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Epitope-based immunoinformatics study of a novel Hla-MntC-SACOL0723 fusion protein from *Staphylococcus aureus*: Induction of multi-pattern immune responses

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ABSTRACT

Staphylococcus aureus infections are now one of the most common causes of surgical drainage, bacteremia, and hospital-acquired infections. The emergence of antibiotic resistance has increased mortality and costs of treatment. The design of a new vaccine against *S. aureus* would have a great beneficial impact on public health. In the current report, we design and introduce a novel epitope-based fusion protein (Hla, MntC and SACOL0723) and investigate its biological activities. Three known antigenic proteins from *S. aureus* were analyzed for the prediction of immunogenic B and T-cell epitopes and validated using bioinformatics tools. The affinity and the map of interactions between the receptor and ligand were evaluated via docking protocols. Functional activity of the recombinant protein was assessed by western blot and opsonophagocytosis tests and determining the bacterial burden from the infected tissues. To determine the type of induced immunity, cytokines profile and isotyping ELISA was performed. Based on *in silico* analysis, seven epitopes rich domain including highly scored T and B-cell epitopes were selected. The study results indicated that the high titer of specific antibodies raised against the vaccine candidate could opsonize the bacteria and decrease the viable bacterial cells. The fusion protein was able to elicit a mixture of Th1, Th2, and Th17 immune responses more towards Th1 and Th17. In conclusion, the fusion protein formulated with alum could be considered as a potential vaccine candidate for protection against *S. aureus* in the near future.

1. Introduction

Staphylococcus aureus (*S. aureus*) is a common hospital-acquired pathogen that causes diverse infections ranging from abscess and carbuncles to life-threating diseases such as endocarditis, pneumonia, osteomyelitis, septic arthritis, and toxic shock syndrome (Kuklin et al., 2006). The pathogen is one of the most common agents of surgical drainage, bacteremia, and intravenous counters infections (Proctor, 2012). Current antibiotic therapies fail to control the infection due to the very rapid emergence of antibiotic-resistant strains (Stranger-Jones et al., 2006). WHO has introduced *S. aureus* as one of the greatest threat to human health in 2017 because of resistant to multiple antibiotics. The methicillin-resistant strain of *S. aureus* was detected for the first time in Britain in 1961 (Harkins et al., 2017). Resistance to the

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antibiotic could lead to increased morbidity, mortality, and rising health costs (Stranger-Jones et al., 2006). The dramatic increase of resistance to methicillin and other commercial antibiotics has caused to accelerate and broaden the interest in developing a new strategy to prevent and combat human infection due to *S. aureus*. Therefore, it seems that vaccination is an affordable and assured method for the prevention or treatment of infectious diseases.

Due to the lack of an effective vaccine against *S. aureus*, there is a need to test different antigens and formulation to develop a suitable vaccine candidate against the pathogen. Cell surface proteins, polysaccharides, and secreted toxins are the major virulence factors of *S. aureus* that could be selected alone or together with others as an efficient vaccine candidate (Bartlett and Hulten, 2010). Subunit vaccines which mostly consist of T and B-cell epitopes have been much considered recently in the field (Faezi et al., 2019; Mehla and Ramana, 2016). The vaccines have some advantages that include reducing side effects as compared to the killed and attenuated vaccines, no risk of pathogenicity due to not replicating in the host and cost-effective production (Hansson and Sta, 2000). Since the antigenic epitopes of a protein can be predictable, the whole protein is not essential to stimulate an immune response. Suitable vaccine candidate must be highly antigenic, conserved, and responsible for pathogenicity (Testa and Philip, 2012). In most studies, humoral, Th1 and Th17 responses are considered to be the most urgent immune response against *S. aureus* infections (Bröker et al., 2016; Brown et al., 2015). But, more accurate studies showed that Th2 response is also necessary for some infections (Uebele et al., 2017). In overall, it seems that a combination response of Th1/Th2 parallel to Th17 is needed for protection against such infection. In such immunologic condition, Th2 cells by induction of antibody response provide bacterial opsonization and complement activation, Th1 by IFN-γ cytokine release increases macrophages, phagocytosis and killing activity and Th17 by IL-17 cytokine secretion provide recruitment of neutrophils at the infection site and faster bacterial elimination (Bröker et al., 2016; Uebele et al., 2017). During the development of a *S. aureus* vaccine, complex pathogenic mechanisms and numerous pathogenic factors should be considered. In this study, we designed a fusion protein based on three virulence factors including Hla, MntC and SACOL0723 proteins. The hemolysin (Hla or α-toxin) is a potent cytolysin for many eukaryotic cells that binds to ADAM10 as a proteinaceous Hla receptor (Tkaczyk et al., 2013). Alpha-toxin stimulates the Th1 and Th17-dependent immune responses (Niebuhr et al., 2011; Breuer et al., 2005). The IL-17A secretion by α-toxin in the skin and soft tissue infection could lead to bacterial elimination and limiting lesion size. Alpha-toxin is conserved among diverse isolates of *S. aureus* and its key role in the pathogenesis of this bacteria made it as a suitable vaccine candidate against *S. aureus* (Tkaczyk et al., 2013; Bhakdi and Tranum-Jensen, 1991). The presence of manganese is essential for ROS (reactive oxygen species) detoxification, repairing oxidative damages and pathogenesis of *S. aureus* (Coady et al., 2015). The MntC is a highly conserved protein among *Staphylococci* which scavenge the metal element. It has been shown that the MntC confer protective immunity in animal model systems of *S. aureus* infections (Yang et al., 2016). Protective effects of MntC is mostly mediated by increased serum IgG levels (Yang et al., 2016). The C-terminal of the SACOL0723 has highly conserved domains and has been introduced as an appropriate candidate vaccine for *S. aureus* (Stapleton et al., 2012). The mechanism of protection by SACOL is mediated by the generation of opsonic antibodies (Foster and Höök, 1998). In the present study, we aimed to design a novel fusion protein (Hla-MntC-SACOL0723 or HMS) containing antigenic epitopes-rich domains. The functional activities of the engineered fusion protein were evaluated by ELISA and opsonic killing activities, and also an investigation of bacterial burden and cytokines assay. We strongly believe that the outcome of the present study will provide a potential vaccine candidate against *S. aureus*.

2. Materials and methods

2.1. Retrieval of protein sequences

Antigens selected for this study included; Hla (Accession No.: Po9616), MntC (Accession No.: Q8VQS9) and SACOL0723 (Accession No.: A0A0H2WYQ0). The full sequences of the proteins were extracted from UniProtKB and then stored as a FASTA format for further analysis.

2.2. Identification of CD4 + T-cells epitopes

For this prediction, we chose HLA class II alleles DRB1*01:01, DRB1*04:01, DRB1*07:01, DRB1*11:01 and DRB1*15:01 from the selection panel and also H2-IAd and H2-Ied. In the vaccine planning step, we considered that the vaccine should be designed for human use but preliminary studies would be done in mice. So for these reasons we tried to choose epitope-rich domains to cover both MHC of human and mouse types. Two 1d sequence-based screening servers (NetMHCpan and RANKPEP) were used to identify T-cell epitopes. NetMHCIIpan (http://www.cbs.dtu.dk/services/NetMHCIIpan/) is ANN-based methods and predict binding to all DRB1 alleles (Dimitrov et al., 2010). The predicted T-cell epitopes having IC50 value less than 50 were considered as potential T-cell epitopes. Another algorithm was RANKPEP (http://imed.med.ucm.es/Tools/rankpep.html) which predicts peptide binders to MHC molecules from protein sequences using the position-specific scoring matrix (PSSM) (Reche and Reinherz, 2007).

2.3. B-cell epitopes (linear) identification

For the prediction of B-cell epitopes, the amino acid sequence was analyzed using BepiPred (http://tools.immuneepitope.org/tools/bcell/iedb_input) and BCpred (http://ailab.ist.psu.edu/bcpred/predict.html) servers. BCPred is a method for predicting linear B-cell epitopes using the subsequence kernel (EL-Manzalawy et al., 2008).

2.4. Antigenicity, allergenicity, and solubility evaluation

VaxiJen v2.0 server was used for prediction of antigenic epitopes (antigenicity) of the designed protein. The VaxiJen classified antigens based on the physicochemical properties of proteins without performing sequence alignment (Doytchinova and Flower, 2007). The prediction of allergenicity for vaccine candidate is very important. The allergenicity of the designed protein was defined by AllerTOP (http://www.ddg-pharmfac.net/AllerTOP/). The AllerTOP method is based on auto cross-covariance (ACC) that describe residue hydrophobicity, size, abundance, α-helix and β-strand forming propensities (Dimitrov et al., 2013). Recombinant Protein Solubility Prediction (RPSP) (http://biotech.ou.edu/) was applied to predict solubility (Diaz et al., 2010).

2.5. The physicochemical parameters

Some parameters such as molecular weight, theoretical pI, estimated half-life, instability index, were evaluated by the ProtParam online server (http://us.expasy.org/tools/protparam.html) (Lebreton et al., 2011).

2.6. Secondary and tertiary structure prediction

The protein secondary structure prediction was performed by GOR V server (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_gor4.html) (Kloczkowski et al., 2002). The tertiary structure was built by I-TASSER (Iterative Threading ASSEmbly Refinement) server that detects similar structure templates from the Protein Data Bank (PDB) by a technique called multiple threading approach LOMETS (Yang et al., 2015).

Table 1
Predicted linear B-cell epitopes from Hla, MntC and SACOL0723 via BCPred and BepiPred.

Antigen	position	Epitope	BCPred score
Hla	144	YGFNGNVTGDDTGKIGGLIG	0.998
	81	IRTKGTIAGQYRVYSEEGAN	0.916
	175	VQPDFKTILESPTDKKVGWK	0.914
	261	DRKASKQQTNIDVIYERVRD	0.859
	123	QISDYYPRNSIDTKEYMSTL	0.814
	225	TRNGSMKAADNFLDPNKASS	0.782
MntC	15	VAACGTGGKQSSDKSNGKLK	0.988
	48	NVGGDNVDIHSIVPVGQDPH	0.983
	230	NTEKQGTPEQMRQAIEFVKK	0.746
SACOL0723	135	GGSGGTTPTATTGSNGNASS	1
	213	QSTPGPYGHVAYVERVNGDG	0.990
	192	AGNAANDGYQVNNTPSVGSI	0.916
	171	FDRRAQAGSPISTYWSDAKY	0.958
	241	YTYGPYNMNYRTIPASEVSS	0.946
			Bepipred
Hla	80	NKKLLVIRTKGTIAGQYRVYS	
	100	KSGLAWPSAFKVQLQLPDNEVAQISDYYPR	
	160	LIGANVSIGHTLKYVQPDFK	
	240	KASSLLSSGFSPDFATVITM	
MntC	1	MKKLVPLLLALLLLVAACG	
	40	ILYDMAK	
	50	GDNVDIHSIV	
	115	VIAVSKDVKPIYLNGEEGNKD	
	245	FVKKHKLKHLLVETSVDKKA	
SACOL0723	95	AGSASSHTVQAGESLNIIASR	
	120	GYLIMPNQTL	
	155	FNHQNLYTAGQCTWYV	
	205	TPSVGS	
	220	IMQSTPGPYG	

2.7. *Tertiary structure refinement and validation*

The selected 3D structure was refined using the Galaxy Refine server (http://galaxy.seoklab.org/cgi-bin/submit.cgi?type = REFINE). Galaxy Refine refined the model by molecular dynamics simulation (Shin et al., 2014). We used the refined model at predictions of discontinuous B-cell epitopes. The final model was validated with ProSA, MolProbity and Ramachandran plot. ProSA web server was used to recognize the errors in the generated 3D models using atomic coordinates of the model. ProSA web checked z-score and a plot of residue energies of proteins. Z-scores that are outside the scope of z-score native proteins demonstrate false structures (Wiederstein and Sippl, 2007). Clash analysis is very important for the validation of proteins. MolProbity is a structure-validation web service that calculates the clashscore, Protein Geometry score, Poor rotamers, Ramachandran plot and MolProbity score (Chen et al., 2010). The Ramachandran plot indicates that torsional angles are allowed, favored or outliers (Oberholser, 2010).

2.8. *Discontinuous (conformational) epitope identification*

For the epitope prediction in 3D structures, ElliPro was employed. ElliPro is implemented as a web-accessible application and accepts two types of input data: protein sequence or structure (Ponomarenko et al., 2008). DiscoTope server was also used for conformational B-cell epitopes prediction from protein 3D structures. DiscoTope method is based on surface accessibility and propensity scores of residues in protein spatial structure. The vaccine protein 3D structures that used in DiscoTope Server, was predicted by I–TASSER server (Haste Andersen et al., 2006).

2.9. *Molecular docking*

To perform protein-receptor docking, 3D structure of the MntC-monoclonal antibody (mAB) (PDB id: 5hdq) were retrieved from RCSB (www.rcsb.org) (Gribenko et al., 2016). The protein-receptor docking was performed by ClusPro 2.0 server (http://nrc.bu.edu/cluster). ClusPro is an automated docking algorithm that rapidly filters docked conformational using compute the pairwise RMSD histogram of all docked conformations. Cluspro includes DOT or ZDOCK algorithms for performing rigid-body docking that both of them are based on the Fast Fourier transform (FFT) correlation method (Vajda et al., 2017). The outputs of the Cluspro and the model with the lowest binding free energy and the largest cluster size was chosen and interaction of amino acids checked by ligplot.

2.10. *Molecular dynamics simulation*

According to previous studies, the MD method is used to examine the stability of the predicted third structure (Negahdaripour et al., 2018). Molecular dynamic (MD) was done by GROMACS MD package 5.0.1. (Abraham et al., 2014) with Amber99sb-ildn force field. The protein structure was settled in 10 A° solvated box that filled with SPC water molecules. This system was neutralized by Na$^+$ and Cl$^-$ ions. Energy minimization was done with steepest descent algorithm that was set to 1000.00 kJ mol^{-1} nm^{-1}. The system was equilibrated at a constant temperature (300 K) and pressure (1 atm). The simulation was carried out to 40 ns.

2.11. *Plasmid construction, expression, and purification of recombinant protein*

The fusion gene was constructed in pET28a vector and synthesized by Biomatik Corporation (Cambridge, Ont., Canada). The designed construct was located between the NcoI site (at the 5′ end) and HindIII site (at the 3′ end). The recombinant vector was transformed into the competent *E. coli* BL21 (DE3) cells. Positive clones were identified by restriction enzyme digestion. Expression of the recombinant protein was induced by adding 1 mM concentrations of Isopropyl-β-D-Thiogalactopyranoside (IPTG) in Tryptic Soy Broth (TSB) medium, evaluated by 12% SDS-PAGE and confirmed by western blot analysis using HRP conjugated His-specific antibody (Sigma, USA) at a dilution of 1:1000. The expressed protein was purified using Ni-NTA column

Table 2
Antigenic epitopes predicted using Rankpep and NetMHCpan.

Antigen	HLA-DR B10101	HLA-DR B10301	HLA-DR B10401	HLA-DR B10701	HLA-DR B11101	HLA-DR B11501	H2-IAd	H2-IEd
Hla	230–239 MKAADNFLD	258–267 ITMDRKASK	110–119 FKVQLQLPD	187–196 TDKKVGWKV	218–227 GNQLFMKTR	127–135 YYPRNSIDT	248–257 SGFSPDFAT	165–174 NVSIGHTLK
	213–222 WNPVYGNQL	278–286 VRDDYQLHW	94–103 YSEEGANKS			248–256 SGFSPDFAT	120–129 EVAQISDYY	
			215–224 PVYGNQLFM			143–152 TYGFNGNVT	259–268 TMDRKASKQ	
MntC	43–52 YDMAKNVGG	259–268 TSVDKKAME	226–235 IWEINTEKQ	246–255 FVKKHKLKH	296–305 YYKMMKSNI	119–128 SKDVKPIYL	242–251 QAIEFVKKH	245–254 EFVKKHKLK
	5-14 VPLLLALLL		151–160 YVKTIQQTF	151–160 YVKTIQQTF		43–53 YDMAKNVGG	217–226 KQYGITPGY	142–151 WLSLDNGIK
	149–158 IKYVKTIQQ		32-41 KLKVVTTNS	1-9 MKKLVPLLL		249-258 KHKLKHLLV		120-129 KDVKPIYLN
	244-253 IEFVKKHKL		14-21 LVAACGTGG					225-234 YIWEINTEK
SACOL0723	122– YLIMPNQTL	169–178 YVFDRRAQA	190–199 YWAGNAAND	162–171 TAGQCTWYV	184–193 YWSDAKYW168-177 WYVFDRRAQ	228–231 VNGDGSILI	218–227 PYGHVAYVE	168–177 WYVFDRRAQ
	250–259 YRTIPASEV		113–122 LMAANNLRG	200–209 YQVNNTPSV		116–125 ANNLRGYLI	88–97 ASSHTVQAG	189–198 KYWAGNAAN
	191–200 WAGNAANDG		169–178 YVFDRRAQA	185–194 WSDAKYWAG				
	169–208 YVFDRRAQA		106–115 YGVSVDQLM					

according to the manufacturer's instruction (Qiagen, Hilden, Germany) under denaturation condition. Purified protein was free of endotoxin using ε-poly-L-lysine-agarose (Pierce High Capacity Endotoxin Removal Spin Column, 0.5 ml, #88,274; Thermo Fisher Scientific, Inc., USA) and was analyzed by 12% SDS–PAGE. The purified protein was dialyzed overnight at 4 °C in PBS with decreasing concentrations of urea from 1 to 0 M, and quantified by Bradford protein assay.

2.12. Animals and immunization

The animal studies were performed according to guidelines of the European Communities Council (86/609/EEC). Six to eight-week-old female BALB/c mice were purchased from the Pasteur Institute of Iran (Karaj, Iran). The inbred mice were assigned into two different groups containing 18 mice in each one as below:

Group I: Fusion protein formulated in alum adjuvant (n = 18).

Group II: PBS (as a control group, n = 18).

Experimental mice were subcutaneously (s.c) immunized on days 0, 14 and 28 with 200 μl containing 35 μg of the vaccine candidate formulated in alum adjuvant. The control group was injected with PBS buffer with the same condition. Two weeks after the last injection, the blood samples were collected, and sera were taken from all mice in each group by centrifugation and stored at −20 °C until immunoassay.

2.13. Assessment of humoral immune responses

Experimental sera were analyzed by an optimized indirect ELISA for the presence of specific total IgG antibodies and IgG isotypes (IgG1 and IgG2a). Briefly, the 96 well ELISA plates (Greiner, Germany) were coated overnight with 100 μl of the recombinant proteins (1 μg/well) at 4°C, then blocked with 5% skimmed milk in PBS (blocking buffer). Afterward, the diluted sera in blocking buffer (1:100 to 1:12,800) were added into the plates and then the 1:10,000 dilution of HRP-conjugated anti-mouse IgG, IgG1, IgG2a (Sigma, USA) were applied as secondary antibodies. After washing, the plates were incubated with the Tetramethylbenzidine (TMB) substrate to visualize antibody reactivity at 450 nm using an ELISA reader (Awareness Stat Fax 2100, USA).

2.14. Opsonophagocytic killing activity

The sera of immunized mice were assessed for Opsonophagocytic killing (OPK) activity (Faezi et al., 2016). Briefly, macrophages were collected from the peritoneal cavity of the naive mice. For OPK assay, first the diluted mice sera (1:2 to 1:16) were incubated with clinical *S. aureus* COL strain (2×10^6 CFU/ml), and then macrophages (1×10^6 cells/ml) and fresh infant rabbit serum (4%) as a complement source were added in the immune complex. The evaluations of the opsonic killing activity of the samples were compared to the PBS group. This test was performed in triplicate for each quantity. The percent opsonic activity of the serum was calculated as follows:

Percentage of killed bacteria = [1 − (CFU of immune serum/CFU of pre-immune serum)] × 100

2.15. Cytokine profile analysis by flow cytometry

Two weeks after last immunization, the mice were killed and the spleens were taken out in a sterile condition and suspended in cold PBS containing 5% FBS, pen/strep and 2 mM L-glutamine. Briefly, the spleen cells (˜ 2×10^6) were cultured in 24-well microtiter plates (Greiner, Germany) and incubated with and without 5 μg/ml of the filtered fusion protein. After 72 h incubation, the supernatants were collected and stored at −70 °C. Cytokines level of IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-17A, IL-17 F, IL-21, IL-22, IFN-γ, and TNF-α, were assessed using a mouse 13 Plex cytokine assay kit according to the

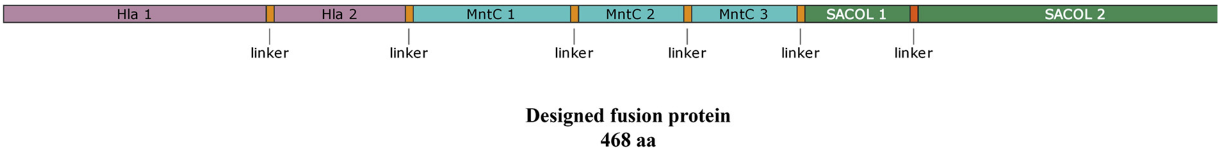

Fig. 1. Schematic diagram of vaccine construct consists of Hla, MntC and SACOL0723 epitopes of the *S. aureus* linked together with AAA linkers.

manufacturer's instruction (BioLegend, USA) using a flow cytometer (BD FACSCalibur™, USA). We compared the level of cytokines between the immunized group and the control group (PBS). Standard curves (ranging from 2.18 to 3484 pg/ml) for all of the analyzed cytokines were included in each run.

2.16. Challenge assay in spleen/kidney of mice

We chose *S. aureus* COL strain for challenge assay in the spleen and kidney of immunized mice. *S. aureus* COL strain was one of the first methicillin-resistant (MRSA) isolates to be identified and has been used extensively in biochemical investigations of methicillin and vancomycin resistance (Gill et al., 2005). Two weeks after the last immunization, the immunized BALB/c mice were infected by intraperitoneal injection of *S. aureus* COL (5×10^9). 72 h after challenge, spleens, and kidneys of mice were harvested, ground and homogenized in 2 ml PBS and cultured in different dilutions on the LB agar medium to determine the CFU/ml of the colonized bacteria.

2.17. Statistical analysis

Statistical analysis of immune responses was performed using One Way Analysis of Variance (ANOVA) followed by Student t-test, and Tukey HSD tests. Kruskal–Wallis and Mann Whitey were used for results of challenge experiments using version 6 Prism (GraphPad) program. P values less than 0.05 were considered statistically significant.

3. Results

3.1. Identification of linear B-cell epitopes

For the linear epitope prediction, BCPred and BepiPred were employed. All predicted B-cell epitopes (20-mer) having a BCPred cut off score > 0.8 were selected. The linear B-cell epitopes that were selected from Hla, MntC and SACOL0723 antigens are summarized in Table 1.

3.2. Identification of CD4 + T-cells epitopes

Rankpep and NetMHCpan predicted different antigenic epitopes from Hla, MntC and SACOL0723 sequence according to DRB1*01:01, DRB1*04:01, DRB1*07:001, DRB1*11:01 and DRB1*15:01 alleles and also H2-IAd and H2-IEd alleles of a mouse. Nine antigenic epitopes with high binding affinity score of < 50 IC_{50} nM were predicted that are summarized in Table 2. Finally, epitopes that were shared between B cell and T cell were selected.

3.3. Selected targets for designing the final vaccine

Probable antigenic epitopes for designing the final vaccine candidate were evaluated by using VaxiJen server. The server showed that the designed fusion protein has potent antigenic epitopes. Based on *in silico* analysis, seven epitope-rich domain including B-cell epitopes and MHC-II epitopes (with the best score), and more conserved epitopes region were extracted and combined together for the generation of multi-epitope protein and joined to each other with a AAA linker (Fig. 1). Final selected epitope-rich domains were listed in Table 3. The designed fusion protein (Hla-MntC-SACOL0723) has 468 amino acids.

3.4. Predicting discontinuous B-cell epitopes

Conformational epitopes are the most prevalent epitopes that are recognized by antibodies which play an important role in the humoral immune response. DiscoTope and Ellipro servers were used for predicting discontinuous B-cell epitopes from the 3D structure of the designed fusion protein. For discontinuous peptides that predict using Ellipro, the score value of 0.7 or more was selected (Fig. 2) and (Table 4).

3.5. Allergenicity, and solubility evaluation

Based on prediction approaches in AllerTop, this protein was not recognized as an allergen. The results of solubility prediction demonstrated that the fusion protein has a solubility chance of 100%.

3.6. The physicochemical parameters

Primary structure analysis showed that our designed protein contains 468 amino acids and has a molecular weight of about 50,542 Da. Theoretical isoelectric point (pI) was 8.67, and the instability index (II) was obtained 22.78, therefore, the designed protein would be a stable protein.

3.7. Protein secondary and tertiary structure prediction and validation

The secondary structure of the designed fusion protein was composed of the alpha helix (30.13%), extended strand (21.15%), and random coil (48.72%). Three-dimensional structures of the designed protein were predicted by I-TASSER server. The best model was selected for further analyses. The Maximum confidence score (C-score) of this model was −1.31. The normal range of C-score is between −5 to 2. The expected template modeling (TM)-score of this model was 0.55 ± 0.15. TM-score measures the structural similarity between two structures with a different sequence on based structural alignment. The ProSA Z-score showed the quality of models based on cα position. The ProSA Z-score of this model was presented in (Fig. 3A). The Z-score of this model was -5.16 that was in the range of native conformations. According to Ramachandran plot, this protein model has 93.3% of the amino acid residues in the favored, 5.8% in the allowed and only 0.9% in the outlier (Fig. 3B). MolProbity analyses clash score of the protein structures. The clash score of all-atoms in this model was 2.41 that was in the normal range.

3.8. Molecular Docking of the designed fusion protein with mAB

Intermolecular interactions between the mAB and recombinant protein were calculated using the program LIGPLOT in which hydrogen bonds and hydrophobic contacts are defined in accordance with standard geometric parameters. The amino acids of mAB that have a role in the formation of the binding interface in the light chain are S^{27}, Y^{27}, Y^{32}, Y^{92}, S^{93} and in heavy chain are D^{27}, Y^{57}, K^{58}, G^{99}, Y^{100} (Fig. 3C). The ligplot result showed that designed protein can interact with these amino acids (Fig. 3D). The amino acids of mAB that in interaction with designed protein from the light chain are S^{27}, Y^{27}, Y^{32}, $S^{93,}$ and heavy chain are G^{99}, Y^{100}, K^{58}. All structural images were created using PyMol (The PyMol Molecular Graphics System, Version 1.1, Schrödinger, LLC). We also demonstrated that the fusion protein also binds to MntC-

Table 3
Seven epitope-rich domain were selected.

Antigen	Position	Antigen score (VaxiJen threshold .4)	Antigenic determinant
Hla	80–180	0.7569	IRTKGTIAGQYRVYSEEGANKSGLAWPSAFKVQLQLPDNEVAQISDYYPRNSIDTKEYMSTLTYGFNGNVTGDDTGKIGGLIGANVSIGHTLKYVQPDFK
	220–270	0.5076	LFMKTRNGSMKAADNFLDPNKASSLLSSGFSPDFATVITMDRKASKQQTN
MntC	1–60	1.1488	MKKLVPLLLALLLLVAACGTGGKQSSDKSNGKLKVVTTNSILYDMAKNVGGDNVDIHSIV
	120–160	0.6579	DVKPIYLNGEEGNKDKQDPHAWLSLDNGIKYVKTIQQTFI
	220–260	0.5016	ITPGYIWEINTEKQGTPEQMRQAIEFVKKHKLKHLLVETS
SACOL0723	90–130	0.6002	HTVQAGESLNIIASRYGVSVDQLMAANNLRGYLIMPNQTL
	140–260	0.4893	TPTATTGSNGNASSFNHQNLYTAGQCTWYVFDRRAQAGSPISTYWSDAKYWAGNAANDGYQVNNTPSVGSIMQSTPGPYGHVAYVERVNGDGSILISEMNYTYGPYNMNYRTIPASEVSS

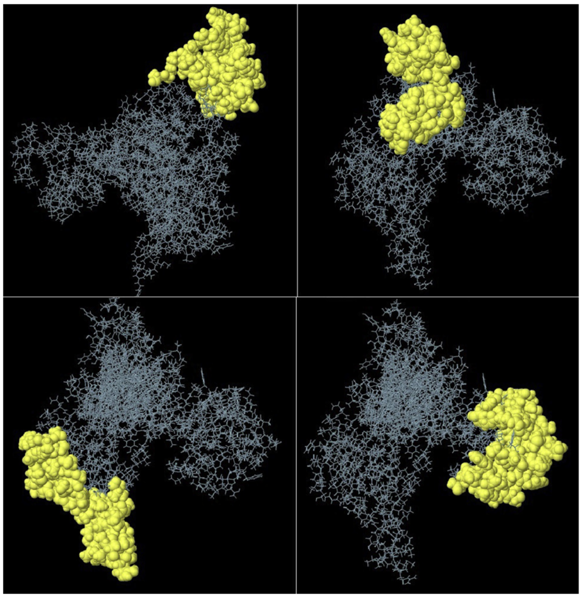

Fig. 2. Three-dimensional representation of conformational or discontinuous epitopes (A–D) of the designed fusion protein. The epitopes are represented by yellow surface, and the bulk of the polyprotein is represented in grey sticks. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

specific antibodies with the ELISA test (data not shown).

3.9. Molecular dynamic

The stability of the docked designed protein and mAB complexes was further investigated by performing molecular dynamics (MD) simulation. The MD result showed that the protein complex reached a stable state in around 40 ns of MD simulation. The RMSD of backbone atoms of this complex showed that this complex had little changes in their tertiary structure during MD. It reached a stable state after 10 ns of simulation and kept this condition until 40 ns (Fig. 3E). The PDB structure of this complex was extracted from a steady state of simulation (Fig. 3F) and the interaction of amino acids was analyzed by LigPlot. The LigPlot result showed a good interaction between designed protein and mAB in the time of MD simulation with amino acids that have a role in the formation of the binding interface in mAB like S^{27}, Y^{27}, Y^{32}, S^{93} form light chain and G^{99}, Y^{100}, K^{58} from the heavy chain (Fig. 3G).

3.10. Expression and purification of recombinant protein

The fusion protein gene was subcloned into the pET-28a vector and overexpressed in *E. coli* BL21 (DE3). The 12% SDS-PAGE analysis showed the presence of a 50 kDa recombinant chimeric protein. The purification of the fusion protein was carried out by Ni-NTA affinity chromatography (Fig. 4A). As the bioinformatics results showed that the fusion protein is soluble, we observed experimentally that after purification and dialysis, this protein was soluble in water and saline. The expression of the fusion protein was confirmed by the detection of the protein by Western blot using anti-His tag antibody (Fig. 4B).

3.11. Specific total IgG responses against recombinant vaccine candidate

Specific total IgG level against the fusion protein assessed two weeks after the last immunization (Fig. 4C). The group of mice that received fusion protein showed that after the third immunization, the specific total IgG level (in 1:200 dilution) significantly increased compared to the control group ($p < 0.0001$). Moreover, the IgG isotypes level (IgG1

Table 4
Conformational epitopes of designed protein as predicted by ElliPro.

NO.	Residues	Number of residues	score
1	A:I1, A:R2, A:T3, A:K4, A:G5, A:T6, A:I7, A:A8, A:G9, A:Q10, A:Y11, A:R12, A:V13, A:Y14, A:S15, A:E16, A:E17, A:G18, A:A19, A:N20, A:K21, A:S22, A:G23, A:L24, A:A25, A:W26, A:P27, A:S28, A:A29, A:F30, A:K31, A:V32, A:Q33, A:L34, A:Q35, A:L36, A:P37, A:D38, A:N39, A:E40, A:V41, A:A42, A:Q43, A:I44, A:S45, A:D46, A:Y47, A:Y48, A:P49, A:R50, A:N51, A:T63, A:Y64, A:G65, A:F66, A:N67, A:G68, A:N69, A:V70, A:T71, A:K184, A:S185	62	0.758
2	A:A404, A:N411, A:N412, A:T413, A:P414, A:S415, A:V416, A:G417, A:S418, A:I419, A:M420, A:Q421, A:S422, A:T423, A:P424, A:G425, A:P426, A:Y427, A:G428, A:H429, A:V430, A:A431, A:Y432, A:V433, A:E434, A:R435, A:V436, A:N437, A:G438, A:D439, A:G440, A:S441, A:I442, A:L443, A:I444, A:S445, A:E446, A:M447, A:N448, A:Y449, A:T450, A:Y451, A:G452, A:P453, A:Y454, A:N455, A:M456, A:N457, A:Y458, A:R459, A:T460, A:I461, A:P462, A:A463, A:S464, A:E465, A:V466, A:S467, A:S468	59	0.739
3	A:V86, A:M106, A:K107, A:T108, A:R109, A:N110, A:G111, A:S112, A:M113, A:K114, A:A115, A:A116, A:D117, A:N118, A:F119, A:L120, A:D121, A:K124, A:S127, A:L128, A:S130, A:S131, A:G132, A:F133, A:S134, A:P135, A:D136, A:F137, A:A138, A:T139, A:V140, A:I141, A:T142, A:M143, A:D144, A:R145, A:K146, A:A147, A:S148, A:K149, A:Q150, A:Q151, A:T152, A:N153, A:A154, A:A155, A:A156, A:M157, A:K158, A:K159, A:P162	51	0.736
4	A:L226, A:E230, A:G231, A:N232, A:K233, A:D234, A:K235, A:Q236, A:D237, A:P238, A:H239, A:L242, A:N246, A:I254, A:Q256, A:T257, A:F258, A:I259, A:A260, A:A261, A:A262, A:I263, A:T264, A:P265, A:G266, A:Y267, A:I268, A:W269, A:E270, A:I271, A:N272, A:T273, A:E274, A:K275, A:Q276, A:G277, A:T278, A:P279, A:E280, A:Q281, A:M282, A:A318, A:S319, A:R320, A:G322, A:V323, A:S324, A:V325, A:D326, A:Q327, A:L328, A:M329, A:A330, A:A331, A:N332, A:N333, A:L334, A:R335, A:G336, A:Y337	60	0.703

and IgG2a) were measured in the serum of immunized mice two weeks after the final immunization. Mice immunized with fusion protein dramatically induced high level of IgG1 and IgG2a responses compared to the control mice (Fig. 4D) ($p < 0.0001$).

4. Opsonophagocytic killing activity

Sera of both experimental groups were examined for phagocytosis killing activity of mouse peritoneal macrophages. *S. aureus* COL strain was used to determine the bioactivity of the specific antibodies. As shown in Fig. 4E. after exposing the opsonized bacteria to the peritoneal macrophages and complement source, the phagocytic capacity of macrophages exposed with a serum of mice immunized with fusion protein showed a significant increase as compared to control group (**** $p < 0.0001$). At 1:2 dilution, the opsonic activity of the fusion protein vaccine group was significantly ($P < 0.05$) higher than other diluted sera in the group and leading to 59.3% reduction in the number of bacteria. In the presence of PBS, as a control group, no opsonic killing activity was observed. These data indicate that the antibodies raised against fusion protein act as a good opsonin for the killing of the COL strain of *S. aureus*.

4.1. Cytokines profile analysis by flow cytometry

Humoral and cellular immunity induced by the fusion protein was evaluated by fluorescence-encoded beads (Fig. 5). Our finding showed that the vaccine candidate is able to elicit a mixture of Th1, Th2, and Th17 immune responses which was more toward the Th1 and Th17. Analysis of cytokines profile of Th2 that including IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 showed a significant increase in the immunized group compared to the control group (****$p < 0.0001$). Th1 secretion cytokines such as IFN-γ, IL-2, and TNF-α were significantly produced in the cultured splenocytes of mice immunized with fusion protein compared to the control group (****$p < 0.0001$). Analysis of cytokines related to IL-17 (IL-6, IL-21, IL-22, IL-17A, and IL-17 F) also showed a dramatic increase (except IL-22) in the group of mice that received the fusion protein compared to the control group (****$p < 0.0001$). We deduced that when the mice are vaccinated with the fusion protein, the $CD4^+$ T cells will be stimulated and polarized the immune responses toward Th1, Th17, and Th2 subsets.

4.2. Vaccine efficacy against kidney and spleen challenge

To determine whether the fusion protein could stimulate protective immunity and reduce bacterial load in spleen and kidney, the challenge experiment with *S. aureus* COL strain was performed in mice groups two weeks after the last immunization. Our data showed that 72 h after challenge, groups of mice immunized with the candidate vaccine significantly reduced the bacterial burden to about 10^1-10^3 CFU in the kidneys (Fig. 6A) (**$p < 0.002$) and spleen (Fig. 6B) (**$p < 0.001$) as compared to the control group.

5. Discussion

Development of a safe and effective vaccine can reduce the rate of *S. aureus* infection; however, so far, several attempts to do so have not been successful (Spellberg and Daum, 2012; Rasmussen et al., 2011). *S. aureus* strains are diverse and their virulence factors could vary markedly from one strain to another. There is a wide range of *Staphylococcal* diseases including skin and soft tissue infections to invasive diseases such as bacteremia, endocarditis, and pneumonia (Kuklin et al., 2006). A successful vaccine against *S. aureus* is a vaccine that comprises of multiple components (multiple targeting), including various proteins, as well as exotoxins. In this study, we designed a new multi-epitope vaccine against *S. aureus* infection by combining the immunodominant regions of three important virulence factors of *S. aureus* (Hla, MntC and SACOL0723) that are expressed by most clinical isolates (Bhakdi and Tranum-Jensen, 1991; Yang et al., 2016; Stapleton et al., 2012). Hla as a α-toxin is released in variable amounts by at least 95% of clinical *S. aureus* isolates and its key role in the pathogenesis of this bacteria made it as a suitable vaccine candidate against *S. aureus* (Bhakdi and Tranum-Jensen, 1991; Otto, 2010). MntC as a manganese transporter is widely conserved in *S. aureus*, including MRSA and VRSA strains. Protective effects of MntC is mostly mediated by increased serum IgG levels and high level of IL-4, IL-17, and IFN-γ (Yang et al., 2016; Yu et al., 2018). SACOL0723 was also identified as a conserved antigen between *Staphylococcal* pathogens (Stapleton et al., 2012). In order to design an effective epitope-based vaccine, we considered both antigens nature and desired immune pattern that implicated for protection. In fact, we hypothesized that to improve the vaccine potency and efficacy, not only conserved epitopes are important but also the immune response pattern that correlates with protection should be considered. Several studies have shown that the protection against *S. aureus* infection needs the various aspects of immune responses and one aspect cannot induce fully protection alone (Bröker et al., 2016; Brown et al., 2015; Uebele et al., 2017; Montgomery et al., 2014; Greenberg et al., 2018).

As mentions above, the fusion protein was produced in the inclusion body form in the *E. coli*. To obtain soluble active proteins from inclusion bodies, we used 8 M urea to denature the protein. The protein in this condition is converted from aggregated form to soluble form. In this study, refolding of the protein to the active form was done by step-wise dialysis with the gradual removal of denaturant from the denatured protein. In this study, we analyzed the protective humoral and cellular immune responses, in an attempt to elucidate the roles of antibodies

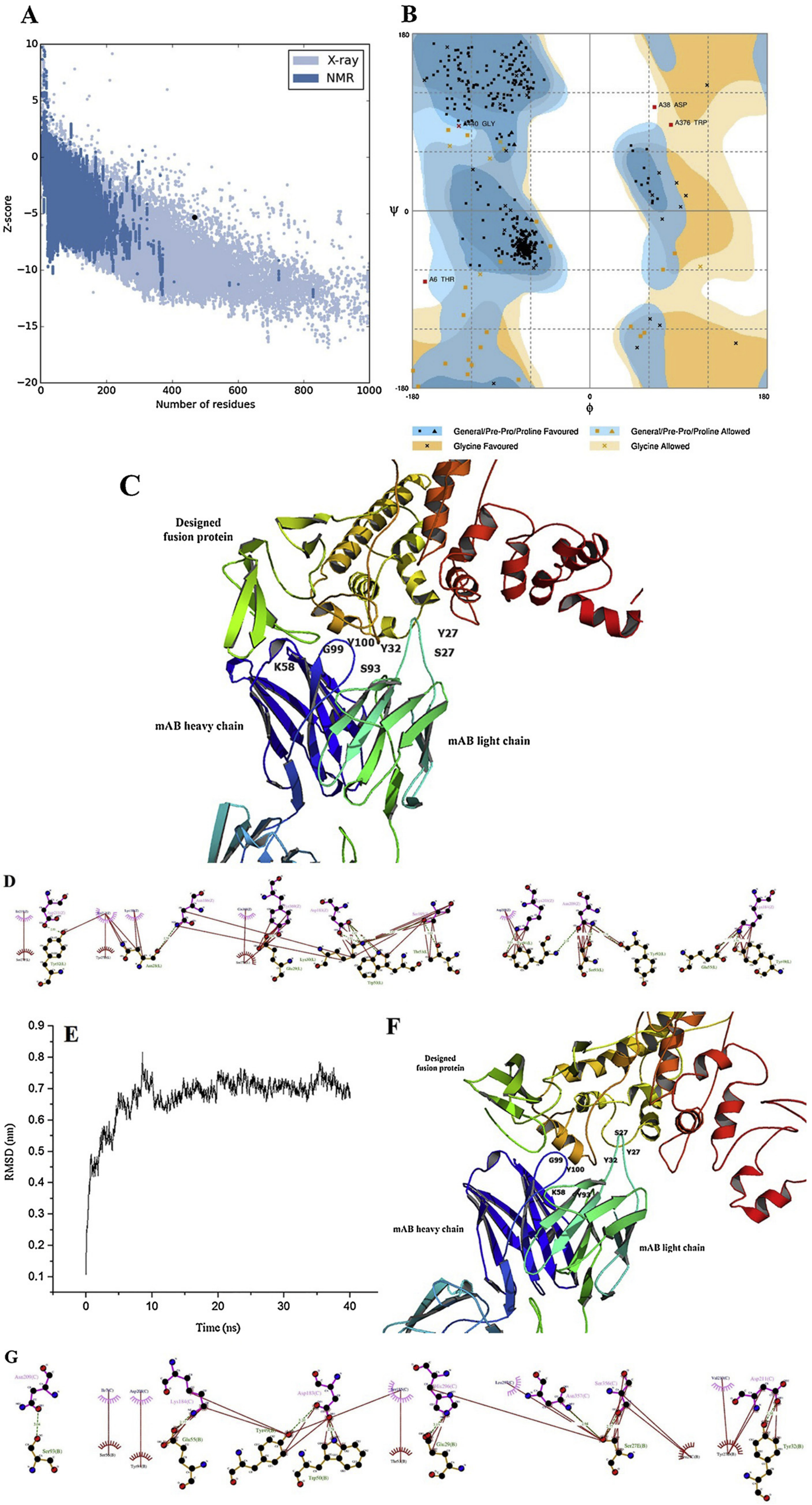

Fig. 3. (A–B) Validation of tertiary structure of the recombinant protein using (A) ProSA web, (B) Ramachandran plot. (C) Molecular model of mAB to MntC protein interactions with designed fusion protein, amino acids that play a role in interface binding site of mAB (Y27, S27, Y 32 S93 from the light chain, and K58, G99 Y100 from the heavy chain) that are in interaction with the designed protein. (D) LigPlot representation of the interaction of mAB and designed protein. Amino acids represented by (H, L, and Z) related to mAB heavy chain, light chain, and designed protein, respectively. (E) RMSD plot of the designed protein-mAB complex after 40 ns MD simulation. RMSD plot of backbone atoms showed that this complex became plateau in 40 ns of MD simulation. (F) PDB structure of designed protein-mAB complex after MD simulation, amino acids that play a role in interface binding site of mAB (Y27, S27, Y 32 S93 from light chain and K58, G99 Y100 from heavy chain) that are in interaction with designed protein after MD simulation. (G) LigPlot interaction of the designed protein-mAB complex after MD simulation.

and cytokines in immunity protection against *S. aureus* infection.

Here, we have chosen Hla, MntC and SACOL0723 proteins, because the previous attempts have been shown that these three important virulence factors which contribute in the pathogenesis and immune responses can control the infection (Tkaczyk et al., 2013; Yang et al., 2016; Stapleton et al., 2012). In addition, these proteins are able to induce a wide range of immune responses including antibody, Th1, Th2 and Th17 pattern (Tkaczyk et al., 2013; Breuer et al., 2005; Yang et al.,

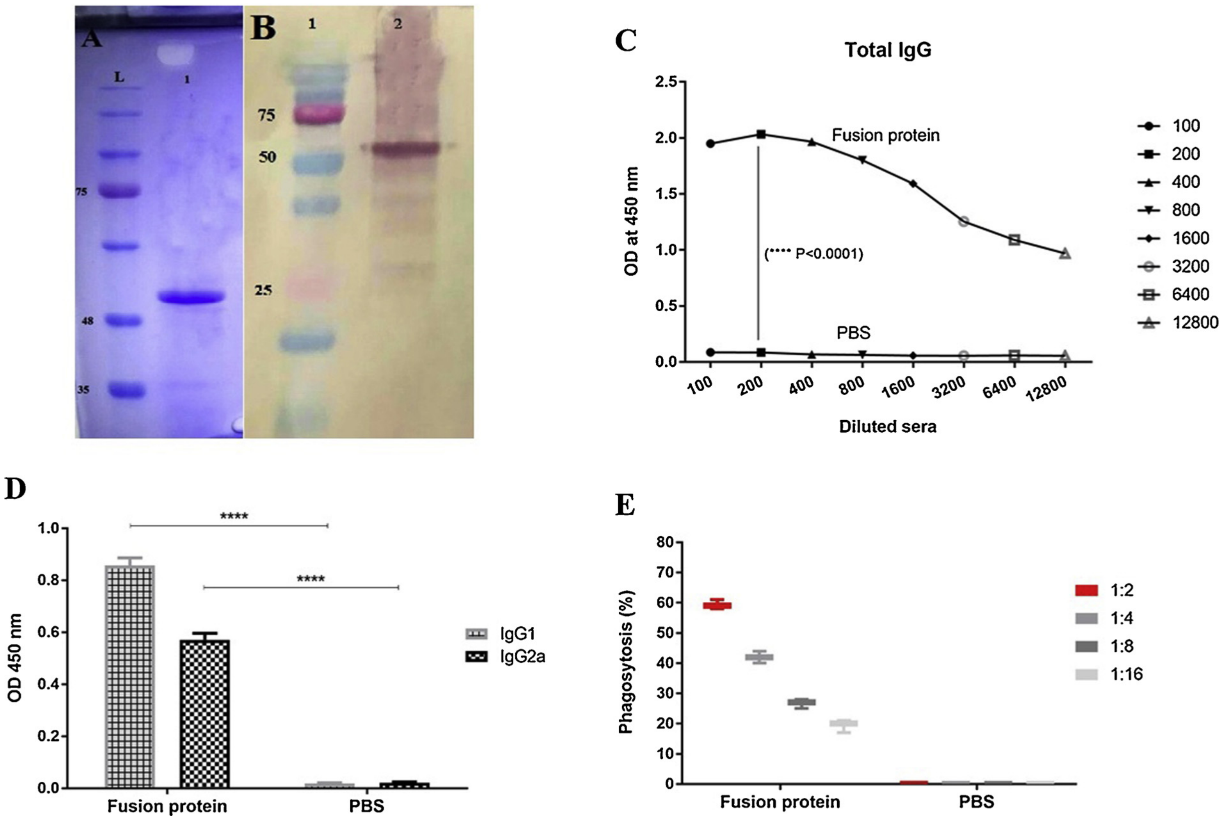

Fig. 4. (A) Purification of chimeric protein by Ni-NTA column. lane L: protein weight marker. lane 1: purified protein (~50 kDa). (B) Western blot analysis of the purified protein: lane 1: protein weight marker, lane 2: purified protein (~50 kDa). (C) Specific total IgG in experimental groups with indirect ELISA. A significant increase was observed in the mice immunized with the fusion protein at 1:100 to 1:12,800 dilution, compared to the PBS control group. (D) The designed fusion protein induced high titer of IgG1 and IgG2a. (E) Comparative analysis of opsonic killing activity. The mouse macrophages dramatically killed the bacteria that opsonized with specific antibodies raised against the fusion protein, compared to the control group.

2016; Foster and Höök, 1998). Each of these proteins induces a specific pathway of the immune response, but we tried to activate different immune response patterns by combining the immunodominant regions of these proteins to provide more adequate protection against *S. aureus*.

In the present study, epitope-prediction tools were used to analyze Hla, MntC and SACOL0723 proteins. Based on *in silico* analysis, seven epitope-rich domains including highly scored and shared epitope between T and B-cell epitopes were selected and joined to each other with AAA linker. Ideally, epitope-based vaccines should contain B-cell epitopes, which are essential for the protective antibody response, and T-cell epitopes that induce cytotoxic T lymphocyte (CTL) and Th immune responses.

Cluspro result of designed fusion protein and mAB complex showed that this recombinant protein would efficiency bond with the binding site of light and heavy chain of fv protein. For assessment of the stability of this complex, MD simulation was done to 40 ns. MD simulation result showed that the desired protein preserved its interaction with the binding site of both light and heavy chain of mAB and made a stable complex with together in a period of time. Based on these results, the selected sequence would support the stable structure of the fusion protein which contained seven domains and can be considered as a suitable immunogenic molecule.

In this study, we analyzed the protective humoral and cellular immune responses, in order to elucidate the roles of antibodies and cytokines in protection against *S. aureus*. The results of the current report demonstrated that the fusion protein evoked high levels of specific total IgG in the sera. In addition, the protein was also able to significantly induce both IgG1 and IgG2a isotypes compared to the PBS control group ($P < 0.0001$), however, the IgG subtype tended to be IgG1. To clear that whether the specific antibodies could mediate immune protection against *S. aureus* infection, we evaluated the bioactivity of these antibodies through opsonophagocytic killing assay. Our finding showed that the antibodies are able to bind to the native protein on the surface of the bacteria and promoted opsonophagocytosis (59.3%) of *S. aureus* COL strain at 1:2 dilution compared to the control group. Opsonic killing activity plays an important role in the clearance of the infectious agents from the infected area. Although these antibodies might contribute to the protection, and cellular immune responses via antibody-dependent cell-mediated cytotoxicity (ADCC).

Cytokines elicited by splenocytes from the immunized mice were evaluated to compare the type of induced immunity. Th1, Th2, and Th17 are important T lymphocytes in the clearance of *S. aureus* infection (Bröker et al., 2016). Our finding showed that the designed vaccine candidate was capable of eliciting a mixture of Th1, Th2, and Th17 immune responses more towards Th1 and Th17. The analysis of cytokines profile of Th2 (IL-4, IL-5, IL-9, and IL13) demonstrated a significant increase in the immunized group compared to the control group. Cytokines related to Th17 (IL-6, IL-21, IL-22, and IL-17) were also analyzed and the results showed a significant increase in IL-17, but the level of IL-22 did not increase, which could confirm the protective effect of IL-17 in bacterial clearance by stimulating of the expression of host defense peptides and the recruitment of neutrophils and T-cells to the sites of infection (Cho et al., 2010). IL-22 induces the expression of IL-17A and both of them are required for optimal host defense against *S. aureus* but they mediate different immune mechanisms (Chan et al., 2015). Th1-secreted cytokines such as IFN-γ, IL-2, and TNF-α activate and recruit macrophages, lymphocytes, and PMNs to destroy the bacterial pathogens (Osugi et al., 1997). In this study Th1 cytokines significantly increased in the immunized group two weeks after the last immunization. The massive production of IL-10 may be involved in the

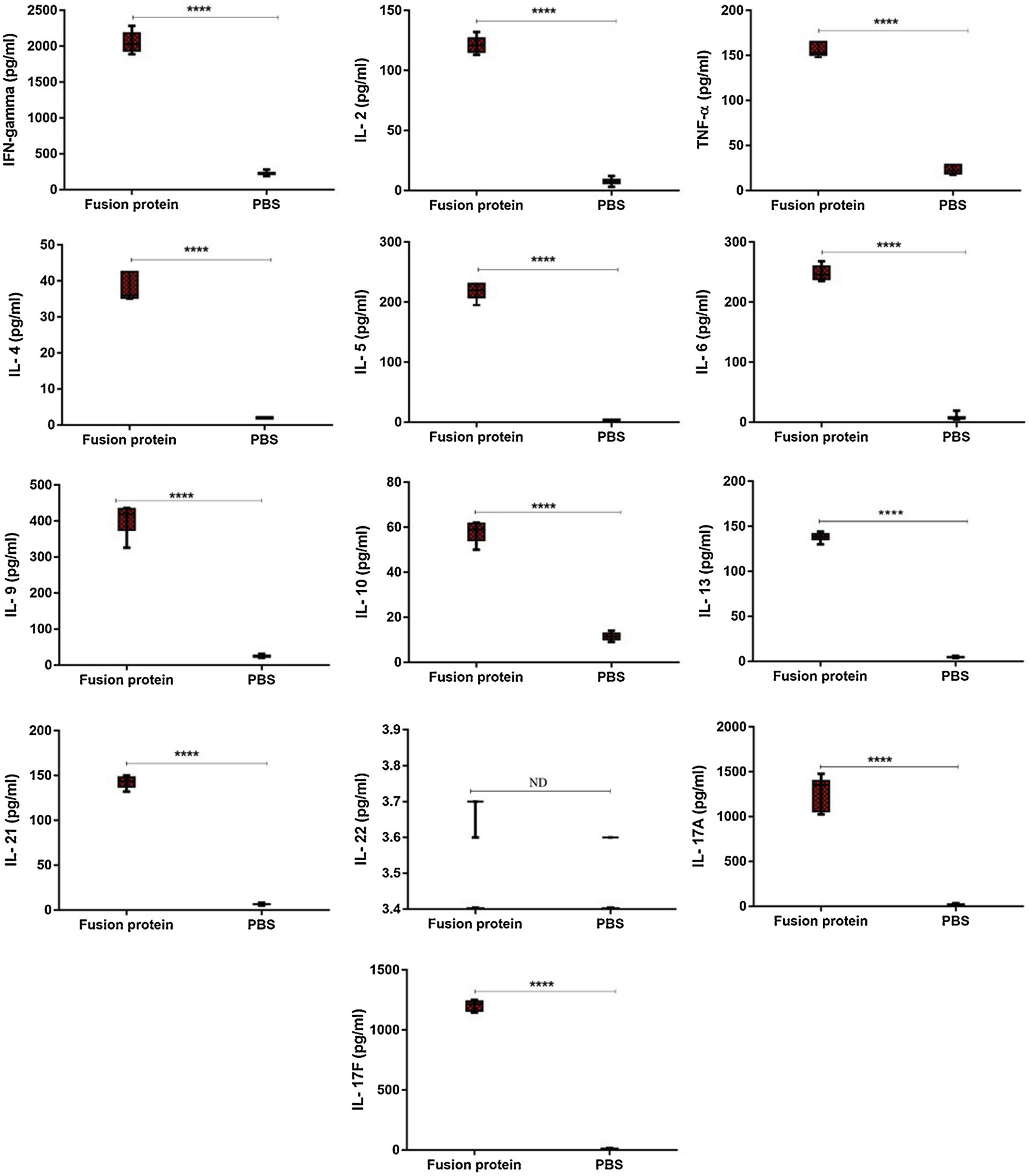

Fig. 5. Cytokines profile following vaccination. Immunized mice were sacrificed 12 days after the last immunization. Splenocytes were prepared and stimulated with the protein for 72 h. The cytokines profile of the Th1 (IFN-γ, IL-2, and TNF-α), Th2 (IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13), and Th17 (IL-6, IL-21, IL-22, IL-17A, and IL-17 F) were assessed. The designed vaccine candidate was able to elicit a mixture of Th1, Th2, and Th17 immune responses more towards Th1 and Th17. ND represents no detectable difference and **** for $p < 0.0001$.

control of the inflammatory induced by bacterial product (Penaloza et al., 2018). Our results were in line with the study conducted by Yu et al. in which immunization of mice with the MntC emulsified with Freund's adjuvant induced high level of IL-4, IL-17 and IFN-γ compared to PBS group (Yu et al., 2018). In the later study, Mancini et al. demonstrated that when *S. aureus* 4C-staph vaccine (detoxified Hla, surface-exposed antigens FhuD2 and Csa1A, and EsxAB) formulated with a novel TLR7-dependent adjuvant, the mice are protected through specific antibodies, effector CD4$^+$ T-cells, and IL-17a (Mancini et al., 2016), which confirmed our results. Other studies in mice have also identified that protection against *S. aureus* infections is Th1/Th17-dependent, which completely in agreement with our study (Lin et al., 2009; Joshi et al., 2012).

We also evaluated the protective efficacy of the multiepitope vaccine candidate against *S. aureus* COL strain in a mouse model. In this challenge, the vaccinated mice reduced the bacterial loads to 10^1-10^3 CFU in the spleen and kidney at 72 h after infection. As there was no protection was observed in the control mice, the protection of immunized mice can be attributed to the vaccine components. The result of the experimental validation of the designed vaccine confirmed immunoinformatics studies.

Overall, we have concluded that preparation of the novel multi-

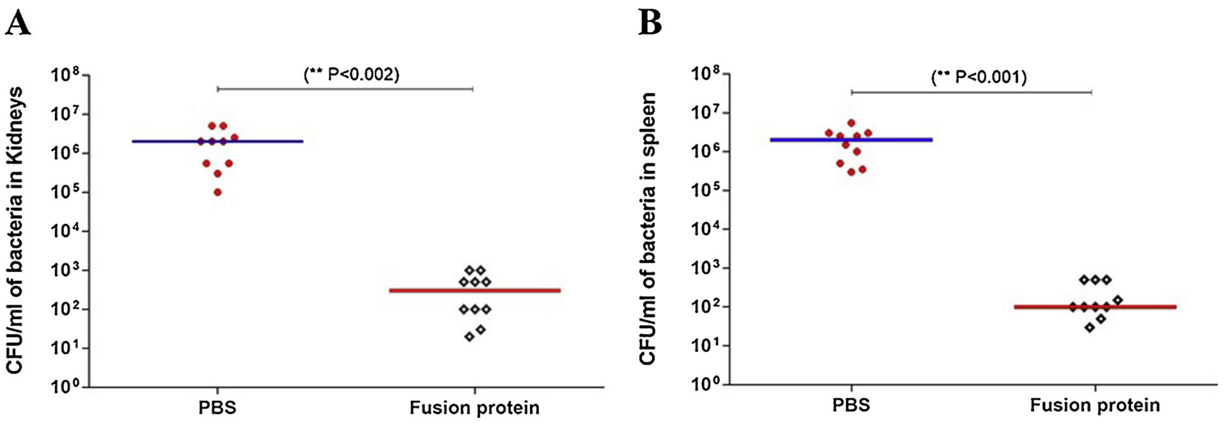

Fig. 6. Functional activity of the specific immune antibodies raised against the designed fusion protein. Two weeks after last immunization, the mice were challenged with *S. aureus* COL strain (5×10^8 CFU) by intraperitoneal injection. 72 h after challenge, the bacterial burden was determined in the kidneys (A) and spleen (B) of mice. The specific antibodies significantly decreased the bacterial load in the infected tissues, compared to control group.

epitope vaccine (Hla, MntC and SACOL0723) against *S. aureus* could elicit high specific IgG titer, and also could induce higher frequencies of Th1, Th2, and Th17 cytokines, but lower percentages of Th2 cytokines. These results suggest that the vaccine candidate have immune stimulatory effects that are distinct, which might play a subtle role in shaping the vaccine-induced immune responses. Also, antisera raised against the fusion protein indicate a desirable influence on the opsonophagocytic killing of the bacterial cells that led to a decrease in the bacterial burden in the spleen and kidneys. According to these findings, the designed vaccine candidate might be suitable for potential protective versus *S. aureus*. More research exploring the protective effect of the candidate vaccine against *S. aureus* in the different mouse model of infection would be welcomed.

Statement of ethics

All experimental procedures and animal care were performed according to guidelines of the European Communities Council (86/609/EEC). Mice were housed for 1 week before the initiation of the experiment.

Competing interests

The authors have no competing interests to declare.

Author contributions

Mehdi Mahdavi and Mohammad Mehdi Aslani conceived and designed the experiments; Khadijeh Ahmadi, Sara Hasanzadeh, and Mehdi Mahdavi performed the experiments; Khadijeh Ahmadi, Ladan Mafakher, and Sobhan Faezi analyzed the data; Khadijeh Ahmadi and Sobhan Faezi wrote the manuscript; Gholamreza Pouladfar, Mehdi Kalani, and Mohammad Reza Pourmand were scientific advisors of the research. All authors read and approved the final manuscript.

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