Recombinant Staphylococcal Antigen-F (r-ScaF), a novel vaccine candidate against methicillin resistant Staphylococcus aureus infection: Potency and efficacy studies

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\textbf{ABSTRACT}

\textit{Staphylococcus aureus} is a human commensal and pathogen, its clinical importance is exacerbated by the spread of multi-drug resistant strains. The potential future failure of antibiotic therapy necessitates the development of novel control regimes, including new immunotherapeutic approaches. \textit{S. aureus} has a large repertoire of surface components with potential for immunological targeting. The aim of this study was to evaluate the efficacy of a novel member of staphylococcal conserved antigen family (ScaF) as a factor to elicit cellular and humoral immunity. To determine the ScaF potential as a vaccine candidate, experimental groups of mice were immunized with recombinant ScaF (r-ScaF) formulated in Freund’s and alum adjuvants or PBS and subsequently challenged in the sepsis model of \textit{S. aureus} disease. The vaccine formulations induced robust cellular cytokines responses, including IFN-\(\gamma\) and IL-17, as well as increased production of IgG2a rather than other subclass of IgGs. Active immunization with r-ScaF with adjuvants led to decreased mortality of infected mice and a lower associated bacterial burden in the internal organs in comparison to the control group. Taken together, our Results indicate to the possibility of the r-ScaF protein to be considered as an important component of a multivalent prophylactic vaccine candidate.

\section{Introduction}

\textit{Staphylococcus aureus} is a commensal bacterium in about one-third of the human population [1,2]. This forms a natural reservoir from which it can cause a variety of infections such as soft tissue infections, endocarditis, osteomyelitis, meningitis, systemic bacteremia, and pneumonia in particular among hospitalized patients [1,2]. \textit{S. aureus} has an ability to acquire antibiotic resistance even against new antibiotics. Methicillin resistant \textit{S. aureus} (MRSA) is considered as a major challenge among infectious diseases around the world [3–5]. This situation is worsened due to emerging vancomycin, daptomycin and linezolid resistant strains [6]. For this reason, the development of effective new therapeutic and/or prophylactic staphylococcal vaccines seem vital [7]. So far, despite a number of clinical trials an effective vaccine has remained elusive [8]. It has been proposed that a vaccine which can induce humoral immunity in parallel to Th1 and Th17 responses may completely eradicate MRSA infections [9]. Although, several antigens have been used as vaccine candidates in animal models limited success has been achieved [10]. The cell wall-associated proteins, MSCRAMM (microbial surface component recognizing adhesive matrix), which are attached covalently to peptidoglycan and ionically bound proteins have been investigated as vaccine candidate [11,12]. Many targets have been tested including fibronectin binding protein (FnBP), collagen binding protein (CnBP), clumping factor B (ClfB), iron-responsive surface determinant B(IsdB) and serine aspartate repeat proteins D & E [SdrD and E] [13–17]. A novel family of staphylococcal conserved antigens A-J (ScaA-J) have been shown to have multiple roles including binding to the host extracellular matrix, cell-wall binding, and cell wall metabolism which shows that having significant roles in the pathogenesis of the MRSA and may be blockade of these types of antigen with immune response.

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responses resulted in elimination of organism and control of infection [18,19]. The Sca family proteins contain putative peptidoglycan hydrolase activity as amidase as well as in one case as a glucosaminidase, all of which associate with the cell wall and can be surface exposed [20,21]. However Sca family proteins have several roles in the life cycle of the bacteria and may be inhibition of these proteins result in control of infection but vaccine study about this protein remained native and according to the structure of physiologic function of Sca family proteins it seems that they can be used as vaccine candidate versus MRSA infection. So, here we hypothesized that may be targeting r-ScaF protein as a vaccine candidate establish protection in the mice after experimental challenge.

In fact, this study concentrated on ScaF, currently of unknown function. We show that r-ScaF is a potential adhesin and is an antigen capable of eliciting protection against S. aureus infection.

2. Materials and methods

Bacterial strain, vector and growth media. The S. aureus COL strain (methylcillin-resistant S. aureus) was obtained from Microbiology lab of Tehran University of Medical Sciences, Tehran, Iran [22]. This strain used for PCR amplification of scaF gene and experimental challenge study. Escherichia coli TOP10F and origami strains were used as preservation and expression hosts. pET21a (+) (Novagen, USA) used as expression vector. Luria Bertani (LB) medium (Merck, Germany), tryptic soy broth (TSB) (Merck) and Blood agar (Merck) were used for preservation and expression hosts. pET21a (+) (Novagen, USA) used as expression vector.

Cloning and Construction of pET21a/scaF expression vector. The scaF gene was amplified from genomic DNA of S. aureus strain COL by using the following primer F 5′-GCGGCCGCTAGCATGTATACGAATGATAGCAAAACATTAG-3′ and R 5′-GCGCGCCTCGAGATGGATGTAAT and selected on LB agar plates containing 100 μg/ml ampicillin. Plasmid clones positive of scaF gene were sequenced for analysis of the sequence integrity.

Expression and characterization of r-ScaF. The colonies containing pET21a/scaF plasmid grown in L of selective LB broth with ampicillin at 37 °C and shaking 200 rpm. While cells reached optical density (OD) at 600 nm (0.6–0.8), the expression of protein was induced by adding 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; Sigma, USA). After 4 h of induction, the cells harvested by centrifugation at 7000 g for 30 min at 4 °C and protein expression was analyzed by 12% SDS-PAGE (Bio-Rad, USA). Also, the separated proteins on the gel was transferred to PVDF membrane (Amersham Biosciences, USA) and characterized by anti His-tag monoclonal antibody conjugated to Horseradish Peroxidase (HRP). The recombinant protein band developed by DAB substrate (Sigma, Saint Louis, MO, USA).

2.1. Purification of r-ScaF protein

The bacteria containing recombinant pET21a/scaF vector grown selective LB broth with ampicillin at 37 °C and shaking 200 rpm to reached optical density (OD) at 600 nm (0.6–0.8). The protein expression was induced by adding 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; Sigma, USA) to the culture and after 4 h, the cells harvested by centrifugation. The prepared pellets used for protein purification under denaturation conditions as a previously described protocol [24]. Briefly, the cell pellets were dissolved in lysis buffer (100 mM NaH2PO4, 10 mM Tris–HCl, and 8 M urea; pH 8). The expressed proteins as inclusion bodies in supernatant were mixed with Ni-NTA resin (QIAGEN, Germany) and then were added to Ni⁺⁺ chelate column. The column was washed with six buffers comprising of a decreasing urea concentration (8, 6, 4, 2, 1, and 0 mM) and recombinant protein was eluted in elution buffer of 50 mM NaH2PO4, 300 mM NaCl, 250 mM imidazole. Recombinant protein fractions were dialyzed against phosphate buffered saline (PBS) to eliminated imidazole. Purity of recombinant protein was checked by 12% SDS-PAGE (Bio-Rad, USA) and protein concentration was measured by Nano Drop spectrophotometer (Thermo Scientific, USA). The purified protein was passed through 0.22 μm filter and then stored at −20 °C until use.

Ligand binding assay. Ligand binding assay was performed as described previously [18]. Briefly, biotinylated human plasma proteins including: fibrinogen, lactoferrin, mucin and fibronectin (Sigma, USA) incubated with r-ScaF to let to permit proteins attachment. Finally, the membranes were exposed with streptavidin conjugated alkaline phosphatase (AP; Sigma, USA) until reactions developed.

Experimental groups and immunization. Six-to-eight week’s old female BALB/c mice (n = 72, Royan Institute; Iran, Tehran) were provided for immunogenicity and efficacy of recombinant vaccine candidate study. Experimental mice were divided into four groups and each one consisting of 18 mice. Mice were immunized three times, with two week intervals, subcutaneously (s.c) with 20 μg of un-formulated purified protein or formulated with Freund’s adjuvant (Sigma, USA) or Alum (Pasteur Institute, Iran) adjuvant. As control group, one group was injected with PBS with same protocol. Two weeks after the last injection, sera of experimental mice were collected and evaluated for specific total IgG antibody and isotyping.

Cell proliferation assay. Two weeks after last immunization, the spleens of immunized mice were removed under sterile conditions and suspended in cold PBS containing 2% fetal bovine serum (PBS; Sigma, USA). RBCs were lysed with lysis buffer (1X RBC Lysis Buffer; eBioscience, USA) and single-cell suspension was adjusted to 4 × 10⁶ cells per milliliters in RPMI 1640 (Gibco, Germany) supplemented with 5% FBS, 4 mM L-glutamine, 0.1 mM non-essential amino acid, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol (2 ME; Sigma, USA), 100 μg/ml streptomycin and 100 IU/ml penicillin. Then 100 μg of cell suspensions were dispensed into 96-well flat-bottom culture plates (Nunc) and stimulated with 2 μg/well of recombinant antigen. Phytohemagglutinin-A (PHA; Gibco, Germany) was used as a positive control and un-stimulated wells were used as negative controls. Cultures were incubated for 72 h at 37 °C in the presence of CO2 (5%). Afterward 20 μl of 5-bromo-2-deoxyuridine (BrdU; Roche, Germany) was added to each well and incubation continued for 18 h. The plates were centrifuged at 300 g for 10 min and supernatant discarded and the plated dried at 60 °C for 30 min. Then the cells permeabilized by adding 200 μl of fixation/permeabilization buffer and then incubated 60 min with 100 μl of anti-BrdU at room temperature. The plates washed 6 times with PBS and 100 μl of TMB was added to the wells and reaction stopped after 5 min with adding 50 μl of 2 N H2SO4. The optical density of each plate measured at 450 nm.

IL-4, IL-17 and IFN-γ cytokines analysis. For cytokines assay, spleen cells suspension of mice adjusted to 4 × 10⁶ cells per milliliters in complete RPMI-1640 (Gibco, Germany) and cultured in 24-well culture plates (Nunc) and stimulated with 10 μg/ml of antigen for 72 h. The quantity of cytokines measured by commercial ELISA kits for interferon-γ (IFN-γ), interleukin-17 (IL-17) and interleukin-4 (IL-4) (Mabtech, Sweden) cytokines according to the company manuals. The standard curve for each cytokine was used to calculate pg/ml of each cytokine.

Specific total IgG and isotyping. Specific antibodies versus vaccine candidate were assessed with an optimized ELISA. The antigen adjusted to 10 μg/ml in PBS and for antigen coating, 100 μl of antigen was added to each well of 96 well-plates and incubated at 4°C for overnight. The plates were washed three times with PBS-T (PBS containing 0.05% tween20) and were blocked with blocking buffer (PBS contain 5% BSA and 0.05% tween20) at 37 °C for 2 h. After washing step, 100 μl of serial dilution of sera (1:100 to 1:25,600) was added to each well and putted at RT. After washing with wash buffer, 100 μl of
HPR-conjugated anti-mouse IgG (Sigma) at a dilution of 1:12000 in blocking buffer was added to each well for 90 min. The plates were washed 5 times and 100 μl of TMB (Sigma, USA) substrate was added to reaction develop. The reaction was stopped by adding 100 μl of 2 N H₂SO₄ and optical density was determined by ELISA reader at 450 nm. The IgG isotypes in mice sera were determined using specific secondary anti mouse IgG1, IgG2a, IgG2b, and IgG3 conjugated to HRP (Sigma, USA) with same protocol.

**Experimental Challenge.** Two weeks after last injection, mice were challenged via tail vein injection of lethal doses of MRSA COL (10⁸ CFUs). Three days after the bacterial challenge, spleen and kidney samples of three mice (per group) were removed and homogenated in aseptically condition. Each homogenate organ was 10-fold serially diluted in PBS and plated onto blood agar containing appropriate antibiotic. Enumeration of bacterial colonies were obtained after 24 h incubation at 37 °C and presented as CFU of organ. The mortality rate of infected mice was monitored daily until day 14 post challenge.

**Statistical analysis.** For statistical analysis, the SPSS 21.0 (SPSS Inc, Chicago, Illinois, USA) and Graph Pad Prism 6 (Graph Pad Prism Software Inc., La Jolla, USA) software’s were used. The ELISA Results and the bacterial loads in the kidney and spleen were analyzed one-way ANOVA, followed by LSD post-test. The survival rate was measured using Kaplan–Meier analysis. A P-value less than 0.05 considered as significant difference.

### 3. Results

**Characterization of r-scaf.** A truncated scaF gene (missing the encoded signal peptide) was amplified, cloned into pET-21a and verified by DNA sequencing (data not shown). After induction using IPTG a protein of approximately 40 kDa was produced and purified by Ni-NTA affinity chromatography approach and verified as having the expected His-tag by Western blot analysis (Fig. 1). Anti His tag monoclonal antibody successfully reacts with r-ScaF. Ligand binding assay Results shown the biological activity of r-ScaF to adhere fibronectin plasma protein but no reaction observed with other same proteins (fibrinogen, lactoferrin and mucin). The strong reactions were observed between obtained serum from cellulitis, UTI and wound infection patients to r-ScaF.

**Lymphocyte proliferation assay.** The lymphocyte proliferation response as shown in Fig. 2 reveals that immunization of mice with r-ScaF formulated in both Freund’s and alum adjuvants significantly increased lymphocyte proliferation compared to the un-formulated r-ScaF and the PBS (P < 0.001) groups. Whatever, there was no statistical difference in the level of lymphocyte proliferation between the r-ScaF in Freund’s and alum adjuvant (P = 0.624) and also the r-ScaF (P = 0.874).

**Results of IL-4, IL-17 and IFN-γ cytokines assay.** IFN-γ, IL-4 and IL-17 cytokines level (Fig. 3A–C) in the supernatant of cultured splenocytes re-stimulated with r-ScaF were evaluated by sandwich ELISA method. As shown in Fig. 3A, mice immunized with the r-ScaF formulated in Freund’s and alum adjuvants produced significantly higher level of IFN-γ cytokine (P = 0.000) versus PBS control group. There was no statistical difference in the level of IFN-γ secretion between Freund’s and alum adjuvants formulation with r-ScaF (P = 0.171). Furthermore, splenocyte from the mice vaccinated with the r-ScaF released higher level of IFN-γ (P = 0.000) compared to PBS control group. Results of IL-17 cytokine showed that, immunization with r-ScaF formulated in Freund’s and alum adjuvants resulted to the higher level secretion of IL-17 versus r-ScaF and PBS groups (P < 0.05) (Fig. 3B). However, there was no statistical difference in the level of IL-17 of between the r-ScaF and PBS control group (P = 0.967). The groups of mice received the r-ScaF formulated in alum and Freund’s adjuvants induced higher level of IL-4 versus PBS control group (P = 0.001) (Fig. 3C). There was no statistical difference in the level of IL-4 cytokine secretion between groups received r-ScaF formulated in Freund’s and alum adjuvants (P = 0.632) and the r-ScaF and PBS control group (P = 0.050).

**Determination of total specific IgG.** Two weeks after the last immunization, mice were bled retro-orbitally and the sera were analyzed by indirect ELISA. As shown in Fig. 4, immunization with r-ScaF formulated in Freund’s adjuvant induced specific antibody until dilution of 1/12800 show significant differences comparison with r-ScaF formulated in alum adjuvants (P < 0.02). The specific total IgG levels of
mice were immunized with r-ScaF formulated in alum adjuvant until dilution of 1/12800 was higher than the PBS control group (P = 0.014). The IgG response in mice that received the r-ScaF with Freund's adjuvant shows significant differences versus other experimental groups.

Specific IgGs Isotyping. The sera of mice immunized with r-ScaF with Freund's adjuvants contained high levels of IgG2a versus other groups (P > 0.001). Serum from r-ScaF with CFA injected mice revealed IgG2b comparison with PBS and r-ScaF alone group (P = 0.001). (Fig. 5A and B). Active immunization of r-ScaF with alum showed high level of IgG1 in comparison to PBS and un-formulated r-ScaF group (P > 0.007) (Fig. 5C). Mice were injected r-ScaF with alum and Freund's adjuvants revealed higher level of IgG3 in comparison to other groups (P > 0.002) (Fig. 5D).

Survival analysis. After last injection, mice were challenged with HA-MRSA strain COL. Immunized mice with r-ScaF/CFA achieved 60% survival as compared with PBS group (P = 0.0045) (Fig. 6). The groups of mice received r-ScaF formulated in alum increased survival (40%) compared to the PBS (10%) groups (P = 0.0475). However two groups of ScaF with alum and or Freund's adjuvants no different between together (P = 0.3241).

Bacterial load in different organs. To determine the efficacy of r-ScaF with or without adjuvants in the diminution of the dissemination of bacteria to the internal organs, 3 day after challenge, kidney and spleen of experimental mice were removed aseptically homogenized mechanically and the bacterial burden then was quantified by the number of colony forming units (CFUs) in each homogenized organ. Immunization of mice with r-ScaF with CFA adjuvants significantly decreased bacterial load in the kidney to mere r-ScaF and PBS groups (P < 0.005) (Fig. 7A). However, there was no statistical difference in the bacterial burden in these organs between the r-ScaF/CFA and the r-ScaF/alum (P > 0.05) groups. In addition, no significant difference observed in the bacterial burden in spleen between the r-ScaF/CFA and the r-ScaF/alum and mere r-ScaF group (P = 0.1) but compared to PBS control were significant (P = 0.000) (Fig. 7B). Immunization of mice with r-ScaF with alum significantly decreased bacterial load in the spleen in comparison to PBS control group (P = 0.001).

4. Discussion

Several antigens can be considered as new vaccine candidates in the prevention and/or treatment of S. aureus infection [25]. In the present work we aimed to determine the protective effects of r-ScaF as potential
vaccine candidate against \( S. \text{ aureus} \) infection. Other aspect of this study was to characterize the attachment of this protein to human plasma proteins. The C-terminal of Sca family proteins between Staphylococcal strains and other Gram positive pathogens are conserved and may be used as vaccine candidate against these pathogens as a universal vaccine [18]. ScaF protein (SACOL0270) belongs to Sca family which is conserved in \( S. \text{ aureus} \) strains [26]. The sacol 0270 is a Staphyloxanthin biosynthesis protein, which is cell wall-associated protein with an unknown function. One of the predicted functions of ScaF is the responsibility for adhesion of bacteria to extracellular matrix [22]. Extra cellular binding proteins have important role in colonization and initiation of pathogenicity by interaction between bacterial and host plasma proteins. Ligand binding assay Results shown r-ScaF may has ability to act as an adhesion factor to fibronectin. Previous studies have shown that Sca family proteins such as ScaB binds to fibronectin and ScaA binds to fibrinogen, lactoferrin, fibronectin and mucin [18]. Based on BLAST analysis the r-ScaF is similar to bacteriolytic proteins [27].

Result of this study demonstrated that active immunization with r-ScaF formulated in adjuvants leads to decrease bacterial burden in the organs in association with better survival against \( S. \text{ aureus} \) infection in murine sepsis model. It has been previously shown that immunization of mice with cell wall associated protein hp2160 has potential to protect mice against \( S. \text{ aureus} \) infection [28].

Furthermore, vaccination of mice with autolysin protein led to significant enhancement of survival rate of mice in comparison to control group, (80% v/v 20% \( P = 0.0437 \), respectively) [29]. Several recent studies have been designed to investigate the role of cellular mediated immune responses against \( S. \text{ aureus} \) infections. Role of IFN-\( \gamma \) cytokine to eradicate \( S. \text{ aureus} \) infection by different mechanisms has been clarified. IFN-\( \gamma \) has shown antagonist effect with immunosuppressive factors that trigger the resistant infections [30]. Cytokine IFN-\( \gamma \) has ability to reduce the load of \( S. \text{ aureus} \) in systemic infections and enhance the bacterium-killing through different ways including: depletion of iron, release of toxic substances or radicals molecules which seems the mechanisms are mainly non-immunologic [31]. Also, IFN-\( \gamma \) cytokine activates macrophages to phagocyte the bacteria and clear the infection.

IL-17A is an important cytokine which produced by Th17 and is able to prevent \( S. \text{ aureus} \) infections. IL-17 cytokine acts as potent neutrophil and macrophage recruitment factor in the kidney and spleen organs by evoke some related chemokine's [32]. Previous studies have suggested that stimulation of Th1/Th17 lymphocytes is essential for protectivity effect of vaccine candidates against \( S. \text{ aureus} \) infection [33]. Lin and co-workers reported that immunization of mice with vaccine formulated in freund's and alum adjuvants have more ability in the stimulation of cellular immune response [34,35]. Other study have been shown vaccine candidates formulated in alum and freund's caused high level of IFN-\( \gamma \) and IL-17A cytokines with same protection result in the \( S. \text{ aureus} \)
IgG2a is representative of cellular immune-response [40] and IgG1 is more related to humoral immunity [41]. Although both subclass antibodies have critical role in phagocytosis [40]. In this study, we found that IgG2a was more than IgG1 and supports the dominancy of Th1 pattern versus humoral immune response as confirmed by IFN-γ cytokine pattern and T lymphocyte proliferation.

In summary r-ScaF skewed the immunity toward a Th1/Th17 response that is urgent for design multicomponent vaccine. Actually, multivalent vaccines should be containing antigens that are immunogenic and have protectivity effect versus experimental infection [42]. Also S. aureus protective vaccine could be established balance between both humoral and cellular immunity. According to our Results the r-ScaF could be used as a protective antigen for designing suitable multivalent vaccine to combat S. aureus infections. But further experiments are still needed to clarify the effect of immunization with r-ScaF on the other aspect of immune response.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micpath.2018.11.036.

References

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