

TARGETING GROUP A STREPTOCOCCUS WITH A RECOMBINANT CHIMERIC VACCINE: INTEGRATING SCPA AND SPEA FRAGMENTS

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**ТАРГЕТНАЯ ВАКЦИНАЦИЯ ПРОТИВ СТРЕПТОКОККА ГРУППЫ А:
РЕКОМБИНАНТНАЯ ВАКЦИНА С ФРАГМЕНТАМИ SCRA И SPEA**

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Abstract

Objective: To develop a vaccine against group A streptococci (*Streptococcus pyogenes*, GAS), the causative agent of a broad spectrum of infections with varying severity.

Methods: The expression vectors pET27 and pQE30 were utilized to clone genes encoding recombinant proteins, which were subsequently affinity-purified. Female mice were immunized subcutaneously twice with the purified polypeptides (20 µg/mouse) formulated with Alum adjuvant (2:1) at three-week intervals. Immune sera were analyzed using ELISA to evaluate antigen-specific responses. Three weeks after the final immunization, mice were challenged intraperitoneally with GAS M1 serotype at a dose of 5×10^7 CFU/mouse. Vaccination efficacy was determined by comparing bacterial clearance in vaccinated versus control animals, assessed by bacterial loads in the spleen at 3 and 15 hours post-infection.

Results: The vaccine candidate is a hybrid recombinant protein comprising fragments from two essential GAS virulence factors: C5a peptidase (ScpA) and SpeA exotoxin. T- and B-cell epitopes from conserved regions, common across multiple GAS serotypes, were identified and included in the construct using bioinformatics tools.

The integration of these epitopes is designed to confer broad-spectrum protection against GAS strains carrying exotoxin, particularly those linked to invasive infections. Immunogenicity studies in mice revealed a robust humoral immune response targeting both components of the hybrid protein. Further evaluation of protective efficacy demonstrated accelerated bacterial clearance in vaccinated animals, with the SpeA fragment playing a significant protective role.

Conclusion: These findings underscore the potential of this recombinant chimeric vaccine as a promising candidate for the prevention of group A streptococcal infections, addressing the critical need for effective prophylactic strategies against GAS-associated diseases.

Keywords: Group A Streptococcus (*S. pyogenes*, GAS), C5a peptidase (ScpA), exotoxin SpeA, recombinant chimeric vaccine, immunogenicity, protectivity.

Резюме

Цель: разработка вакцины против стрептококков группы А (*S. pyogenes*, СГА) – возбудителя широкого спектра инфекций различной степени тяжести.

Методы: Для клонирования генов рекомбинантных белков использовали экспрессионные вектора рЕТ27 и рQE30. Для иммунизации использовали аффинно-очищенные полипептиды. Самок мышей иммунизировали дважды подкожно полипептидами в дозе 20 мкг/мышь с адьювантом Alum (2:1) с интервалом в 3 недели. Образцы иммунных сывороток исследовали методом ИФА. Для оценки специфической защитной эффективности иммунного ответа через 3 недели после последней инъекции мышей внутрибрюшинно заражали СГА М1 серотипа в дозе 5×10^7 КОЕ/мышь. Защитную эффективность вакцинации оценивали путем сравнения скорости бактериального клиренса у вакцинированных и контрольных животных, о чем судили по бактериальной нагрузке в селезенке через 3 и 15 часов после заражения.

Результаты: Конструкция вакцины представляет собой гибридную молекулу, состоящую из фрагментов, полученных из двух ключевых белков СГА: пептидазы С5а (ScpA) и экзотоксина SpeA. Используя биоинформатический анализ, мы идентифицировали и выбрали для включения в состав рекомбинантного белка Т- и В-клеточные эпитопы в консервативных регионах, общих для разных серотипов СГА.

Посредством стратегии интеграции этих фрагментов предполагается усилить защиту от штаммов СГА, несущих экзотоксин, особенно тех, которые связаны с инвазивными инфекциями.

Оценка иммуногенности на моделях мышей продемонстрировала сильный гуморальный иммунный ответ после парентеральной вакцинации, направленный на оба компонента гибридной молекулы. Последующая оценка защитной эффективности против внутрибрюшинного введения СГА выявила ускорение бактериального клиренса у вакцинированных животных, при этом фрагмент SpeA проявлял значительный защитный эффект.

Заключение: Представленные результаты демонстрируют потенциал полученной рекомбинантной химерной вакцины как многообещающего кандидата для разработки вакцины против стрептококков группы А, удовлетворяющей острую потребность в эффективных профилактических стратегиях против патологий, вызванных СГА.

Ключевые слова: Стрептококк группы А (*S. pyogenes*, СГА), С5а пептидаза (ScpA), экзотоксин SpeA, рекомбинантная химерная вакцина, иммуногенность, протективность.

1 Introduction

Group A Streptococcus (*S. pyogenes*, GAS) is widely distributed and is responsible for a spectrum of diseases in humans, exhibiting diverse clinical presentations and varying degrees of severity [25, 30, 20, 38]. These include tonsillitis, pharyngitis, acute respiratory infections, otitis, impetigo, scarlet fever, erysipelas, rheumatic fever, glomerulonephritis, and vasculitis. Less common manifestations encompass necrotizing fasciitis and myositis, enteritis, focal lesions within internal organs, and toxic shock syndrome. According to estimates from the World Health Organization (WHO), GAS infections afflict over 100 million individuals annually, culminating in more than half a million deaths each year due to associated diseases [46].

While GAS remains susceptible to antibiotics, antibiotic therapy alone is insufficient as a primary treatment for rheumatic fever and rheumatic heart disease. It merely reduces the duration of the illness and alleviates symptoms. Furthermore, the widespread use of antibiotics has contributed to the emergence of antimicrobial resistance in GAS [47, 9, 10, 17, 34, 29], further complicating the situation and underscoring the need for new vaccine-based prevention strategies [11, 39].

As of now, licensed vaccines targeting Group A Streptococcus (GAS) antigens are not available; however, significant efforts are underway towards their development [27]. The M protein, a key virulence factor of GAS, represents a primary target for vaccine research and development. Numerous vaccine candidates, predominantly centered around the M protein or its N- and/or C-terminal regions, have been under investigation. Encouragingly, certain candidates, such as a 6-, 26-, and 30-valent M protein-based vaccines, have demonstrated promising outcomes in preclinical assessments and early-stage clinical trials [16, 32].

Other Group A Streptococcus (GAS) surface proteins are also being studied as vaccine candidates. These include proteins such as streptococcal pyrogenic exotoxin (SpeA), fibronectin binding proteins (FBI), *Streptococcus pyogenes* cell wall proteinase (SpyCEP), streptococcal C5a peptidase (ScpA), arginine deiminase (ADI), trigger factor (TF), and streptolysin O (SLO). To date, most of these developments remain at the preclinical research stage [45, 44, 22].

The phenomenon of antigenic drift under the influence of the immune system, leading to constant variability in the antigenic properties of bacteria, necessitates the inclusion of conserved segments of bacterial molecules in vaccines and/or the development of multispecific vaccine compositions. In this regard, the use of recombinant proteins opens up prospects for the development of effective universal vaccine preparations. These pre-designed proteins can ensure good immunogenicity of the vaccine and reduce the risk of immune evasion [41].

Unlike multicomponent vaccines, chimeric vaccines simplify manufacturing processes by eliminating the need to separately produce individual vaccine components. This not only reduces production costs but also improves the efficiency of vaccine production overall. In addition, chimeric constructs can be tailored to target specific factors of the immune system, such as inducing neutralizing antibodies or enhancing T-cell immunity.

Incorporation of specific amino acid regions into chimeric proteins may reduce the risk of toxicity compared to full-length proteins derived from pathogenic bacteria, which may be inherently toxic. Additionally, chimeric proteins offer versatility and can be used in combination vaccines targeting multiple pathogens, highlighting their potential in the fight against co-infectious diseases.

In this article, we present the results of a study of a combined recombinant molecule consisting of a fragment of peptidase C5a and the erythrogenic toxin SpeA of *S. pyogenes* (Dick toxin). Through the strategic integration of these moieties, we expect to achieve enhanced protection against exotoxin-carrying GAS strains, especially those associated with invasive infections. The structure of this chimeric recombinant molecule, designated ScpA-SpeA, was determined through preliminary bioinformatics analysis. The recombinant ScpA-SpeA protein included conserved and immunogenic epitopes of the surface serine protease ScpA and the erythrogenic toxin SpeA.

Following two subcutaneous immunization of mice with the chimeric recombinant molecule, a systemic humoral immune response was elicited. Analysis of specific serum IgG revealed the presence of antibodies targeting both components of the combined molecule in circulation. Furthermore, quantitative assessment of *S. pyogenes* content in the spleens of mice after intraperitoneal infection demonstrated a significantly higher rate of bacterial elimination from the bloodstream of vaccinated mice compared to that in control, non-immunized animals.

The implications of these results are discussed in the context of the potential utility of the developed construct for the prevention of GAS infections.

2 Materials and Methods

Bacterial Strains, Culture Media, and Growth Conditions

The *Escherichia coli* strains M15 and BL21 sourced from the collection of the Institute of Experimental Medicine (St. Petersburg, Russia) were utilized in this study. *E. coli* M15 cells were cultivated in LB medium (Luria Broth) supplemented with kanamycin at a final concentration of 25 µg/ml, and incubated at 37°C with vigorous agitation overnight. For the production of recombinant protein, *E. coli* M15 transformants were cultured in Terrific Broth medium (prepared according to Maniatis) supplemented with ampicillin (100 µg/ml) and kanamycin (25 µg/ml), and incubated at 37°C with vigorous agitation.

Similarly, *E. coli* BL21 cells were cultured in LB medium at 37°C with vigorous agitation overnight. For the production of recombinant protein, *E. coli* BL21 transformants were cultured in Terrific Broth medium (prepared according to Maniatis) supplemented with ampicillin (100 µg/ml), and incubated at 37°C with vigorous agitation.

Streptococcus pyogenes serotype M1, obtained from the collection of the Institute of Experimental Medicine (St. Petersburg, Russia), was cultured in THB (Todd-Hewitt Broth) medium (HiMedia, India) for 24 hours at 37°C under aerobic conditions. Bacterial cells were subsequently washed three times with PBS by centrifugation at 3500 rpm for 20 minutes and concentrated if necessary. The

88 resulting suspension was utilized for peritoneal infection.

89 Method for Quantitative Determination of Bacteria

90 To quantitatively assess bacterial concentration, 10 µl of 10-fold dilutions of
91 the sample were inoculated onto the surface of 5% blood agar in triplicate. The
92 inoculated plates were then incubated for 24 hours at 37°C. For dilutions where
93 colony counting is feasible, the average number of colonies formed was determined.
94 Considering the dilution factor, this number was extrapolated to represent the
95 bacterial concentration per milliliter of the original sample. The bacterial
96 concentration was expressed in colony-forming units per milliliter (CFU/ml) of the
97 sample.

98 Animal Procedures

99 Female inbred Balb/c mice, aged 10 weeks, were obtained from the
100 Rappolovo laboratory animal nursery in the Leningrad region, Russia. The mice
101 were housed under standard laboratory conditions with ad libitum access to food and
102 water.

103 Experimental procedures adhered to the principles outlined in EU Directive
104 2010/63/EU for animal experiments and were conducted in accordance with
105 guidelines and under the supervision of the local biomedical ethics committee.
106 Approval for these experiments was obtained during an ethics committee meeting
107 held on January 28, 2021, as documented in Meeting Minutes 1/21.

108 The mice were humanely euthanized by qualified personnel using the cervical
109 dislocation method, in accordance with institutional ethical guidelines and approved
110 animal care protocols. The health status of the live vaccine- challenged mice was
111 monitored and recorded once a day for ten days post-vaccination. No animal showed
112 any signs of illness following vaccine strain infection. No animals died (without
113 euthanasia) as a result of the experimental procedures.

114 Immunization Protocols

115 Proteins were administered subcutaneously into the dorsal area of mice at a
116 dose of 20 µg/mouse, with two injections given at a three-week interval. Prior to
117 injection, the protein was emulsified in 0.2 ml of PBS along with 0.1 ml of Imject
118 Alum (Thermo Scientific, USA). Blood samples were collected from the
119 submandibular vein on day 18 and on day 39 and centrifuged at 1500×g for 10
120 minutes. The collected sera were then stored at -20°C.

121 Study of the Protective Effectiveness of Vaccination

122 To assess the specific protective efficacy of the immune response, mice were
123 intraperitoneally infected 3 weeks after the final injection with GAS at a dose of 5 x
124 10⁷ CFU/mouse.

125 Spleens were collected in 3 and 15 hours post infection and homogenized in
126 PBS using a Retsch MM-400 vibrating ball mill. Serial 10-fold dilutions of the
127 homogenates were prepared in PBS, and aliquots of the dilutions were plated on
128 solid nutrient medium (Columbia agar with 5% human red blood cells). The plates
129 were then incubated at 37°C for 14–16 hours, and colonies were subsequently
130 counted under a microscope. The bacterial load in CFU per organ was calculated
131 and expressed as log₁₀.

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA analysis was conducted following a previously described protocol [13]. Maxisorb 96-well plates (Nunc; Denmark) were coated with 0.5 µg/ml of the corresponding protein in 0.1 M sodium carbonate buffer, pH 9.3, overnight at 4°C. Samples (100 µl) were added to duplicate wells in a series of two-fold dilutions and incubated for 1 hour at 37°C. Following each step, the plates were washed with blocking buffer (0.05% Tween 20 in PBS). Serum and reagents were diluted using the same buffer. HRP-conjugated goat anti-mouse IgG antibodies (Sigma, USA) were added at a volume of 100 µl per well. After incubation at 37°C for 1 hour, the plates were developed with 100 µl per well of TMB substrate (BD Bioscience, The Netherlands). Color development was observed after 20 minutes of incubation, and the reaction was halted by adding 30 µl of 50% sulfuric acid. ELISA endpoint titers were determined as the highest dilution yielding an optical density at 450 nm (OD450) greater than the mean OD450 plus 3 standard deviations of the negative control wells.

Bioinformatics Analysis

Analysis of Group A Streptococcus (GAS) DNA and proteins was conducted utilizing the NCBI non-redundant (nr) database and the BLAST tool [6], both of which are freely accessible resources. DNA primer design was facilitated by the Primer 3.0 computer program. Protein sequence analysis for B cell and T cell epitopes was performed using the Immune Epitope Analysis Database and Resource (IEDB), a freely available tool.

Genetic Engineering Procedures

A chimeric gene, *scpA-speA*, 1536 bp in length, was chemically synthesized with built-in BamHI and HindIII restriction sites and initially cloned into the vector plasmid DNA pAL2-T (Evrogen, Russia).

Fragments of the *scpA* and *speA* genes were obtained via polymerase chain reaction (PCR) using the primers listed in Table 1. The primer sequences were designed to incorporate BamHI and SacI restriction endonuclease sites for efficient cloning.

Subsequently, the resulting *scpA-speA* gene and *scpA* gene fragment were cloned into the expression vector pQE-30 (The QIAexpress System, Qiagen, USA). Additionally, the *speA* gene fragment was cloned into the expression vector pET-27.

Purification of Recombinant Proteins:

Cultures of *E. coli* strains M15 and BL21 were grown in Terrific Broth medium and induced by the addition of an IPTG solution. Following induction, cells were lysed using an ultrasonic disintegrator (Soniprep 150plus, MSE, UK). The resulting cell lysate was then applied to a Ni-Sepharose column for the purification of recombinant proteins. Following purification and determination of protein concentration using the Lowry method, the product was stored at -20°C.

Immunoblotting Analysis

Immunoblotting was performed according to standard protocols. Briefly, protein samples were separated by SDS-PAGE using a 12% polyacrylamide gel and transferred onto a nitrocellulose membrane using a semi-dry transfer system. The

membrane was then blocked with 5% non-fat milk in Tris-buffered saline with 0.1% Tween-20 (TST) for 1 hour at room temperature. After blocking, the membrane was incubated with primary antibodies (sera obtained from mice immunized with recombinant protein ScpA, SpeA or ScpA-SpeA) against the target protein overnight at 4°C. Following primary antibody incubation, the membrane was washed three times with TST and then incubated with horseradish peroxidase-conjugated secondary antibodies (Sigma, USA) for 1 hour at room temperature. After three additional washes with TST, protein bands were visualized using staining HRP substrate - 3, 3'-diaminobenzidine (DAB).

Statistical Data Analysis

Data normality was assessed using the Shapiro–Wilk test, and statistical significance (p-value) was determined using a Student's t-test. Results are presented as mean \pm SEM. Statistical analysis was conducted using the statistical module of GraphPad Prism 6 software (GraphPad Software, Inc., San Diego, CA, USA). p-values < 0.05 were considered significant.

3 Results

The chimeric recombinant molecule investigated in this study was engineered from two components corresponding to the immunogenic regions of streptococcal proteins. It comprised a fragment of peptidase C5a (ScpA), an enzyme expressed on the bacterial surface that cleaves and deactivates human complement factor C5a, and a segment of the SpeA gene encoding the erythrogenic toxin of group A streptococci. Both proteins are recognized as virulence factors for group A streptococci.

The efficacy of antibacterial polypeptide vaccines is widely acknowledged to hinge upon the quality and specificity of the induced immune response. The specificity of a vaccine can be broadened by incorporating conserved regions into the vaccine molecule that are shared across a diverse array of serotypes of pathogenic bacteria.

With these considerations in mind, a preliminary bioinformatic analysis was conducted, facilitating the identification of conserved regions within the amino acid sequences of ScpA and SpeA. These regions were found to harbor T and B cell-dependent epitopes, thereby enhancing the potential specificity and efficacy of the vaccine.

The amino acid sequences used for bioinformatic analysis were retrieved from the NCBI Protein database [28] using the following accession numbers: WP_011055051.1, WP_011054794.1. These sequences correspond to the proteins ScpA and SpeA from GAS strain MGAS315. The NCBI Protein database version used was the latest available at the time of retrieval. Conserved regions of the proteins were selected based on sequence alignment using BLAST (Basic Local Alignment Search Tool) [6] with a conservation threshold of 100% identity.

The selected ScpA and SpeA protein sequences corresponded to the ScpA and SpeA sequences at positions 42–366 and 57–229, respectively (Figure 1).

Subsequently, within the identified regions, analysis using the IEDB immune epitope database revealed the presence of top-ranking B-cell epitopes, alongside T-

cell-dependent epitopes that exhibited a predicted strong binding affinity to MHC II molecules (Figure 2).

During the construction of the vaccine molecule, functionally active regions were intentionally excluded from the sequences of streptococcal proteins. Specifically, the conserved active site of ScpA, characterized by three amino acid residues (Ser-Asp-His), was altered due to the absence of serine residue No. 512 [14]. Additionally, the selected SpeA structure lacked Glu-33, which is essential for the formation of a zinc binding site by the SpeA protein, necessary for MHC class II recognition [3]. These alterations were made to prevent the retention of functional activity in the vaccine molecules. The two amino acid sequences were linked together using the linker region GGGGGGSSS (Table 2).

The amino acid sequence of the chimeric recombinant protein ScpA-SpeA was translated into a nucleotide sequence optimized for expression in *E. coli*. The synthesis of the nucleotide sequence of the gene encoding the chimeric protein, the principle of its cloning into the pQE-30 plasmid, the selection of transformant clones, the preparation of the chimeric recombinant protein, and its purification are detailed in the "Materials and Methods" section. The resulting chimeric recombinant protein ScpA-SpeA exhibited a molecular weight of 70 ± 0.5 kDa (Figure 3).

Strains producing components included in the chimeric protein ScpA-SpeA were constructed, and recombinant ScpA and SpeA proteins were obtained as described in the "Materials and Methods" section (Figure 3).

In an experiment conducted on Balb/c mice, the immunogenicity of the chimeric recombinant protein was assessed in comparison to its constituent fragments, ScpA and SpeA. The experimental protocol is illustrated in Figure 4.

Mice were subcutaneously immunized with the three proteins, and the IgG antibody levels in the blood serum were evaluated using ELISA after single and double immunization. ELISA analysis of the humoral immune response revealed that both the GAS chimeric proteins and recombinant analogs of its components, when administered twice, induced the accumulation of IgG antibodies in the blood serum. Importantly, the immune response to the chimeric molecule resulted in the production of antibodies specific to both components of the chimera - C5a and SpeA. Furthermore, antibodies to C5a and SpeA in the ELISA assay interacted with the chimeric molecule immobilized on the bottom of the plate (Figure 5).

The ELISA findings were corroborated through immunoblotting (Figure 6A). The results indicate that the serum of mice immunized with the chimeric protein harbors antibodies capable of binding not only to the chimeric protein itself but also to the recombinant fragments of the peptidase ScpA and SpeA. Additionally, sera obtained from mice immunized with individual components of the combined protein exhibited binding to it in immunoblotting (Figure 6B).

A comparative investigation into the protective efficacy of the chimeric protein and its individual recombinant components against *S. pyogenes* infection was conducted through intraperitoneal administration of a suspension of GAS serotype M1 at a dose of 5×10^7 per mouse, followed by monitoring the bacterial count in the spleen of mice at 3 and 15 hours post-infection (Figure 7).

Mice subjected to standard immunization were intraperitoneally infected with a bacterial suspension. *S.pyogenes* load in the spleens was assessed at 3 and 15 hours post-infection. For detailed procedures, refer to the Materials and Methods section. Bacterial load were expressed as log10 and presented as mean \pm SEM on the ordinate axis, (*)— $p < 0.05$.

After 15 hours following the onset of infection, a notable increase in the rate of clearance of intraperitoneally administered GAS was observed in mice vaccinated with both the GAS chimera and its components – ScpA and ScpA.

Thus, it has been demonstrated that the combined protein ScpA-SpeA, comprising fragments of C5a peptidase and erythrogenic toxin, displays immunogenicity upon subcutaneous administration twice to mice, eliciting a robust humoral immune response. IgG antibodies, specific to the chimeric protein itself, as well as its individual components, accumulated in the blood serum. Following intraperitoneal infection caused by GAS serotype M1, accelerated elimination of streptococcus from the bodies of mice immunized with the chimeric protein and its individual components was reliably observed.

4 Discussion

The World Health Organization (WHO) has articulated the need to create an effective vaccine against Group A Streptococcus (GAS), which has led to increased research in this area [46]. The investigation of the M protein and its components as vaccine candidates has advanced to the stage of clinical trials [32]. Additionally, progress has been made in the development of other non-M protein vaccines [45, 44, 22].

To ensure reliable neutralization or elimination of GAS, researchers frequently test multicomponent combinations of various streptococcal proteins [36, 37, 35, 5, 12]. An alternative to mixing vaccine proteins into a single formulation is the production of combined recombinant proteins. These are composed of specially selected fragments of various GAS proteins that possess specific properties such as varied specificity, immunogenicity, conservativeness and safety.

We constructed a two-component protein consisting of tandem fragments of two GAS proteins: C5a peptidase (ScpA) and SpeA. Both proteins have been extensively studied as vaccine candidates due to their critical roles as major virulence factors for GAS [31].

Protein fragments were carefully selected to include conserved regions of the molecules containing T- and B-cell epitopes. This approach ensures the elicitation of a robust immune response following vaccination.

The production of composite recombinant vaccine formulations represents a prominent avenue in the advancement of protein vaccine manufacturing technologies [7].

Among protein GAS vaccines, polyvalent recombinant formulations comprising tandem variable sequences of N-terminal fragments of the M protein from various GAS serotypes occupy the forefront. However, given that the literature has described over 200 M serotypes of *S. pyogenes* to date, the feasibility of

developing a vaccine capable of targeting all serological variants of GAS appears challenging [1].

Expansion of the serological specificity of the vaccine preparation can be achieved by including conserved protein regions in the vaccine molecule that are present in all or the most virulent and epidemiologically relevant strains of streptococci. The C5a peptidase shows a significant degree of conservation and is found in all members of the different GAS serotypes. C5a peptidase is a surface protein of GAS that plays an important role in immune evasion by cleaving the host chemotactic peptide C5a and interfering with the development of the host inflammatory response [23, 19].

The amino acid sequence of C5a peptidase shares 98% similarity between *S. pyogenes* and *Streptococcus agalactiae*, suggesting the potential for vaccine-mediated prevention, including against Group B streptococcus (GBS) infections [22].

Conversely, SpeA, encoded by temperate bacteriophages, is prevalent in the genome of the majority of GAS strains, contributing to the invasive properties of streptococci [21, 26].

Incorporation of SpeA, a known streptococcal pyrogenic exotoxin produced by GAS, into the vaccine molecule is expected to provide protection against the risk of invasive infections.

Regions exhibiting a high degree of homology, characterized by significant sequence conservation or alignment scores, were identified in the amino acid sequences of ScpA and SpeA using the BLAST tool. These regions were incorporated into the corresponding proteins of GAS serotypes associated with increased virulence across various clinical settings (serotypes M1, M3, M12, M18, M89).

The composite molecule was formulated to mitigate the risk of enzymatic activity associated with ScpA and the mitogenic activity linked to SpeA. Notably, the selected conservative fragment of ScpA lacked amino acid sequences responsible for the formation of the enzyme's active center [14].

A mutation was introduced into the nucleotide sequence encoding the SpeA fragment to induce an amino acid substitution at Glu 33. This mutation, as indicated by Baker et al. (2001), results in a significant reduction in the mitogenic properties of the toxin [3].

According to bioinformatics analysis, the recombinant polypeptides ScpA and SpeA, as well as the linear sequence of the studied combined molecule, contained a substantial number of B- and T-dependent epitopes essential for eliciting a robust T-dependent immune response. This was corroborated by the results of immune response assessments following the first and second immunizations.

Just two weeks after the initial subcutaneous administration, IgG antibodies to the test antigens were detected in the blood of mice. Subsequent immunizations led to increased antibody levels (Figure 5), indicating the T-dependent nature of the immune response to all test antigens.

Specific IgG targeting epitopes of ScpA and SpeA were observed in the blood of mice immunized with the chimeric protein (Figure 5). This was demonstrated in ELISA assays, where both the chimeric protein and its components were adsorbed onto the plate surface.

The data were corroborated by the results of immunoblotting. In immunoblotting, IgG antibodies present in the serum of mice vaccinated with the chimeric molecule showed binding affinity not only to the chimera but also to both individual polypeptides ScpA and SpeA (Figure 6A). IgG antibodies targeting ScpA and SpeA interacted with the chimeric protein molecule (Figure 6B). For practical application of the ScpA-SpeA vaccine molecule, additional assessment of the safety of antibodies formed against spatial determinants resulting from the secondary and tertiary folding of this artificial protein will be necessary.

The protective efficacy of the induced immune response was evaluated using a model of peritoneal GAS infection in CBA mice. GAS M1 serotype expressing SpeA was selected for the assessment of the vaccine protectivity. For this purpose we have chosen the peritoneal infection model. The reason for this choice was based on the inability of rodent models to establish significant oropharyngeal colonization of GAS or exhibit symptomatic infection. Since GAS is a human-specific pathogen, the lack of a native animal model for studying GAS pathogenesis has also posed challenges for GAS vaccine development [2].

The protective efficacy of vaccination was evaluated by assessing the rate of bacterial clearance from the bodies of vaccinated and control animals, as indicated by the bacterial load in the spleen. A comparative analysis of the protective effectiveness was conducted not only for the chimeric vaccine but also for its individual components.

Three hours post intraperitoneal administration, no significant differences were observed in the spleen infection levels among the studied groups, although there was a trend towards accelerated bacterial elimination in mice vaccinated with ScpA (Figure 7). However, after 15 hours, a significant reduction in bacterial load was evident in the spleens of mice across all three immunized groups compared to the control.

Anti-C5a peptidase antibodies play an essential role in the immune response to GAS infection by neutralizing the ScpA enzyme, thereby restoring the function of C5a. This leads to enhanced recruitment and activation of immune cells, improved phagocytosis, and overall more effective clearance of the bacteria, reducing the pathogen's ability to evade the host immune system [23].

In line with classical concepts of infectious immunology, bacterial elimination primarily occurs through effective opsonophagocytosis, which serves as the principal mechanism for limiting bacterial infection [24].

There is conflicting information regarding the opsonizing potential of ScpA-specific antibodies. Evidence suggests that immunization with the five-component vaccine preparation Combo5, which includes ScpA, leads to the formation of non-opsonizing antibodies [36]. Conversely, other data indicate that antibodies generated by a five-component vaccine of similar composition possess opsonizing

properties [4]. Additionally, according to Cheng et al., IgG against the C5a peptidase of GBS are effective opsonins [8, 33].

This classical logic is less straightforward when considering the protective efficacy of the immune response elicited by vaccination with a pyrogenic exotoxin, which is an excreted protein.

Several studies have explored the potential use of exotoxins as vaccine components. There is clinical observations documented in the literature that antibodies that neutralize streptococcal toxins protect patients from toxic shock [15, 18].

There is experimental evidence indicating that immunization with SpeA prevents the development of experimental nasopharyngeal infection of mice. According to authors, the proposed mechanism of infection suppression they recorded possesses a specific nature. ScpA targets and activates V β -specific T cells, leading to the remodeling of the nasopharyngeal environment essential for initiating the early stages of GAS colonization. Specific antibodies capable of neutralizing SpeA prevent the formation of this environment, thereby impacting the early stages of colonization and preventing infection development [48].

Previously R.G. Ulrich described a protein vaccine based on a recombinant fusion protein of streptococcal pyrogenic exotoxin B (SpeB) and streptococcal pyrogenic exotoxin A (SpeA) [43, 42].

Vaccination of susceptible to GAS infection HLA-DQ8 transgenic mice with the SpeA-SpeB fusion protein protected against a challenge with the wild-type SpeA that was lethal to naïve controls. Vaccinated mice were protected from a lethal *S. pyogenes* infection. The authors recorded the protective effect based on mortality rates in the vaccinated and control groups and did not monitor bacterial infection.

The authors of the aforementioned studies discussed the protective mechanisms following SpeA immunization within the context of neutralizing the free pyrogenic toxin with antibodies and mitigating its pathogenetic effects. In our investigation, the intraperitoneal administration of GAS simulated an invasive infectious process. Bacteria within the peritoneal cavity could enter the bloodstream, either directly through compromised blood vessels or indirectly via the lymphatic system, ultimately reaching the spleen. Remarkably, after just 15 hours, a marked reduction in bacterial content was observed in the spleens of SpeA-immunized mice compared to controls, indicating an accelerated clearance of bacteria in transit through the bloodstream toward the spleen. The accelerated clearance of GAS from the circulation in the SpeA-vaccinated group could be attributed to the process of opsonophagocytosis.

It can be hypothesized that during the excretion process, the pyrogenic exotoxin SpeA remains on the bacterial cell surface for a certain duration, ample enough for opsonization to occur.

Indeed, it is plausible that SpeA could transiently associate with various components of the GAS cell wall, including peptidoglycan, teichoic acids, lipoteichoic acids, as well as adhesins and surface proteins. Such interactions may facilitate its retention on the bacterial surface, thereby extending the window of

exposure for potential opsonization and subsequent clearance by the immune system [40]. We suggest that the planctonic variants of GAS which are dominating during to the invasive process might be more sensitive to such opsonization and clearance. A confident statement will require direct evidence however from our observations, it can be cautiously assumed that the protective potential of SpeA in the vaccine preparation may be realized not only through neutralizing the pyrogenic exotoxin with specific antibodies but also by accelerating the elimination of *S. pyogenes* via opsonophagocytosis.

4 Conclusions

Our results suggest that the ScpA-SpeA chimeric protein could be a valuable vaccine candidate for controlling GAS infections. The presence of conserved ScpA and SpeA fragments in the studied molecule implies the effectiveness of the immune response against multiple isolates of *S. pyogenes* due to extensive antibody cross-reactivity. This aspect warrants further exploration.

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ТАБЛИЦЫ

Table 1. Oligonucleotide Primers.

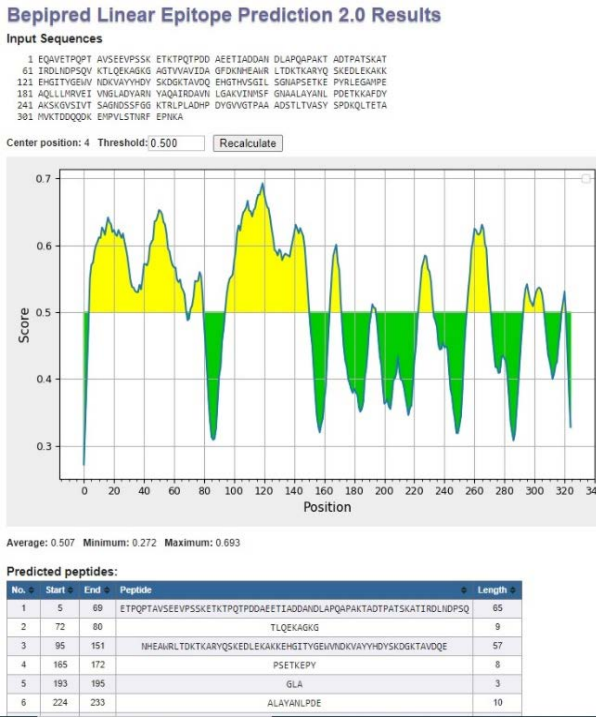
Primer Name	Sequence
Scpafor	TCC TGG ATC CGA ACA AAC CGT AGA AAC TCC AC
Scparev	TCT TGG AGC TCG GCT GTT TTG ACC GTA GCA GT
spefor	GGA TCC AAG CCA ACT TCA CAG ATC TAG T
sperev	GTA AGC TTC AAT TTG GCT TGT GTT TGA

Table 2. Amino Acid Sequences of Recombinant Polypeptides.

Recombinant Polypeptide	Amino Acid Sequences
ScpA-SpeA	MRGSHHHHHHGGSEQAVETPQPTAVSEEVPSSKETKTPQTPDDAEETIADDANDLAPQAPAKTADTPATSKATIRDLNDPSQVKTLQEKAGKGAGTVVAVIDAGFDKNHEAWRLTDKTKARYQSKEDLEKAKKEHGITYGEWVNDKVAYYHDYSKDGKTAVDQEHGTHVSGILSGNAPSETKEPYRLEGAMPEAQLLLMRVEIVNGLADYARNYAQAIRDAVN LGAKVINMSFGNAALAYANLPDETKKAFDYAKSKGVSIVTSAGNDSSFGGKTRLPLADHPDYGVVGTPAAADSTLTVASYSPDKQLTETAMVKTDDQQDKEMPVLSTNRFEPNKAGGGGGGGSSSGDPVTHE NVKSVDQLLSHDLIYNVSGPNYDKLKTTELKNQEMATLFDKKNVDIYGVEYYHLCYLCENAE RSACIYGGVTNHEGNHLEIPKKIVVKVSIDGIQSLSFDIETNKKMVT AQELDYKVRKY LTDNKQLYTNGPSKYETGYIKFIPKNKESFWFDFFPEPEFTQSKYLMISLIS
ScpA	MRGSHHHHHHGGSTPQPTAVSEEAPSSKETKTPQTPDDAGETVADDANDLAPQAPAKTADTPATSKATIRDLNDPSQVKTLQEKAGKGAGTVVAVIDAGFDKNHEAWRLTDKTKARYQSKEDLEKAKKEHGITYGEWVNDKVAYYHDYSKDGKTAVDQEHGTHVSGILSGNAPSETKEPYRLEGAMPEAQLLLMRVEIVNGLADYARNYAQAIRDAIN LGAKVINMSFGNAALAYANLPDETKKAFDYAKSKGVSIVTSAGNDSSFGGKTRLPLADHPDYGVVGTPAAADSTLTVASYSPDKQLTEILKLN
SpeA	MKYLLPTAAAGLLLLAAQPAMAMDIGINSDPSQLHRSSLVKNLQNIYFLYEGDPVTHENVKSVDQLLSHDLIYNVSGPNYDKLKTTELKNQEMATLFDKKNVDIYGVEYYHLCYLCENAE RSACIYGGVTNHEGNHLEIPKKIVVKVSIDGIQSLSFDIETNKKMVT AQELDYKVRKY LTDNKQLYTNGPSKYETGYIKFIPKNKESFWFDFFPEPEFTQSKYLMYKDNETLDSNTSQIRKLAAALEHHHHHH

Figure 1. Bepired liner epitope prediction of ScpA and SpeA.

ScpA



SpeA

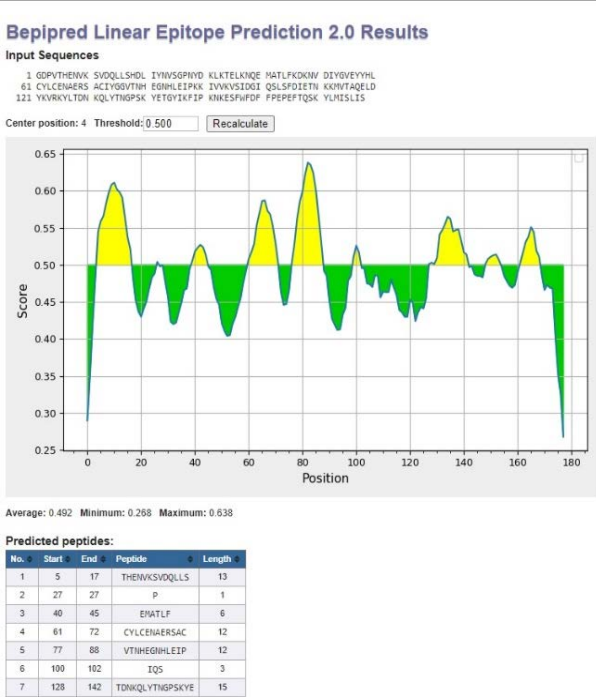
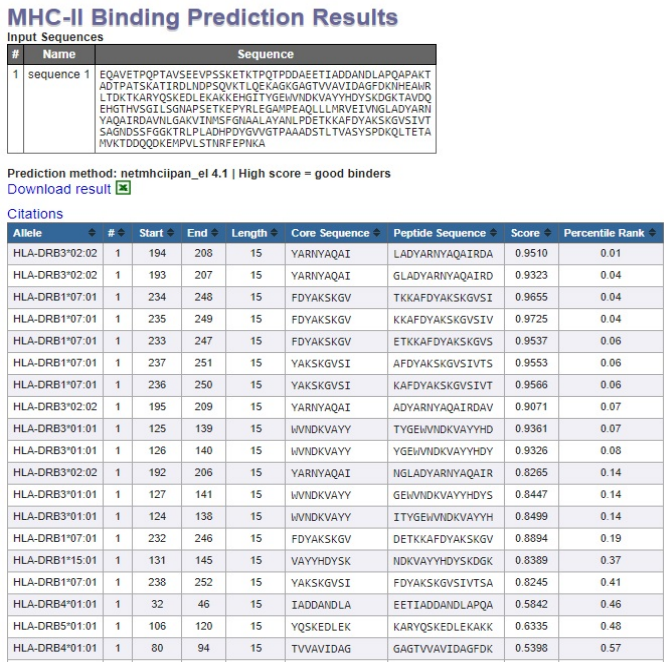


Figure 2. The IEDB immune epitope database analysis of ScpA and SpeA



SpeA

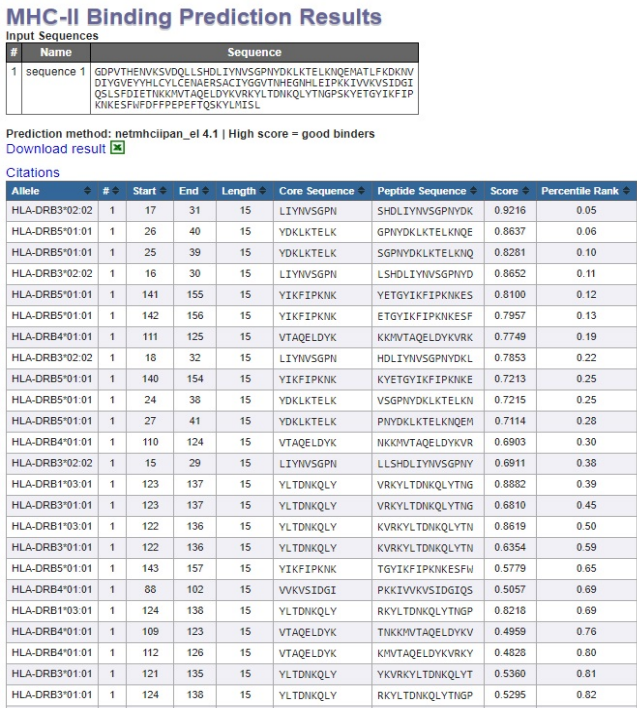


Figure 3. SDS-PAGE of Recombinant Polypeptides.

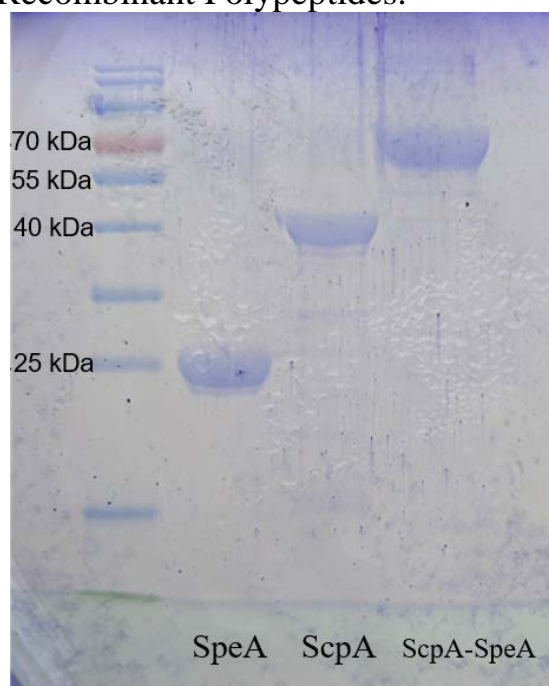


Figure 4. Schematic representation of the immunization protocol for mice followed by evaluation of the protective efficacy of the induced immune response. For detailed procedures, refer to the "Materials and Methods" section.

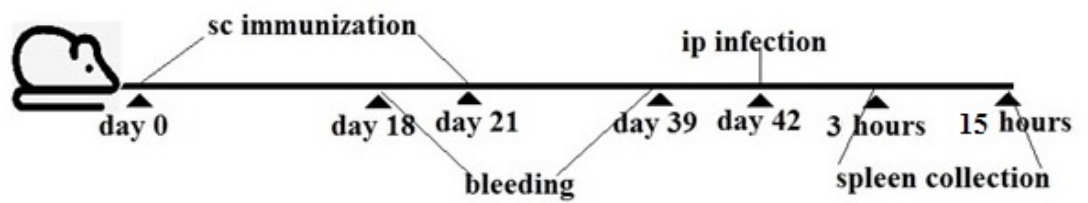


Figure 5. Sera analysis post-immunization: specific IgG response to ScpA-SpeA molecule detected in ELISA.

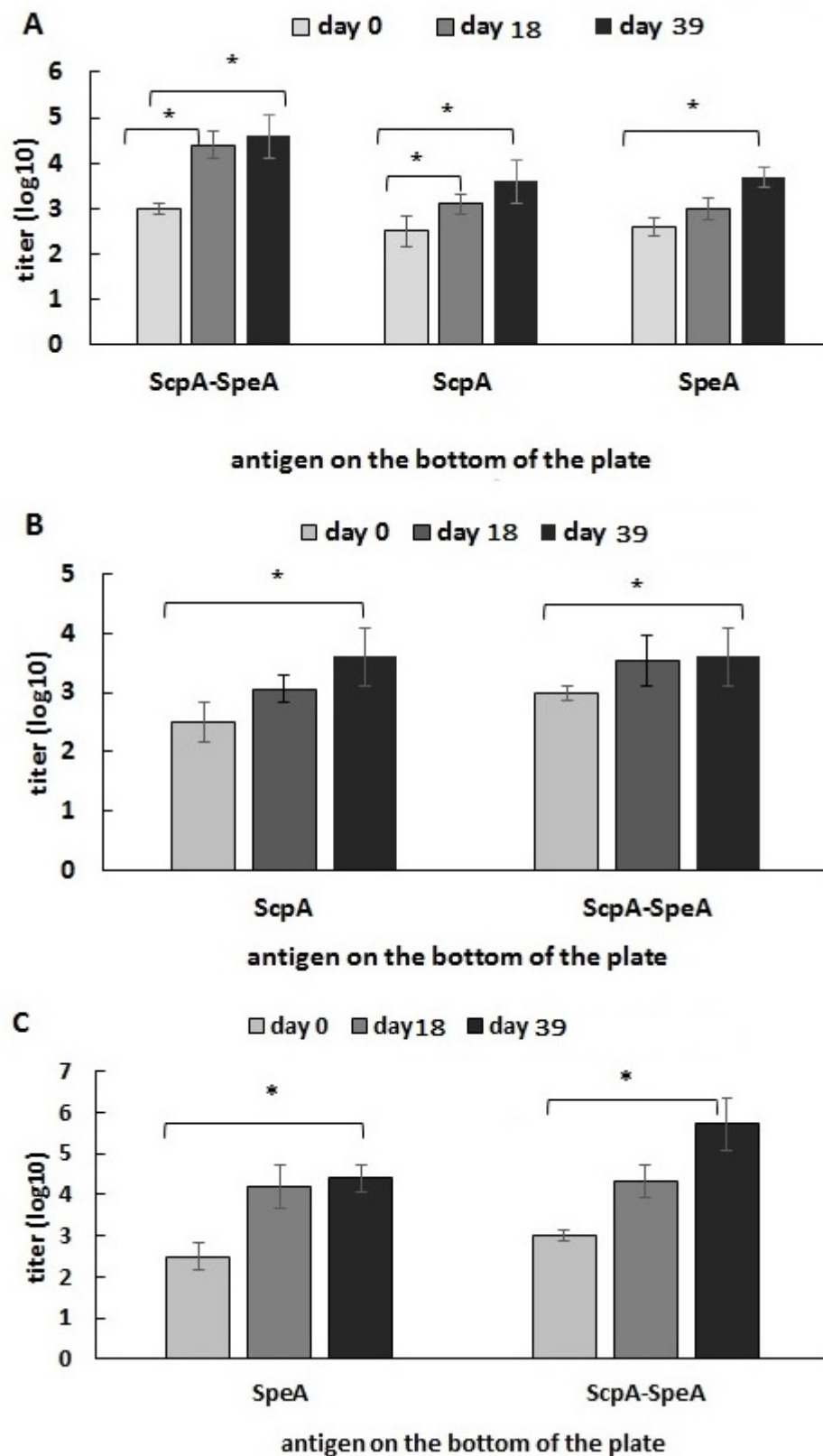
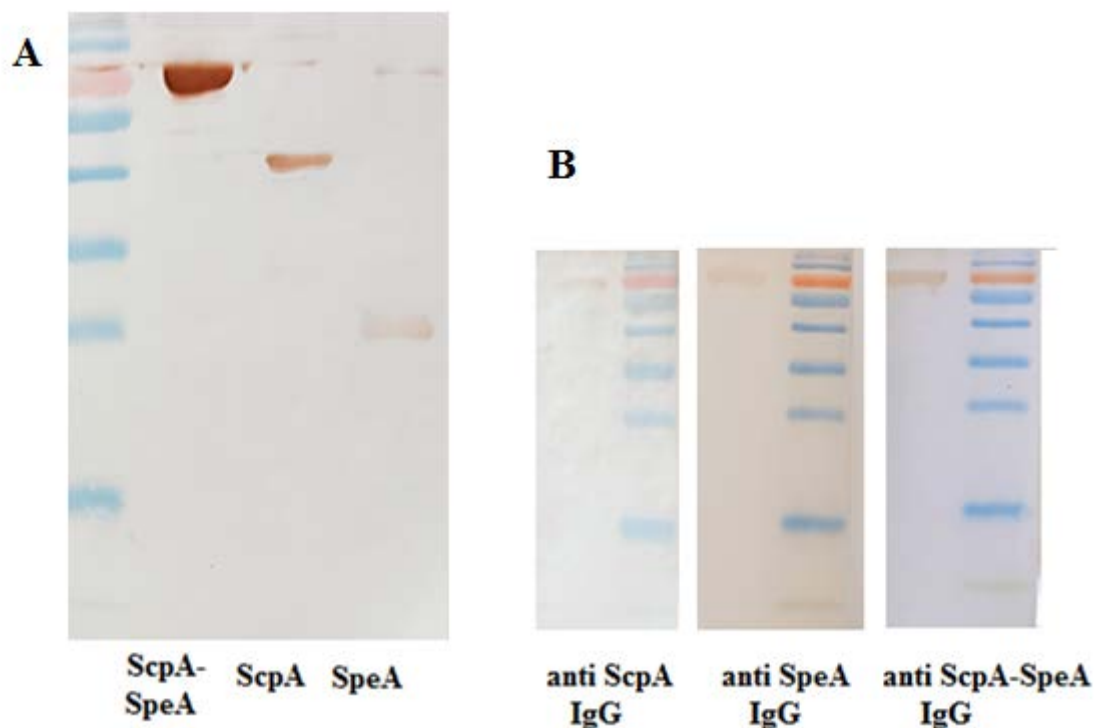


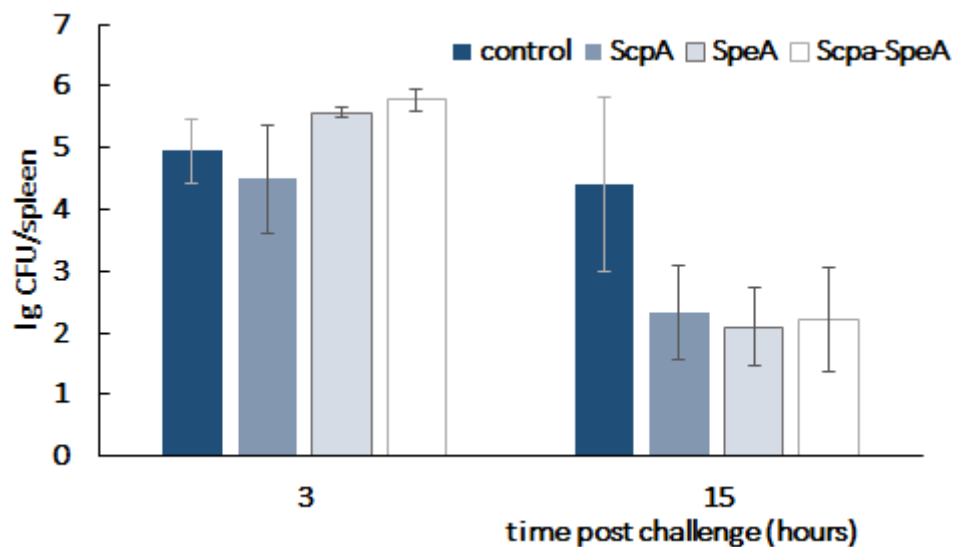
Figure 6. Immunoblotting Analysis of Serum Specificity.



A. Electrophoresis was performed on a 12% polyacrylamide gel (PAGE) to separate the proteins ScpA-SpeA, ScpA, and SpeA, followed by transfer to nitrocellulose paper and treatment with serum obtained from mice immunized with the chimeric protein ScpA-SpeA.

B. The chimeric protein ScpA-SpeA was subjected to 12% PAGE electrophoresis in triplicate, followed by transfer to nitrocellulose paper and treatment with sera obtained from mice immunized with proteins ScpA, SpeA, and the chimeric ScpA-SpeA, respectively. For detailed procedures, refer to the Materials and Methods section.

Figure 7. Assessment of Protective Efficacy of the Immune Response.



ТИТУЛЬНЫЙ ЛИСТ_МЕТАДАННЫЕ

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Блок 3. Метаданные статьи

TARGETING GROUP A STREPTOCOCCUS WITH A RECOMBINANT
CHIMERIC VACCINE: INTEGRATING SCpA AND SPEA FRAGMENTS
ТАРГЕТНАЯ ВАКЦИНАЦИЯ ПРОТИВ СТРЕПТОКОККА ГРУППЫ А:
РЕКОМБИНАНТНАЯ ВАКЦИНА С ФРАГМЕНТАМИ SCpA И SPEA

Сокращенное название статьи для верхнего колонтитула:

GAS RECOMBINANT CHIMERIC VACCINE

РЕКОМБИНАНТНАЯ ХИМЕРНАЯ ВАКЦИНА ПРОТИВ СГА

Keywords: Group A Streptococcus (*S. pyogenes*, GAS), C5a peptidase (ScpA),
exotoxin SpeA, recombinant chimeric vaccine, immunogenicity, protectivity.

Ключевые слова: Стрептококк группы А (*S. pyogenes*, СГА), С5а пептидаза
(ScpA), экзотоксин SpeA, рекомбинантная химерная вакцина,
иммуногенность, протективность.

Оригинальные статьи.

Количество страниц текста – 12,

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