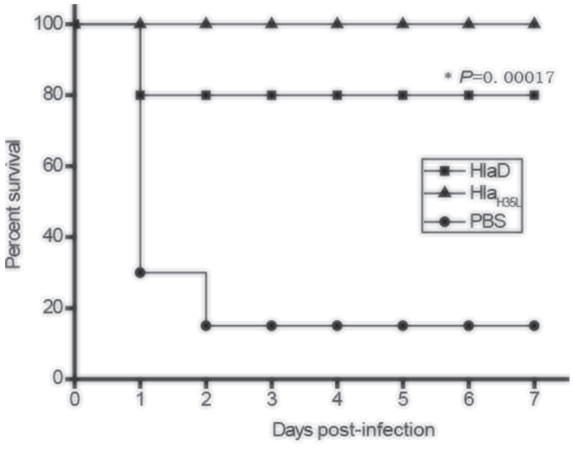

Figure 4. Effective protection of HlaD against *S. aureus* Newman infection. Percent survival after challenge i.n. with the lethal dose of Newman cells (3×10^8 CFU) was calculated. Survival of mice was monitored for 7 days The asterisk indicate P values (*P<0.05).

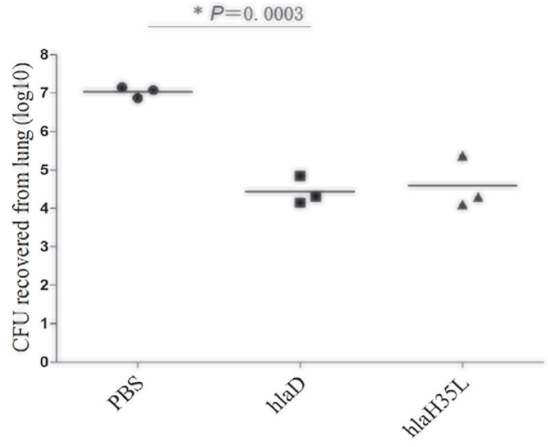

Figure 5. Bacterial burden in lung of vaccinated mice after challenge. Mice vaccinated with HlaD after infection from i.n. with the sub-lethal dose of *S. aureus*. The asterisk indicate P values (*P<0.05).

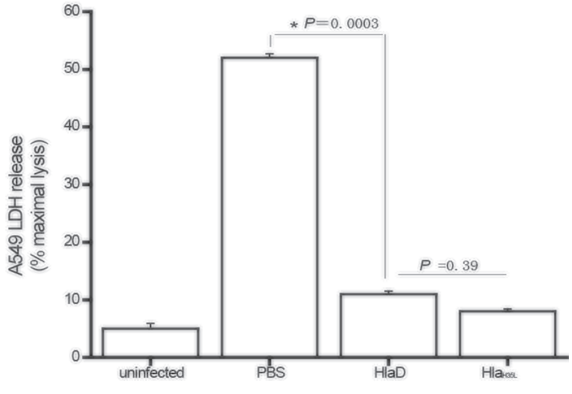

Figure 6. Assessment of LDH release assay by A549 cells that were lysed after antiserum was co-incubated with **α**-hemolysin. No significant reduction in LDH release in HlaD was observed compared with Hla_{H35L}. The significant difference was found between PBS group and HlaD group (*P<0.05).

Furthermore, the addition of mice anti-sera to HlaD (at a consistent concentration of 20 nM) also protected against the *S. aureus* cell injury, and no significant difference was found compared with the HlaD group and Hla_{H35L} group (Figure 6). Evaluated results of injury of lung tissue suggested that HlaD and Hla_{H35L} performed the same function in stimulating an immune response in mice.

DISCUSSION

The *S. aureus* is one of the major human and animal pathogens associated with various infection types. The effective prophylaxis or treatment against the *S. aureus* disease is urgently needed, yet, partly hampered due to the variety of virulence factors produced by the *S. aureus.* Therefore, the preparation of multivalent vaccines against various virulence factors of the *S. aureus* is particularly critical. Previously promising vaccine against the *S. aureus* such as Glycovaxine from GSK is a multivalent vaccine containing hemolysin Hla_{H35L}. Multivalent vaccines containing Hla_{H35L} are being studied and promoted today, such as Glycovaxine from GSK and PentaStaph from Nabi and USUHS, and 4C-Staph from Novartis, etc. [17]. The α-hemolysin played a key role in the pathogenesis and was a core genome-coded toxin present in virulently all community-associated MRSA strains (CA-MRSA) [18]. So the α-hemolysin represents a prime component of a multi-antigen *S. aureus* vaccine or an essential biomarker for the isolated *S. aureus* detection.

Insights from existing research have elucidated the α-hemolysin as a prime vaccine target. The evidence that an attenuated α-hemolysin mutant (H35L) mediated neutralization of α-hemolysin in vitro, has been shown to prevent the *S. aureus* infections [2]. Several truncation

mutants of α-hemolysin were constructed using a structure-based approach and validated their efficacy in two models of the *S. aureus* infections [18]. Recent reports suggested that a linear neutralization site was found in the N-terminal 19 amino acids of α-hemolysin by in vivo localization technique and in vitro neutralization test [19]. A single conformationally specific neutralizing epitope has been identified in Foletti's paper [20]. Despite all this, little is known regarding the presence or location of other linear or conformational neutralizing epitopes or T cell epitopes based on the structural data of α-hemolysin. In the current study, we employed a strategy incorporating multiple antigens into one fusion, and constructed a chimeric fusion based on the *S. aureus* α-hemolysin mutant which may replace the full-length α-hemolysin. Our results showed that HlaD, an immunodominant chimera fused possible B cell and T cell determinant regions of α-hemolysin, has the antigenic completeness of full-length α-hemolysin, and was potentially suitable as one component of a multi-antigen *S. aureus* vaccine or detected biomarker.

The crystal structure of Hla demonstrated that the residual span (I_1-S_{72}) existed as predominantly random coil and β-sheet, and the residual span (E_{153}-S_{213}) is a predominantly region interacting with cells [21]. In the present work, we designed a flexible linker ($(G_4S)_3$) that maintained the original confirmation of the possible B and T cell epitope regions. Humoral and cellular responses were evaluated to assess the protection conferred by HlaD. As to the humoral response and antibody isotyping, the total IgG titers and primary immunoglobulin subclass in HlaD vaccinated group showed consistency with that in Hla_{H35L} vaccinated group. The study has shown that IL-17 and the Th1 cytokines IFN-γ and TNF-α can induce the production of chemokines and activate the immune function of neutrophils and phagocytes [22]. The Th2 cytokine IL-4 is considered an anti-inflammatory molecule. Thus, a mixed Th1/Th2 /Th17 response can be assessed in immune defense against the *S. aureus* infections. Our results for cytokine detection showed that HlaD elicited significantly greater cytokine production, which similarly functions with the Hla_{H35L} mutant. Compared with the data shown in the previous study, the survival percentage of mice vaccinated with HlaD was slightly higher [18]. Human alveolar epithelial cell A549 has been widely used as a model for lung epithelial cells in many physiological and immunological studies [23]. So with the addition of results of LDH release and bacterial burden in the lung, the protective effect induced by HlaD was similar to Hla_{H35L}.

Taken together, the results presented here demonstrated that a designed fused antigen could be useful in multiple antigen vaccines or pathogen identification when B/T cell determinants are unclear or incompletely identified.

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Conflict of Interest: None declared.

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