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Research article

# Exploiting immunopotential PAPI-1 encoded type IVb major pilin targeting *Pseudomonas aeruginosa*

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ABSTRACT

*Pseudomonas aeruginosa (P. aeruginosa)* significantly contributes to nosocomial infections and necessitates research into novel treatment methods. For the first time, this research evaluated the immunoprotective potential of recombinant PAPI-1 encoded type IV pili targeting *P. aeruginosa* in BALB/C mice. The target sequence was identified, and a PilS2-encoding vector was constructed. The vector was then expressed and purified in *E. coli* BL21 (DE3). The PilS2 protein was inoculated into BALB/C mice in four groups, with or without alum, to measure total IgG, its subclasses, and cytokines. MTT and opsonophagocytosis tests were used to examine the immunological response. PilS2, especially when paired with alum, boosts the humoral immune response by enhancing IgG and IL-4 levels. However, PilS2 did not affect IL-17 or IFN-γ and only increased lymphocyte proliferation. Antibodies targeting PilS2 increased phagocytic cell death of *P. aeruginosa* by over 95 %, indicating possible therapies for *P. aeruginosa* infections. Our study on the immunopotentiation of *P. aeruginosa* PilS2 paves the way for pilin-based vaccines and immunotherapy targeting this pathogen.

## 1. Introduction

*Pseudomonas aeruginosa (P. aeruginosa)* is an important nosocomial infection-causing bacterium. Due to its versatile arsenal of pathogenic factors and remarkable ability to acquire antibiotic resistance, *P. aeruginosa* has earned a seat on the World Health Organization's priority list among the top life-threatening human pathogens, mandating urgent research into innovative treatment methods [1]. In the last three decades, even with the introduction of new types of antibiotics, there has been a rise in multidrug resistance to *P. aeruginosa* in Asian, European (especially Greek), and Latin American nations [2]. *P. aeruginosa*'s ability to thrive in various habitats is attributed to its intricate composite chromosomal makeup of ~5–~7 Mb and mosaic genome architecture [3–6]. This makeup enables the bacteria to cause high mortality rates, especially for people with burn injuries, acute and chronic illnesses, AIDS, cancer, and chronic obstructive pulmonary disease [7–9]. Extended hospital stays increase the likelihood of *P. aeruginosa* colonization and are connected to well-respected healthcare-associated acute and chronic infections. These infections include ventilator-associated pneumonia, chronic obstructive pulmonary disease, and infections related to burned skin and tissue [2].

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*P. aeruginosa*'s multifaceted pathogenic factors encompass an intricate network of lipopolysaccharides and exopolysaccharides, extracellular DNA, an extensive repertoire of secretion machinery, lytic enzymes, flagella, and a tripartite classification of type IV pili. These factors collectively empower the pathogen to survive and proliferate in multiple anatomical locations [10–13]. Among a large panel of strains identified from this species, the extremely virulent *P. aeruginosa* burn wound isolate UCBPP-PA14 emerged as a prominent member of the most prevalent *P. aeruginosa* global genetic lineage [5,14]. This strain demonstrates a significant degree of genome divergence attributed to integrating pathogenicity islands, such as pKLC102 or PAPI-1 –81-108 kb islands [15]. Horizontal gene transfer is a primary evolutionary mechanism contributing to the virulence properties of numerous bacterial pathogens [16]. Carter et al. outlined that the transformation of PAPI-1 into recipient *P. aeruginosa* occurs through a conjugative mechanism encouraged by a type V pilus encoded in PAPI-1 by a ten-gene cluster [17]. To date, the coexistence of three variants of multifunctional type IV pili fibers, IVa, IVb, and tight-adherence (Tad), within a single strain has been documented in *P. aeruginosa* [18]. The type IVb fiber, like other fimbriae, has an unbranched filamentous structure consisting of 176-amino-acid major pilin subunits, PilS2, which is encoded by the PAPI-1 genomic island [19,20]. With the emergence of multidrug-resistant variants and the limited efficacy of conventional therapeutic approaches, researchers have investigated alternative strategies to attenuate pathogenesis through virulence factor targeting [21–24]. Surface-exposed epitopes on *P. aeruginosa* pili have been shown to elicit immunological responses in both acute and chronic infection models. These antibodies might impair bacterial adhesion and colonization, acting as an anti-infection strategy. Furthermore, they can act as opsonin and boost pathogen clearance. Despite extensive endeavors to formulate a reliable immunotherapeutic strategy against *P. aeruginosa*, no relevant antigen functioning as a potent immunogenic agent or an antiserum targeting this omnipresent pathogen is currently available. The heterogeneity in immune responses and antigenic variation observed in type IVa pili major subunit among different strains of *P. aeruginosa* pose a formidable obstacle in the quest for effective vaccine development. Previous studies on other *P. aeruginosa* pili components have shown a promising approach for targeting this bacterium [21,25–27]. For instance, we demonstrated the immunogenic capabilities of other compartments within the type IV pilin apparatus, such as the PilQ secretin, or engineered synthetic Flp pilin as a robust stimulator of both cellular and humoral immune responses against multiple strains of *P. aeruginosa* [28–31]. The type IVb pilin fiber, known as PilS2 pilin, functions as a bacterial fimbria and features a hair-like structure that does not branch out. This structure comprises oligomeric major pilin subunits weighing approximately 15–20 k Da [2]. Notably, PilS2 is encoded by many *P. aeruginosa* strains, including PA2192, PA7, C3710, PACS2, and PSE9 (PAGI-5), and is highly conserved in the virulent strain PA14 [31]. Passive immunization treats infectious diseases using ready-made antibodies, offering an alternative to conventional antibiotics, especially when it focuses on *P. aeruginosa*. Despite extensive efforts to develop accurate *P. aeruginosa* immunotherapy, there are likely several reasons why previous *P. aeruginosa* immunotherapy did not target this organism [2]. The ineffectiveness of the *P. aeruginosa* vaccine can be attributed to the lack of highly immunogenic virulence factors and the inability of the immune system to recognize them to stimulate a robust immune response, making it difficult to effectively control the bacteria before they transition from acute to pathogenic chronic stages of infection [32]. Studies have shown that flagellum and pilin can protect against acute and chronic *P. aeruginosa* infection [2]. Given the unique characteristics of the PilS2 protein and its potential role in the pathogenesis of highly virulent strains of *P. aeruginosa*, this investigation stands out for its innovative work in demonstrating the immunopotentiation of the PilS2 protein targeting *P. aeruginosa*, which may have significant implications for the generation of antigen-specific antibodies and the creation of subsequent vaccination strategies. In this research, BALB/c mice were studied because BALB/c mice display sensitivity to infection and are commonly utilized to assess therapeutic efficacy in cases of acute disease. The vaccination program started after dividing the mice into test and control groups. Two weeks after the first injection, two booster doses were administered two weeks apart, and two weeks after the last injection, the final examinations were started.

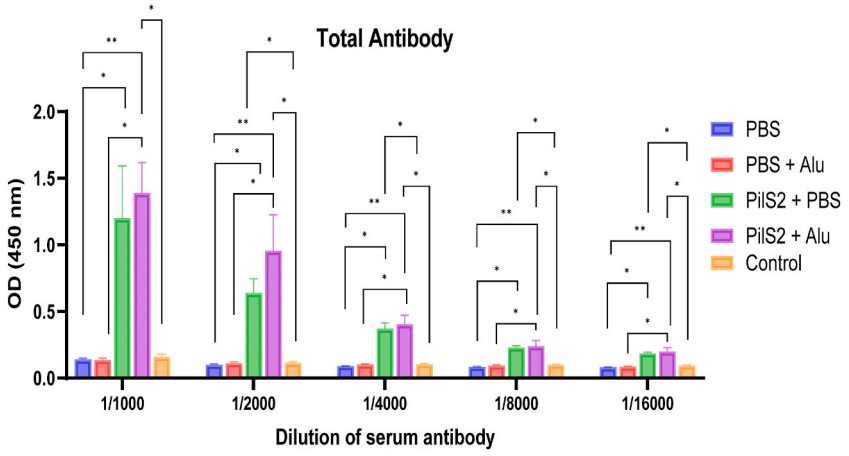


**Fig. 1.** Analysis of the 42-day total antibody response. The data was presented as mean SEM. *p < 0.05, **p < 0.01, and ***p < 0.001 denote significant difference.

## 2. Results

### 2.1. Total antibody analysis

Total body and isotype body tests were used to measure the effects of the recombinant production vaccine on antibody production in the vaccinated mice. The recombinant PilS2 protein and alum adjuvant were combined for subcutaneous inoculation in BALB/c mice three times, once every two weeks. Our study focused on the impacts of various treatments on mice, including the effects of (PBS + alum), (PBS + PilS2), and (PilS2 + alum) therapies. To validate the outcomes, five sera dilutions (1/1000, 1/2000, 1/4000, 1/8000, and 1/16000) were carried out. The (PBS + PilS2) and (PilS2 + alum) groups significantly escalated optical density values in response to protein treatments. The (PBS + PilS2) and (PBS + alum) groups had substantially greater levels ($P < 0.05$ and $P < 0.01$, respectively) over the dilution range of 1/1000 to 1/16000 (Fig. 1).

### 2.2. Antibody isotypes analysis

To explore the antibody response's immunoglobulin isotype and determine the activation of the humoral immune system prompted by the recombinant PilS2 protein, our study examined the induction of subclass antibodies (IgG1, IgG2a, IgG2b, and IgG3) in the serum of the treated mice. The results revealed that whether injected alone or administered with alum adjuvant, chimeric PilS2, created from a unique genetic sequence for the chimeric PilS2 derived from the *P. aeruginosa* PA14 strain (AAP84205.1) after the codon optimization approach, demonstrated a remarkable ability to trigger a robust immunological response spanning all subclasses of IgG (IgG1, IgG2a, IgG2b, and IgG3 isotypes) antibodies compared to the control group ($p < 0.05$). Notably, the IgG2b and IgG3 levels were higher in the PilS2 + alum groups than in the PilS2 alone groups (Fig. 2).

### 2.3. The PilS2's cell activation and proliferation potential

Assessment of cell-mediated immunity frequently involves using lymphocyte proliferation assays. We evaluated the stimulation index of splenic T cells from the vaccinated mice using the 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test (MTT) to observe whether PilS2 chimeric protein could cause lymphocyte proliferation (Fig. 3. (The results demonstrated a marked enhancement in splenocyte proliferation in groups receiving PilS2 protein, with the highest stimulation index observed in the group treated with the combination of PilS2 and alum adjuvant. Specifically, the stimulation index for the PBS control group was significantly lower than those of the treatment groups, with statistical analyses revealing p-values of less than 0.01 and 0.0001 for the PilS2 and PilS2 + alum groups, respectively. These findings indicate that both PilS2 protein and its combination with alum adjuvant significantly

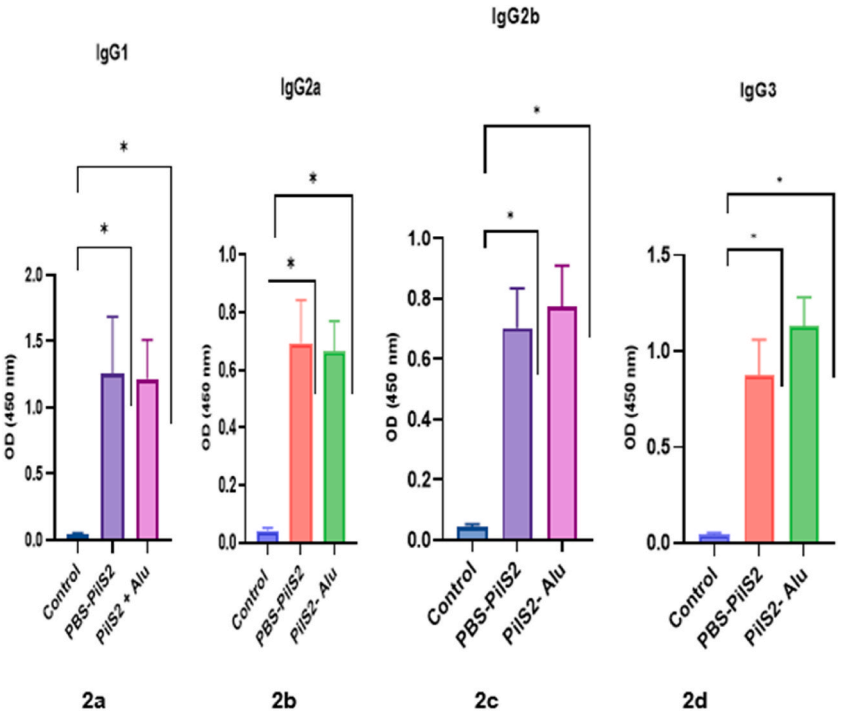


**Fig. 2.** The comparison of the titer of IgG subclasses (IgG1 (2a), IgG2a (2b), IgG2b (2c), IgG3 (2d)) induced by Pils2 and PilS2-Alum. Data were expressed as mean ± SD. Significant difference: *$p < 0.05$, **$p < 0.01$.

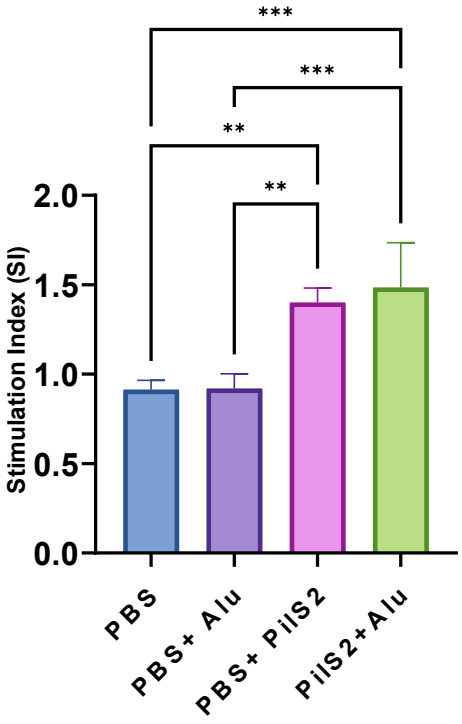


**Fig. 3.** Splenocyte proliferative responses of different mice groups. Data were expressed as Mean ± SD. **p < 0.01 mean ± SD, ****p < 0.0001.

boost immune cell activation, highlighting their potential as effective vaccine components.

Two weeks following the last immunization, the levels of IL-4, IFN-, and IL-17 were measured using the ELISA technique to induce a particular cellular immune response and assess the immunological types. In contrast to the control group receiving PBS alone, the groups vaccinated with PBS + alum, PilS2, or PilS2 + alum elicited a robust IL-4 response ($p < 0.0001$) (Fig. 4), which confirms the positive effect of the PilS2 protein and alum adjuvant on increasing the IL-4 level. The distinctive cytokine released by Th17 cells, IL-17, encourages neutrophil emigration to the infection site and inflammation. The IFN- and IL-17 levels in groups treated with PilS2 or

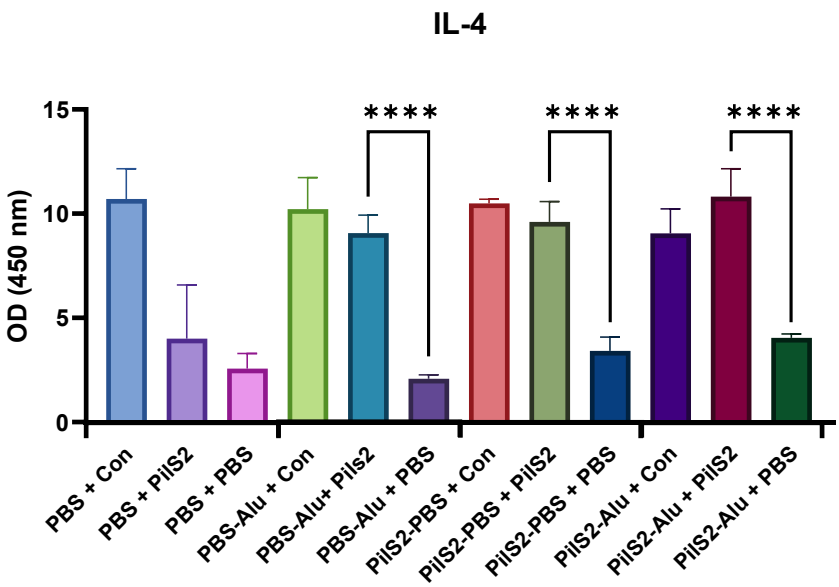


**Fig. 4.** X-axis shows sera of four treated groups of mice (PBS/PBS-Alu/PilS2-PBS/PilS2-Alu), while each group was once exposed to concanavalin (Con) as a positive control, once exposed to PilS2 and once exposed to PBS as a negative control for 72 h. Data were expressed as Mean ± SD. p ****<0.0001.

PilS2 + alum did not significantly increase compared to those in the control group.

### 2.4. Evaluation of opsonophagocytic activity of PilS2-Specific antibodies

We assessed the capacity of PilS2-specific antibodies in immune sera to induce opsonophagocytic death in *P. aeruginosa*. The results demonstrated a robust immune response, with phagocytosis rates reaching 97 % for PilS2 alone. Notably, when PilS2 was combined with alum, phagocytic activity surged to an impressive 99.98 %, as illustrated in Fig. 5. These findings underscore the potential of PilS2-specific antibodies in enhancing activity against *P. aeruginosa*, supporting the central thesis of our manuscript. The data presented in Fig. 5 correlate with our experimental design, providing compelling evidence of the effectiveness of the immune response elicited by PilS2.

## 3. Discussion

The emergence of antibiotic resistance in *P. aeruginosa* underscores the urgent need to explore alternative therapeutic strategies, particularly immunotherapies that specifically target this pathogen [33]. While traditional vaccination efforts have succeeded against various infectious diseases, gaps remain for several severe conditions, including infections caused by *P. aeruginosa* [21]. *P. aeruginosa* expresses various types of fimbriae, particularly type IVa pili, which have garnered significant attention in vaccine research [18]. Despite some promising findings regarding the efficacy of immunotherapy against *P. aeruginosa*, challenges such as serological diversity and variations in amino acid sequences often hinder the development of effective type IV pilin-based vaccines [34–36]. Our study is the first to comprehensively explore the immunopotentiation effects of the PilS2 subunit in a BALB/c mouse model, paving the way for future research on vaccine development and potential immunological strategies against this opportunistic pathogen. Previous studies have shown that *P. aeruginosa* pili can significantly improve survival rates in infected animal models and reduce bacterial colonization in vital organs such as the lungs [37]. For example, pilin and exotoxin A combinations have elicited robust neutralizing antibody responses, providing a solid foundation for further exploring pilin-based vaccination strategies [38]. It is noteworthy that the chimeric pilin from *P. aeruginosa* demonstrated the capacity to trigger both humoral and cell-mediated immune responses, presenting a promising avenue for combating infections [31].

Korpi et al. (2015) used a chimeric PilA protein in an active immunization program against *P. aeruginosa* infection in a mouse burn wound model [39]. They also discovered that immunization with flagellin and pilin effectively protected mice in the burn wound sepsis model against multi-drug-resistant *P. aeruginosa* infections [40]. Immunization with r-PilA, alum + Naloxone, and two booster shots elicited strong Th1 and Th2 responses, producing IgG1 and IgG2a antibodies. This response reduced bacterial load in the lungs, increased survival in challenged mice, and provided protection against acute *P. aeruginosa* lung infection [41]. It has the potential to trigger a robust immune reaction against *P. aeruginosa*. This response encompasses both the production of antibodies and the activation of immune cells, making it a promising candidate for combating this versatile pathogen [42]. Analyzing cytokine responses further elucidated the immune mechanisms at play. IL-4's involvement in directing naive CD4(+) T cells toward a Th2 phenotype enhances antibody production, particularly IgG1, critical in conferring protection against *P. aeruginosa* [43,44]. Our findings suggested that the

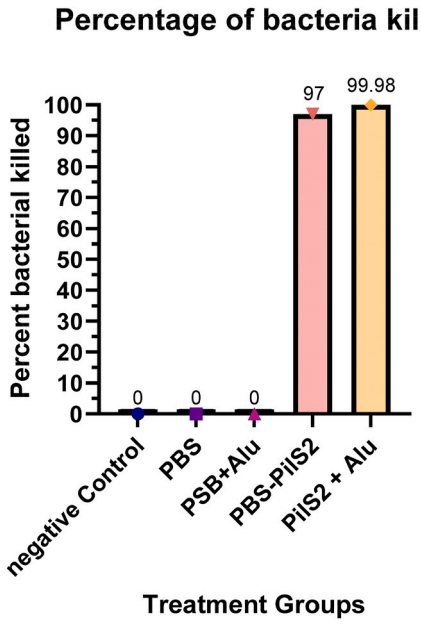


**Fig. 5.** Opsonic killing activity of mice sera inoculated with *P. aeruginosa* (PA14). For doing this test, PA14 were incubated with anti PilS2 sera and mouse macrophage in the presence of rabbit complement.

PilS2 chimeric protein, alongside alum adjuvant, prompted a robust Th2-dependent humoral immune response without significantly enhancing IFN-γ levels. This response indicated a targeted immune activation strategy that could be advantageous in developing effective treatments. The significant elevation in total antibody levels, especially IgG1 and IgG3, in the serum of the immunized mice further supports the efficacy of the PilS2 protein when formulated with aluminum. Such antibody subtypes are fundamental for pathogen clearance and effective opsonization. Our results are consistent with previous findings that showed similar antibody profiles enhancing the clearance of *P. aeruginosa* during acute lung infections. In this study, we noted that the PilS2 protein could not stimulate substantial production of IFN-γ cytokine. IFN-γ and IL-4 are two cytokines that work against one another to create and control immune responses. IL-4 enhances T helper type 2 (Th2) differentiation and stability while inhibiting Th1-cell differentiation, whereas IFN-γ inhibits Th2-cell development and promotes Th1-cell differentiation [45]. As demonstrated by our additional investigation of cytokine profiles in splenic cells, PilS2 had a limited potential to efficiently induce the production of IL-17, a Th17-associated cytokine. IgG1 induction is assumed to be regulated by Th2 cytokines and is associated with Th2 cell growth and humoral immunity in mice, whereas IgG2a, IgG2b, and IgG3 triggering are associated with a Th1 response [46]. IgG1 and IgG3 antibodies are crucial for pathogen clearance by macrophages, opsonization of invading microorganisms, and complement activation [47,48]. Following the administration of the recombinant PilS2, we observed an increase in the levels of IgG1, IgG2a, IgG2b, and IgG3 subtypes in the serum of the immunized groups. Previous studies showed that in an acute lung infection rat model, passive immunization with *P. aeruginosa*-specific IgG antibodies, comprising both IgG1 and IgG2a, and IgG3 dramatically enhanced *P. aeruginosa* clearance from the pulmonary system [48]. Specifically, the stimulation index for the PBS control group showed a significantly lower value compared to the treatment groups. These results suggest that both the PilS2 protein and its combination with alum adjuvant effectively enhance the activation of T lymphocytes, demonstrating their potential as valuable components in vaccines.

## 4. Conclusion

Our investigation into the immunogenicity of the *P. aeruginosa* PilS2 chimeric protein, combined with alum, provides promising evidence of its potential as an effective candidate for immunotherapy. The resulting immune responses encompass both humoral and cellular components, highlighting the viability of this approach against *P. aeruginosa* infections.

While the prospect of utilizing type IV pili in vaccine development presents inherent challenges, our findings encourage future research focusing on refining pilin-based immunotherapies. Additionally, strategies aimed at optimizing adjuvant formulations and exploring advanced delivery systems may further enhance the immunogenicity of pilin-based vaccines. Considering mucosal administration methods, such as targeting respiratory tracts, could potentially bolster local immune responses, a critical factor in combating respiratory pathogens like *P. aeruginosa*. These future directions warrant cautious exploration, as they may significantly improve prevention strategies in high-risk populations.

## Methods

### *Sequence analysis and construct design*

The current experiment utilized the full-length PilS2 sequence sourced from *P. aeruginosa* PA14 (accession number AAP84205.1). The FASTA format sequence was obtained from the NCBI protein database (https://www.ncbi.nlm.nih.gov/protein). The amino acid sequence corresponding to PilS2 was used in a BLAST search against the NCBI server's non-redundant protein sequence (Nr) database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences with similarity and coverage greater than 90 % and an E-value less than $10^4$ were chosen. Subsequently, the selected sequences were aligned using the European Bioinformatics Institute's Multiple Sequence Alignment service. The protein sequence of PilS2 was then aligned with the protein sequences of 9 clinically significant *P. aeruginosa* strains encompassed various clinical contexts: the respiratory tract infection isolate PA1 (WP_023518442.1), the non-respiratory multidrug-resistant human isolate PA7 (WP_003120996.1), the toxigenic strain PA103 (WP_003099765.1), cystic fibrosis isolates including CF77 (WP_003120996.1), CF127 (WP_003099765.1), SCV20265 (WP_023122673.1), and DK2 (WP_003099765.1), along with the human eye infection isolate BL13 (WP_003120996.1) and the bloodstream isolate E2 (WP_003099765.1). Utilizing the ProtParam server (http://web.expasy.org/protparam), we evaluated the fundamental chemical and physical characteristics of the PilS2 sequence. The analysis included an examination of several different aspects, such as the composition of amino acids, molecular weight estimation, aliphatic index assessment, extinction coefficient calculation, theoretical isoelectric point prediction (pI), anticipated half-life prediction, instability index estimation, and calculation of the grand average of hydropathicity. After the codon optimization approach, we created a unique genetic sequence for the chimeric PilS2 derived from the *P. aeruginosa* PA14 strain (AAP84205.1). Using the SnapGene 5.3.1 design program, we placed this constructed genetic sequence into the pET28a cloning expression vector and synthesized it at Biomatik Corporation (Kitchener, Ontario, Canada). In our previous study [2], the specifications and the sequence of the PilS2 protein were given. In short, the protein had 176 amino acids, the molecular weight was 17918.23, and the Theoretical pI was 9.03.

### *PilS2 chimeric antigen expression and purification*

In this experiment, the recombinant PilS2 protein was expressed using the pET-28a(+) vector, which includes a His-tag for purification purposes. This vector was chosen due to its strong T7 promoter, facilitating high-level expression in *E. coli* BL21 (DE3) cells. The chimeric PilS2 protein was expressed by introducing the designed vector into *E. coli* BL21 (DE3). An overnight bacterial culture

was introduced into a fresh LB medium. Chimeric PilS2 expression was induced using Isopropyl-beta-thiogalactopyranoside (Sigma Chemical Co., USA), followed by incubation at 37 °C for 6 h. After sonication of the bacterial pellet, the recombinant protein was purified using Ni-NTA agarose column chromatography [49]. The isolated chimeric protein was dialyzed in PBS (pH 7.4) with 0.2 mM PMSF at 4 °C overnight to remove imidazole. The Bradford technique and 15 % SDS-PAGE gel electrophoresis were used for quality testing and quantification, respectively. Western blotting was used to test the immunoreactivity of the recombinant PilS2 protein with a rabbit anti-6X His tag antibody (1:1000) (Abcam, USA) and a horseradish peroxidase antibody (Sigma) at a dilution of 1:1000. An electrochemiluminescent substrate (GE Healthcare, Uppsala, Sweden) was used to view the antibody-bound protein.

*Mice immunization with PilS2 recombinant protein*

A total of 24 female BALB/c mice, aged 6–8 weeks and weighing 20–22 g, were divided into two groups: the test group and the control group, each consisting of six mice. The mice were acclimated for a week with unlimited access to food and water. All experiments were conducted following the animal care and use policies of Shahid Beheshti University of Medical Sciences in Tehran, Iran. The recombinant PilS2 protein (10 μg) was mixed with alum adjuvant (100 μl) for subcutaneous inoculation. To enhance absorption, the mixture was gently rotated overnight at 4 °C. The control group received an injection of PBS alone, while the second group was injected with an equivalent volume of alum adjuvant (Sigma, St. Louis, MO). The third group received a single injection of 10 μg of the PilS2 protein, and the fourth group was administered the chimeric PilS2 along with an equal volume of alum adjuvant. On days 14 and 28, booster doses of the same vaccine formulations were administered to each group. Additionally, two weeks after the third injection, serum samples were collected via orbital sinus puncture, and total IgG levels, along with IgG isotypes against anti-Pseudomonas pili protein, were analyzed. It should be noted that mice were given an injection of 0.2 ml of sterile water containing 1.3 mg/ml of xylazine and 6.7 mg/ml of ketamine prior to vaccination.

*Total and subclass antibody analysis*

To measure the total antibody levels in the mouse antisera (day 0 to day 42), a modified indirect enzyme-linked immunosorbent assay (ELISA) was used. Briefly, 100 μl of the recombinant PilS2 protein (10 μg/ml in PBS) was coated onto 96-well microtiter plates (Sigma-Aldrich) [31]. Non-fat dry milk was used to block the plates for 30 min. After adding the horseradish peroxidase at a dilution of 1:4000 and incubating for 2 h at 37 °C, the immunoreaction was blocked, and enzyme activity was measured at 450 nm using an ELISA plate reader (Biotech XS2). An indirect ELISA was used to differentiate IgG isotypes (IgG1, IgG2a, IgG2b, and IgG3) responses in mice sera (1:1000–1:16000) using goat anti-mouse IgG subclasses (1:1000) and rabbit anti-goat antibodies (1:1000) at 450 nm.

*Mouse splenic lymphocyte Isolation and enrichment*

Mice splenocytes were isolated two weeks after the last vaccination. The mice were euthanized with a 60 mg/kg intraperitoneal pentobarbital injection. Following the manufacturer's guidelines, splenic lymphocytes were isolated via density gradient centrifugation with Lymphoprep (specific gravity 1.077) from Sigma-Aldrich for the experiment. To obtain a final concentration of $4 \times 10^6$ cells/mL, single splenocyte suspensions were prepared in complete RPMI 1640 supplemented with 10 % fetal bovine serum and 1 % penicillin-streptomycin L-glutamine from Gibco (Carlsbad, CA, USA). The isolated cells were cultured for three days at 37 °C and 5 % CO2 [31].

*Assessing cell proliferation and cytokine responses*

To assess cell proliferation, we prepared spleen cells at a density of $2 \times 10^4$ cells per well and stimulated them with 10 μg/ml of the recombinant PilS2 protein. For the MTT assay, we added 100 μL of this cell suspension to each well. After a 72-h incubation at 37 °C with 5 % CO2, we introduced 20 μL of MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Vybrant®, Molecular Probes) and allowed it to incubate for another 4 h.

Following this incubation, we centrifuged the plates at 2000 rpm for 5 min to separate the supernatant. To dissolve the resulting formazan crystals, we added 50 μL of dimethyl sulfoxide (DMSO) to each well. The absorbance was then measured at 540 nm using a microplate reader (Biotek, PowerWave XS2). For controls, we included wells with 25 μg/ml of concanavalin A (Gibco) as a positive control and unstimulated cell suspensions as negative controls. The blank wells contained only the complete culture medium. The stimulation index was determined by comparing the average absorbance of stimulated cells to the mean absorbance of unstimulated cells.

In addition to the proliferation assessment, we also measured cytokine responses. The cultured splenocytes were stimulated with the recombinant PilS2 protein (10 μg/ml), with positive control wells receiving concanavalin A (25 μg/ml) and negative controls containing only the complete culture medium. After a 48-h incubation at 37 °C with 5 % CO2, we used ELISA kits (Sigma-Aldrich, USA) to quantify the levels of interleukin-4 (IL-4), interferon-gamma (IFN-γ), and interleukin-17 (IL-17). The absorbance for these assays was measured at 450 nm using an ELISA microplate reader (Biotek, PowerWave XS2), following the manufacturer's guidelines.

*Opsonophagocytic killing activity*

The opsonophagocytosis assay was performed following the protocol established by Ames et al. [50], with minor modifications. In

brief, the bacterial suspensions of *P. aeruginosa* strain PA14 were mixed with an equal volume of diluted and heat-inactivated antiserum (56 °C for 30 min) and incubated at 22 °C for 60 min, achieving a bacterial concentration of $2 \times 10^7$ CFU/ml. Mouse macrophages were resuspended in RPMI-1640 medium at $1 \times 10^7$ cells/ml. Serum was obtained from a 3-week-old rabbit (Pasteur Institute, Karaj, Iran) and pooled after bleeding. The complement activity in the antisera was inactivated by heating at 56 °C for 30 min. Each microtiter plate well was treated with 100 μl of the macrophage suspension, 100 μl of mouse serum (from two selected mice per group), and 100 μl of 4 % rabbit serum in RPMI medium. The plate was incubated at 37 °C for 90 min. Following incubation, 2 μl and 10 μl samples from each well were cultured on nutrient agar plates using the spread plate method. Bacterial colonies were counted, and the percentage of bacterial killing was calculated using the formula:

OP activity (%) = [1 − (bacterial CFUs of pre-immune serum at 90 min/bacterial CFUs of immune serum at 90 min)] × 100.

### *Statistical analysis*

The cytokine assay and antibody determination data were analyzed using GraphPad Prism software, version 9.4.0. For nonparametric data, Kruskal-Wallis analysis was performed, followed by Dunn's multiple comparison tests. ANOVA and Tukey's multiple comparison tests were used to assess the parametric data. Statistical significance was defined as a p-value of 0.05.

### *Data availability statement*

Data will be made available on request.

## Ethical approval

The protocol of this study received approval from the Ethics Committee of the Shahid Beheshti University of Medical Sciences in Tehran, Iran (IR.SBMU.AEC.1402.110).

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## CRediT authorship contribution statement

**Mojgan Arefian Jazi:** Writing – review & editing, Writing – original draft, Software, Methodology, Formal analysis. **Bahareh Hajikhani:** Writing – review & editing, Writing – original draft, Visualization, Validation, Data curation. **Mehdi Goudarzi:** Writing – review & editing, Writing – original draft, Resources, Project administration, Funding acquisition, Conceptualization. **Gholamhossein Ebrahimipour:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Conceptualization.

## Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Mehdi Goudarzi reports financial support was provided by Shahid Beheshti University of Medical Sciences. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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