Synthetic selenium nanoparticles as co-adjuvant improved immune responses against methicillin-resistant *Staphylococcus aureus*

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Received: 15 August 2022 / Accepted: 1 November 2022 / Published online: 19 November 2022
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Abstract
Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the leading causes of hospital-acquired infections worldwide, which is resistant to many antibiotics, resulting in significant mortality in societies. Vaccination is a well-known approach to preventing disease. Autolysin, a surface-associated protein in *S. aureus* with multiple functions, is a suitable candidate for vaccine development. As a co-adjuvant, selenium nanoparticles (SeNPs) can increase the immune system, presumably resulting in increased vaccine efficacy. The present study evaluated the immunogenicity and defense of recombinant autolysin formulated in SeNPs and Alum adjuvants against MRSA. r-Autolysin was expressed and purified by the Ni-NTA affinity chromatography. SeNPs were synthetically obtained from sodium dioxide, followed by an assessment of shape and size using SEM and DLS. Balb/c mice were injected subcutaneously with 20 mg of r-autolysin formulated in Alum and SeNps adjuvants three times with the proper control group in 2 weeks intervals. Cytokine profile and isotyping ELISA were conducted to determine the type of induced immunity. Opsonophagocytosis tests assessed the functional activity of the vaccine, and the bacterial burden from the infected tissues was determined. Results showed that mice receiving SeNps and r-Autolysin had higher levels of total IgG and isotypes (IgG1 and IgG2a) and increased cytokine levels (IFN-γ, TNF-α, IL-12, and IL-4) as compared with those only receiving autolysin and PBS as a control. More importantly, mice immunized with SeNps and r-Autolysin exhibited a decrease in mortality and bacterial burden compared to the control group. We concluded that SeNps could stimulate immune responses and can be used as an adjuvant element in vaccine formulation.

Keywords MRSA · Autolysin · Immunogenicity · Synthetic selenium nanoparticle · Alum

Introduction
Multiple-antibiotic-resistant *Staphylococcus aureus* is one of the leading worldwide causes of infections, causing significant mortality in societies (Gould 2005). There are several helpful anti-*staphylococcal* antibiotics targeting bacterial cell walls, including methicillin (in addition to other β-lactams), vancomycin, and distamycin; however, *S. aureus* has developed resistance to all these agents, leading to methicillin-resistant *S. aureus* (MRSA). More importantly, the strains show decreased susceptibility to vancomycin (VISA) and daptomycin (Tiwari et al. 2018), which can cause more problems in treating patients infected with this microorganism.

Due to the antibiotic resistance of MRSA and the incidence of multidrug-resistant strains (Cascioferro et al. 2021), Non-antimicrobial approaches to control MRSA have recently attracted potential interest in immunotherapy (Schaffer and Lee 2008). In this regard, vaccination,
as an old and well-known approach, is one of the possible approaches to preventing resistant *S. aureus*. Successful experience in controlling various infectious diseases in human beings showed that vaccination might be useful in handling this problem. A wide variety of vaccines against MRSA, exerted by targeting different proteins on the organism, have been developed and assessed in animal models or even in humans, none of which showed protection against MRSA. The surface proteins are considered crucial factors for *S. aureus* colonization and virulence. Therefore, recombinant cell wall-anchored antigens have been recommended as potential *S. aureus* vaccine candidates (Kalali et al. 2019).

Autolysin, a surface-associated protein, has both enzymatic (amidase and glucosaminidase) and adhesive functions (Biswas et al. 2006; Heilmann et al. 2005; Houston et al. 2011); it also makes the connection of the cells to a polymer surface (Heilmann et al. 2005), excretion of cytoplasmic proteins (Pasztor et al. 2010), biofilm formation (Heilmann et al. 2003), and separation of daughter cells after cell division (Biswas et al. 2006; Heilmann et al. 2003, 2005; Houston et al. 2011). Autolysin can bind to vironectin (Vn), suggesting its role in colonizing polymer surfaces and colonizing host factor-coated materials and host tissues. Recent studies have well reported the importance of autolysin in *S. aureus* pathogenicity and protective immunity to *S. aureus* infections. Active immunization with Autolysin protein could lead to the development of specific antibodies and humoral immune responses, resulting in decreased bacterial loads and inflammation response and an improved survival rate of experimental mice (Kalali et al. 2019; Haghighat et al. 2017a, b).

Adjuvants, another vaccine component, are critical in promoting the desired type of immune response and protection. While traditional adjuvants, such as Alum, have been exclusively employed clinically to stimulate immune responses, recent studies have shown that nanoparticles (NPs) can improve the effectiveness of vaccines as co-adjuvants (Barnowski et al. 2019; Kang et al. 2019; Kim et al. 2019; Kye et al. 2019; Lim et al. 2020; Tan and Jiang 2019; Vijayan et al. 2019). NPs can increase the immune system against microorganisms and protect the antigens inside them against harsh conditions such as low pH, bile salts, and enzyme activity as a co-adjuvant in vaccine development (Amini et al. 2017; Najminejad et al. 2019; Khatami et al. 2019). Also, frequent bacterial infections and excessive inflammation are still major challenges in treating nosocomial infections. Therefore, antibacterial and anti-inflammatory nanoparticles are always attractive for treating these infections (Ananth et al. 2019; Ren et al. 2022). In recent years, extensive studies have been conducted about nanoparticles and their antibacterial and anti-inflammatory effects on the common agents of nosocomial infections such as MRSA (Ananth et al. 2019; Ren et al. 2022; Xu et al. 2021; Hu et al. 2018).

Selenium nanoparticles (SeNPs), one of the most extensively studied NPs, exhibit immunomodulatory effects and co-adjuvant activity in several vaccine models (Mahdavi et al. 2017). SeNPs have gained attention recently due to their excellent biological properties, similar to selenium ions but in even lower doses and with less toxicity (Shakibaie et al. 2013). SeNPs were demonstrated to increase immune responses in mice (Yazdi et al. 2012). Also, SeNPs has antibacterial activity on some important human pathogens such as *S. aureus* and *P. aeruginosa* (Ananth et al. 2019).

In the present study, we aimed to evaluate the co-adjuvant activity of synthetic SeNPs in combination with Alum to induce cellular and humoral immune responses in the recombinant Autolysin (r-Autolysin) vaccine model of MRSA. Lastly, the animal challenge study investigated the newly-developed vaccine.

**Materials and methods**

**Recombinant autolysin (r-autolysin) production and purification**

The r-Autolysin protein was previously expressed in a bacterial expression system in *Escherichia coli* BL-21 (DE3) and then purified under denaturing conditions using Ni-NTA affinity chromatography. SDS–PAGE and Western blot characterized the expressed protein. The Limulus amebocyte lysate assay (Lonza, USA) was carried out according to the manufacturer’s directions to detect the presence of any remaining endotoxin in the samples. Then the purified proteins were dialyzed against phosphate-buffered saline, and the solution was filtered. After the determination of the protein concentration with the Bradford assay, the samples were stored at −20 °C before use (Kalali et al. 2019; Haghighat et al. 2017a, b).

**Synthetic selenium nanoparticles (SeNPs)**

To synthesize SeNPs, a solution of 5.2 mM selenium dioxide (Merck, Germany) was prepared. An aqueous ascorbic acid solution (5.2 mM) was slowly added to the reaction with continuous stirring at 300 rpm on a magnetic stirrer. After that, the mixture was centrifuged and washed three times with double-distilled water. The physical properties of isolated SeNPs were examined by scanning electron microscope (SEM) and dynamic light scattering (DLS) (Microtrac, Bluewave model, Germany) to evaluate the shape and size of NPs. The zeta potential of the purified NPs was determined using a Zeta sizer MS2000 (Malvern...
Instruments, UK). Finally, a stock solution of SeNPs was prepared (1 mg/mL) in sterile PBS and stored at 4 °C for further use.

**Vaccine formulation**

For vaccine formulation, r-Autolysin was formulated in Alum adjuvant (aluminum hydroxide, Razi Vaccine, and Serum Research Institute, Iran). For this purpose, 10 µg of the vaccine was formulated in 200 µg of Alum adjuvant in water for injection at room temperature to absorb the recombinant protein on the alum gel. After 1 h of mixing the reaction, SeNPs was added to the vaccine, and the response continued for 30 min. At the end of the formulation, the reaction was adjusted with PBS. In the formulated vaccine, 200 µL of vaccine contained 10 and 100 µg of r-Autolysin and SNPs, respectively.

**Experimental groups and immunization**

Six- to eight-week-old Female BALB/c mice were purchased from the Razi Vaccine and Serum Research Institute of Iran (Karaj, Iran). Mice were kept for 1 week before the experiment, fed with food and water ad libitum, and in light/dark (12 h/12 h) conditions according to the animal ethics of the Department of Pharmaceutical Sciences, Islamic Azad University. The mice were divided into three groups, each containing 16 mice. The first group was immunized subcutaneously on day 0 with 200 µL having 10 µg of the vaccine candidate formulated in Alum, SNPs, and r-Autolysin. The second group was immunized subcutaneously with a 200 µL vaccine formulated in Alum adjuvant containing 10 µg of r-Autolysin. The third group, as a control group, was injected with PBS buffer in the same condition. The mice were boosted twice at 2-week intervals (on days 14 and 28). It should be noted that the mice were immunized subcutaneously by ARRIVE guidelines.

Two weeks after the last injection, blood samples were taken from the mice, and the serum was separated using a centrifuge (10,000×g for 10 min) and stored at −20 °C.

**Evaluation of specific total IgG and IgG1/IgG2a isotypes**

An optimized enzyme-linked immunosorbent assay (ELISA) was used to determine the presence of specific antibodies in the sera of the immunized mice. r-Autolysin was coated in 96-well plates (Greiner, Germany) at a concentration of 10 µg/mL in PBS and incubated overnight at 4 °C. The plates were washed three times with washing buffer (PBS containing 0.05% Tween 20, PBS-T) and then blocked with PBS having 2% skimmed milk and 0.05% Tween 20 as blocking buffer for 60 min at 37 °C. The mouse sera were diluted in blocking buffer (1:25 to 1/51,200), and 100 µL of sera were loaded into the plates and then incubated for 90 min at 37 °C. After washing five times, HRP-conjugated anti-mouse IgG (Sigma, USA) diluted 1:8000 was added as an s secondary antibody for 90 min at 37 °C. After then, the plates were washed five times, and tetramethylbenzidine (TMB, Sigma, USA) was used as a substrate to develop the reaction in the dark for 30 min. The reaction was stopped by adding 2NH2SO4, and color density was measured at 450 nm with an ELISA plate reader (Awareness Stat Fax 4200, USA). In addition, the specific IgG isotypes (IgG1 and IgG2a) were assessed with the isotyping kit (goat anti-mouse IgG1, IgG2a) (Sigma, St. Louis, USA) according to the manufacturer’s instruction.

**ELISA for IFN-γ, TNF-α, IL-12 and IL-4 cytokines**

The sera were collected from the mice and used for the cytokine assay. The quantity of IFN-γ, TNF-α, IL-12, and IL-4 cytokines were assessed by ELISA Kits (Mabtech, Sweden) according to the manufacturer’s instruction. According to the standard curve, each mouse’s cytokine quantity was calculated as pg/mL. Furthermore, the ratio of cytokines was calculated by dividing IFN-γ and IL-12 by IL-4 in each mouse group. Additionally, The IFN-γ/TNF-α ratio was also assessed in the disease index.

**Opsonophagocytic assay**

The bioactivity of specifically-developed antibodies was assessed in the opsonization process. For this purpose, the response ingredients, including S. aureus strains COL (OD = 0.2; at 650 nm) (~ 108 CFU/mL in 1% BSA); mouse macrophages (2 × 107/mL); diluted serum samples (1:2 to 1:16) and 4% baby rabbit serum (Iran Blood Transfusion Organization) were incubated at 37 °C for 90 min. The assessment of the opsonic killing activity of the immune sera was reconciled with those of the pre-immune serum. The test was performed in triplicate for each quantity. The percentage of opsonic activity of experimental sera was calculated using the following formula:

Percentage of killed bacteria = \[1 - \frac{\text{CFU of Immune serum}}{\text{CFU of pre-immune serum}}\] × 100.
Challenge studies

Two weeks after the vaccine’s last injection, the mice were intraperitoneally injected with 0.5 mL of endotoxin-free PBS containing $5 \times 10^8$ CFU (three times LD50 dose according to our setup) of *S. aureus* (COL strain). Mice were monitored daily for survival rate up to 30 days after the challenge.

Bacterial burden in the internal organs and pathology

Four mice from each group were sacrificed to assess bacterial burden in internal organs. Seventy-two hours after the challenge, spleens, liver, and kidneys were separated from the mice, ground and homogenized in 1 mL of PBS, and cultured in different dilutions on the LB agar medium to determine the CFU/mL of the colonized bacteria. The second part of the organs was fixed in formalin (10% phosphate-buffered formalin) and sent to the p.a. pathobiology laboratory, where four micrometer-thick sections were prepared and stained with hematoxylin and eosin (H&E) for microscopic examination.

Statistical analysis

Statistical analysis of immune responses was performed using One Way Analysis of Variance (ANOVA), followed by Tukey HSD tests. Kaplan–Meier survival curves and the log-rank test were used for results from challenge experiments using version 8 Prism (GraphPad Software, San Diego, CA, USA) program. The data were represented as mean ± S.D of three separate experiments. All data in this study have been expressed as mean ± SD p values less than 0.05 and were considered statistically significant.

Results

Expression and purification of autolysin

As shown in Fig. 1, results from SDS–PAGE analysis revealed that the highest levels of the r-autolysin protein were produced in *E. coli* BL21 (DE3) by the induction of 1 mM IPTG at 37 °C for 4 h. The expression product of the protein was approximately 43 kDa in molecular size. The Ni-NTA affinity column chromatography performed the protein purification under denaturation conditions. Also, the endotoxin content in the sample was in the normal trend and safe (EU/mL).

Fig. 1 Assay of r-autolysin on SDS–PAGE (12% w/v). Columns 1 and 2, purified autolysin from Ni-NTA agarose chromatography, and column M, the standard protein size marker (kDa). Arrows showed the r-autolysin proteins

Characterization of selenium nanoparticles (SeNPs)

The shape and size of the SeNPs were measured using SEM and DLS. SEM showed that SeNPs had a spherical surface and DLS [under the following conditions: laser wavelength 780 nm, water refractive index 1.33 at room temperature (25 °C)] exhibited the average size of the particle was in the range of 93.87(d.nm) with 0.369 polydispersity index (PDI), demonstrating that the particle size range is below 100 nm (Fig. 2). The surface charge of the synthesized Se NPS was −23.5 as determined by Malvern Zetasizer.

Humoral immune response for specific total IgG

Results from the total antibody titration after the final immunization showed that mice immunized with r-autolysin + SeNPs + Alum had a significant increase in the total IgG concentration at a dilution of 1/25 to 1/25,600, as compared with those receiving PBS (p < 0.0001 to p = 0.0228), and mice immunized with r-autolysin + Alum exhibited a significant increase in the total IgG concentration at a dilution of 1/25 to 1/800, as compared with the control group (p = 0.0001 to p = 0.0287). Furthermore, our result showed that mice immunized with combination r-autolysin + SeNPs + Alum displayed a significant increase in the total IgG concentration at a dilution of 1/25 to 1/25,600, as compared with those vaccinated with the combination r-autolysin + Alum (p < 0.05) (Fig. 3).
Humoral immune response from the specific IgG1 and IgG2a isotypes

Because IgG1 and IgG2a are dependent on the Th2 and Th1 subclasses, respectively, IgG antibody isotypes of r-autolysin were evaluated in experimental groups. The mice receiving r-autolysin + SeNPs + Alum and r-autolysin + Alum had a higher antigen-specific IgG1 isotype, compared with those receiving PBS (p < 0.0001). In addition, the level of IgG1 isotype in the mice immunized with r-autolysin + SeNPs + Alum significantly increased when compared to those immunized with r-autolysin + Alum (p = 0.006) (Fig. 4A). Evaluation of the IgG2a level in mice showed that mice receiving r-autolysin + SeNPs + Alum (p < 0.0001) and r-autolysin + Alum (p < 0.05) had significantly higher IgG2a levels as compared with PBS. Additionally, the IgG2a level in mice receiving r-autolysin + SeNPs + Alum significantly increased compared to those receiving the r-autolysin + Alum (p = 0.0067). It seems that SeNPs in the vaccine formulation improved both IgG1 and IgG2a antibodies responses (Fig. 4B).

Cytokine assay and cellular immune response

IFN-γ, TNF-α, IL-12, and IL-4 cytokines were measured using a quantitative ELISA method. IFN-γ, as a major cytokine in the Th1 network, affects the induction of NK and T cell reaction. Mice immunized with r-autolysin + SeNPs + Alum and r-autolysin + Alum produced high levels of IFN-γ, as compared with those immunized with PBS (p < 0.0001 and p = 0.001, respectively); in addition, a significant difference was found in the level of IFN-γ between the mice receiving r-autolysin + SeNPs + Alum and r-autolysin + Alum (p < 0.0001) (Fig. 5A).
Serum analysis for TNF-α showed that mice receiving r-autolysin + SeNPs + Alum and r-autolysin + alum had high levels of TNF-α, demonstrating a significant difference as compared with those receiving PBS (p < 0.0001 and p = 0.0004 respectively); however, no significant differences were found in the TNF-α levels between mice immunized with r-autolysin + SeNPs + Alum versus r-autolysin + Alum (p = 0.5575) (Fig. 5B).
Serum analysis for IL-12 showed that mice immunized with r-autolysin + SeNPs + Alum produced high levels of the IL-12, showing a significant difference as compared with those receiving PBS (p = 0.0006); however, mice immunized with r-autolysin + alum showed no significant difference with those immunized with PBS (p = 0.6964). In addition, a significant difference was detected in IL-12 levels between r-autolysin + SeNPs + alum and r-autolysin + alum groups (p = 0.0029) (Fig. 5C).

Mice immunized with r-autolysin + SeNPs + alum, and r-autolysin + alum produced high levels of IL-4 as compared with those immunized with PBS (p < 0.0001 and p = 0.0016, respectively); additionally, a significant difference was found in the level of IL-4 between mice receiving r-autolysin + SeNPs + alum and r-autolysin + alum was (p = 0.0012) (Fig. 5D).

**The ratio of IFN-γ/IL-4 is an essential ratio of Th1/Th2 balance**

Mice immunized with r-autolysin + SeNPs + alum and r-autolysin + alum showed higher IFN-γ/IL-4 (p < 0.0001, p = 0.0025 respectively) as compared with those immunized with PBS, showing a significant difference between r-autolysin + SeNPs + alum and r-autolysin + alum in the IFN-γ/IL-4 ratio (p = 0.0235). This indicated cellular immunity (TH1) development in mice immunized with r-autolysin + SeNPs + alum (Fig. 6A).

The IL-12/IL-4 ratio measured showed an increase in mice immunized with the r-autolysin + SeNPs + alum group compared with those immunized with r-autolysin + alum and PBS (p = 0.0344 and 0.0381, respectively), indicating a Th1 response. No significant differences were detected in the IL-12/IL-4 ratio between the r-autolysin + alum and the control group (p = 0.9984) (Fig. 6B).

Because IFN-γ and TNF-α indicate Th1 and inflammation profiles, respectively, the increased IFN-γ/TNF-α ratio showed greater and safer cellular immunity in the absence of inflammation. The IFN-γ/TNF-α ratio in mice immunized with r-autolysin + SeNPs + alun compared with those immunized with r-autolysin + alum and PBS (p = 0.0006, p < 0.0001, respectively), showing no significant differences between mice immunized with r-autolysin + alum and PBS (p = 0.0692) (Fig. 6C).

**Opsonophagocytic assay**

Using the antisera taken from immunized mice, the opsonic killing activity was assessed in vitro (Fig. 7). At dilutions (1/2 up to 1/8), the opsonic activity of vaccinated groups was significantly higher when compared with the control group (p < 0.0001). A significant difference in the opsonic activity (dilutions of 1/2) was observed between mice immunized with r-autolysin + SeNPs + Alum and r-autolysin + Alum (p < 0.05). At serum dilutions ranging from 1:2 to 1:16, sera from mice immunized with r-autolysin + SeNPs + Alum displayed 59–28% opsonic killing activity, respectively.
**Bacterial burden in the internal organs and pathology**

To evaluate the vaccine’s effectiveness, we measured the bacterial load of the three internal mice organs (spleen, kidneys, and liver) 72 h after intraperitoneal challenge with *S. aureus* strain COL. The CFUs were calculated in three dilutions after enumerating the number of colonies on each LB plate. Mice immunized with r-autolysin + SeNPs + alum showed decreased bacterial load in the spleen, kidneys, and liver compared to those immunized with PBS (p < 0.0001). In addition, the bacterial load was significantly decreased in all three internal organs of the mice immunized with r-autolysin + SeNPs + alum compared with those immunized with PBS (p < 0.0001). Mice immunized with r-autolysin + SeNPs + alum and r-autolysin + alum exhibited a significant difference in the bacteria load in the spleen and liver (p = 0.0443 and p = 0.0143, respectively). There was no significant difference between the bacteria load in kidneys among mice immunized with r-autolysin + SeNPs + alum and r-autolysin + alum (p = 0.1431) (Fig. 8).

Histological analysis of internal organs after the challenge with *S. aureus* showed that the risk of MRSA infection is lower in mice immunized with the combination of synthetic SeNPs, r-autolysin, and alum, as compared with other groups. In addition, symptoms, such as inflammation and immune cell infiltration, were significantly lower in mice immunized with synthetic SeNPs, r-autolysin, and alum than in other groups. There were no signs of infection, especially in the kidneys and spleen of this group. Pathological results indicated that the vaccine protects against MRSA (Fig. 9).

**Survival rate**

Two weeks after the last immunization, mice were challenged with an infectious dose of COL strain (5 × 10^8 CFU) and monitored daily for a survival rate of up to 30 days. Results showed that mice immunized with
r-autolysin + SeNPs + Alum reached an 80% survival rate as compared with those immunized with PBS (p < 0.005); in addition, mice receiving r-autolysin + Alum reached a 50% survival rate as compared with the control group (p < 0.001). There was a significant difference in the survival rate between mice immunized with r-autolysin + SeNPs + Alum and r-autolysin + Alum (p < 0.005). Our results showed that there was a significant difference between the mortality rate in the vaccine (20%) and control (100%) groups within 5 days after the challenge (p < 0.005) (Fig. 10).

Discussion

Methicillin-resistant Staphylococcus aureus (MRSA), with the capability of causing various infections such as bacteremia, endocardium, meninges, and skin, and bone infection, is one of the significant strains of Staphylococcus, which is resistant to beta-lactam antibiotics (Kramer et al. 2019). S. aureus is currently considered the most common cause of nosocomial infections and one of the leading causes of death in hospitalized patients (Cascioferro et al. 2021). Because the MRSA epidemic is life-threatening and goes beyond antibiotic therapy, it is essential to develop an alternative approach, such as vaccines, to treat this challenge in an immunoprophylaxis setting (Søe et al. 2017).

There are now several formulation platforms for producing a vaccine against S. aureus, but they suffer from some obstacles. Various reasons can be considered in the form of limitations and shortcomings in developing S. aureus vaccines. Pathogenicity factors are among antigen candidates for developing S. aureus vaccines; however, when used as a single component in the production of vaccines, they cannot provide an adequate immunity against S. aureus infections. The use of killed and attenuated live vaccines, although seeming to be a promising approach, suffers from problems such as the need for appropriate adjuvants in the killed vaccine and ensuring the patient’s safety when attenuated live vaccines are used (Sandi et al. 2015).
In the present study, autolysin was allocated for vaccine structures. Autolysin is an essential protein in *S. aureus* with multiple functions, including cell isolation, cell lysis, hydrolysis of cell surface peptidoglycan, isolation of daughter cells after cell division and biofilm formation, making it a successful potential candidate for vaccine development with the remarkable ability to provide acceptable levels of protection (Kalali et al. 2019; Haghighat et al. 2017a, b; Singh 2014; Foster 1995). Also, various studies on autolysin showed their protective roles in mouse models (Kalali et al. 2019; Haghighat et al. 2017a, b; Nair et al. 2015).

Adjuvant selection for vaccine development is crucial to increasing immunogenicity and stimulating innate immunity, developing the appropriate protective response to combat the microorganisms (Marques Neto et al. 2017). Recent studies have shown that using NPs as adjuvants for increased immune responses is at the center of vaccine researchers’ attention (Barnowski et al. 2019; Kang et al. 2019; Kim et al. 2019; Kye et al. 2019; Lim et al. 2020; Tan and Jiang 2019; Vijayan et al. 2019). SeNPs, as an adjuvant, can significantly improve the parameters of the immune system (Han et al. 2021; Lin et al. 2021). In this study, we investigated the effects of autolysin and SeNPs on cellular and humoral immunity in a mouse model of the *S. aureus* vaccine.

SeNPs, as an adjuvant, enhance the response of total IgG antibodies (Raahati et al. 2020; Yazdi et al. 2015). In this study, the total amount of antibodies after immunization with r-autolysin + SeNPs + alum (at a dilution of 1/25 to 1/25,600) and r-autolysin + alum (at a dilution of 1/25 to 1/800) additives, as compared with the control group, showing a significant increase. In addition, there was a significant difference between mice immunized with r-autolysin + SeNPs + alum and r-autolysin + alum up to a dilution of 1/25,600, demonstrating improved humoral immunity when combined r-autolysin + SeNPs were used (Fig. 3).

IgG1 and IgG2a are representative indexes for Th2 (Mohammadi et al. 2021; Pimentel et al. 2020; Mortazavi et al. 2020) and Th1 (Mortazavi et al. 2020; Paydarnia et al. 2020) responses. Our findings showed that high IgG1 and IgG2a antibodies were obtained in mice immunized with *S. aureus* surface proteins formulated with different adjuvants compared with other experimental groups (Haghighat et al. 2017a, b; Mortazavi et al. 2020). Our results showed that mice receiving the r-autolysin + SeNPs + Alum vaccine had higher levels of IgG1 and IgG2a as compared with those receiving r-autolysin + Alum and PBS group (Fig. 4), representing the effective combination of r-autolysin and SeNPs as the antigen and co-adjuvant in humoral immunity and polarization into a mixture of Th1/Th2 immune responses.

IFN-γ, one of the measured cytokines in this study effective in regulating Th1/Th2 balance, increasing antigen supply, and eliminating pathogenic bacteria by activating macrophages (Osugi et al. 1997; Ahmadi et al. 2021), is a crucial cytokine to determine the type of immune responses against foreign pathogens, which is the main cytokine to prevent and quench infection (Vahdani et al. 2021). Results from this study showed that mice immunized with r-autolysin + SeNPs + alum significantly increased IFN-γ in their serum as compared with other groups (Fig. 5A). In a study, Yu et al. pointed to the role of selenium compounds in increasing IFN-γ in the fight against H1N1 Influenza (Yu et al. 2011).

TNF-α, as an internal pyrogen with a critical role in regulating immune cells, can induce inflammation, fever, cachexia, and apoptotic cell death and respond to sepsis through IL-6 and IL-1-producing cells (Grivennikov et al. 2005; Gough and Myles 2020). Our results showed that the level of TNF-α was significantly higher in mice receiving r-autolysin + SeNPs + alum significantly increased IFN-γ in their serum as compared with other groups (Fig. 5A). In a study, Yu et al. pointed to the role of selenium compounds in increasing IFN-γ in the fight against H1N1 Influenza (Yu et al. 2011).

IL-12, known as a T cell-stimulating factor, can stimulate the growth and function of T cells and the production of IFN-γ and TNF-α from T cells and NK cells (Zheng et al. 2016). Nguyen et al. showed that IL-12 is necessary for
survival from infection with MRSA and that IL-12-mediated protection depends on IFN-γ expression (Nguyen et al. 2015). Our findings demonstrated the effect of SeNPs (as a co-adjuvant) with r-autolysin on increasing the IL-12 level because mice immunized with r-autolysin + SeNPs + Alum had significantly higher levels of IL-12 as compared with the control group. However, mice immunized with r-autolysin + alum group exhibited no significant difference from the control group (Fig. 5C).

IL-4, with various roles, including differentiating B cells into plasma cells and stimulating activated B-cell and T-cell proliferation, is a critical regulator in humoral and adaptive immunity. IL-4 induces B-cell class switching to IgE and up-regulates MHC class II production (Sandova et al. 2020; Silva-Filho et al. 2014). Our results showed that the IL-4 level in mice immunized with r-autolysin + SeNPs + Alum was significantly higher than those vaccinated with r-autolysin + Alum and PBS, indicating the effect of SNP and r-autolysin combination on the induction of the IL-4 cytokine (Fig. 5D).

The ratio of IFN-γ/IL-4, as an essential ratio of Th1/Th2 balance, exhibited a high IFN-γ/IL-4 ratio, indicating the dominance of cellular immunity response. Increased Th1/Th2 balance showed a shift from humoral immunity to cellular immunity. Our results (Fig. 6A) showed that the IFN-γ/IL-4 ratio in mice immunized with r-autolysin + SeNPs + Alum was significantly higher than those immunized with r-autolysin + Alum and PBS, which may indicate the superiority of Th1 and strengthen the cellular immune system through the cytokine network.

IL-12 and IL-4 are the polarizing cytokines of Th1 and Th2, respectively. An increase in the IL-12/IL-4 ratio indicated a Th1 response (Li et al. 2018; Sirenko 2018). Results from this study showed that this ratio in mice receiving r-autolysin + SeNPs + Alum was significantly higher than in those receiving r-autolysin + Alum and PBS, which can indicate the superiority of Th1 response in r-autolysin and SeNPs exposure (Fig. 6B).

IFN-γ enhances Th1 responses, and TNF-α stimulates inflammatory cells and causes inflammation (Osugi et al. 1997; Gough and Myles 2020). An increased IFN-γ/TNF-α ratio indicates that cellular immunity has increased without inflammation, which can be considered a safe immune response. In this study, the IFN-γ/TNF-α ratio in mice immunized with r-autolysin + SeNPs + Alum was significantly increased compared to the r-autolysin + Alum and control groups. While the difference in this ratio between the r-autolysin + Alum and the control group was not significant (Fig. 6C). SeNPs, in addition to increases in the IFN-γ and TNF levels, we’re able to help macrophage activity through increment opsonization; importantly mice receiving vaccine formulation containing SeNPs showed a significantly decreased mortality rate, as compared with the other groups. In addition, the amount of bacterial load in the internal organs (The Liver, Spleen, and Kidney) of mice immunized with r-autolysin + SeNPs + alum was significantly decreased as compared with the control group, and examination of pathological slides from these organs showed a decrease in symptoms of inflammation and bacterial infiltration.

Conclusion

Results from this study showed that although r-Autolysin in MRSA vaccine candidates promotes both cellular and humoral immunity, the addition of SeNPs, as co-adjuvant, to this vaccine formulation significantly enhances cellular and humoral immunity. Due to the decreased bacterial load in the internal organs and increased survival rate, the use of autolysin and SeNPs is highly suggested as a potential candidate for the clinical study of vaccines against MRSA infections.

Acknowledgements

This work is supported by the Tehran University of Medical Sciences and Tehran Medical Sciences, Islamic Azad University, Tehran, Iran.

Author contributions

Experiments were performed by AR. Data analyses were performed by AR and SH. The study was supervised by SH, MHY, and SAB. The original draft was written by AR and SH. The manuscript was reviewed and edited by SH and MHY. The authors read and approved the final manuscript.

Declarations

Competing interests

The authors declare that they have no competing interests.

Ethical approval

Animal study protocols were by the ethical principles and the national norms and standards for conducting Medical Research in Iran. All experimental procedures were approved by Islamic Azad Tehran Medical Sciences University-Pharmacy and Pharmaceutical Branches Faculty.

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