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Investigation of the Effect of Adjuvant Positioning within a FliC-derived Multi-Epitope Construct on TLR5 Engagement and Epithelial Immune Modulation

Saeide Mirsharifi[1], Mohammad Reza Asadi Karam[2]*, Fatemeh Foroohi[1]*, Mehri Habibi[3], Touraj Rahimi[4,5]

[1]Department of Microbiology, ShQ.C., Islamic Azad University, Shahr-e Qods, Iran. [2]Department of Molecular Biology, Pasteur Institute of Iran, Tehran, Iran. [3] Department of Medical Biotechnology, Biotechnology Research Center, Pasteur Institute of Iran, Tehran, Iran. [4]Department of Plant Production and Genetics, ShQ.C., Islamic Azad University, Shahr-e Qods, Iran
[5]Research Center for Medicinal Plants, ShQ.C., Islamic Azad University, Shahr-e Qods, Iran.

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***Corresponding Authors:**

Mohammad Reza Asadi Karam,;Department of Molecular Biology, Pasteur Institute of Iran, Pasteur Ave, Tehran 13164, Iran
Email: m_asadi12@yahoo.com
Tel/Fax: +98 21 64112223 / +98 21 66492619

Fatemeh Foroohi; Department of Microbiology, ShQ.C., Islamic Azad University, Shahr-e Qods, Iran.
E-mail: drforoohi@iau.ac.ir
Tel/Fax: +98 21 46896000/ +98 21 46896432

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ABSTRACT

Introduction: Rational vaccine design is a promising strategy for combating bacterial infections. Multi-epitope-based vaccines as a modern platform need to improvement by immunostimulants. Flagellin from *Salmonella typhimurium* acts as a potent Toll-like receptor 5 (TLR5) ligand. The incorporation of vaccine antigens into different sites, including the N- and C-terminal regions, as well as the hypervariable domains of FliC influences receptor docking and immune signaling. This study evaluates the effect of adjuvant positioning in a FliC-derived construct on TLR5 docking and epithelial IL-8 modulation. **Methods:** Variable D2 and D3 domains of FliC were substituted with antigenic regions of PapG II and FimH from *Escherichia coli*, connected via rigid and flexible linkers to generate three constructs differing in adjuvant position (N-terminal, central, and C-terminal). Computational analyses including 3D structure prediction and TLR5 docking were assessed. The optimal construct was expressed in *E. coli*, purified, and applied to HT-29 cells to measure IL-8 secretion using ELISA. **Results:** Docking analyses revealed that central placement of FliC in the multi-epitope construct (FlFPFl) provided the highest TLR5 binding affinity and stable interaction. Functional assays confirmed that FlFPFl significantly modulated IL-8 secretion compared to untreated cells ($p < 0.05$), demonstrating the critical role of adjuvant positioning in epithelial immune activation. **Conclusion:** This study highlights the importance of adjuvant topology in multi-epitope vaccine design. Based on *in silico* analyses, structural optimization of flagellin-based constructs derived from *S. typhimurium* improved receptor-accessible motif exposure and folding stability. Central adjuvant placement enhanced interaction with TLR5 and FlFPFl model showed the most favorable docking performance.

INTRODUCTION

Vaccination represents one of the most powerful and cost-effective interventions for the prevention of infectious diseases, significantly decreasing global morbidity and mortality. In addition to protecting vaccinated individuals, immunization programs promote herd immunity and substantially reduce pathogen circulation within populations. However, the emergence of novel and drug-resistant bacterial strains has intensified the demand for vaccines that are not only safe and durable but also capable of inducing precisely tailored immune responses. These challenges have driven a paradigm shift in vaccine development, moving from traditional empirical methods toward more rational, mechanism-based, and structure-informed design strategies [1, 2]. Within this modern framework, rational vaccine design focuses on constructing structurally optimized antigens that can direct specific and effective immune modulation, particularly against drug-resistant bacterial pathogens. Approaches such as structural vaccinology and multi-epitope vaccine engineering have therefore gained prominence as innovative platforms for next-generation immunotherapeutics [3]. In parallel, the incorporation of potent molecular adjuvants has become essential for enhancing vaccine efficacy. Among these, flagellin derived from *Salmonella typhimurium* has attracted significant attention due to its strong ability to activate innate immune pathways and amplify immune responses [4].
Flagellin functions as a pathogen-associated molecular pattern and is primarily recognized by Toll-like receptor 5 (TLR5), which is highly expressed on intestinal epithelial and immune cells [4]. The interaction between flagellin and TLR5 activates downstream NF-κB and MAPK signaling pathways, resulting in the secretion of pro-inflammatory mediators, particularly interleukin-8 (IL-8), a key chemokine involved in neutrophil

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recruitment and mucosal immune activation [4, 5]. Moreover, flagellin-mediated immune sensing is further amplified by the activation of the NLRC4 inflammasome, strengthening innate immune signaling cascades [6, 7]. Genetic fusion of target antigens to flagellin has been shown to enhance immunogenic responses, and considerable research is ongoing to identify the minimal functional flagellin regions, mainly the conserved D0 and D1 domains, that are required to preserve adjuvant activity [4, 3]. Structurally, flagellin consists of four domains (D0–D3). The conserved N- and C-terminal regions form the D0 and D1 domains, which are primarily responsible for TLR5 recognition and signal initiation, respectively. The D1 domain contains a major TLR5-binding hotspot enriched with arginine-centered motifs that facilitate electrostatic complementarity and stable receptor–ligand interactions, thereby promoting efficient docking with TLR5. In contrast, the D0 domain contributes to receptor dimerization and signaling activation, whereas the surface-exposed and highly variable D2 and D3 domains play limited roles in TLR5-mediated immune stimulation [8-10].

Recent advances in immunoinformatics have highlighted that vaccine efficacy is determined not only by antigen selection but also by the three-dimensional topology of multi-epitope constructs. The spatial arrangement of functional domains influences epitope accessibility, receptor-docking geometry, and downstream signaling efficiency. Therefore, the precise positioning of adjuvant sequences is a critical factor in recombinant vaccine design for maintaining folding stability and enhancing receptor-binding exposure. Computational methods, including molecular docking and molecular dynamics simulations are widely used to evaluate the structural stability, binding affinity, and interaction behavior with immune receptors, such as TLR5 [11].

Based on these considerations, the present study aimed to investigate the effect of adjuvant positioning within a FliC-derived multi-epitope construct on TLR5 engagement and epithelial immune modulation. Integrated bioinformatics and experimental approaches were employed to design and evaluate recombinant constructs to identify an optimal candidate capable of modulating IL-8 secretion in human intestinal epithelial cells. This topology-guided strategy was implemented to explore the relationship between structural engineering and functional immunogenicity in rational-vaccine design.

MATERIALS AND METHODS

Retrieval of Protein Sequences

The amino acid sequences of PapG II (UniProt: O86476-1) and FimH (UniProt: P08191) from *E. coli* subsp. CFT073 and flagellin (FliC) from *Salmonella enterica* serovar typhimurium (UniProt: P06179) were obtained from the UniProt database (https://www.uniprot.org/) [12]. To focus on functionally relevant regions, residues 46–128 of FimH lectin-binding domain and residues 70-150 of PapG II carbohydrate-binding domain were selected for constructing engineered proteins. For FliC, the conserved D0 and D1 regions corresponding to residues 1-173 (N-terminal) and 401–495 (C-terminal) were selected for the vaccine construct design.

Design of the Multi-Epitope Vaccine Construct

To improve structural stability and remove poorly conserved or unstable segments, the conserved regions of the FliC including D0 and D1 domains were replaced with selected PapG II and FimH antigenic sequences, respectively. Rigid EAAAK and flexible GGGGSGGGGS linkers were incorporated to preserve domain separation, maintain conformational flexibility, and reduce steric interference between functional motifs.

Three recombinant chimeric constructs were generated to evaluate the impact of adjuvant positioning on their structural integrity and immunogenicity. In the first design, the adjuvant sequence was fused to the N-terminus (FlFlFP); in the second, it was placed at the center (FlFPFl); and in the third, it was positioned at the C-terminus (FPFlFl). All candidate sequences were subjected to comprehensive computational screening.

Tertiary Structures Prediction and Validation of Models

The three-dimensional (3D) structures of the designed constructs were predicted using the Rosetta Modeling Platform [13]. Multiple structural models were generated for each sequence, and the model with the highest TM score (closer to 1, indicating greater structural confidence) was selected for further analysis. Structural refinement was subsequently performed using GalaxyRefine [14] to enhance side-chain packing and overall conformational stability. Global structural quality was evaluated using ProSA-web Z-score analysis (https://prosa.services.came.sbg.ac.at/prosa.php) [15] and Ramachandran plot using the PROCHECK tool of SAVES v6.1 (https://saves.mbi.ucla.edu/) [16].

Selection of the Optimal Vaccine Construct, Based on TLR5 Docking

To identify the most promising candidate, all three constructs were subjected to molecular docking against TLR5 (PDB ID: 3J0A). Docking simulations were performed using ClusPro 2.0 (https://cluspro.bu.edu/login.php) [17] and ZDOCK (http://zdock.umassmed.edu/) servers [18]. The constructs were treated as ligands and TLR5 as the receptor. The docking results were compared based on the cluster distribution, interaction energy, and binding interface characteristics. Complex structures were visualized using Discovery Studio, and residue-level interactions were analyzed using PDBsum (https://www.ebi.ac.uk/thornton-srv/software/PDBsum1/) [19].

Expression and Purification of the Recombinant Protein

The codon-optimized synthetic gene was cloned into the pET28a expression vector. The recombinant plasmid was transformed into competent *E. coli* BL21 (DE3) cells and positive clones were screened using double restriction digestion with *Nco*I and *Hind*III and Sanger sequencing [20]. Protein expression was induced using isopropyl β-D-1-thiogalactopyranoside (IPTG) and expression was confirmed using Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis using an anti-His monoclonal antibody (Sigma, USA). Purification of the expressed protein was performed using Ni-NTA affinity chromatography (Qiagen, Germany). A lipopolysaccharide (LPS) removal kit (Thermo Fisher Scientific, Lithuania) was applied to remove LPS contamination from the purified recombinant protein. The purified protein was dialyzed and protein purity and concentration were determined using the Bradford assay kit [20].

HT-29 Cell Culture and Treatment

Human colorectal adenocarcinoma HT-29 cells (National Cell Bank of Iran) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco) and antibiotics penicillin and streptomycin (Gibco) at 37°C in a humidified atmosphere containing 5% CO_2. The

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cells were incubated overnight with the filter-sterilized recombinant protein at concentration of 10 µg/ml.

Measurement of IL-8 Secretion by ELISA

IL-8 concentration in the culture supernatants was quantified using a human IL-8 ELISA kit (Abcam, Cambridge, MA, USA) following the manufacturer's protocol.

Statistical Analysis

Experimental data are presented as mean ± standard deviation (SD) from three independent experiments performed in triplicates. Statistical comparisons were performed using One-way analysis of variance (ANOVA), followed by Tukey–Kramer post hoc test. Statistical significance was set at $p < 0.05$.

RESULTS

Comparison of the Vaccine Constructs and Docking Analysis

Three multi-epitope vaccine constructs were designed, differing exclusively in the positional arrangement of the FliC sequence within the engineered protein. In the first construct (FlFlFP), the FliC was fused to the N-terminus; in the second construct (FlFPFl), it was inserted into the central region; and in the third construct (FPFlFl), it was positioned at the C-terminus. All three constructs were subjected to the tertiary structure prediction, followed by molecular docking analysis with TLR5 to evaluate receptor-binding behavior. The results of the 3D predicted structures of the constructs before and after refinement are mentioned in Fig. 1.

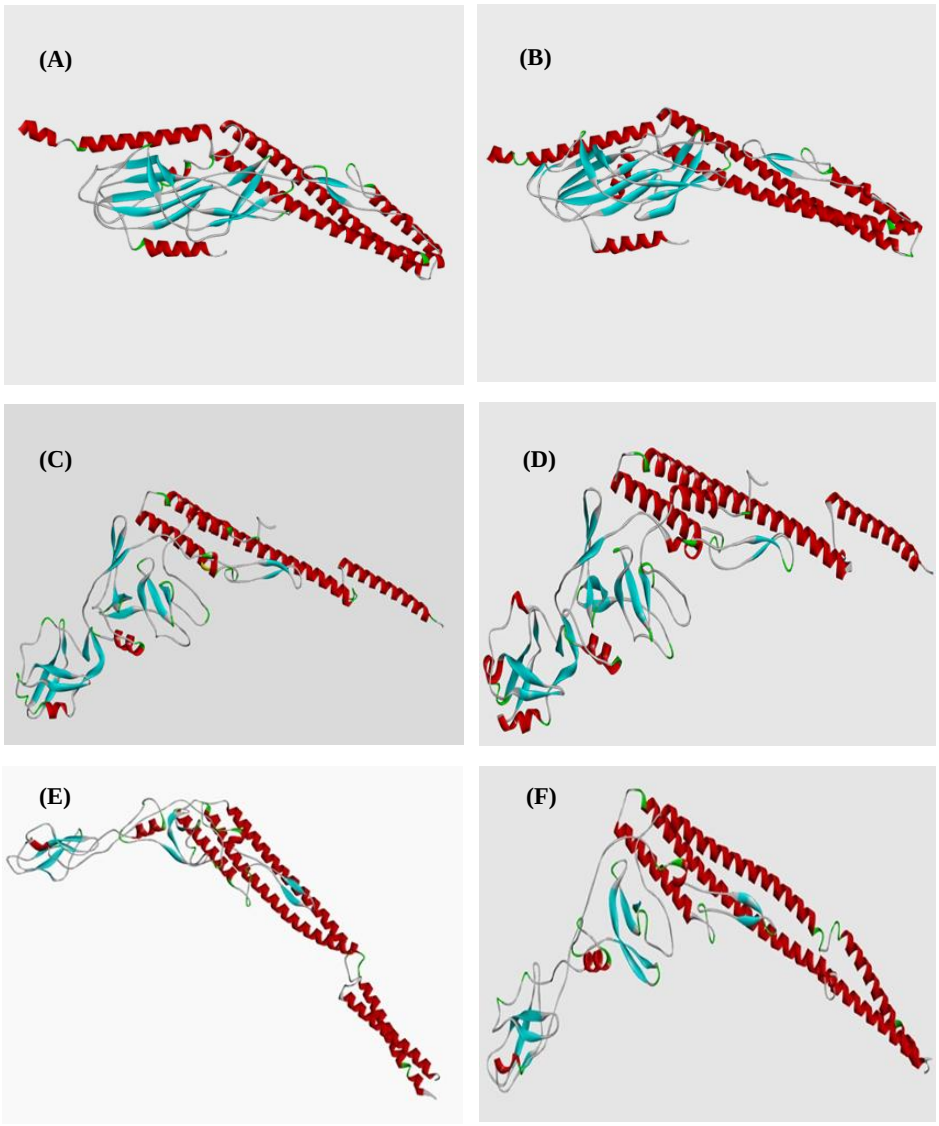

Fig. 1. Results of the predicted 3D structures of the constructs before and after refinement. (A) FPFlFl before refinement. (B) FPFlFl after refinement. (C) FlFlFP before refinement. (D) FlFlFP after refinement. (E) FlFPFl before refinement. (F) FlFPFl after refinement.

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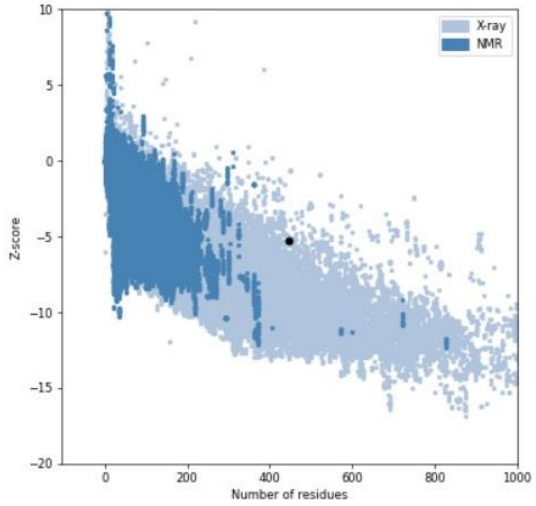
Structural validation of the selected construct supported the reliability of the predicted model. Ramachandran plot analysis indicated that 87.8% of the residues from FlFPFl were found within the most favored regions, 8.7% within the additionally allowed regions, 1.7% within the generously allowed regions, and only 1.7% (7 residues) fell in the disallowed regions. In addition, the ProSA Z-score of -8.18 fell within the range characteristic of native proteins of comparable size, further confirming structural soundness (Fig. 2). The Ramachandran plot and the ProSA web results related to the FPFlFl construct and FlFlFP are mentioned in Table 1.

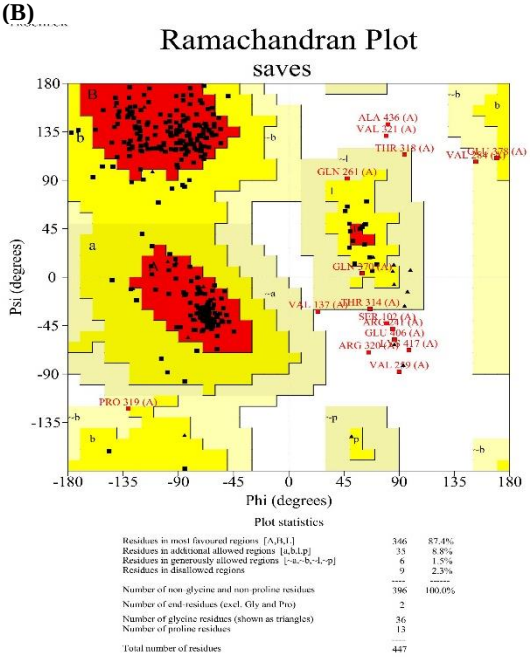
Table 1. Structural validation of the selected constructs of FPFlFl and FlFlFP.

	FPFlFl	FlFlFP
Most favored regions	92.4%	87.4%
Additionally allowed regions	8.8%	5.8%
Generously allowed regions	1.5%	0.5%
Disallowed regions	2.3%	1.3%
ProSA Z-score	-7.2	-5.28

(A)



(B)



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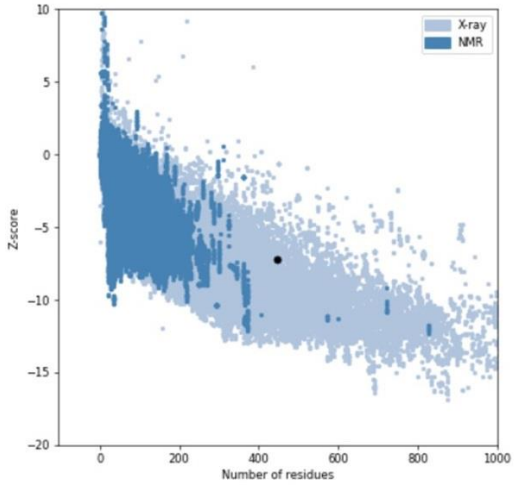

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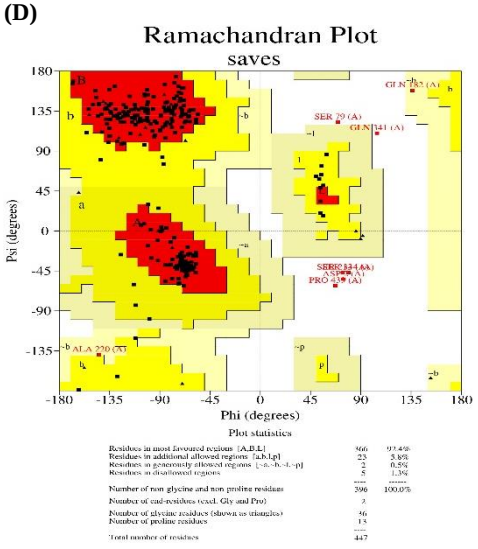

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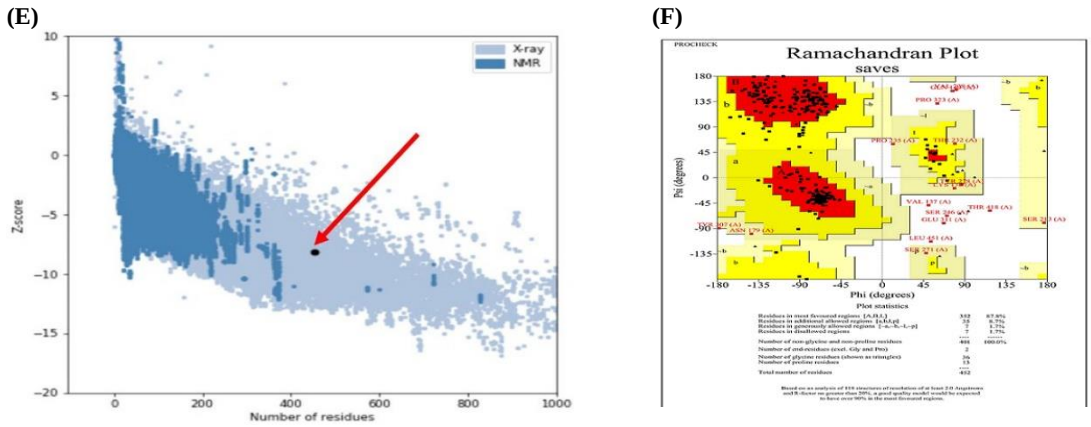

Fig. 2. The results of validation of vaccine structures. (A) ProsA Z-score of FlFlFP. (B) Ramachandran plot analysis of FlFlFP. (C) ProsA Z-score of FPFlFl. (D) Ramachandran plot analysis of FPFlFl. (E) ProsA Z-score of FlFPFl. (F) Ramachandran plot analysis of FlFPFl.

Evaluation of the docking of the vaccine constructs with TLR5

Comparative molecular docking analysis demonstrated distinct interaction patterns between the three engineered vaccine constructs and TLR5. Quantitative evaluation of docking outputs revealed significant variation in the predicted binding affinities. Among the constructs, the construct containing the centrally positioned adjuvant (FlFPFl) exhibited the most favorable docking characteristics, including higher predicted binding affinity and formation of a more stable receptor–ligand complex. In contrast, the N-terminal (FlFlFP) and C-terminal (FPFlFl) FliC configurations demonstrated comparatively weaker and less stable interaction patterns with TLR5 (Fig. 3). According to the results presented in Table 2, FlFPFl construct showed the most favorable interaction profile, characterized by the lowest ClusPro binding energy and the highest ZDOCK score. Given the critical role of TLR5 in initiating innate immune signaling cascades, the superior receptor-binding profile of FlFPFl supports its prioritization for subsequent studies.

Table 2. Comparative docking scores of vaccine constructs with TLR5 receptor

Receptor	Vaccine construct	Cluspro	ZDOCK Score
TLR5	FlFPFl	-1255.9	2186.716
TLR5	FlFlFP	-1189.1	2182.373
TLR5	FPFlFl	-1129.5	1872.161

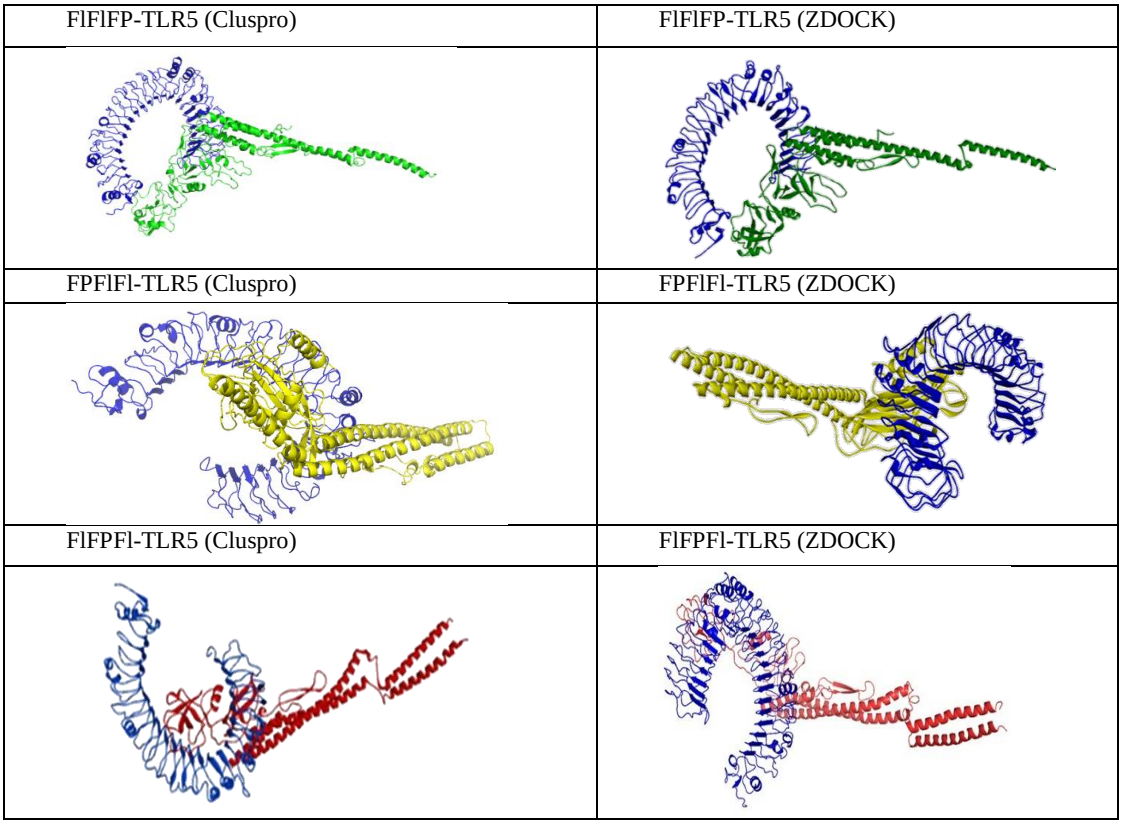

Fig. 3. Docking results of the vaccine constructs with TLR5. Detailed view of the interaction interface shows ClusPro binding energy and ZDOCK score.

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Expression and purification of the multi-epitope protein

After cloning and transformation of the multi-epitope gene, the recombinant clones were confirmed successfully by double enzyme digestion and sequencing. Under optimized induction conditions, SDS-PAGE analysis revealed a distinct protein band at approximately 53 kDa (Fig. 4), consistent with the theoretical molecular weight of the recombinant construct. Western blot analysis confirmed the specificity of expression by detecting a single immunoreactive band at the corresponding molecular weight (data not shown). Affinity purification resulted in a highly enriched ~53 kDa protein in the eluted fractions, with minimal nonspecific background (Fig. 4), indicating efficient purification. Applying the LPS removal kit reached the LPS concentration < 1 EU/ml, as measured by the Limulus amebocyte lysate (LAL) assay. Protein integrity was preserved following dialysis, and the final concentration of the purified recombinant protein was approximately 700 µg/mL.

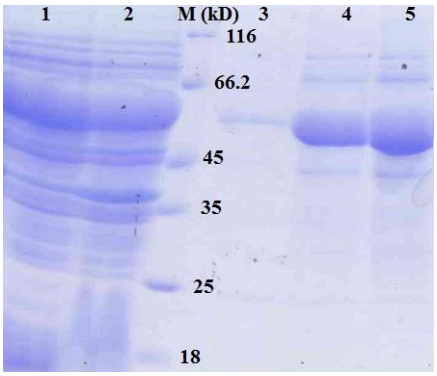

Fig. 4. Analysis of the expressed protein by SDS-PAGE. The recombinant protein was expressed in pET28a-BL21 system and purified by Nickel resin. Lane 1: Expressed protein (0.5 mM IPTG), Lane 2: Expressed protein (1 mM IPTG), Lane 3: Purified protein (elution 1), Lane 4: Purified protein (elution 2), Lane 5: Purified protein (elution 3). M: Protein marker.

Production and Secretion of IL-8

The results of ELISA indicated that treatment of HT-29 cells with the recombinant protein (10 µg/ml) significantly enhanced IL-8 production compared with the untreated control group (p=0.001) (Fig. 5).

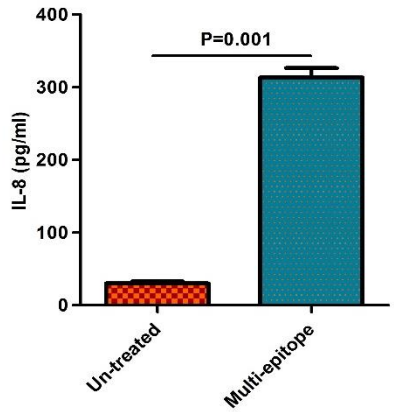

Fig. 5. Measurement of IL-8 in the cell line. The HT-29 cell lines were incubated with the multi-epitope and the level of IL-8 was measured in the supernatant. Statistical analysis confirmed a significant difference in IL-8 secretion between the two groups (p=0.001). Data are presented as mean ± SD of three independent experiments performed in triplicate.

DISCUSSION

The present findings further reinforce that the immunological performance of flagellin-based multi-epitope constructs is primarily governed by structure–function coupling rather than by sequence composition alone. In rational vaccine design, flagellin serves as a molecular immune amplifier, whose adjuvant potency depends on the conformational accessibility of conserved receptor-interacting motifs rather than simple incorporation into the recombinant framework [21, 22]. In this study, the centrally positioned FlFPFl construct demonstrated an optimal balance between structural rigidity and conformational adaptability, enabling stable receptor engagement while preserving biologically relevant molecular flexibility.

TLR5 recognition by flagellin is highly sensitive to the spatial presentation of functional motifs, particularly within the D1 domain, which contains conserved arginine-centered residues that form the primary receptor-binding interface. These positively charged residues facilitate electrostatic complementarity and hydrogen bonding with the extracellular domain of TLR5, promoting stable ligand–receptor complex formation. Central adjuvant localization appears to distribute the mechanical strain more uniformly across the protein backbone, minimizing the structural distortion near the receptor-binding hotspot. In contrast, terminal fusion configurations may introduce localized conformational instability or excessive rotational freedom, potentially reducing receptor affinity and signaling efficiency [8-10].

Beyond receptor binding, the flagellin–TLR5 interaction activates downstream innate immune signaling pathways, including NF-κB and MAPK cascades, which regulate epithelial cytokine secretion and antimicrobial immune responses. IL-8 plays a central role in neutrophil recruitment and early mucosal defense. The observed moderate but significant IL-8 induction in HT-29 epithelial cells indicates that structural optimization enabled controlled immunostimulation rather than excessive inflammatory amplification, which is particularly important in mucosal vaccine design, where immune activation should be balanced with epithelial barrier integrity [23, 24, 5].

The biological rationale for employing *S. typhimurium* flagellin as an adjuvant is further supported by its natural ability to interact with the immune receptors of intestinal epithelial cells. Given the basolateral localization of TLR5 in intestinal epithelial cells, engineered vaccine constructs should preserve the motif orientation and structural accessibility to ensure efficient receptor engagement. The superior docking profile of FlFPFl suggests that topology-guided engineering can improve the ligand orientation within the TLR5 binding pocket, thereby enhancing the interaction stability and signaling probability [24, 5]. The linker architecture also plays a crucial role in maintaining the structural integrity and functional independence of vaccine domains. The combined application of rigid EAAAK and flexible GGGGSGGGGS linkers enabled controlled interdomain spacing while minimizing steric interference and structural collapse. Rigid linkers contributed to the geometric stabilization of the recombinant construct, whereas flexible linkers provided limited conformational freedom required for antigen processing and immune recognition. This dual-linker strategy aligns with contemporary multi-epitope vaccine engineering principles aimed at optimizing folding kinetics and reducing domain–domain structural conflicts [8, 25, 22].

Although molecular docking provides mechanistic insights into potential receptor–ligand interactions, it represents a static approximation of biological behavior. Therefore, the docking

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results should be interpreted as predictive indicators of binding feasibility rather than as definitive measures of immune activation. Nevertheless, the favorable binding energy and stable clustering patterns observed for FlFPFl support the hypothesis that structural topology significantly influences receptor interaction thermodynamics and ligand recognition probability [26]. Experimental validation using HT-29 cells further confirmed the biological functionality of this optimized construct. The controlled pattern of IL-8 modulation suggests the successful translation of topology-guided structural design into measurable epithelial immune activity. Importantly, a moderated cytokine response may represent a desirable immunomodulatory profile for mucosal vaccine candidates, as excessive inflammatory signaling may disrupt epithelial homeostasis. Similar regulatory immune patterns have been reported in host–commensal microbial interactions, where selective cytokine modulation contributes to the mucosal immune balance [27-29].

In conclusion, despite certain limitations—namely, the absence of molecular dynamics (MD) analysis of the recombinant protein, the limited scope of *in vitro* cytokine evaluation, and the lack of *in vivo* efficacy testing in an animal model—this study underscores the critical importance of adjuvant topology in the design of multi-epitope vaccines. Importantly, our findings demonstrate that the immunological efficacy of flagellin-based constructs is strongly influenced by structural configuration rather than by primary sequence composition alone. Central placement of the flagellin adjuvant within the recombinant scaffold enhanced the exposure of receptor-interacting motifs, improved global folding stability, and generated more favorable interaction geometry with TLR5. Among the designed constructs, FlFPFl exhibited the most stable docking behavior, indicating that spatial optimization of adjuvant domains can significantly improve ligand–receptor compatibility and interaction stability. These findings support topology-guided vaccine design as a promising strategy for developing next-generation mucosal immunotherapeutics that target epithelial immune pathways.

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CONFLICT OF INTEREST

The authors declare they have no conflict of interest.

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