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Identification of Th cell epitopes on ClfA adhesin of *Staphylococcus aureus* and characterization of their role in immunity

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Abstract: [**Objective**] The ClfA adhesin of *Staphylococcus aureus* is an excellent vaccine candidate antigen. CD4 + T cells play central roles during immune responses, but their functional contributions to *Staphylococcus aureus* in fection have yet to be evaluated. [**Methods**] By using the SYFPEITHI prediction algorithm, we identified and characterized four Th epitopes within the ClfA adhesin. [**Results**] Peptide C335 was I-Ed restricted Th1 type epitopes; peptides C214, C286, and C436 were I-Ad restricted Th2 type epitope. [**Conclusion**] The identification of these epitopes is important to evaluate and optimize the vaccine-primed protection against *Staphylococcus aureus* infection.

Keywords: *Staphylococcus aureus*, ClfA, CD4 + T cell, Epitope

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Staphylococcus aureus, a gram-positive bacterium, is an important opportunistic pathogen that causes a variety of diseases. While most effective treatment for *S. aureus* are antibiotics, the potential multi-antibiotic resistance initiate a pronounced problem[1-3]. Consequently, vaccination has been an alternative approach to control the *S. aureus* infection. Many vaccines against *S. aureus* have been reported to incorporate native components [4]. These treatments are based on a natural form of the pathogen. Such vaccines are safer than whole cell vaccines. Though recent studies indicate that the host triggers vigorous

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humoral and cellular immune responses to *S. aureus* infection [5-7], the infection persists, indicating that the immune responses triggered by *S. aureus* are not eliminating the pathogen. The immune responses evoked by natural infection or subunit vaccine immunization are not favorable and should be improved and modified [8]. Modified immunotherapy might be an effective way to combat *S. aureus* infection. ClfA adhesin mediated *S. aureus* has been shown to adhere to host tissue [9-10]. Mapping the antigenic epitope of ClfA adhesin is one of the essential steps for the development of antibodies against *S. aureus* [11-13].

Epitope vaccines represent immunotherapeutic approach which is based on the observation that in some examples. The advantages of the approach include a specific immune response, increased safety, increased potency and breadth of rationally engineered epitopes and focusing on immune responses elicited by conserved epitopes [14]. Rational choices are made to isolate the components desired for the responses. The premise of these efforts is the identification of the appropriate epitopes [15].

The requirement of MHC class II for protection in mice implies an important role of CD4 + T cell [16-18]. CD4 + T cells respond to processed antigens presented by MHC class II molecules expressed on APCs. CD4 + T cells are critically important for memory B-cell responses and the affinity maturation of antibodies [19]. In addition to enhancing humoral immunity, CD4 + T cells may also contribute to forms of protection that are directly mediated cellular immune response [20-22]. These findings support an important role for CD4 + T cell in acquired immunity to *S. aureus* infection and suggest that a successful immunotherapeutic strategy should include CD4 + T cell epitopes. Identification of the epitopes recognized by CD4 + T cells would enable researchers to evaluate and optimize the contributions of CD4 + T cells to vaccine-primed protection against *S. aureus* infection.

In the current study, there is no information available about CD4 + T cells epitopes from ClfA. The epitopes in ClfA and their correlation with protection is limited primarily to the B cell epitopes. We report the identification of ClfA epitopes recognized by CD4 + T cells in the context of the murine class II MHC molecule. The present study aimed to identify the Th epitopes of ClfA and the findings should pave the way for decisive evaluations of roles for CD4 + T cells during vaccine-mediated protection against *S. aureus* infection.

1 Materials and methods

1.1 Epitope prediction and synthesis of candidate peptides

The protein sequence of ClfA was retrieved from GenBank database (Accession No. AEK94092.1). Potential H2-Ad and H2-Ed restricted epitopes of ClfA were predicted by the SYFPEITHI algorithm [23]. The software provides scores based on the amino acids at certain positions of the MHC class II binging sites. The five highest-scored H2-Ad restricted and five highest-scored H2-Ed restricted epitopes were selected for identification. The selected epitopes were synthesized and purified (GL Biochem, Shanghai, China). The purity of peptides was assessed by HPLC and molecular weight was identified by mass spectrometry. Recombinant ClfA adhesin was expressed in *Escherichia coli* previously [26]. In brief, the recombinant ClfA protein was purified by His tag Ni affinity chromatography. The rClfA proteins before and after purification were analyzed by 10% SDS-PAGE, and a pronounced band with the molecular weight of approximately 59.5 kDa was examined in the supernatant of cell lysate, suggesting that the fusion protein was successfully expressed in the bacterial cells [26].

1.2 Immunization of mice

Six to eight week old female BALB/c mice were purchased from the Experimental Animal Center of The Jilin University during the study. All experiments were conducted in accordance with the Institutional Animal Care and Use Committee guidelines. For rClfA immunization, mice received 100 μg rClfA protein and adjuvant (CFA; Sigma). The immunizations were

given two weeks later with the same protein and adjuvant (IFA; Sigma). For epitopes immunization, mice received 100 μg peptide and adjuvant (IFA; Sigma) with the same procedure. Two weeks after immunization the mice were used for further experiments. Control groups received PBS instead of the protein or peptides.

1.3 Isolation of splenic antigen presenting cells (APCs)

Spleens were removed from mice and the single cell suspension was harvested through screens. Erythrocytes in the spleen cell suspension were lysed in Tris-NH_4Cl. The spleen cells were irradiated by Co60, then used as APCs for the further experiments.

1.4 Assay for proliferation in CD4 + T lymphocytes

One week after the last immunization, mice were sacrificed and their spleen cells were harvested. Single cell suspensions were prepared from homogenizing spleens. The erythrocytes were removed using the Ammonium Chloride Lysing Reagent (BD Biosciences, PharMingen). CD4 + T cells were negatively isolated from either single cell spleen preparations or single cell preparations of a mixture of spleen and peripheral lymph node using the DynalR Mouse CD4 Negative Isolation Kit. The resultant cell populations were routinely > 95% CD4 + positive as determined by flow cytometry. CD4 + T cells were incubated with irradiated syngeneic splenic APCs in the presence of epitopes or rClfA proteins with complete RPMI 1640 media. Negative control were the groups that the cultures without any antigen. The cultures without antigen were used as negative control. The irradiated APCs incubated with ConA and CD4 + T cells incubated with epitopes were used as other controls. The cultures were set up in triplicate and incubated for 7 days. During the final 16 h, cultures were pulsed with 1 μCi of ^3H-thymidine per well for 4 h of incubation. The radioactive thymidine was specifically incorporated into DNA which correlated with cell proliferation. Radioactivity was measured by liquid scintillation couting of samples dried on glass-fiber filters. The cellular stimulation index (SI) was calculated by comparing ^3H-thymidine incorporation; stimulation index = mean cpm of peptide containing triplicate wells / mean cpm of control triplicate wells, without peptide. The ten synthesized peptides were pooled in different combination to stimulate CD4 + T cells for detecting the interaction among the peptides.

1.5 MHC restriction

To discuss MHC restriction in antigen presentation, murine monoclonal antibodies against the murine I-Ad, I-Ed, and MHC class I (H-2d) molecules were placed in the cultures to examine their ability to inhibit peptide specific proliferation of ClfA primed lymphocytes. Briefly, splenic lymphocytes were incubated by murine monoclonal antibodies, peptides were then mixed. A negative control assay was done with medium incubated with peptides alone.

1.6 Cytokine measurement

Levels of IL-2, IFN-γ and IL-4 in the CD4 + T lymphocyte culture supernatants were measured by using commercial ELISA kits (Pierce Biotechnology), according to the manufacturer's instructions. The sensitivity of the ELISA was determined with a calibrated standard. Results were corrected for dilution of the sample to yield the sample concentration in pg/ml.

1.7 Real-time quantitative reverse-transcriptase PCR (qRT-PCR) to quantify mRNA expression

Total RNA was extracted using kits from Qiagen according to the manufacturer's instructions. qRT-PCR was performed by TaKaRa Biotechnology Corp. (TaKaRa, Dalian) for the mRNAs of IL-2, IL-4, IFN-γ and GAPDH (as control). Datas were analyzed by relative quantification. In relative quantification, the two delta Ct (threshold cycle) method was used to assess the mRNA levels of IL-2, IL-4 and IFN-γ [24]. In brief, the means and standard deviations were calculated based on technical replicates. Using the Ct values of technical replicates, the delta-Ct value was calculated for each experimental group, thereby helping to eliminate data anomalies. The two delta Ct values were calculated after examining average Ct values for each experimental group.

1.8 Statistical analysis

Figures represent data from two or three independent experiments, and the data were expressed as the mean ± standard deviation and were processed by using Student's t test on a SPSS 13.0 statistical program.

2 Results

2.1 Prediction of potential ClfA Th epitopes and peptides synthesis

Our goal was to identify the potential binding motifs for I-Ad and I-Ed in amino acid sequence of ClfA. We began by running ClfA protein through the SYFPEITHI software. Overall, combining the prediction results using the software algorithm, we selected five I-Ad restricted and five I-Ed restricted epitopes with highest scores (Table 1). We attemped to synthesize the selected peptides and the purities of these peptides were all ≥90%.

Table 1. Potential Th epitopes identified within the ClfA by using the SYFPEITHI algorithm

Peptide	MHC restriction	Sequence	Score
C214	I-Ad	R A F S L A A V A A D A P V A	32
C286	I-Ad	N G V T S T A K V P P I M A G	26
C436	I-Ad	Y K V D N A A D L S E S Y F V	25
C151	I-Ad	S N D T N T V S S V N S P Q N	24
C83	I-Ad	N N G E T S V A Q N P A Q Q E	23
C335	I-Ed	M P A Y I D P E N V K K T G N	26
C2	I-Ed	N M K K K E K H A I R K K S I	22
C247	I-Ed	T T V Y P H Q A G Y V K L N Y	20
C463	I-Ed	N I T F P N P N Q Y K V E F N	18
C203	I-Ed	N Q A V N T S A P R M R A F S	14

Note: C214 represents the amino acid 214 - 228 of ClfA, the same to others. The peptides were synthesized based on the ClfA sequence (strain 8325).

2.2 CD4 + T cell proliferation responses to peptides derived from ClfA protein

To better characterize the epitopes within ClfA, CD4 + T cells were separated from BALB/c mice immunized with rClfA. We cultured these cells along with Co^{60} irradiated syngeneic splenic APC and synthetic peptides. As shown in Figure 1-A, proliferative responses (SI > 2) to peptides C214 (aa 214 - 228), C286 (aa 286 - 300), C436 (aa 436 - 450), and C335 (aa 335 - 349), and rClfA were observed in the CD4 + T cells isolated from rClfA vaccinated mice, but not from PBS treated mice. As shown in Figure 1-B, CD4 + T cells without APC did not proliferate (SI < 2). So we can infer that the proliferative response of CD4 + T cells is dependent on the presence of peptides, not non-specific response. Furthermore, these antigen-specific responses were confirmed, as the cells were primed by rClfA (Figure. 1-A). Our results indicate that the four peptides (C214, C286, C436, and C335) contain the Th cell epitopes for BALB/c ($H\text{-}2^d$) mice.

2.3 MHC restriction of the lymphoproliferative response to synthetic peptide

The peptide MHC class II complexes are recognized by CD4 + T cells. In contrast, CD8 + T cells recognize peptides presented by MHC class I molecules. The interaction between T cell receptor, MHC and accessory molecules can be blocked by the addition of mAbs against surface molecules. MHC class II molecule includes I-Ad and I-Ed types in BALB/c mice. To analyse the MHC restriction of the proliferative response to C214, C286, C436, and C335, we used mAbs against MHC class I, anti-I-Ad and anti-I-Ed mAbs. The specific lymphoproliferative response could be blocked by I-Ad-specific mAb to C214, C286, and C436, while anti-I-Ed and anti-MHC I mAbs had no effects (Figure 2). The I-Ed-specific mab inhibited T-cell responses to C335. In conclusion, peptides C214, C286, C436, and C335 were able to stimulate CD4 + T cell and also indicate that the four peptides are CD4 + T cell epitopes.

2.4 Cytokine production induced by ClfA derived peptides

We studied the IL-2, IL-4, IFN-γ cytokines to determine the subset of CD4 + T cells induced by each peptide. As shown in Figure. 3, three profiles of cytokines were found: (A) the C214 and C286 peptides induced strong Th2 cytokine (IL-4); (B) the C335 peptide preferentially induced Th1 cytokine

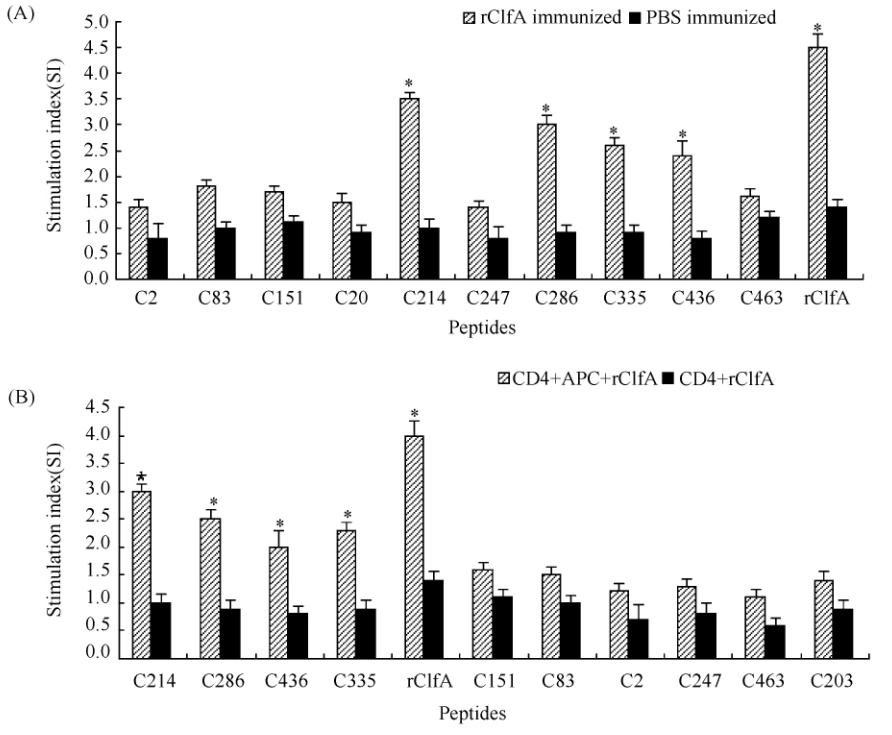

Figure 1. Proliferation of epitopes specific CD4 + T cell in mice immunized with rClfA. Lymphoproliferative responses to the antigens were expressed as the mean stimulation index (SI). The SI indicated is the mean value obtained from three independent experiments ± S. D. SI ≥ 2 is considered positive. (A) To determine whether the responses were rClfA-specific, CD4 + T cells treated with PBS served as controls. (B) CD4 + T cells proliferation assays for responses to synthetic peptides (2 μg/ml), rClfA (20 μg/ml) and control medium. CD4 + T cell pretreated with rClfA in absent of APC did not respond to synthetic peptides. (*) SI ≥ 2 at the 95% confidence level.

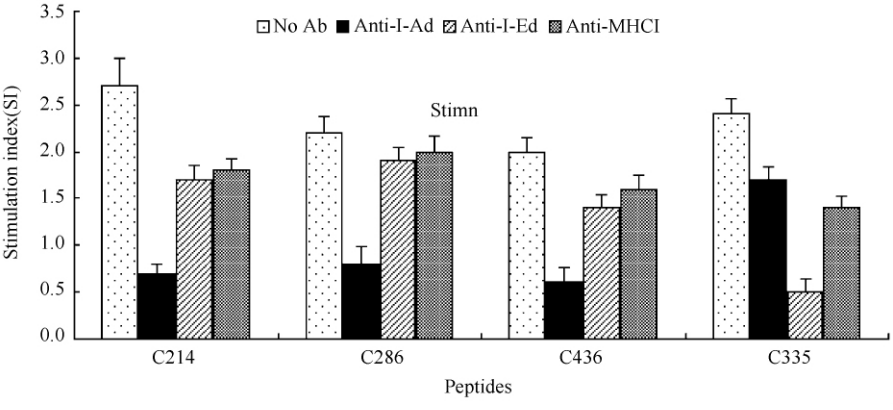

Figure 2. Inhibition of lymphocyte proliferation by anti-MHC II monoclonal antibody. Lymphocytes obtained from BALB/c mice primed with rClfA and were stimulated with peptides (2 μg/ml) with presence of monoclonal antibody for I-Ad, I-Ed, or MHC I molecules. As control served cultures incubated with peptides in the absent of monoclonal antibody. Proliferation was measured in triplicate by 3H-TdR incorporation.

(IFN-γ and IL-2); and (C) finally, the C436 peptide induced a mixed IFN-γ-IL-4 response, since both cytokines were induced to a comparable extent. We compared the expression of IL-4, IFN-γ and IL-2

mRNA level of peptide-primed lymphocytes by qRT-PCR. Both ELISA and qRT-PCR produced similar results, the C214 and C286 peptides induced a strong IL-4 response and the C335 peptide induced production of IL-2 and IFN-γ ($P < 0.05$, Figure 4). These results demonstrated that peptides C214 and C286 elicited a polarized Th2 immune response and C335 preferentially a polarized Th1 immune response.

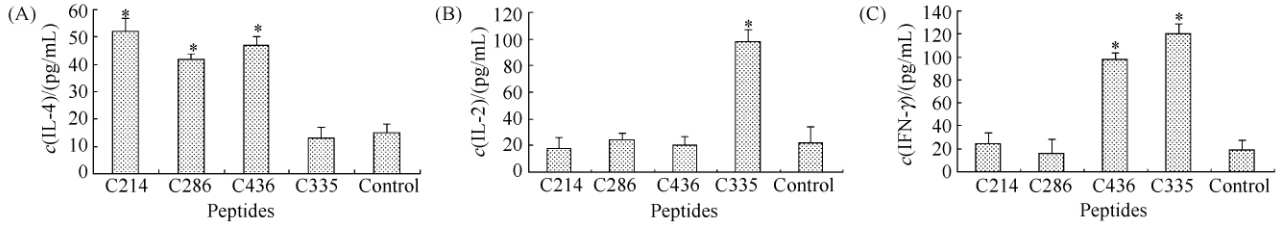

Figure 3. Production of cytokine (A, IL4; B, IL2; C, IFN-γ) by lymphocytes in reponse to peptides. CD4 + T lymphocyte derived from rClfA-immunized mice were incubated with the indicated doses of peptides and cell culture supernatants were analyzed for cytokines by ELISA technique. As control served cultures in the absent of antigen. The datas shown are representative of three independent experiments and the results were expressed as means ± S. D. of triplicate wells (* $P < 0.05$, vs. control group).

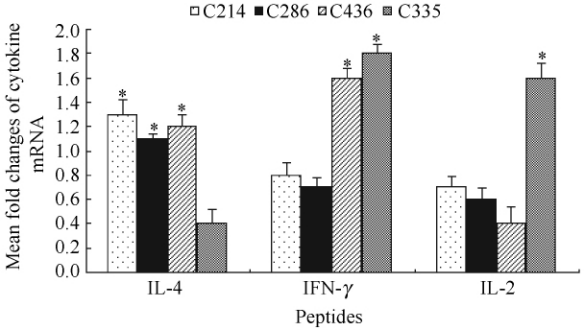

Figure 4. Relative mRNA levels of cytokine in mice induced by ClfA-derived peptides. Analysis of relative gene expression by qRT-PCR and the IL-2, IL-4 and IFN-γ were measured in culture supernatants. We use the delta delta Ct method to calculate the fold change in gene expression. Significant differences are indicated by * $P < 0.05$.

2.5 Priming of T cell responses by pools of peptides

We pooled versus individual peptides in different combination and compared the lymphocyte responses side by side to investigate if there was any agonistic or synergistic interaction between the peptide epitopes. Immunization with a pool of C214, C286 and C436 peptides generated significantly more elevated T cell responses specific to each peptide ($P < 0.05$, Table 2). Each peptide induced a lower response when evaluated individually (Table 2).

Table 2. Synergistic interaction between epitopes. Negative control: no antigen in the cultures.

Peptide	Mean cpm
C214	1893 ± 165
C286	1786 ± 241
C436	1824 ± 189
C335	2207 ± 261
C214 + C286	3712 ± 323[a,b]
C214 + C436	3625 ± 246[a,c]
C214 + C335	3810 ± 314[a,d]
C286 + C436	3683 ± 192[b,c]
C286 + C335	3891 ± 364[b,d]
C436 + C335	3903 ± 259[c,d]
C214 + C286 + C436	4632 ± 276[a,b,c]
C214 + C286 + C335	4185 ± 233[a,b,d]
C214 + C436 + C335	4019 ± 342[a,c,d]
C286 + C436 + C335	4465 ± 318[b,c,d]
C214 + C286 + C436 + C335	4593 ± 421[a,b,c,d]
rClfA	4362 ± 268
No antigen	867 ± 159

Positive control: rClfA in the cultures. a: $P < 0.05$ vs. C214. b: $P < 0.05$ vs. C286. c: $P < 0.05$ vs. C436. d: $P < 0.05$ vs. C335.

2.6 Immunization with peptides evokes ClfA specific CD4 + T cell responses

Having identified CD4 + T cell epitopes in ClfA protein, we next evaluated whether immunizing mice with peptides could prime ClfA specific CD4 + T cell responses. For this purpose, we procured highly purified peptides and then immunized mice with these

peptides emulsified in IFA. The responses of CD4 + T cells were assessed in vitro. In parallel, we evaluated the responses of CD4 + T cells isolated from mice vaccinated with PBS in IFA as controls. CD4 + T cells isolated from BALB/c mice immunized with peptides C214, C286, C436, and C335 responded specifically in vitro stimulation with immunizing peptide and rClfA respectively (SI > 2, Figure 5). T cells from the control mice injected with PBS did not respond to any of the peptides and rClfA (SI < 2). Thus, these peptides can elicit memory T cell response and ClfA-derived peptides induced CD4 + T cells react to rClfA. FACS analyses were used to measure the relative percentage of CD4 + T cells stimulated with peptides. The percentage of CD4 + T cells from peptides (C214, C286, C436, and C335) immunized mice were higher than PBS control ($P < 0.05$, Table 3). These datas demonstrate that the peptides can activated the CD4 + T cells response.

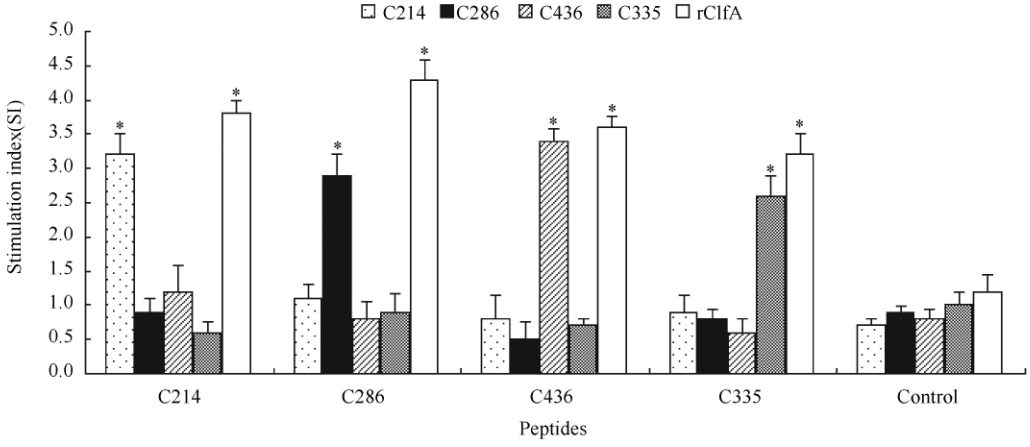

Figure 5. Immunization with epitopes recruit CD4 + T cell responses. Peptide-based immunization elicited CD4 + T cells responses in a proliferation assay to synthetic peptides (2 μg/ml), rClfA (20 μg/ml) and control medium. CD4 + T cells treated with PBS were used as negative controls. Response to epitopes was expressed as the mean SI of three independent experiments ± S.D. (*) Rusults were considered positive when SI ≥ 2 at the 95% confidence level.

Table 3. Phenotype analysis of CD4 + T lymphocytes induced by peptides

Peptide	CD4 + T cell percentages
C214	31.06 ± 2.97*
C286	27.96 ± 3.13*
C436	29.14 ± 2.41*
C335	33.17 ± 1.52*
Control	21.62 ± 2.46

Control group treated with PBS. * $P < 0.05$ vs. control.

3 Discussion

During the last decade, *S. aureus* vaccine has primarily focused on various forms of recombinantly proteins. ClfA has emerged as an excellent candidate Ag for inducing a protective immunity. Furthermore, CD4 + T cells also mediate the protective immune responses in *S. aureus* infection. CD4 + T cells recognize peptides bound to MHC class II molecules on APC. Upon activation, CD4 + T cells play a critical role in immune maturation, development and maintenance of humoral and cellular immune responses. CD4 + T cells play an important role in supporting both B and CD8 + T cell function. Identification and characterization of CD4 + T cell epitope of ClfA would contribute to a better understanding of the immune protection to *S. aureus* infection. In this paper, we propose approachs using the MHC class II prediction software for predicting Th epitopes in ClfA protein. We identified four Th cell epitopes (C214, C286, C436, and C335) by this approach. The present findings may be valuable for developing effective immunotherapeutic and

immunoprophylactic strategies against *S. aureus* infection.

The identification of Th epitopes has been done either by eluting peptides bound to specific class II MHC molecules from APCs or by screening panels of overlapping peptides derived from the antigen. Both the two methods have their own benefits and shortcomings. The method of eluting and sequencing peptides bound to specific MHC class II molecules from APCs has been used to identify MHC class II epitopes. This method is potentially cumbersome and identified epitopes from multiple processed antigens but not specifically a single antigen under study. The other method has identified Th epitopes by screening panels of overlapping peptides. The benefits of this approach are that the results are exhaustive and efficient in eliciting Th proliferation. But it needs massive experiments to identify, making it an expensive, laborious and time-consuming process. In addition, this method would have been less feasible in our system though getting the relative exhaustive result by spanning the entire length of the antigens. The results of this method possibly miss the junctional epitopes that might be present in the overlapping regions [25]. Therefore, we purposefully chose to use an algorithm prediction to identify the Th epitopes. Although this method may certainly have missed potential epitopes, it represents a quick and effective way to identify target epitopes. In the current study, we identified MHC class II restricted epitopes by SYFPEITHI software prediction, combined with biological analysis. We selected ten potential epitopes according to the results of SYFPEITHI software, of which four were tested to be Th epitopes. It demonstrates the feasibility and utility of this method. The datas prove that the combination of the prediction is a useful strategy for identifying target epitope from the sequence of immunogenic protein.

Based on their pattern of cytokine production and functional responses, CD4 + T cells can polarize to Th1 or Th2 cells. Th1 cells responses produce mainly IFN-γ, interleukin IL-2, and tumour necrosis factor-beta, which initiate the body's cellular immune response. In humoral immunity, Th2 cells responses produce mainly produce IL-4. Some investigation demonstrated that the immune response with mixed Th1-Th2 phenotype are correlated with the protective immunity. In our results, the four epitopes that we identified seem to differentially stimulate CD4 + T cells. Epitope C214, C286, and C436 were found to preferentially induce the Th2 subset of CD4 + T cells. And the epitope C335 induced Th1 subset of CD4 + T cells. Characterization of Th1 and Th2 immune responses may help us to develop effective multiple Th1 and Th2 epitope vaccines.

The advantages of epitope-based vaccine are the possibility of including multiple immunodominant epitopes. Epitope vaccine may offer an opportunity to elicit immune responses superior to that induced following immunization by proteins. The four epitopes we selected in this study induced potent specific CD4 + T cells response to rClfA, with production of high levels of IFN-γ, IL-2, and IL-4 which play a significant role in host response to *S. aureus*. In addition, we demonstrated an enhancement in the immunogenicity of peptides injected as a pool, showing a synergistic effect among the four epitopes and supporting the concept that combining potent immunogenic epitopes may be a practical way to prevent or treat *S. aureus* infection. In conclusion, we have defined four Th cell epitopes (C214, C286, C436, and C335) in ClfA protein. Peptide C335 was I-Ed restricted Th1 type epitopes, peptides C214, C286, and C436 were I-Ad restricted Th2 type epitope. Furthermore, we will develop the epitope-based vaccine against *S. aureus* infection and we think our results will provide information for further study of the epitope vaccine.

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金黄色葡萄球菌 ClfA 蛋白 Th 表位鉴定及其免疫原性分析

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摘要:【目的】ClfA 蛋白是金黄色葡萄球菌粘附于宿主细胞的最主要粘附因子之一,筛选鉴定 ClfA 蛋白的 Th 表位,研制基于表位的联合疫苗将成为该菌疫苗新的发展方向。【方法】本研究采用生物信息学软件预测 ClfA 可能的 H-2d 限制性 Th 表位,并以 CD4 + T 淋巴细胞增殖实验及流式细胞分析进行筛选和鉴定。【结果】获得 BALB/c 小鼠 H-2d 限制性 Th1 表位(C335)和 Th2 表位(C214,C286 和 C436)。【结论】筛选出优势性 Th 细胞表位,并对表位的免疫学特性进行实验免疫研究,为最终研制保护作用强、安全性高的新型疫苗奠定基础。

关键词:金黄色葡萄球菌, ClfA, CD4 + T cell, 表位

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