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In Silico Immunoinformatics Design and Evaluation of a Protein Based Vaccine Candidate Against Type 1 Diabetes Mellitus

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ABSTRACT

Background: *Type 1 diabetes mellitus (T1DM) results from autoimmune destruction of pancreatic β-cells, and no licensed vaccine currently prevents or halts this process. Computational immunology offers a rapid approach for identifying tolerogenic antigen combinations that may modulate autoimmune responses. Objective: To design and evaluate an in silico multi-epitope vaccine candidate incorporating the CTB–GM1 system to promote antigen-specific immune tolerance in T1DM.* ***Methods***: *Protein sequences were retrieved from NCBI and assessed using ProtParam, VaxiJen, AllerTOP, ToxinPred, and SolPro. T- and B-cell epitopes were predicted through IEDB, filtered by antigenicity, allergenicity, toxicity, and population coverage, and assembled using appropriate linkers and an adjuvant. Structural modelling was performed using I-TASSER, with validation by Ramachandran and ERRAT analyses. Molecular docking with GM1 was conducted via ClusPro, and immune responses were simulated using C-IMMSIM.* ***Results***: *The final construct incorporated five MHC-I, five MHC-II, and four B-cell epitopes and demonstrated favourable predicted antigenicity (VaxiJen score 0.62), non-allergenicity, and non-toxicity. Structural modelling indicated a stable conformation (ERRAT >95%). Docking predicted strong interaction with GM1 (best score –1050.7). Immune simulations suggested a Th2-leaning profile with increased IL-4, IL-5, IL-13 and generation of memory subsets; however, these findings are theoretical.* ***Conclusion***: *This CTB–GM1 multi-epitope construct shows promising computational characteristics, but all findings are predictive only and require comprehensive in vitro, in vivo, and clinical validation before any therapeutic relevance can be established.*

Keywords
Type 1 diabetes; CTB-GM1; in silico vaccine; epitope prediction; molecular docking; immune simulation.

INTRODUCTION

Chronic Type 1 Diabetes Mellitus (T1DM) is characterised by immune-mediated destruction of insulin-secreting pancreatic β-cells, leading to a progressive decline in endogenous insulin production and persistent hyperglycaemia (1). Uncontrolled hyperglycaemia damages multiple organ systems, including the cardiovascular, renal, neurological and visual pathways. T1DM is an autoimmune disease that typically manifests in childhood or early adulthood, whereas type 2 diabetes is primarily a metabolic disorder associated with obesity and insulin resistance and can occur at any age. Genetic susceptibility, particularly certain HLA class II alleles such as HLA-DR3 and HLA-DR4, together with environmental triggers, substantially increases the risk of developing T1DM rather than directly causing obesity or metabolic syndrome (2). Despite advances in insulin formulations and delivery technologies, many individuals with T1DM continue to experience hypoglycaemic episodes, long-term complications and a high treatment burden. T1DM therefore remains incurable with current standard care.

Immunotherapeutic approaches seek to modulate dysregulated immune responses against β-cell antigens, but their translation into routine clinical practice is still limited and requires further optimisation (3). Immunoinformatics-guided vaccine design offers a complementary strategy by identifying antigenic determinants that might either elicit protective immunity or induce antigen-specific tolerance. In T1DM, pathway-targeted or antigen-specific vaccines aim to induce immune tolerance to β-cell antigens, thereby slowing or halting autoimmune destruction (3,4). A key conceptual challenge is to design interventions that maintain overall immune homeostasis and protection against pathogens while selectively dampening autoreactive responses. Genetic heterogeneity, environmental influences and variability in disease onset across populations further complicate vaccine development and may necessitate population-tailored strategies (5,6). In preclinical models, vaccination of autoimmune-prone or at-risk animals before overt hyperglycaemia can delay or prevent disease onset, but safe translation to humans requires rigorous preclinical and clinical evaluation (6,7).

Cholera toxin B subunit (CTB) has emerged as a promising mucosal carrier and adjuvant for tolerogenic vaccination. CTB is the non-toxic B subunit of cholera toxin, which binds with high affinity to the GM1 ganglioside expressed on intestinal epithelial cells, neurons and various immune cells (8). Through this interaction, CTB can efficiently deliver linked antigens to mucosal immune inductive sites, enhancing antigen uptake and presentation while generally retaining a favourable safety profile compared with the holotoxin (8). Experimental studies suggest that CTB-antigen conjugates can promote regulatory T cell (Treg) responses, skew T helper responses towards a Th2-biased profile and enhance mucosal IgA production, thereby modulating autoimmune and allergic inflammation (9–11). In the context of T1DM, CTB-based constructs coupled to β-cell-related antigens have been investigated as potential tools to enhance tolerance to pancreatic β-cell epitopes and protect against autoimmune attack

in preclinical models (9–11). Effective adjuvant choice is particularly critical for chronic autoimmune diseases such as T1DM, where durable yet well-controlled immunological memory is required.

In parallel, in silico vaccine design has become an attractive, cost-effective and time-efficient approach for pre-laboratory screening of vaccine candidates. Bioinformatics, molecular modelling and immunoinformatics tools can be used to predict B- and T-cell epitopes, assess their antigenicity, allergenicity and toxicity, model three-dimensional vaccine structures, explore receptor interactions through docking, and simulate immune responses, thereby reducing the likelihood of failure in subsequent in vivo and clinical studies (12). Traditional drug and vaccine development is slow and expensive, whereas computational pipelines can prioritise the most promising constructs for experimental validation. Insulin injections remain the cornerstone of T1DM management but do not address the underlying autoimmunity or prevent progressive β-cell loss. There is therefore a strong need for immunomodulatory interventions that can preserve or restore β-cell function (13). Against this background, the present study aimed to design and in silico evaluate a CTB-based multi-epitope protein vaccine candidate targeting T1DM. Specifically, we sought to (i) retrieve and analyse CTB-derived sequences, (ii) predict and select B- and T-cell epitopes with favourable antigenicity, non-allergenicity and non-toxicity profiles, (iii) assemble a chimeric construct incorporating a 50S ribosomal protein adjuvant, appropriate linkers and a His-tag, (iv) model and validate the three-dimensional structure, and (v) assess receptor binding and immune response profiles using molecular docking and immune simulation tools (14). All findings are based on computational predictions and are intended to provide a rational framework for subsequent in vitro and in vivo evaluation of CTB-based tolerogenic vaccine candidates for T1DM.

MATERIALS AND METHODS

Multiple computational immunoinformatics tools were used to design and evaluate a CTB-based multi-epitope protein vaccine candidate targeting T1DM. The workflow included sequence retrieval, physicochemical and immunological profiling, epitope prediction, vaccine construct assembly with a 50S ribosomal protein adjuvant, structural modelling and validation, receptor docking, normal mode analysis and in silico immune simulation. Unless otherwise stated, web-based tools were used with their default parameters.

Sequence retrieval and protein selection

The design process began with selection of an appropriate cholera toxin B subunit (CTB) protein sequence as the base for epitope prediction and vaccine construction. CTB protein sequences were retrieved from the NCBI database (https://www.ncbi.nlm.nih.gov/), which provides curated protein records. Among the available entries, one CTB sequence associated with previously reported GM1 binding and suitable length for multi-epitope engineering was selected as the primary template for subsequent analyses.

Protein sequence analysis

The selected CTB amino acid sequence was analysed to characterise its basic structural and immunological properties. Secondary structure elements (α-helices, β-strands and coils) were predicted using the PSIPRED server, providing an initial indication of exposed and potentially antigenic regions. Physicochemical properties, including sequence length, molecular weight, theoretical isoelectric point (pI), amino acid composition, instability index, aliphatic index and grand average of hydropathicity (GRAVY), were calculated using the ExPASy ProtParam tool (https://web.expasy.org/protparam/).

To estimate its potential suitability as a vaccine component, the antigenicity of the CTB sequence was predicted using VaxiJen (https://www.ddg-pharmfac.net/vaxijen/), applying the recommended default model and threshold (0.4) for classification of a sequence as "probable antigen." Allergenicity was evaluated using AllerTOP (https://www.ddg-pharmfac.net/AllerTOP/) to classify the sequence as allergen or non-allergen. These analyses guided subsequent epitope selection from regions predicted to be antigenic and non-allergenic.

T-cell epitope prediction

T-cell epitopes were predicted to identify CTB-derived peptides with strong binding affinity to human MHC class I and II molecules. The CTB sequence was submitted to the Immune Epitope Database (IEDB; https://www.iedb.org/) T-cell epitope prediction tools. For MHC class I, peptide binding predictions were generated against a panel of common HLA class I alleles using IEDB-recommended prediction methods, and epitopes with predicted IC_{50} values below a pre-defined threshold (e.g., <200 nM) were considered high-affinity candidates. For MHC class II, similar predictions were performed for a representative set of HLA-DR, -DP and -DQ alleles, and peptides with strong predicted binding were shortlisted. To evaluate potential global applicability, the IEDB population coverage tool was used to estimate theoretical population coverage of the selected MHC class I and II epitopes across different geographic and ethnic populations. Shortlisted epitopes were further screened for antigenicity using VaxiJen and for allergenicity using AllerTOP to retain only those predicted to be antigenic, non-allergenic and thus more suitable for inclusion in the vaccine construct.

B-cell epitope prediction

Linear B-cell epitopes were predicted to identify CTB regions likely to be recognised by B cells and to contribute to antibody generation. The CTB sequence was analysed using the IEDB B-cell epitope prediction tools, applying consensus-based methods to identify continuous peptide segments with favourable physicochemical and structural properties (e.g., surface accessibility, flexibility, hydrophilicity). Predicted linear B-cell epitopes were subsequently evaluated using VaxiJen and AllerTOP to assess their antigenicity and allergenicity, respectively. Only epitopes classified as probable antigens and non-allergens were prioritised. Where appropriate, predicted B-cell epitopes were also assessed for their potential coverage across different human populations using the IEDB population coverage tool.

Vaccine construct assembly

A chimeric multi-epitope vaccine construct was then designed by combining the selected CTB-derived B-cell and T-cell epitopes with an N-terminal adjuvant domain and a C-terminal purification tag. The 50S ribosomal protein was used as an adjuvant moiety and fused to the N-terminus

of the construct to enhance immunogenicity and improve immune system activation. Epitope-rich regions from CTB were organised in a rational order to optimise presentation of both MHC class I– and class II–restricted epitopes as well as B-cell epitopes.

Three types of peptide linkers were employed to maintain structural flexibility, reduce junctional immunogenicity and facilitate proper processing: an EAAAK linker was used to couple the adjuvant domain to the first epitope module; GPGPG linkers were used primarily to connect B-cell and T-cell epitopes and to enhance helper T-cell stimulation; and AAY linkers were incorporated between cytotoxic T-cell epitopes to promote proteasomal processing and MHC class I presentation. A hexahistidine (6×His) tag was added at the C-terminus of the final sequence to enable affinity purification and detection of the recombinant protein. The final construct sequence was checked to ensure reading frame integrity and absence of stop codons within the designed protein.

Antigenicity, allergenicity, toxicity, solubility and physicochemical profiling of the vaccine construct

After assembling the full multi-epitope construct, its overall antigenic, allergenic, toxic and physicochemical properties were reassessed. VaxiJen was used to predict the antigenicity of the entire chimeric protein and to confirm that the construct retained a global antigenicity score above the selected threshold. AllerTOP was applied to the full sequence to verify that the construct remained non-allergenic. Potential toxicity of the multi-epitope protein was predicted using ToxinPred, retaining constructs classified as non-toxic.

Protein solubility upon overexpression in a heterologous host was predicted using the SOLpro server, which provides a probability score for a sequence being soluble. The physicochemical characteristics of the final construct, including molecular weight, theoretical pI, instability index, aliphatic index and GRAVY, were evaluated again using ExPASy ProtParam to judge its stability and suitability for recombinant expression.

Three-dimensional structure prediction and validation

The three-dimensional (3D) structure of the multi-epitope vaccine construct was modelled using the I-TASSER server, a widely used platform for protein structure prediction based on iterative threading and fragment assembly. The full vaccine amino acid sequence was submitted to I-TASSER, and the top-ranked model(s) were selected based on the C-score and template modelling score.

Model quality and stereochemical properties were evaluated using Ramachandran plot analysis to assess backbone dihedral angle distributions and to ensure that the majority of residues fell within favoured and allowed regions. Additional structural validation was performed using ERRAT to examine non-bonded atomic interactions and overall model reliability. Only models with acceptable Ramachandran and ERRAT statistics were retained for downstream interaction studies.

Molecular docking with a GM1-binding receptor

To explore the potential interaction between the CTB-based vaccine construct and a GM1-binding receptor, protein–protein docking simulations were performed using the ClusPro server (https://cluspro.bu.edu/). The validated 3D structure of the multi-epitope vaccine served as the ligand, and a GM1-binding receptor or receptor surrogate structure was used as the docking partner. ClusPro generates multiple docked complexes by sampling relative orientations and then clustering low-energy poses.

The resulting docked complexes were ranked according to the ClusPro energy-based scoring functions, and the best-scoring cluster(s) were inspected visually to identify plausible binding interfaces and contact residues. The top-ranked model, with the most favourable predicted interaction energy, was selected as the representative complex for subsequent stability analysis and interpretation of potential receptor engagement.

Normal mode analysis of vaccine flexibility

The dynamic behaviour and intrinsic flexibility of the docked vaccine–receptor complex were examined using the iMODS server, which performs normal mode analysis (NMA) in internal coordinates. The selected docked complex was submitted to iMODS, and low-frequency normal modes were analysed to characterise collective motions, deformability, and overall structural stability.

Parameters such as deformability profiles (per-residue mobility), eigenvalue (reflecting the stiffness of the mode), variance, covariance maps and elastic network models were evaluated. Deformability plots helped identify flexible regions of the vaccine construct, while the eigenvalue provided an indication of the energy required for deformation. Covariance and elastic network analyses were used to assess correlated motions and internal connectivity, respectively, providing insight into whether the vaccine–receptor complex adopted a stable yet sufficiently flexible conformation in silico.

In silico immune simulation

To predict the potential immune response elicited by the CTB-based multi-epitope vaccine, in silico immune simulations were conducted using the C-IMMSIM server. The amino acid sequence of the final construct was provided as antigen input, and simulations were configured to mimic repeated vaccine doses over time. C-IMMSIM uses a position-specific scoring matrix–based method to process epitopes and models cellular and humoral immune responses using an agent-based representation of B cells, T helper cells, cytotoxic T cells and antigen-presenting cells.

The simulation tracked key immune events, including antigen uptake, T-cell activation, B-cell activation, antibody (IgM, IgG and IgA) production, and the generation and maintenance of memory B and T cells. Cytokine profiles and cell population dynamics were monitored across simulation time-steps to assess whether the construct was predicted to induce robust but controlled humoral and cellular responses consistent with a tolerogenic and protective profile. The resulting immune kinetics, antibody titres and memory cell levels were subsequently interpreted in the context of the vaccine's proposed role in modulating autoimmunity in T1DM.

RESULTS

The development of a CTB-based multi-epitope vaccine candidate for T1DM was initiated by selecting a cholera toxin B subunit (CTB) protein sequence as the base scaffold. Relevant CTB sequences were retrieved from the NCBI protein database, and a single CTB sequence (GenBank ID: AMR44217.1) was chosen for subsequent analyses based on its completeness and canonical GM1-binding characteristics. The amino acid sequence used for immunoinformatics analysis is shown in Table 1.

Table 1. CTB protein sequence (GenBank ID: AMR44217.1) used as the template for epitope prediction.

MIKLKFGVFFTVLLSSAYAHGTPQNITDLCAEYHNTQIHTLNDKIFSYTESLAGKREMAIITFKNGATFQ VEVPGSQHIDSQKKAIERMKDTLRIAYLTEAKVEKLCVWNNKTPHAIAAISMAN

Protein sequence analysis

ProtParam analysis of the CTB sequence showed a length of 124 amino acids with a calculated molecular weight of 13,919.09 Da and a theoretical pI of 8.91. The protein contained 1,967 atoms with a molecular formula of $C_{624}H_{989}N_{167}O_{181}S_{6}$. The instability index was 35.0, indicating that the protein is predicted to be stable in vitro. Antigenicity prediction using VaxiJen yielded a score of 0.5291, which is above the recommended threshold of 0.4 for probable antigens, suggesting that CTB has intrinsic vaccine-relevant antigenic potential. Allergenicity prediction using AllerTOP classified the sequence as non-allergenic, supporting its suitability as a vaccine scaffold. Secondary structure analysis performed with PSIPRED indicated that the CTB sequence comprises alternating α-helices and β-strands flanked by coil regions, with several predicted helices and strands clustered in the central region of the protein. Residue property mapping showed a mixture of hydrophobic and polar amino acids, with hydrophobic residues enriched in the core and polar residues more frequent on the surface, consistent with a soluble, secreted protein.

Protein structure prediction for CTB

The tertiary structure of the CTB template was predicted using the I-TASSER server. The top-ranked model adopted the characteristic CTB fold, with a β-sandwich core flanked by α-helices and an extended flexible tail region (Figure 1A). The predicted structure provides a basis for understanding the spatial distribution of potential epitopes and for subsequent comparison with the multi-epitope vaccine construct.

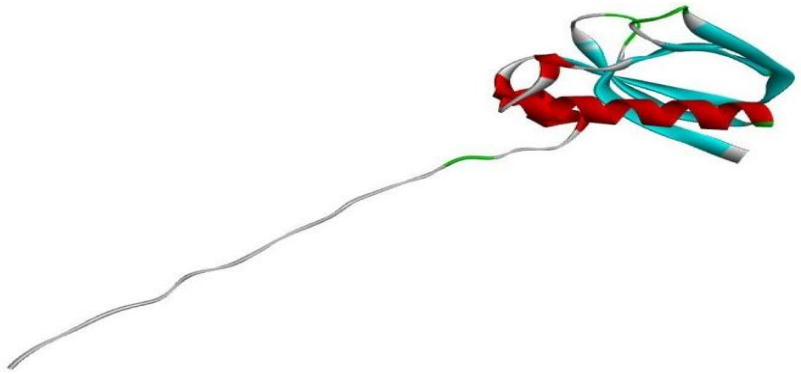

Figure 1. I-TASSER-predicted three-dimensional structures. (A) Model of the CTB scaffold (GenBank ID: AMR44217.1) showing α-helices (red), β-strands (cyan) and loops (grey/green). (B) Model of the final multi-epitope vaccine construct (see Section 3.8), illustrating the CTB-like core and extended epitope/linker regions.

T-cell epitope analysis > 1 MHC class I epitopes

To identify CTB-derived peptides with strong binding to human MHC class I alleles, the CTB sequence was analysed using IEDB prediction tools. A total of 182 candidate 9–11-mer peptides were initially predicted with IC_{50} values below 500 nM. From these, 10 epitopes with the strongest predicted binding (IC_{50} < 200 nM) and broad allele coverage were shortlisted.

These 10 candidates were then filtered based on antigenicity (VaxiJen score ≥0.8), predicted non-allergenicity (AllerTOP) and non-toxicity (ToxinPred), as well as IEDB population coverage. After this stepwise refinement, five MHC class I epitopes were retained as final candidates with an estimated cumulative global population coverage of approximately 91%. The final CTB-derived MHC I epitopes and their positions are summarised in Table 3.

Table 3. Final CTB-derived MHC class I epitopes selected for inclusion in the multi-epitope vaccine construct.

Start	End	Peptide	Length	Representative HLA class I alleles*
1	9	MIKLKFGVF	9	HLA-B*08:01, HLA-B*15:01
3	12	KLKFGVFFTV	10	HLA-A*02:01, HLA-A*02:03, HLA-A*02:06
46	56	FSYTESLAGK	11	HLA-A*11:01, HLA-A*68:01, HLA-A*03:01
56	63	REMAIITFK	8	HLA-A*31:01, HLA-A*11:01, HLA-A*03:01
70	79	TPHAIAAISM	10	HLA-B*07:02

*Alleles listed are examples of those with the strongest predicted binding in IEDB.

All five epitopes were predicted to be antigenic (VaxiJen score ≥0.8), non-allergenic and non-toxic, and were therefore considered suitable for inclusion in the vaccine design.

MHC class II epitopes

For MHC class II, IEDB analysis identified 1,627 conserved predicted epitopes (typically 15–17-mers) with IC_{50} values below 200 nM across a representative panel of HLA-DR, -DP and -DQ alleles. From these, 500 peptides with broader allele binding profiles were examined in greater detail. Ten epitopes that bound to at least five different HLA class II alleles were shortlisted and further evaluated.

Filtering based on antigenicity (VaxiJen ≥0.8), non-allergenicity, non-toxicity and population coverage resulted in five final MHC class II epitopes with an estimated global coverage of approximately 89%. These epitopes, their positions and representative HLA class II restrictions are shown in Table 4.

Table 4. Final CTB-derived MHC class II epitopes retained after antigenicity, allergenicity, toxicity and population coverage filtering.

Start	End	Peptide	Length	Representative HLA class II alleles*
4	18	LKFGVFFTVLLSSAY	15	HLA-DPA1*02:01/DPB1*01:01, HLA-DPA1*01:03/DPB1*02:01, HLA-DRB1*12:01, HLA-DRB1*01:01
43	57	KIFSYTESLAGKREM	15	HLA-DRB5*01:01, HLA-DRB1*07:01, HLA-DRB1*04:01, HLA-DRB1*01:01
42	58	DKIFSYTESLAGKRE	17	HLA-DRB5*01:01, HLA-DRB1*04:01, HLA-DRB1*07:01, HLA-DRB1*01:01
54	68	GKREMAIITFKNGAT	15	HLA-DRB1*15:01, HLA-DRB1*08:02, HLA-DRB5*01:01, HLA-DRB4*01:01, HLA-DRB1*11:01
14	27	LLSSAYAHGTPQNIT	14	HLA-DQA1*05:01/DQB1*03:01, HLA-DRB1*01:01

*Representative alleles with high predicted binding in IEDB.

All five MHC II epitopes were predicted to be antigenic, non-allergenic and non-toxic and were therefore incorporated into the final vaccine design.

B-cell epitope analysis

Linear B-cell epitopes within CTB were predicted using IEDB B-cell tools to identify regions likely to be recognised by B cells and able to induce antibody responses. A consensus-based approach was applied, integrating multiple physicochemical criteria (surface accessibility, flexibility and hydrophilicity). Four linear B-cell epitopes were selected based on favourable prediction scores and location in exposed surface regions (Table 5). Each of the four epitopes showed VaxiJen scores above the antigenicity threshold and was predicted by AllerTOP to be non-allergenic, supporting their inclusion in the multi-epitope construct. These epitopes span N-terminal, central and C-terminal regions of CTB, providing broad surface coverage.

Table 5. Predicted CTB-derived linear B-cell epitopes selected for inclusion in the vaccine construct.

Start	End	Peptide	Length
20	39	HGTPQNITDLCAEYHNTQIH	20
46	56	FSYTESLAGKR	11
71	90	VEVPGSQHIDSQKKAIERMK	20
109	118	WNNKTPHAIA	10

Vaccine construction

The final multi-epitope vaccine construct was assembled by combining 4 CTB-derived B-cell epitopes, 5 MHC class I epitopes and 5 MHC class II epitopes with an N-terminal 50S ribosomal protein adjuvant and a C-terminal 6×His tag. An EAAAK linker was used to fuse the 50S ribosomal protein adjuvant to the first B-cell epitope at the N-terminus, providing structural rigidity and functional separation between the adjuvant and epitope regions.

GPGPG linkers were used to connect B-cell epitopes and to bridge B-cell epitopes with helper T-cell epitopes, aiming to promote efficient antigen processing and helper T-cell activation. AAY linkers were inserted between cytotoxic T-cell epitopes to facilitate proteasomal cleavage and MHC class I presentation. The C-terminal hexahistidine tag (6×His) was incorporated to facilitate downstream purification of the recombinant protein.

An excerpt of the epitope–linker arrangement within the vaccine construct is shown in Table 6, illustrating the order of epitopes and linkers in the non-adjuvant region.

Table 6. Schematic representation of epitope–linker arrangement in the non-adjuvant region of the multi-epitope vaccine construct.

EAAAK-HGTPQNITDLCAEYHNTQIH-FSYTESLAGKR-VEVPGSQHIDSQKKAIERMK-WNNKTPHAIA-GPGPG-KLKFGVFFTV-MIKLKFGVF-TPHAIAAISM-REMAIITFK-FSYTESLAGK-AAY-GKREMAIITFKNGAT-KIFSYTESLAGKREM-LKFGVFFTVLLSSAY-DKIFSYTESLAGKRE-LLSSAYAHGTPQNIT-HHHHHH

Vaccine stability, antigenicity, allergenicity, solubility and toxicity profiling

Global immunological and physicochemical profiling of the full multi-epitope construct showed that the chimeric vaccine retained favourable properties. VaxiJen predicted the construct to be antigenic with a score of 0.6246, exceeding the 0.4 threshold and indicating good potential to stimulate immune responses. AllerTOP classified the full construct as non-allergenic, while ToxinPred predicted it to be non-toxic, suggesting a low risk of allergenicity and toxicity in silico.

SOLpro predicted the vaccine to be soluble with a probability score of 0.528, consistent with acceptable solubility upon heterologous expression. ProtParam analysis indicated that the chimeric protein has physicochemical parameters compatible with a stable recombinant antigen, including an instability index below the threshold for instability and an aliphatic index compatible with moderate thermostability. Together, these predictions support the suitability of the designed construct for further experimental evaluation.

Vaccine structure prediction and validation

The three-dimensional structure of the complete multi-epitope vaccine construct (adjuvant, CTB-derived epitopes, linkers and His tag) was predicted using I-TASSER. The best-ranked model displayed a compact CTB-like core formed by α-helices and β-strands, with the adjuvant and epitope/linker regions extending as flexible tails (Figure 2B and Figure 2).

Structural validation using a Ramachandran plot showed that 96.6% of residues were located in favoured or additionally allowed regions, indicating good stereochemical quality. ERRAT analysis produced an overall quality factor greater than 95%, consistent with a reliable model of the vaccine construct. These data suggest that the designed protein can adopt a physically plausible and stable tertiary structure in silico (Figure 2).

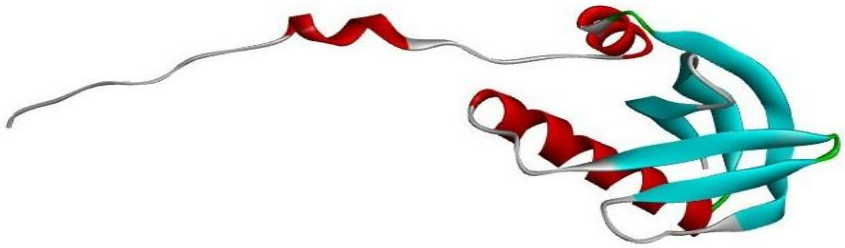

Figure 2. Predicted three-dimensional structure of the CTB-based multi-epitope vaccine construct obtained from I-TASSER. The CTB-like core is shown as β-strands (cyan) and α-helices (red), while extended linker/epitope regions are represented as flexible loops (grey/green).

To assess the ability of the vaccine construct to interact with a GM1-binding receptor, protein–protein docking was performed using ClusPro. The validated vaccine model was docked against a GM1-binding receptor surrogate, and docked complexes were clustered based on their interaction energies. The top electrostatically favoured model exhibited a ClusPro energy score of approximately −1050.7, while the hydrophobic-favoured model scored around −13,309, indicating highly favourable interaction energies for both scoring schemes. A combined van der Waals–electrostatic model also showed a substantially negative score (~−233.8). Visual inspection of the top-ranked complex revealed that the CTB-like core of the vaccine construct engages the receptor surface in a manner consistent with GM1-recognition regions, while the extended epitope segments project away from the interface (Figure 4). These findings suggest that the designed construct is capable of maintaining receptor-binding capacity in silico.

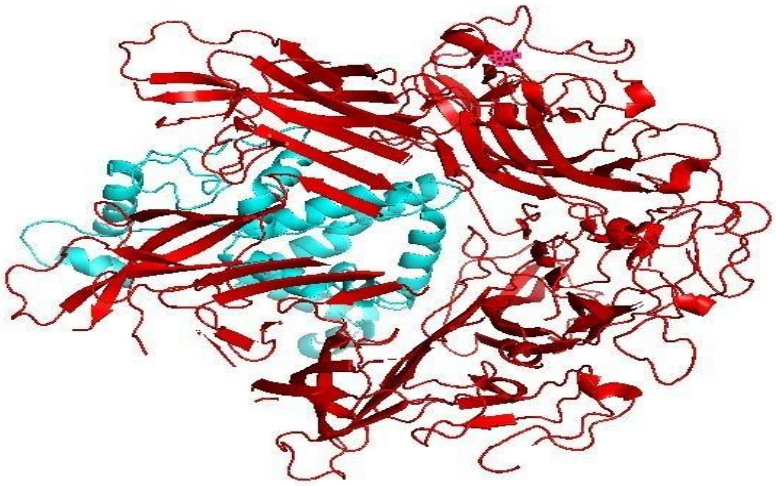

Figure 3. Docked complex between the CTB-based multi-epitope vaccine construct (cyan helices and strands) and the GM1-binding receptor surrogate (red) as predicted by ClusPro. The model shown corresponds to the top-ranked cluster based on interaction energy.

Normal mode analysis of vaccine–receptor complex

The dynamic stability and flexibility of the docked vaccine–receptor complex were evaluated using normal mode analysis (NMA) in iMODS. Deformability plots indicated generally low to moderate per-residue mobility across most of the complex, with higher flexibility confined mainly to terminal regions and loop segments of the vaccine construct (Figure 5, upper left).

The first eigenvalue associated with the dominant normal mode was 6.397183×10^{-5}, indicating that relatively low energy is required for collective motions, yet the overall complex remains structurally coherent (Figure 5, upper right). The predicted B-factor profile derived from NMA agreed with the deformability analysis, highlighting limited fluctuations in the core interface region (Figure 5, lower left). Variance analysis revealed that a small number of low-frequency modes account for the majority of the collective motion, as shown by the cumulative variance curve approaching a plateau across the first 20 modes (Figure 5, lower right). Collectively, these data support a stable but flexible vaccine–receptor complex in silico.

Figure 4. Normal mode analysis of the docked vaccine–receptor complex generated by iMODS. Panels show (top left) per-residue deformability, (top right) eigenvalues for the first 20 normal modes (with eigenvalue(1) = 6.397183 × 10 $^{-5}$), (bottom left) predicted B-factor profile and (bottom right) individual and cumulative variance associated with each mode.

In silico immune simulation

The immunogenic potential of the CTB-based multi-epitope construct was explored using the C-IMMSIM server. Simulations were configured to mimic repeated vaccine administrations over a 30–35-day period.

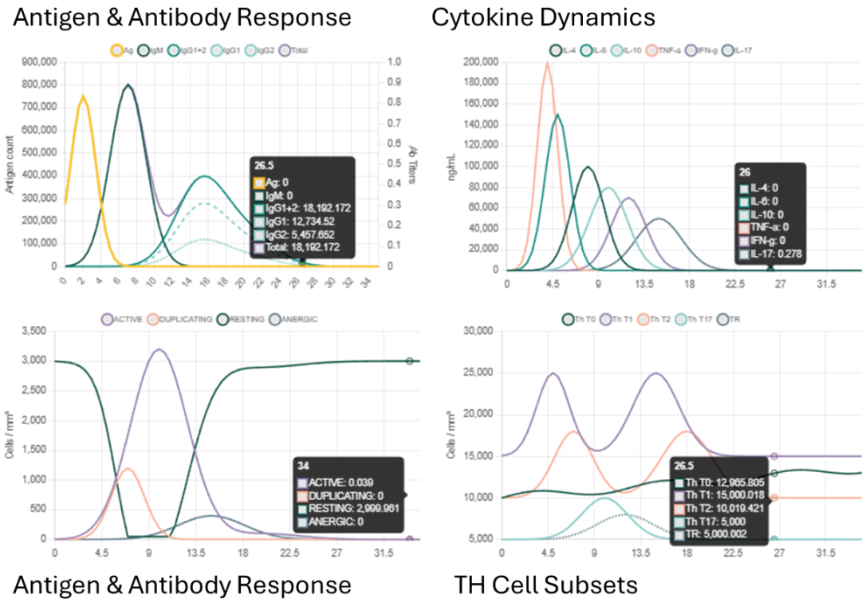

Figure 5. C-IMMSIM immune simulation outputs for the CTB-based multi-epitope vaccine construct. Top left: antigen load and antibody isotype kinetics over 35 days (Ag, IgM, IgG subclasses, IgA). Top right: cytokine concentration profiles (e.g., IFN-γ, IL-2, IL-4, IL-10, TNF-α). Bottom left: Th cell population per activation state (active, duplicating, resting, anergic). Bottom right: total Th cells and Th1, Th2, Th17 and regulatory T-cell subset dynamics and relative proportions over time.

Antigen concentration (Ag) peaked immediately after administration and declined rapidly, with near-complete clearance by approximately day 8–10 (Figure 6, top left). A primary IgM response emerged first, peaking shortly after antigen exposure, followed by class-switched IgG responses (notably IgG1 and IgG2) and a detectable IgA response. Antibody titres showed a characteristic rise-and-fall pattern with persistence of IgG isotypes beyond the decline of antigen. Cytokine profiles indicated robust early production of IFN-γ and IL-2, accompanied by detectable levels of other cytokines such as IL-4, IL-10 and TNF-α (Figure 6, top right). These patterns are consistent with activation of both cellular and humoral arms of the immune system in silico.

Helper T-cell dynamics showed a rapid expansion of active Th cells, reaching a plateau of approximately 3,000–3,500 cells/mm ^{3} by about day 10 and remaining relatively stable thereafter, with declining proportions of duplicating and anergic cells (Figure 6, bottom left). Subset analysis demonstrated that total Th cell counts increased to around 60,000–70,000 cells/mm ^{3}, with Th1 cells predominating and contributions from Th2, Th17 and regulatory T-cell (T $_{R}$) subsets (Figure 6, bottom right). The proportion plot suggested a stable Th subset distribution after the initial expansion phase. Overall, the C-IMMSIM simulations predicted that the designed multi-epitope construct can elicit a multi-wave immune response characterised by initial IgM production, class-switched IgG and IgA responses, activation of Th subsets and the development of immunological memory cells. These findings, while purely computational, support the potential of the CTB-based vaccine candidate to induce sustained cellular and humoral responses that warrant further experimental validation.

DISCUSSION

This study used an immunoinformatics pipeline to design and in silico evaluate a CTB-based multi-epitope protein vaccine candidate that could, in principle, be used to modulate autoimmune responses in Type 1 Diabetes Mellitus (T1DM). CTB, the non-toxic B subunit of cholera toxin, binds with high affinity to the GM1 ganglioside expressed on intestinal epithelial cells and antigen-presenting cells, making it an attractive carrier for mucosal delivery of linked antigens (15–17). When coupled to disease-relevant peptides, CTB has been reported in preclinical models to enhance antigen uptake in gut-associated lymphoid tissue, promote regulatory T cell (Treg) responses and, in some settings, reduce autoimmune or allergic inflammation (16,17). Our work builds on this concept by identifying CTB-derived T- and B-cell epitopes with favourable in silico properties and assembling them into a chimeric construct that retains predicted GM1-binding capacity while presenting multiple epitopes in a single protein.

The epitope prediction and filtering steps yielded a focused panel of five MHC class I, five MHC class II and four linear B-cell epitopes from the CTB sequence. These epitopes were selected based on high predicted binding affinity to common HLA alleles, broad theoretical population coverage and favourable antigenicity, non-allergenicity and non-toxicity profiles. The inclusion of both class I and class II T-cell epitopes is intended to support coordinated activation of $CD8^+$ cytotoxic and $CD4^+$ helper T cells, while B-cell epitopes provide targets for antibody generation. The theoretical population coverage values suggest that the selected epitope set could be relevant across diverse human populations, although this remains to be validated experimentally.

Structurally, the designed multi-epitope construct combined the CTB-derived epitope set with a 50S ribosomal protein adjuvant at the N-terminus and a C-terminal His tag, linked via rationally selected spacers (EAAAK, GPGPG, AAY). I-TASSER modelling indicated that the chimeric protein can adopt a plausible three-dimensional conformation in which a CTB-like core is preserved and epitope/linker segments extend outward as flexible regions. Ramachandran and ERRAT analyses supported the stereochemical quality and overall reliability of the model. These findings are important because maintenance of the CTB fold is expected to be critical for GM1 binding and mucosal targeting (15–17).

Protein–protein docking using ClusPro suggested that the vaccine construct is capable of forming a stable complex with a GM1-binding receptor surrogate, with favourable interaction energies for electrostatic, hydrophobic and combined scoring models. Visual inspection showed that the CTB-like domain of the construct engaged the receptor surface while the engineered epitope regions remained largely solvent-exposed. Normal mode analysis using iMODS indicated that the docked complex is dynamically stable, with limited deformability in the core interface and greater flexibility in terminal loops. Together, these results provide in silico support for the hypothesis that epitope engineering did not abolish the capacity of the CTB region to interact with a GM1-binding receptor, although this must be tested empirically.

In silico immune simulations using C-IMMSIM predicted that the multi-epitope construct can elicit a multi-phase immune response characterised by an early IgM peak followed by class-switched IgG and IgA responses, expansion of helper T-cell populations and generation of memory cells. The cytokine profile included IFN-γ and IL-2, together with regulatory and Th2-associated cytokines such as IL-4 and IL-10, suggesting a mixed but sustained response. For a tolerogenic T1DM vaccine, complete Th1 suppression is unlikely to be desirable; rather, a balanced profile with adequate regulatory and non-inflammatory components may be advantageous (18–21). However, because C-IMMSIM simulations rely on generic immune parameters and do not incorporate T1DM-specific autoantigen contexts, these outputs should be interpreted qualitatively as indicators of immunogenic potential rather than definitive evidence of a particular Th1/Th2/Treg balance.

Our findings align with earlier experimental work showing that CTB-based conjugates can enhance oral tolerance or modulate autoimmune responses in animal models (16,17,22). The present study extends this concept by applying a systematic immunoinformatics framework to epitope selection, construct design and pre-screening. Such computational approaches can help prioritise candidates for laboratory testing by excluding sequences predicted to be poorly antigenic, allergenic or toxic, and by providing early information on structural feasibility and potential receptor interactions (23–25). At the same time, it is important to emphasise that in silico predictions cannot substitute for experimental data; they are best viewed as a triage and hypothesis-generation tool within a broader translational pipeline.

This work has several important limitations. First, all data presented here are purely computational. No in vitro MHC-binding assays, T-cell activation assays, B-cell epitope mapping, or in vivo studies in T1DM models were performed, and the true immunological behaviour of the construct may differ substantially from predictions. Second, epitope selection was restricted to CTB-derived sequences; β-cell–specific autoantigens (e.g., insulin, GAD65, IA-2) were not explicitly incorporated. The current construct therefore represents a CTB-based platform rather than a fully developed β-cell autoantigen vaccine, and future designs could integrate epitopes directly derived from T1DM-relevant antigens. Third, the docking studies used a GM1-binding receptor surrogate rather than a complete cell-surface context, and the iMODS analysis provides normal mode behaviour rather than full atomistic molecular dynamics in explicit solvent. The stability and binding modes inferred from these analyses should thus be validated using higher-resolution simulation methods and experimental binding studies. Fourth, the C-IMMSIM simulations use generic human immune parameters and do not account for patient-to-patient variability, HLA diversity beyond the selected alleles, or the complex immunopathology of T1DM.

Despite these limitations, the study illustrates how an integrated immunoinformatics workflow can generate a coherent CTB-based multi-epitope construct with favourable predicted antigenicity, structural plausibility, receptor binding and immune stimulation profiles. Future work should focus on several key steps: cloning and expression of the designed construct; empirical assessment of solubility, stability and oligomerisation; in vitro evaluation of MHC binding and T-cell activation; assessment of Treg induction and cytokine profiles in relevant immune cell cultures; and ultimately, testing in appropriate animal models of T1DM to evaluate safety, tolerogenic capacity and effects on β-cell preservation. Comparative studies with other antigen-specific interventions, including peptides derived from insulin and other β-cell antigens, will be needed to determine the relative advantages of this CTB-based design.

In summary, while the present work does not provide clinical evidence or claim therapeutic efficacy, it offers a structured, data-rich starting point for experimental development of CTB-based multi-epitope vaccines aimed at modulating autoimmunity in T1DM.

CONCLUSION

In this study, we used an immunoinformatics-driven approach to design and in silico evaluate a CTB-based multi-epitope protein vaccine candidate for Type 1 Diabetes Mellitus. CTB-derived T- and B-cell epitopes with favourable predicted antigenicity, non-allergenicity and non-toxicity were combined with a 50S ribosomal protein adjuvant and rational linkers to form a chimeric construct. Structural modelling suggested that the construct

can adopt a stable CTB-like fold while maintaining accessible epitope regions and preserving predicted GM1-binding capacity. Docking, normal mode analysis and immune simulations supported the potential of the construct to interact with a GM1-binding receptor and to elicit coordinated humoral and cellular responses in silico.

These findings should be regarded as preliminary and hypothesis-generating. Experimental validation is essential to confirm expression, structural integrity, GM1 binding, immunogenicity, safety and, critically, any capacity to induce antigen-specific tolerance or protect pancreatic β-cells in vivo. Nonetheless, the present work underscores the value of integrating epitope prediction, structural modelling and immune simulation as an efficient first step in the rational design of candidate vaccines for complex autoimmune diseases such as T1DM.

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