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In-Silico Design of a PIN1/ABCC5-Targeted Multi-Epitope Vaccine for Nasopharyngeal Cancer

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HIGHLIGHTS

- PIN1/ABCC5 multi-epitope vaccine construct has a predicted antigenic, non-allergenic, non-toxic construct with favorable solubility (<40)
- Immune simulation indicates the PIN1/ABCC5 multi-epitope vaccine construct produces robust CTL/HTL and IFN-γ responses
- Strong predicted binding of the PIN1/ABCC5 multi-epitope vaccine construct to human TLR4 and stable docked complexes
- The designed PIN1/ABCC5 multi-epitope vaccine for NPC has a broad HLA coverage across Indonesian alleles (≈ 95% population)

ABSTRACT

Nasopharyngeal Carcinoma (NPC) is a rare malignancy. However, it is the fourth most common cancer affecting both sexes in Indonesia, and research on NPC remains limited. NPC is related to Epstein-Barr Virus (EBV) infection, making it a potential target for immunotherapy strategies. Developing a multi-epitope-based NPC vaccine targeting tumor-overexpressed antigens may evoke an immune response against cancer cells and benefit patients with advanced cancer stages or those resistant to treatment. Four cytotoxic (CTL) and one helper T-cell (HTL) epitopes from PIN1 were identified using NetMHCpan and NetMHCIIpan algorithms, respectively. Whereas from ABCC5, five CTL and four HTL epitopes were identified using the same algorithms. These epitopes were found to have good coverage across the Indonesian population, with population coverage analysis showing 99% coverage for human leukocyte antigen (HLA) Class I and 95% for HLA Class II. Having fulfilled other criteria such as immunogenicity, IFN-γ-inducing ability, and non-homology to human peptides, the epitopes were assembled into a vaccine construct together with *E. coli* and *Bacillus* as adjuvants and appropriate linkers. The construct was shown to have good physicochemical characteristics and the ability to induce CTL and HTL responses, which stem from the engagement of the vaccine with toll-like receptor 4 (TLR4) as revealed by docking simulations.

INTRODUCTION

On top of the soft palate, the nasopharynx connects the nose to the oropharynx and serves as part of the airway to the lungs. In the nasopharynx, benign (non-cancerous) and malignant (cancerous) tumors can form. Squamous cell carcinomas are the most common tumors found in this area and behave differently from other head and neck cancers. The fossa of Rosenmüller, also known as the pharyngeal recess, is the most common site of origin (Sinha et al., 2024).

Nasopharyngeal carcinoma (NPC) is the most frequent cancer of the nasopharynx. Carcinoma is a type of cancer that begins in the cells that line the internal and external surfaces of the body (epithelial cells). NPC, previously known as lymphoepithelioma, is a rare type of cancer that affects the region of the throat that connects the back of the nose to the back of the mouth (the pharynx). This cancer is a unique kind of head and neck cancer that originates from epithelial cells that line the nasopharyngeal surface as well as its inner wall (Xu et al., 2016).

NPC is rare and affects people worldwide, with a prevalence of less than 1 in 100,000 (Adham et al., 2012). However, it is endemic to various parts of the globe, most notably Southeast Asia, and has been shown to have a bad prognosis. In Indonesia, NPC is the fourth most common cancer of both sexes, preceded by breast cancer, cervical cancer, and hematopoietic and reticuloendothelial system malignancy (Gondhowiarjo et al., 2019). Little is known about NPC in the country, despite the recorded mean prevalence of 6.2/100,000 and 13,000 new cases of NPC each year.

Histological investigation is used to diagnose NPC, specifically immunohistochemical detection for Epstein–Barr virus-encoded small RNA (EBER). Staging is usually based on the International Union Against Cancer (UICC) and the American Joint Committee on Cancer (AJCC) staging systems. The disease is mostly related to Epstein-Barr virus (EBV) infection. Sir Epstein and associates found EBV in cells cultured from the explants of African Burkitt lymphoma tumors using electron microscopy, and the virus was later found to infect more than 90% of the world's population. EBV was the first human tumor virus to be discovered. The relations with EBV, along with many genetic abnormalities, escalate the intricacy of NPC research. This is especially true in Indonesia; NPC is common among distinct local peoples in a country with a population of 225 million, who are ethnically diverse and pose significant socio-economic problems. Regrettably, a lot of these cases go undocumented as there is limited medical as well as nationwide awareness and a lack of hospital facilities, cancer diagnostic, and registration systems.

For treatment, the early-stage cancers are treated with radiation therapy aimed at the tumor. The latter stages would usually get chemoradiation. Additionally, viral antigen expression in NPC makes this disease an attractive target for immunotherapy. In normal conditions, the immune system's intolerance to the self-antigens is prevented; otherwise, it can lead to autoimmune disease. However, it is known that under the conditions of cancer, the immune response can fight against self-antigens. These immune responses may be initiated by changes in the tumor that increase the immunogenicity of self-antigens (Fonseca & Dranoff, 2008). Some antigens have been related to tumor cells and identified as tumor-associated antigens (TAAs). Immune responses can be divided into two categories: cell-mediated and humoral immunity. Both CD8+ and CD4+ T cells recognize peptides displayed on the surface of cancer cells by major histocompatibility complex (MHC) class 1 and class 2, respectively, contributing to cell-mediated immunity (Delves & Roitt, 2000). MHC, or human leukocyte antigen (HLA) in humans, is a molecule that binds peptide fragments derived from pathogens and displays them to the appropriate T cells. The T cells will then produce granzyme B and perforin to kill the cancer cell (Janeway et al., 2001).

MHC class 1 presents endogenous antigens, meaning it presents antigens from the cytoplasm; MHC class 2, however, presents exogenous antigens. In humoral immunity, antibodies specific to antigens are produced by B cells. Stimulating immune responses shows great potential for treating cancer. Because of this, immunotherapy, which boosts the immune system's ability to recognize, engage, and eliminate cancer cells, appears to be showing very promising results in cancer care as of late. Immunotherapy can take the form of cytokine treatments, monoclonal antibodies, or vaccinations, among other approaches (Kamta et al., 2017). The majority of research has been focused on therapeutic vaccinations. Tumor-associated antigens (TAAs) create immune responses that preferentially target cancer cells and are up-regulated in cancer cells; therefore, the vaccine will target TAAs on nonviral tumors (Melief et al., 2015). The antigens can be overexpressed, underexpressed, or not expressed at all by tumor cells, thereby demonstrating their potential to prime the immune system to recognize tumor cells (Turk et al., 2002).

Peptide-based vaccines are made of defined, small-peptide antigens that are manipulated to evoke peptide-specific B- or T- cell responses (Nevagi et al., 2018). Unfortunately, this kind of vaccine may have a limitation in immunogenicity because it is made from self-antigens. Nonetheless, there are different approaches to mitigate this problem, such as the introduction of immunostimulatory adjuvants (Malonis et al., 2019). The effectiveness of peptide-based cancer vaccines depends on whether the peptide is presented by HLA molecules for recognition by T cells.

As with most cancers, NPC shows elevated levels of peptidyl-prolyl cis-trans isomerase (PIN1). This protein identifies and isomerizes the phosphorylated Serine/Threonine-Proline motif. PIN1 is involved in many cellular processes, and its dysregulation may lead to neoplastic diseases (Chen et al., 2018). Several studies have been conducted on the role of PIN1 in NPC development, including one by Xu et al. (2016). They concluded that PIN1 overexpression promotes tumor cell proliferation by upregulating cyclin D1. Another gene highly expressed in cancer is ATP-binding cassette subfamily C member 5 (ABCC5). It is overexpressed in breast cancer, prostate cancer, in addition to NPC, as reported by Du & Yao (2008). ABCC5 overexpression has also been reported in paclitaxel-resistant NPC cells (Hou et al., 2017).

Thus far, the NPC vaccine has targeted EBV. In clinical trials, several therapeutic EBV vaccines have shown promising benefits (Taylor & Steven, 2016). Developing a multi-epitope-based NPC vaccine might be helpful for patients with more advanced stages of the cancer and who are resistant to treatment. Targeting overexpressed antigens around the tumor area is predicted to evoke an immune response against cancer cells.

MATERIALS AND METHODS

Sequence retrieval

The protein sequences of PIN1 (accession Q13526 and proteome UP000005640) and ABCC5 (accession O15440 and proteome UP000005640) were obtained from the UniProtKB online database on (www.uniprot.org, accessed 2025-07-21). The first step was inserting the query terms that consist of the human proteome, then exact files on the result page. Then, choose the FASTA format, as it allows retrieval of all sequences from the query result list.

Prediction of B-cell epitope

B-cells epitope prediction

The epitopes of interest were identified. The algorithms for determining flexibility, hydrophilicity, antigenicity, and surface accessibility were evaluated using B-cell epitope prediction. PIN1 and ABCC5 were assessed using BepiPred linear epitope prediction, surface accessibility prediction by Emini, alongside

Kolaskar and Tongaonkar antigenicity programs. All assessment tools use the default value based on the standard prediction set by each tool (http://tools.iedb.org/bcell, accessed 2025-07-21) (Galanis et al., 2021).

Surface accessibility prediction

IEDB's Emini surface accessibility prediction (http://tools.iedb.org/bcell, accessed 2025-07-21) was utilized to predict the accessible surface of PIN1 and ABCC5. The calculation is based on the surface accessibility scale of a product. A peptide with surface probability greater than the default threshold of 1.000 shows an increased probability for the antigen to be found on the surface of the cell (Emini et al., 1985).

Epitopes antigenicity prediction

The Kolaskar & Tongaonkar antigenicity prediction from IEDB was utilized to predict the antigenic sites of the proteins PIN1 and ABCC5. The antigenic sites of both proteins were separated by passing the default threshold value of 1.037 (http://tools.iedb.org/bcell, accessed 2025-07-21).

Allele frequency analysis

The major alleles in Indonesia were identified using the Allele Frequency Net Database (AFND) (http://allelefrequencies.net/hla.asp, accessed 2025-07-21). Most of the HLA allele data for the Indonesian population listed in AFND came from one study conducted in the Javanese and Sundanese Javanese populations. This data is regarded as representative of the Indonesian population. The population was set on the website for: all populations, regions for all regions, set the country as Indonesia, and the population standard for all. Alleles with a frequency higher than 5% were chosen (Gonzalez-Galarza et al., 2020).

Prediction of T-cell epitope

Prediction of cytotoxic T-lymphocytes epitopes

The binding peptides in MHC class I molecules were analyzed using NetMHCpan-4.1 (https://services.healthtech.dtu.dk/services/NetMHCpan-4.1, accessed 2025-07-25). This approach can be used to predict CTL epitopes restricted by any MHC molecules in a known protein sequence. The parameters for the weight placed on C-terminal cleavage and the antigen transport efficiency were set to the default values of 0.225 and 0.025, respectively. Epitopes that have a percentage rank lower than 1.0 were chosen (Sarma et al., 2022). The percentile rank is proportionally related to the binding affinity of the peptide to the HLA molecule.

Epitopes immunogenicity prediction

The immunogenicity of MHC-I peptides was predicted using IEDB tools with default parameters (http://tools.iedb.org/immunogenicity, accessed 2025-07-25). This is done to choose potential MHC-I peptides for vaccine design, as they are responsible for developing continuing cellular immunity. The epitopes will be chosen based on their immunogenicity score because a positive score indicates immunogenicity and may provoke an immune response.

Helper T-lymphocytes epitopes prediction

MHC-II binding peptides were analyzed using NetMHCIIpan-4.0 (https://services.healthtech.dtu.dk/services/NetMHCIIpan-4.0, accessed 2025-07-25). Epitopes with a percentage rank below 2.0 were chosen. The predicted peptides were divided into three categories: strong,

intermediate, and non-binders, with threshold values of 2, 10, and > 10%, respectively, based on the NetMHCII pan server's percentile rank concept (Jensen et al., 2018).

IFN-γ epitope prediction

MHC-II peptides' capability to produce interferon-gamma cytokine was predicted with the IFN epitope. The prediction was based on the Motif and SVM model, which is the default setting on the website, which is accessible at http://crdd.osdd.net/raghava/ifnepitope/predict.php (accessed 2025-07-25).

Population coverage

The HLA alleles were calculated from the distribution of the populations among the MHC-I and MHC-II epitopes. IEDB tools (https://tools.iedb.org/population, accessed 2025-07-25) were utilized to analyze this information. Using the default parameters, the coverage was assessed against the HLA-I and HLA-II binding alleles.

Vaccine development

Vaccine construct

Epitope selection to be included in the construct followed several criteria: (1) Epitopes should be promiscuous so that they can be presented by many HLA alleles and therefore generate a high-population coverage of the vaccine (Fleri et al., 2017). (2) Epitopes presented by HLA Class I should be immunogenic to ensure the presence of T-cells within the repertoire that will be able to respond to the peptides. (3) Epitope presented by HLA Class II should have the ability to induce IFNγ responses so that the vaccine will be able to activate the Th1 responses needed for antiviral immune responses. (4) Epitopes should not have homology with the human peptides to avoid autoimmune responses triggered while ensuring the immunogenicity of the vaccine construct. The vaccine was designed by joining individual epitopes into a polypeptide. Additionally, the adjuvants *E. coli* and *Bacillus* for the vaccine were selected. Adjuvant, HTL, and CTL were connected using linkers EAAAK, GPGPG, and AAY (Deepthi et al., 2025).

Epitopes antigenicity test

The antigenicity of the chosen peptide and the new vaccine design were assessed using VaxiJen (https://ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html, accessed 2025-07-31) with the default parameters. VaxiJen is a bioinformatics tool used to analyze the alignment-free *in silico* identification of antigen methods that classify antigens based on physicochemical properties of proteins without recourse to the sequence alignment (Flower et al., 2017). Additionally, the AntigenPro server (http://scratch.proteomics.ics.uci.edu/explanation.html#ANTIGENpro, accessed 2025-07-31) was used to test vaccine antigenicity, and the test was done without changing the settings (Magnan et al., 2010).

Epitopes retention verification after construct assembly

The new vaccine design will then be analyzed again using NetMHCpan-4.1 and NetMHCIIpan-4.0, respectively, using default values (https://services.healthtech.dtu.dk/services/NetMHCpan-4.1 and https://services.healthtech.dtu.dk/services/NetMHCIIpan-4.0, accessed 2025-07-31). The use of default values ensures that the chosen epitopes remain present and bind to their respective MHC molecules (Stranzl et al., 2010).

Antigenicity test after construct assembly

VaxiJen double-checked the antigenicity of the new vaccine design. In addition, the AntigenPro server (http://scratch.proteomics.ics.uci.edu/explanation.html#ANTIGENpro, accessed 2025-07-31) was

utilized to test vaccine antigenicity (Flower et al., 2017). Both VaxiJen and AntigenPro were used without changing the default value previously set by the software.

Allergenicity test

The vaccine cannot cause sensitization or an allergic reaction. The AllerTop server was used in its standard settings to test the new vaccine's allergenicity, as it is the performing model for allergen prediction (https://www.ddg-pharmfac.net/AllerTOP, accessed 2025-08-01) (Dimitrov et al., 2013).

Physicochemical characteristics

The physicochemical properties, such as amino acid composition (379), molecular weight (41123.47), pI (4.98), instability index (35.48), and grand average of hydropathicity of -0.206 (GRAVY) of the new vaccine construct with *Bacillus* as adjuvant were checked with Protparam tools using its default parameters (Zaib et al., 2023). The physicochemical properties of the new vaccine with *E.coli* as adjuvant, such as amino acid composition (594), molecular weight (64446.71), pI (8.68), instability index (22.59), and grand average of hydropathicity of -0.222 (GRAVY) were also checked with Protparam tools. This tool showed whether or not a protein is stable by looking at the functional and structural characteristics of a protein (https://web.expasy.org/cgi-bin/protparam/protparam, accessed 2025-08-01) (Garg et al., 2016).

BLASTP analysis

BLASTP analysis tools from NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed 2025-08-03) were used to check the similarity of the peptides with other peptides across the human proteome. The peptide sequences were input into the BlastP server and checked against non-redundant human protein sequences (taxid:9606). The blast parameters were set as follows: word size 2, expectation value/ threshold 30,000, PAM30 matrix, low complexity filter was turned off, and composition-based statistics were left unchanged. Hit-table was generated and evaluated further with Microsoft Excel to screen for peptides aligned to 9 amino acids with no gap and no mismatch residues between the query sequences (vaccine peptides) and the subject sequences (peptides from human proteome) (Gustiananda et al., 2021).

***In silico* simulation of the immunological response**

The immunological responses of the vaccine design were studied *in silico* using the C-ImmSim website (https://kraken.iac.rm.cnr.it/C-IMMSIM, accessed 2025-08-03). The simulation was carried out using the HLA haplotype with the highest prevalence in the Indonesian population, such as haplotype HLA-A*3401, HLA-B*1521, HLA-DRB1*1502 (4.6%), and HLA-A*2407, HLA-B*3505, HLA-DRB1*1202 (4.3%) (Yuliwulandari et al., 2009). The simulation was run for 1000 phases. Three injections of 1000 units of vaccine were given at one-month intervals (days 0, 28, then 56), corresponding to time-steps 1, 84, and 168. Eight injections were given four weeks apart to induce recurrent antigen exposure (Hossain et al., 2021).

Structural modeling, validation, and molecular docking

The 3D models of the vaccine were generated by the SWISS-MODEL website with its standard parameters (https://swissmodel.expasy.org/interactive, accessed 2025-08-03). Next, the Ramachandran plot analysis was performed to ensure that the predicted model's structure was valid without changing any settings in the software. The SWISS-MODEL website (https://swissmodel.expasy.org/interactive, accessed 2025-08-03) was also utilized to evaluate the modeled structure and generate the Ramachandran plot (Obaidullah et al., 2021). The overall quality of the constructed vaccination model is validated by the Ramachandran plot analysis. The interaction between the vaccine construct and TLR4 was evaluated after

the modeling and validation. Molecular docking was done via ClusPro software. TLR-4 (PDB ID: 3FXI) was selected as the target protein against the constructed vaccine candidates. ClusPro 2.0 server (https://cluspro.org) was used for this purpose by predicting the binding energy between TLR-4 and the vaccine construct. The experiment was performed by uploading PDB files of receptors and ligands into the server and submitted with default parameters (accessed 2025-08-03).

RESULTS

Sequence retrieval

The protein sequences of PIN1 and ABCC5 were obtained using the UniProtKB online database. The sequences below in **Table 1** are shown in FASTA format.

Table 1. PIN1 and ABCC5 sequences from UniProtKB in FASTA format

Protein	Sequence (FASTA)
PIN1	>sp|Q13526|PIN1_HUMAN Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1 OS=Homo sapiens OX=9606 GN=PIN1 PE=1 SV=1 MADEEKLPPGWEKRMSRSSGRVYYFNHITNASQWERPSGNSSSGGKNGQGEPARVRCSHLVKHSQSRRP SSWRQEKITRTKEEALELINGYIQKIKSGEEDFESLASQFSDCSSAKARGDLGAFSRGQMQKPFEDASFALRTG EMSGPVFTDSGIHIILRTE
ABCC5	>sp|O15440|MRP5_HUMAN ATP-binding cassette sub-family C member 5 OS=Homo sapiens OX=9606 GN=ABCC5 PE=1 SV=2 MKDIDIGKEYIIPSPGYRSVRERTSTSGTHRDREDSKFRRTRPLECQDALETAARAEGLSLDASMHSQLRILDE EHPKGKYHHGLSALKPIRTTSKHQHPVDNAGLFSCMTFSWLSSLARVAHKKGELSMEDVWSLSKHESSDVN CRRLERLWQEELNEVGPDAASLRRVVWIFCRTRLILSIVCLMITQLAGFSGPAFMVKHLLEYTQATESNLQYSL LLVLGLLLTEIVRSWSLALTWALNYRTGVRLRGAILTMAFKKILKLKNIKEKSLGELINICSNDGQRMFEAAAVG SLLAGGPVVAILGMIYNVIILGPTGFLGSAVFILFYPAMMFASRLTAYFRRKCVAATDERVQKMNEVLTYIKFIK MYAWVKAFSQSVQKIREEERRILEKAGYFQSITVGVAPIVVVIASVVTFSVHMTLGFDLTAAQAFTVVTVFNS MTFALKVTPFSVKSLSEASVAVDRFKSLFLMEEVHMIKNKPASPHIKIEMKNATLAWDSSHSSIQNSPKLTPK MKKDKRASRGKKEKVRQLQRTEHQAVLAEQKGHLLLDSDERPSPEEEEGKHIHLGHLRLQRTLHSIDLEIQEG KLVGICGSVGSGKTSLISAILGQMTLLEGSIAISGTFAYVAQQAWILNATLRDNILFGKEYDEERYNSVLNSCCLR PDLAILPSSDLTEIGERGANLSGGQRQRISLARALYSDRSIYILDDPLSALDAHVGNHIFNSAIRKHLKSKTVLFV THQLQYLVDCDEVIFMKEGCITERGTHEELMNLNGDYATIFNNLLLGETPPVEINSKKETSGSQKKSQDKGPK TGSVKKEKAVKPEEGQLVQLEEKGQGSVPWSVYGVYIQAAGGPLAFLVIMALFMLNVGSTAFSTWWLSYWI KQGSGNTTVTRGNETSVSDSMKDNPHMQYYASIYALSMAVMLILKAIRGVVFVKGTLRASSRLHDELFRRIL RSPMKFFDTTPTGRILNRFSKDMDEVDVRLPFQAEMFIQNVILVFFCVGMIAGVFPWFLVAVGPLVILFSVLH IVSRVLIRELKRLDNITQSPFLSHITSSIQGLATIHAYNKGQEFLHRYQELLDDNQAPFFLFTCAMRWLAVRLDLI SIALITTTGLMIVLMHGQIPPAYAGLAISYAVQLTGLFQFTVRLASETEARFTSVERINHYIKTLSLEAPARIKNKA PSPDWPQEGEVTFENAEMRYRENLPLVLKKVSFTIKPKEKIGIVGRTGSGKSSLGMALFRLVELSGGCIKIDGV RISDIGLADLRSKLSIIPQEPVLFSGTVRSNLDPFNQYTEDQIWDALERTHMKECIAQLPLKLESEVMENGDNF SVGERQLLCIARALLRHCKILILDEATAAMDTETDLLIQETIREAFADCTMLTIAHRLHTVLGSDRIMVLAQGQV VEFDTPSVLLSNDSSRFYAMFAAAENKVAVKG

Predicted B-cell epitopes

BepiPred linear epitope prediction, Emini surface accessibility prediction, and Kolaskar and Tongaonkar antigenicity software were used to extract and analyze the complete PIN1 and ABCC5 protein sequences.

Linear epitope prediction

The result of BepiPred linear prediction identified six possible regions of the PIN1 protein that can bind to B-cell receptors. The default threshold was 0.500, and all values above it were determined as potential linear epitopes. The average value was 0.533, with the lowest value of 0.260 and the highest of 0.688. **Table 2** and **Figure 1** showed the location and length of predicted B-cell linear peptides.

For ABCC5, the same steps were repeated. The result of BepiPred linear prediction identified 35 possible regions of ABCC5 protein that can bind to B-cell receptors. The default threshold was 0.500, and all values above the threshold were determined as potential linear epitopes. The average value was 0.457, with the lowest value of 0.240 and the highest of 0.681. **Table 3** and **Figure 2** showed the location and length of predicted B-cell linear peptides.

Surface accessibility prediction

The Emini surface accessibility test revealed four distinct regions on the PIN1 protein surface that can interact with B-cell receptors. The default threshold was 1.000, and all the values above the threshold corresponded to the region on the surface of the PIN1 protein. With the lowest score of 0.064 and the highest score of 4.097, the average value was 1.000. **Table 4** and **Figure 3** showed the location and length of the predicted peptide.

Likewise, Emini surface accessibility analysis was performed for the ABCC5 sequence. The results showed 19 specific regions on the surface of the ABCC5 protein that bind to B-cell receptors. However, peptides that consist of fewer than seven amino acids were eliminated. The default threshold was 1.000, and all the values above the threshold were the region that was on the surface of the ABCC5 protein. With the lowest score of 0.038 and a high score of 9.915, the average value was 1.000. **Table 5** and **Figure 4** showed the location and length of the predicted peptide.

Antigenicity prediction

The result of Kolaskar and Tongaonkar's prediction showed four potential antigenic regions of the PIN1 protein. However, peptides containing fewer than seven amino acids were eliminated. The default threshold was 0.995, and all the values above the threshold were determined as potential antigenic regions. The average value was 0.995, with the lowest value of 0.885 and the highest score of 1.192. **Table 6** and **Figure 5** showed the locations and lengths of predicted immunogenic peptides.

ABCC5 Kolaskar and Tongaonkar prediction showed 41 potential antigenic regions. However, peptides that consist of fewer than seven amino acids were eliminated. The default threshold was 1.038, and all the values above the threshold were determined as potential regions that were antigenic. The average value was 1.038, with the lowest value of 0.866 and the highest score of 1.259. **Table 7** and **Figure 6** showed the locations and lengths of predicted immunogenic peptides.

Chosen B-cell epitopes

The B-cell epitopes are chosen based on linearity, surface accessibility, and antigenicity. These parameters ensure the resulting construct will be easily recognizable, located on the surface (active sites of

protein are on the surface) for accessibility, and able to induce a specific immune response. Four B-cell epitopes are chosen from PIN1 and ABCC5, shown in **Table 8.**

Allele frequency analysis

The alleles with a frequency of 5% or higher were considered major alleles in Indonesia. There were 15 MHC-I alleles as well as 6 MHC-II alleles identified **(Table 9).**

Table 2. Predicted linear B-cell epitopes of PIN1 using BepiPred

No.	Start	End	Peptide	Length
1	5	40	EKLPPGWEKRMSRSSGRVYYFNHITNASQWERPSGN	36
2	43	57	SGGKNGQGEPARVRC	15
3	61	87	LVKHSQSRRPSSWRQEKITRTKEEALE	27
4	96	102	IKSGEED	7
5	110	133	FSDCSSAKARGDLGAFSRGQMQKP	24
6	142	148	RTGEMSG	7

Note: BepiPred linear epitope prediction identifies potential linear epitopes accessible on the protein surface that can bind to B-cell receptors.

Table 3. Predicted linear B-cell epitopes of ABCC5 using BepiPred

No.	Start	End	Peptide	Length
1	5	103	DIGKEYIIPSPGYRSVRERTSTSGTHRDREDSKFRRTRPLECQDALETAAR AEGLSLDASMHSQLRILDEEHPKGKYHHGLSALKPIRTTSKHQHPVDN	99
2	125	144	KGELSMEDVWSLSKHESSDV	20
3	146	165	CRRLERLWQEELNEVGPDAA	20
4	264	273	KLKNIKEKSL	10
5	345	356	RKCVAATDERVQ	12
6	382	388	QKIREEE	7
7	473	489	MEEVHMIKNKPASPHIK	17
8	500	562	DSSHSSIQNSPKLTPKMKKDKRASRGKKEKVRQLQRTEHQAVLAEQKG HLLLDSDERPSPEEE	63
9	647	653	KEYDEER	7
10	671	688	PSSDLTEIGERGANLSGG	18
11	764	772	RGTHEELMN	9

No.	Start	End	Peptide	Length
12	786	842	LGETPPVEINSKKETSGSQKKSQDKGPKTGSVKKEKAVKPEEGQLVQLE EKGQGSVP	57
13	886	911	QGSGNTTVTRGNETSVSDSMKDNPHM	26
14	962	968	FFDTTPT	7
15	1071	1084	QEFLHRYQELLDDN	14
16	1168	1189	KTLSLEAPARIKNKAPSPDWPQ	22
17	1258	1264	ISDIGLA	7
18	1288	1298	LDPFNQYTEDQ	11
19	1313	1322	AQLPLKLESE	10
20	1324	1330	MENGDNF	7

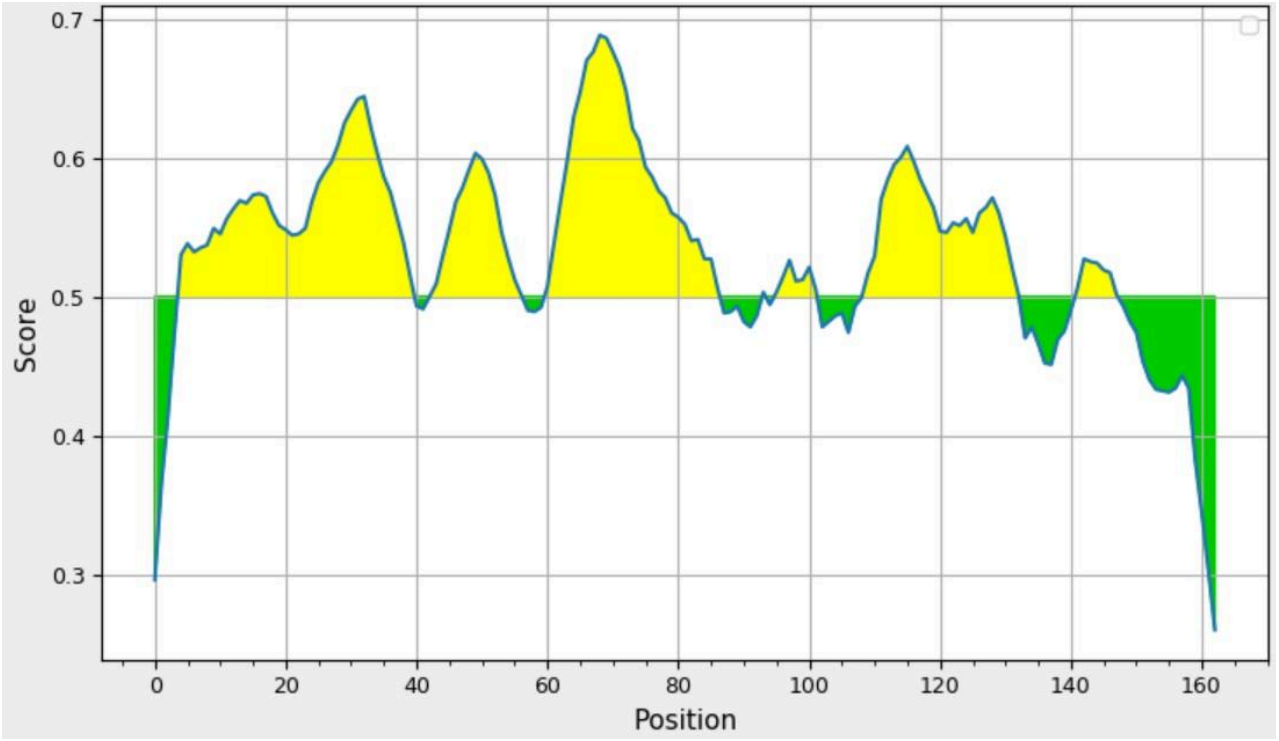

Figure 1. BepiPred linear B-cell epitope prediction for PIN1. Yellow regions represent predicted epitopes located on the protein surface, while green regions indicate non-epitope areas

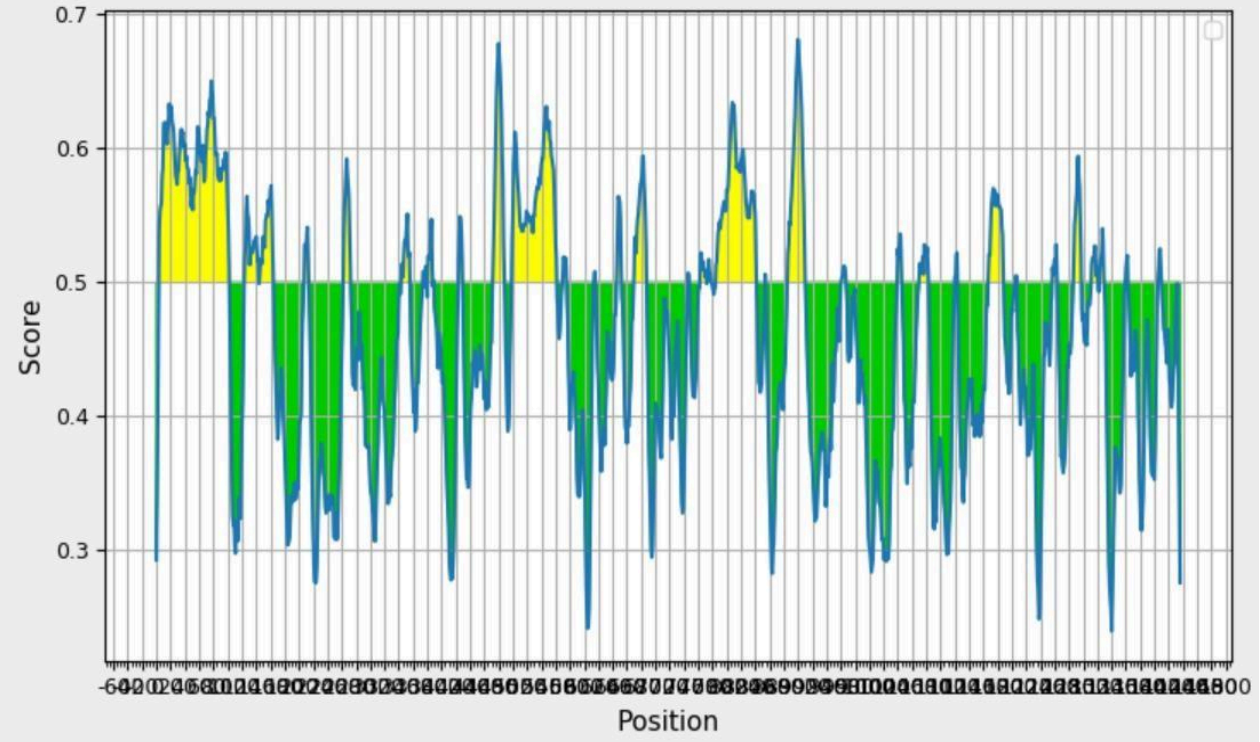

Figure 2. BepiPred linear B-cell epitope prediction for ABCC5. Yellow regions represent predicted epitopes located on the protein surface, while green regions indicate non-epitope areas

Table 4. PIN1 peptides identified by Emini surface accessibility prediction

No.	Start	End	Peptide	Length
1	10	16	GWEKRMS	7
2	32	38	SQWERPS	7
3	64	84	HSQSRRPSSWRQEKITRTKEE	21
4	128	134	GQMQKPF	7

Note: Emini surface accessibility prediction identifies regions located on protein surface that are available for B-cell receptor binding.

Table 5. ABCC5 peptides identified by Emini surface accessibility prediction

No.	Start	End	Peptide	Length
1	15	42	PGYRSVRERTSTSGTHRDREDSKFRRTR	28
2	74	82	EEHPKGKYH	9
3	90	100	PIRTTSKHQHP	11
4	206	215	EYTQATESNL	10
5	266	272	KNIKEKS	7

No.	Start	End	Peptide	Length
6	351	358	TDERVQKM	8
7	382	391	QKIREEERRI	10
8	505	538	SIQNSPKLTPKMKKDKRASRGKKEKVRQLQRTEH	34
9	552	565	DSDERPSPEEEEGK	14
10	647	654	KEYDEERY	8
11	763	769	ERGTHEE	7
12	795	827	NSKKETSGSQKKSQDKGPKTGSVKKEKAVKPEE	33
13	903	913	DSMKDNPHMQY	11
14	974	980	RFSKDMD	7
15	1073	1085	FLHRYQELLDDNQ	13
16	1150	1156	ASETEAR	7
17	1176	1191	ARIKNKAPSPDWPQEG	16
18	1198	1205	AEMRYREN	8
19	1289	1298	DPFNQYTEDQ	10

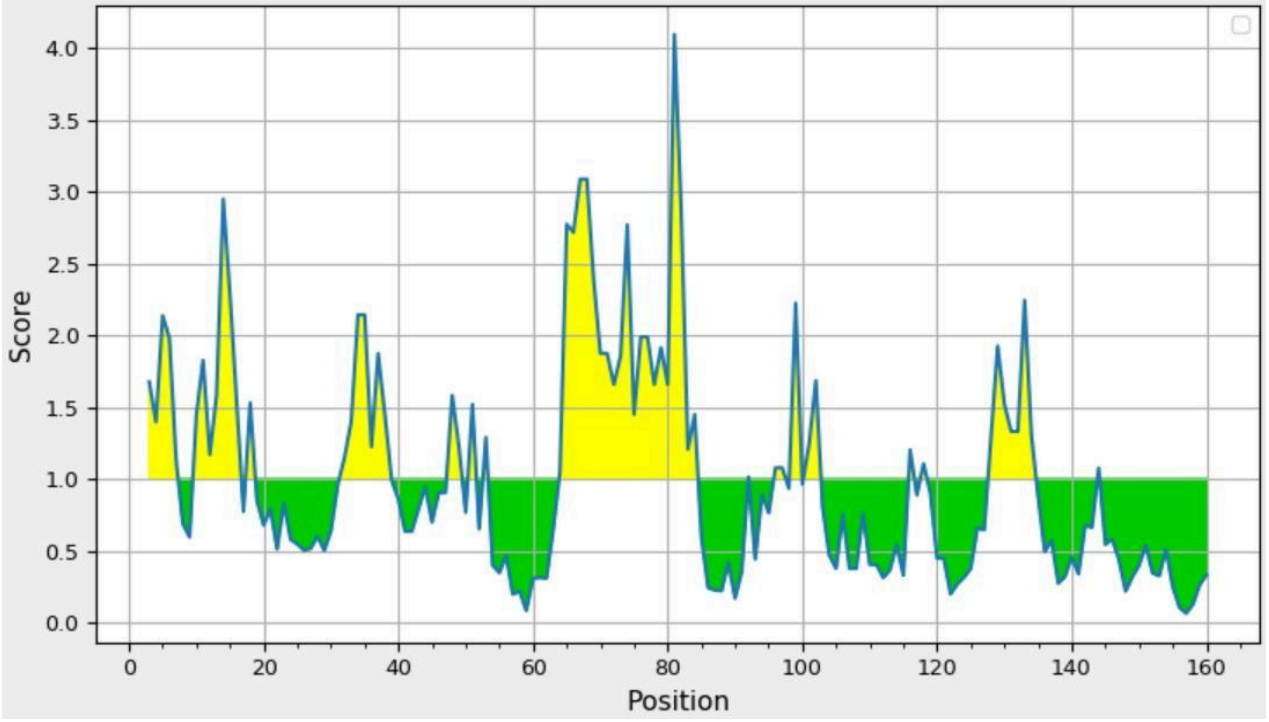

Figure 3. Emini surface accessibility prediction for PIN1. Yellow regions correspond to potential B-cell epitopes localized to the protein surface, while the green regions represent inaccessible peptides

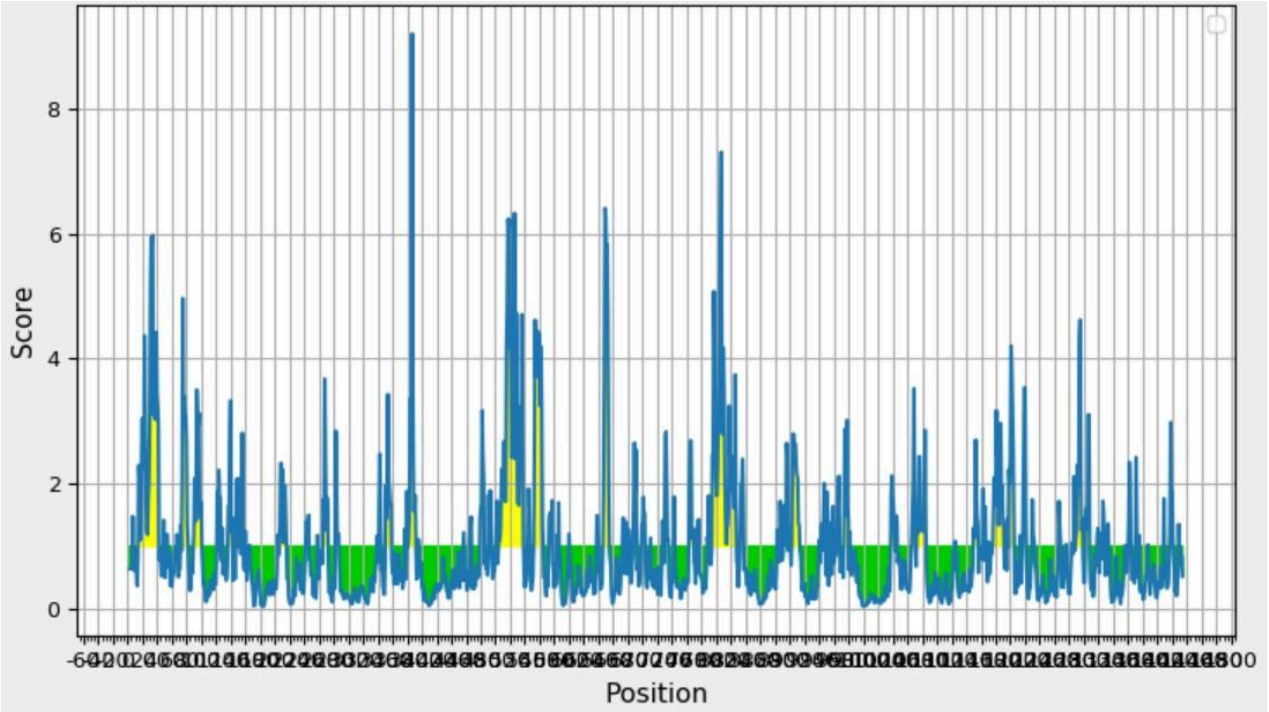

Figure 4. Emini surface accessibility prediction for ABCC5. Yellow regions correspond to potential B-cell epitopes localized to the protein surface, while the green regions represent inaccessible peptides

Prediction of T-cell epitope

Prediction of cytotoxic T-lymphocytes epitope and epitopes immunogenicity prediction

A preferable prophylactic vaccine should mimic the natural immunity caused by infection, resulting in long-lasting adaptive immunity, with both CTL and HTL epitopes playing key roles (Testa & Philip, 2012). CTL epitopes are responsible for the development of long-lasting cellular immunity capable of eradicating circulating viruses and virus-infected cells (Doherty et al., 1992). HTL epitopes, on the other hand, are critical in the formation of both humoral and cellular immune responses. These epitopes trigger a CD4+ helper response, which is required not only for the generation of protective CD8+ T-cell memory but also for B-cell activation and antibody production (Panina-Bordignon et al., 1989). As a result, a successful vaccination candidate should have epitopes specific to the CTL and HTL receptors. The predicted epitopes were submitted to several immunological filters in order to pick the best epitopes, and those with high binding affinity to MHC-I and MHC-II alleles were chosen **(Tables 10 & 11)**. The epitopes had to meet the following criteria: they had to be promiscuous, antigenic, and immunogenic.

Helper T-Lymphocytes epitopes and IFN-γ epitope prediction

The IFN-γ scores were analyzed to identify which peptides with strong or weak binding to MHC-II; peptides with strong binding showed a percentage rank below 2.0. This analysis showed that PIN1 epitopes had the highest score of 0.33614053, and ABCC5 epitopes had the highest score of 0.8438963.

Chosen predicted cytotoxic T-lymphocytes epitopes

From the identified PIN1 and ABCC5 peptides by NetMHCpan, nine peptides were chosen for further use in vaccine construction **(Table 12)**. These peptides were selected based on their immunogenicity score and their ability to interact with major alleles in the Indonesian population.

Chosen predicted helper T-lymphocytes epitopes

From the identified PIN1 and ABCC5 peptides by NetMHCIIpan-4.0, five peptides were chosen for further use in vaccine construction **(Table 13)**. These peptides were chosen based on the Interferon-gamma scores and their ability to interact with major alleles in the Indonesian population.

Population coverage

The population coverage research revealed that MHC-I had 99.27% coverage and MHC-II had 95.26% coverage, indicating a high percentage. These findings indicate that the developed multi-epitope vaccination can be employed to combat NPC in the Indonesian population **(Table 14)**. The population coverage of both MHC-I and MHC-II is 99.97% **(Figure 7)**.

Antigenicity test

One criterion in choosing a peptide is antigenicity. Antigenicity refers to the capacity of the epitopes to bind to specific antibody molecules. To test the antigenicity of epitopes, VaxiJen software was used. The result showed that the overall antigen score was 0.5125, meaning that the epitopes are a probable antigen.

Table 6. Predicted antigenic regions in PIN1 using Kolaskar and Tongaonkar predictions

No.	Start	End	Peptide	Length
1	19	27	SGRVYYFNH	9
2	52	65	PARVRCSHLLVKHS	14
3	85	96	ALELINGYIQKI	12
4	104	116	ESLASQFSDCSSA	13

Table 6. presents four potential regions of the protein that are antigenic. The average value obtained was 0.995 with a minimum score of 0.885 and a maximum score of 1.192.

Table 7. Predicted antigenic regions in ABCC5 using Kolaskar and Tongaonkar predictions

No.	Start	End	Peptide	Length
1	82	89	HHGLSALK	8
2	115	122	LSSLARVA	8
3	167	174	LRRVVWIF	8
4	176	189	RTRLILSIVCLMIT	14
5	200	207	MVKHLLEY	8
6	216	230	QYSLLLVLGLLLTEI	15
7	233	239	SWSLALT	7
8	290	296	AAAVGSL	7
9	298	307	AGGPVVAILG	10

No.	Start	End	Peptide	Length
10	309	316	IYNVIILG	8
11	322	331	GSAVFILFYP	10
12	344	350	RRKCVAA	7
13	373	382	WVKAFSQSVQ	10
14	400	419	SITVGVAPIVVVIASVVTFS	20
15	433	440	AFTVVTVF	8
16	446	457	ALKVTPFSVKSL	12
17	459	465	EASVAVD	7
18	566	572	HIHLGHL	7
19	590	597	LVGICGSV	8
20	624	632	TFAYVAQQA	9

Table 7. presents 20 of the 41 antigenic regions in ABCC5. The average value was 1.038, with the lowest value of 0.866 and the highest score of 1.259.

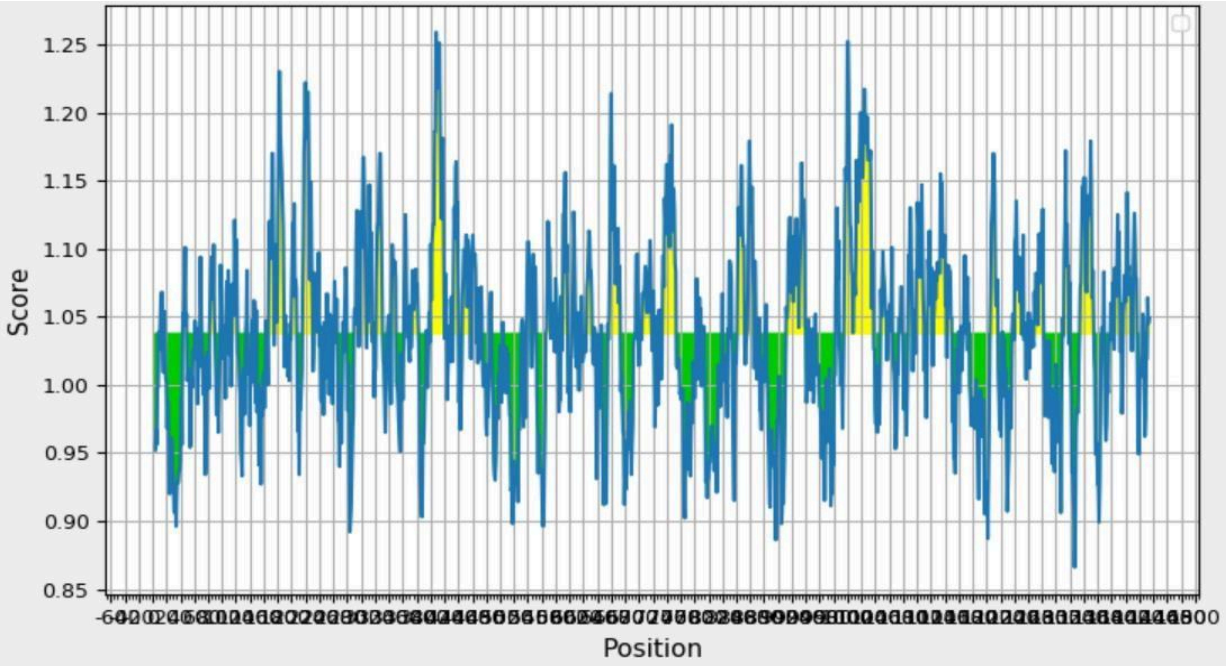

Figure 5. Kolaskar and Tongaonkar prediction for PIN1. Yellow regions indicate potential antigenic sites, while the green regions represent non-potential antigenic sites

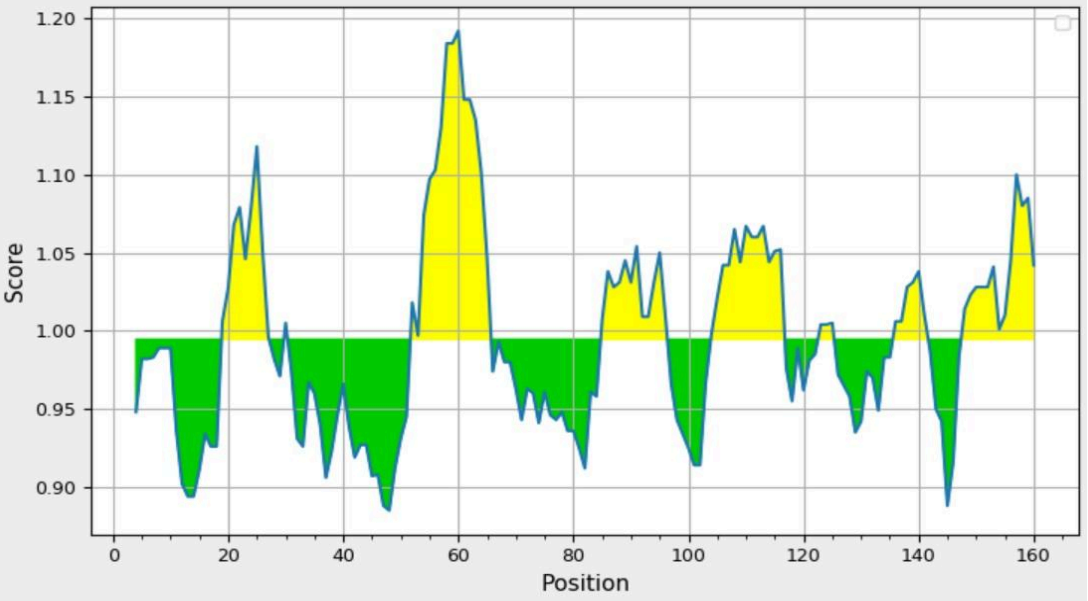

Figure 6. Kolaskar and Tongaonkar prediction for ABCC5. Yellow regions indicate potential antigenic sites, while the green regions represent non-potential antigenic sites

Table 8. List of chosen B-cell epitopes

No.	Start	End	Peptide	Length
1	19	27	SGRVYYFNH	9
2	85	96	ALELINGYIQKI	12
3	82	89	HHGLSALK	8
4	344	350	RRKCVAA	7

Table 9. List of 15 MHC-I and 6 MHC-II alleles in Indonesia recognized by Allele Frequency Net Database

No.	Class I	Frequency	No.	Class II	Frequency
1	A*02:01	6,6%	1	DRB1*07:01	13.6%
2	A*02:03	6.9%	2	DRB1*11:01	8.9%
3	A*11:01	16.4%	3	DRB1*12:02	53.4%
4	A*24:02	14.4%	4	DRB1*15:01	12%
5	A*24:07	26.4%	5	DRB1*15:02	47.9%
6	A*33:03	16.9%	6	DRB1*16:02	5%
7	A*34:01	8.3%			
8	B*15:02	12.2%			
9	B*15:13	12.5%			

No.	Class I	Frequency	No.	Class II	Frequency
10	B*15:21	11.1%			
11	B*18:01	6,4%			
12	B*35:05	9%			
13	B*38:02	6%			
14	B*44:03	9.3%			
15	B*58:01	6%			

Table 10. Predicted MHC-I binding peptides and immunogenicity score of PIN1

No.	Start	End	Epitopes	Alleles	Score
1	24	32	ITNASQWER	HLA-A*33:03	0.01257
2	13	21	RSSGRVYYF	HLA-A*24:02, HLA-A*24:07, HLA-B*58:01	0.06438
3	53	61	DSGIHIILR	HLA-A*33:03	0.40754
4	84	92	EALELINGY	HLA-A*34:01	0.22597
5	117	125	KARGDLAF	HLA-B*15:02	0.11352

Table 11. 10 of the 85 predicted MHC-I binding peptides and immunogenicity score of ABCC5

No.	Start	End	Epitopes	Alleles	Score
1	110	118	MTFSWLSSL	HLA-A*02:01, HLA-A*02:03, HLA-A*33:03, HLA-A*34:01, HLA-B*15:02, HLA-B*15:13, HLA-B*15:21, HLA-B*58:01	0.41264
2	128	136	LSMEDVWSL	HLA-A*02:01, HLA-B*15:13, HLA-B*38:02, HLA-B*58:01	0.35256
3	294	303	SLLAGGPVV	HLA-A*02:01	0.33543
4	304	312	AILGMIYNV	HLA-A*02:01	0.3223
5	321	328	FLGSAVFIL	HLA-A*02:01	0.31726
6	357	365	KMNEVLTYI	HLA-A*02:01	0.31333
7	456	464	SLSEASVAV	HLA-A*02:01, HLA-A*02:03	0.30327
8	471	479	FLMEEVHMI	HLA-A*02:01, HLA-A*02:03, HLA-B*38:02	0.29897
9	613	621	TLLEGSIAI	HLA-A*02:01, HLA-A*02:03	0.28232
10	620	628	AISGTFAYV	HLA-A*02:01, HLA-A*02:03	0.27485

Table 12. List of chosen CTL epitopes

No.	Start	End	Epitopes	Alleles	Score	Pop. coverage %
1	13	21	RSSGRVYYF*	HLA-A*24:02, HLA-A*24:07, HLA-B*58:01	0.06438	63.36%
2	53	61	DSGIHIILR*	HLA-A*33:03	0.40754	29.13%
3	84	92	EALELINGY*	HLA-A*34:01	0.22597	13.66%
4	117	125	KARGDLGAF*	HLA-B*15:02	0.11352	22.07%
5	110	118	MTFSWLSSL**	HLA-A*02:01, HLA-A*02:03, HLA-A*33:03, HLA-A*34:01, HLA-B*15:02, HLA-B*15:13, HLA-B*15:21, HLA-B*58:01	0.41264	81.72%
6	128	136	LSMEDVWSL**	HLA-A*02:01, HLA-B*15:13, HLA-B*38:02, HLA-B*58:01	0.35256	47.62%
7	471	479	FLMEEVHMI**	HLA-A*02:01, HLA-A*02:03, HLA-B*38:02	0.29897	28.72%
8	840	848	SVPWSVYGV**	HLA-A*02:01, HLA-A*02:03, HLA-A*34:01	0.25943	32.37%
9	70	78	RILDEEHPK**	HLA-A*11:01	0.15926	29.64%

Pop. coverage % = population coverage %, * for PIN1 peptides and ** for ABCC5 peptides.

Table 13. List of chosen HTL epitopes

No.	Start	End	Epitopes	Alleles	Score	Pop. coverage %
1	75	89	ITRTKEEAL*	DRB1_0701	0.33614053	20.49%
2	394	408	YFQSITVGV**	DRB1_0701, DRB1_1602	0.30455579	25.31%
3	239	253	YRTGVRLRG**	DRB1_1101	0.8438963	4.83%
4	412	426	VHMIKNKPA**	DRB1_1202, DRB1_1501, DRB1_1502, DRB1_1602	0.67684557	84.51%
5	1423	1437	FAAAENKVA**	DRB1_1602	0.43674678	5.41%

Pop. coverage % = population coverage %, * for PIN1 peptides and ** for ABCC5 peptides.

Table 14. Population coverage of epitopes binding to MHC-I and MHC-II alleles in Indonesia

No.	Start	End	Epitopes	Alleles	Pop. coverage %
1	13	21	RSSGRVYYF*	HLA-A*24:02, HLA-A* 24:07, HLA-B*58:01	63.36%
2	53	61	DSGIHIILR*	HLA-A*33:03	29.13%
3	84	92	EALELINGY*	HLA-A*34:01	13.66%
4	117	125	KARGDLGAF*	HLA-B*15:02	22.07%
5	110	118	MTFSWLSSL**	HLA-A*02:01, HLA-A*02:03, HLA-A*33:03, HLA-A*34:01, HLA-B*15:02, HLA-B*15:13, HLA-B*15:21, HLA-B*58:01	81.72%
6	128	136	LSMEDVWSL**	HLA-A*02:01, HLA-B*15:13, HLA-B*38:02, HLA-B*58:01	47.62%
7	471	479	FLMEEVHMI**	HLA-A*02:01, HLA-A*02:03, HLA-B*38:02	28.72%
8	840	848	SVPWSVYGV**	HLA-A*02:01, HLA-A*02:03, HLA-A*34:01	32.37%
9	70	78	RILDEEHPK**	HLA-A*11:01	29.64%
10	75	89	ITRTKEEAL*	DRB1_0701	20.49%
11	394	408	YFQSITVGV**	DRB1_0701, DRB1_1602	25.31%
12	239	253	YRTGVRLRG**	DRB1_1101	4.83%
13	412	426	VHMIKNKPA**	DRB1_1202, DRB1_1501, DRB1_1502, DRB1_1602	84.51%
14	1423	1437	FAAAENKVA**	DRB1_1602	5.41%

Pop. coverage % = population coverage %, * for PIN1 peptides and ** for ABCC5 peptides.

MHC class	Coverage	Average hit	PC90
combined	99.97%	5.92	3.51

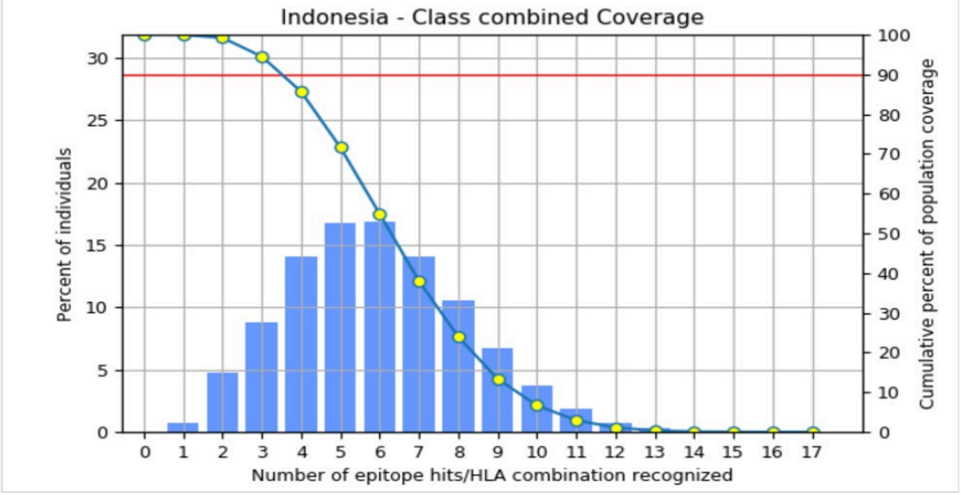

Figure 7. Population coverage in Indonesia for both MHC-I and MHC-II epitopes

Vaccine development

Vaccine construct

The chosen B-cell and T-cell vaccines were used in vaccine development. The epitopes were linked using three linkers: EAAAK, GPGPG, and AAY. Maltose-binding proteins from *E. coli* and *Bacillus* were also included as adjuvants, respectively. Shown in **Table 15** below is the sequence of the new *E. coli* and *Bacillus* adjuvant vaccines with the proteins color-coded for distinction (Kolla et al., 2021).

Table 15. Population coverage in Indonesia for both MHC-I and MHC-II epitopes

Adjuvant	Vaccine Sequences*
E. coli	**MKIKTGARILALSALTTMMFSASALAKIEEGKLVIWINGDKGYNGLAEVGKKFEKDTGIKVTVEHPDKLE EKFPQVAATGDGPDIIFWAHDRFGGYAQSGLLAEITPDKAFQDKLYPFTWDAVRYNGKLIAYPIAVEAL SLIYNKDLLPNPPKTWEEIPALDKELKAKGKSALMFNLQEPYFTWPLIAADGGYAFKYENGKYDIKDVG VDNAGAKAGLTFLVDLIKNKHMNADTDYSIAEAAFNKGETAMTINGPWAWSNIDTSKVNYGVTVLPT FKGQPSKPFVGVLSAGINAASPNKELAKEFLENYLLTDEGLEAVNKDKPLGAVALKSYEEELAKDPRIAAT MENAQKGEIMPNIPQMSAFWYAVRTAVINAASGRQTVDEALKDAQTRITKEAAAKALELINGYIQKIE** AAAKSGRVYYFNHEAAAKRRKCVAAEAAAKHHGLSALKEAAAKITRTKEEALGPGPGYFQSITVGVGPGP GYRTGVRLRGGPGPGVHMIKNKPAGPGPGRSSGRVYYFAAYDSGIHIILRAAYEALELINGYAAYKARGDL GAFAAYMTFSWLSSLAAYSVPWSVYGVAAYRILDEEHPK

Bacillus	**MKKGFSLLSLITMFLMIILLAACAPEREEEAVTTDTNDGEADQPEELTIWANDREEQLEAIEKIANDYTE QTGINVKVETKPMMDQLQELSLAGPEGNGPDLFFQPHDQIGNIVAQGLADPLTLSDDELSNYASSSID AVTYEFEGETDIYGIPAVIETYGIFYNKEIVPEAPETIRIRSLEAAAKALELINGYIQKIEAAAKSGRVYYFNHE** AAAKRRKCVAAEAAAKHHGLSALKEAAAKITRTKEEALGPGPGYFQSITVGVGPGPGYRTGVRLRGGPGP GVHMIKNKPAGPGPGRSSGRVYYFAAYDSGIHIILRAAYEALELINGYAAYKARGDLGAFAAYMTFSWLSS LAAYSVPWSVYGVAAYRILDEEHPK

*Adjuvant (**bold**), EAAAK linker (blue), B-cell epitopes (yellow), GPGPG linker (red), HTL epitopes (purple), AAY linker (orange), and CTL epitopes (green).

Re-evaluation of epitopes

The new vaccine sequence was run again in NetMHCpan- 4.1 and NetMHCIIpan-4.0. The result showed that the chosen T-cell epitopes for the vaccine are still present and bound to the respective MHC molecules. Interestingly, two of the CTL epitopes, namely LSMEDVWSL and FLMEEVHMI, were previously found to be bound with MHC molecule HLA-B*38:02. However, after the vaccine is constructed, they no longer bind to said MHC Class molecules. Another epitope that lost its ability to bind to MHC molecules is this HTL epitope FAAAENKVA, which no longer binds to DRB1_1602. Therefore, the two CTL and one HTL epitope were not included in the subsequent investigation.

Antigenicity test

After vaccine construction, the antigenicity test was run by the AntigenPro software on the new vaccine sequence. The score was 0.757329, indicating higher antigenicity for the vaccine construct with *E. coli* as the adjuvant. The *Bacillus* adjuvant, on the other hand, had a score of 0.447263, which indicated a lower antigenicity for the vaccine construct.

Allergenicity test

AllerTop software was used to conduct the allergenicity test for the new vaccine. This was done to ensure that, once in the body, the vaccine candidate does not cause any allergic reactions. The findings revealed that neither of the developed vaccine constructs were allergens.

Physicochemical characteristics

The vaccine's physicochemical characteristics were assessed using a Protparam server. To construct the vaccine sequence with *E. coli* as the adjuvant, 594 amino acids were used, with a molecular weight of 64446.71. The result shows that the vaccine with *E. coli* adjuvant was stable.

The software was run again to analyze physicochemical characteristics of the vaccine, this time around with *Bacillus* as the adjuvant. To make up the vaccine sequence with *Bacillus* as the adjuvant, 379 amino acids were used, which have a molecular weight of 41123.47. This also shows that the vaccine with *Bacillus* adjuvant was stable.

Homology with other human peptides

Sequences producing significant alignments with a lot of the vaccine peptides originated from PIN1 or the ABCC5 protein. However, some peptides were found not to have originated from the selected antigens. This indicates that the peptides chosen for vaccine construction are not as specific and will probably induce responses against proteins other than PIN1 and/or ABCC5.

***In silico* immune simulation**

The server simulated the immune responses at three different immunologic compartments, namely the bone, the lymphatic organ, and the thymus. In **Figures 8** through **12**, the simulation was run to test the ability of vaccine construct to induce various types of adaptive immune cells such as B-cell population, Plasma B lymphocyte (PLB) population, B-cell population per state, number of effector helper T-cells (TH cells) and cytotoxic T-cells (CT cells); alongside adaptive immune cells such as natural killer cells (NK cells), macrophages, dendritic cells, and production of the effector molecules such as Immunoglobulins and cytokines.

At each dose of vaccine administration, B-cell isotypes IgM, IgG1, and IgG2, as well as B-cell memory was created in response to the vaccine administration. The active B-cell population increased in response to each dose of vaccine administration, and the level remained steady throughout the remaining

duration of the simulation. At each dose of vaccine administration, the B-cell proliferation was notable. These B-cells also internalized the antigen and presented the peptide antigens using MHC Class II molecules. The number of effector helper T-cells and cytotoxic T-cells (TC), as well as the memory T-cell populations, also increased upon administration of the vaccine. The activity of NK cells, on the other hand, remains steady. Enhanced activity of macrophages that internalized the vaccine component and presented the antigen on MHC Class II was seen upon administration of each vaccine dose. The internalization of vaccine by dendritic cells was observed along with the increase in the number of dendritic cells presenting the antigen via MHC class I and Class II, notably presentation by MHC Class I is higher than class II. Following the injection of the first dose of vaccination, IgM is produced and keeps increasing with subsequent booster administration. IgG levels increased upon administration of the second dose of the vaccine, indicating that antibody isotype switching occurs during secondary responses. The IgG level increased again after the third vaccination. The increase in IgM and IgG levels was concomitant with the decrease in the concentration of the antigen. In addition, the vaccination can produce large quantities of IFN-, which is essential for an effective immune response against cancer cells (N). The inset in P also shows successful activation of T-cells, as the presence of danger signal is needed for the maturation of dendritic cells, and as IL-2 is produced by activated T-cells.

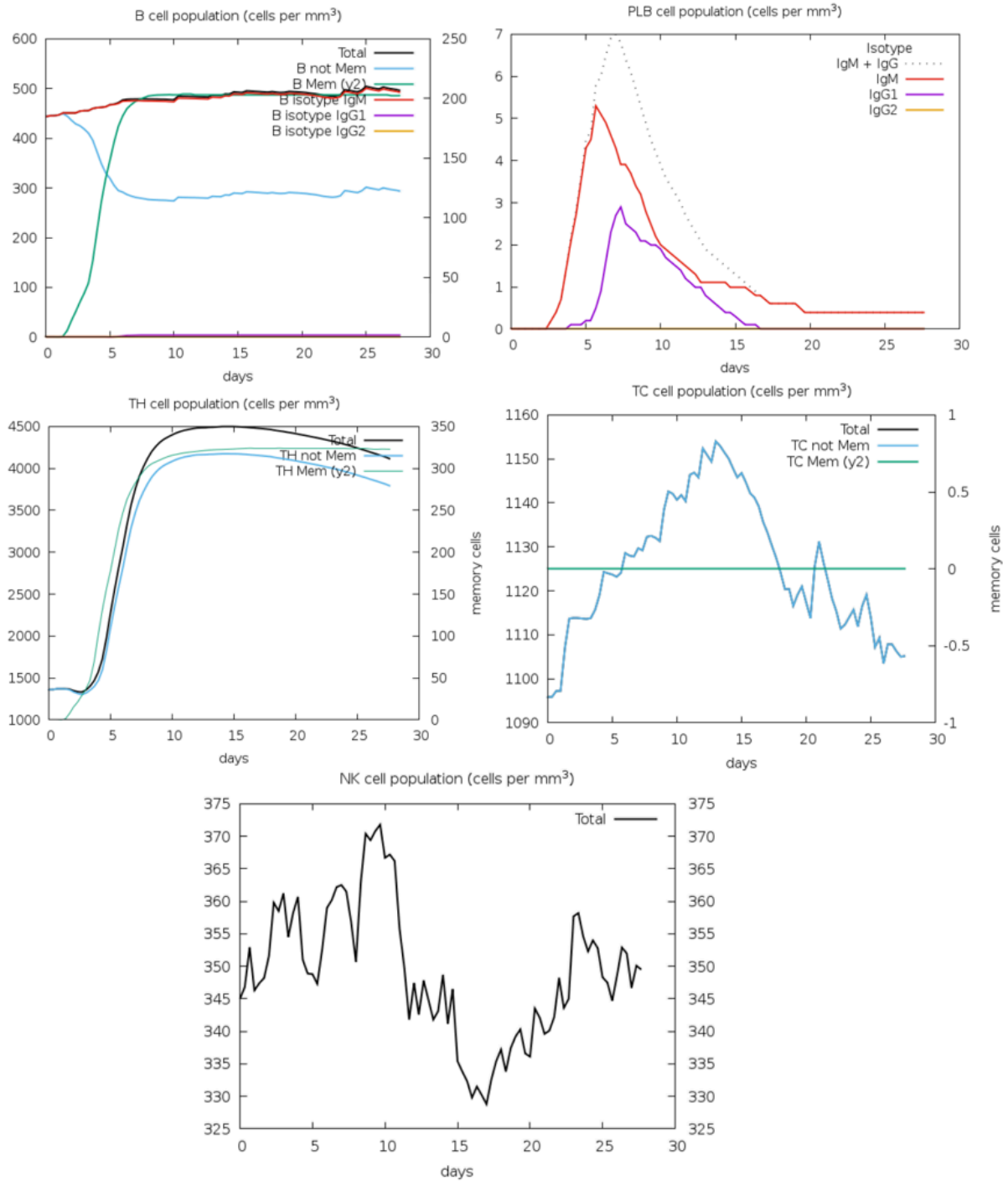

Figure 8. *E. coli* adjuvant vaccine construct. The profile of immune responses after primer-boost vaccination by the multi-epitope vaccine. Showing the cell counts of the B-cell population, PLB cell population, TH cell population, TC cell Population, and NK cell population.

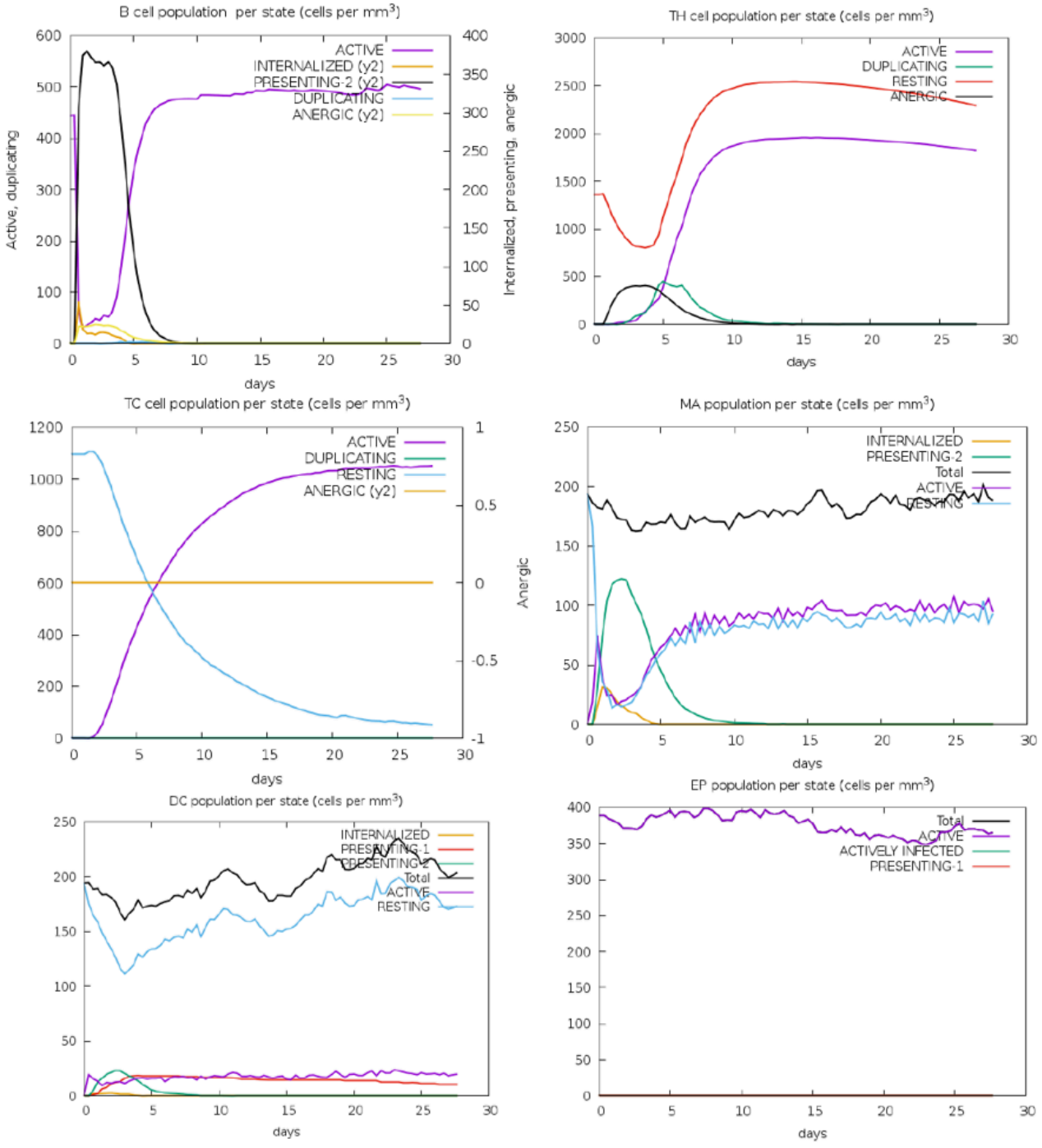

Figure 9. *E. coli* adjuvant vaccine construct. Cell counts. Act=active, Intern=internalized the Ag, Pres II = presenting to MHC-II, Dup = mitotic cycle, Anergic = anergic, Resting = inactive. The profile of immune responses after primer-boost vaccination by the multi-epitope vaccine. Showing the cell counts of B-cell population per state, TH cell population per state, TC cell population per state, MA population per state, DC population per state, EP population per state.

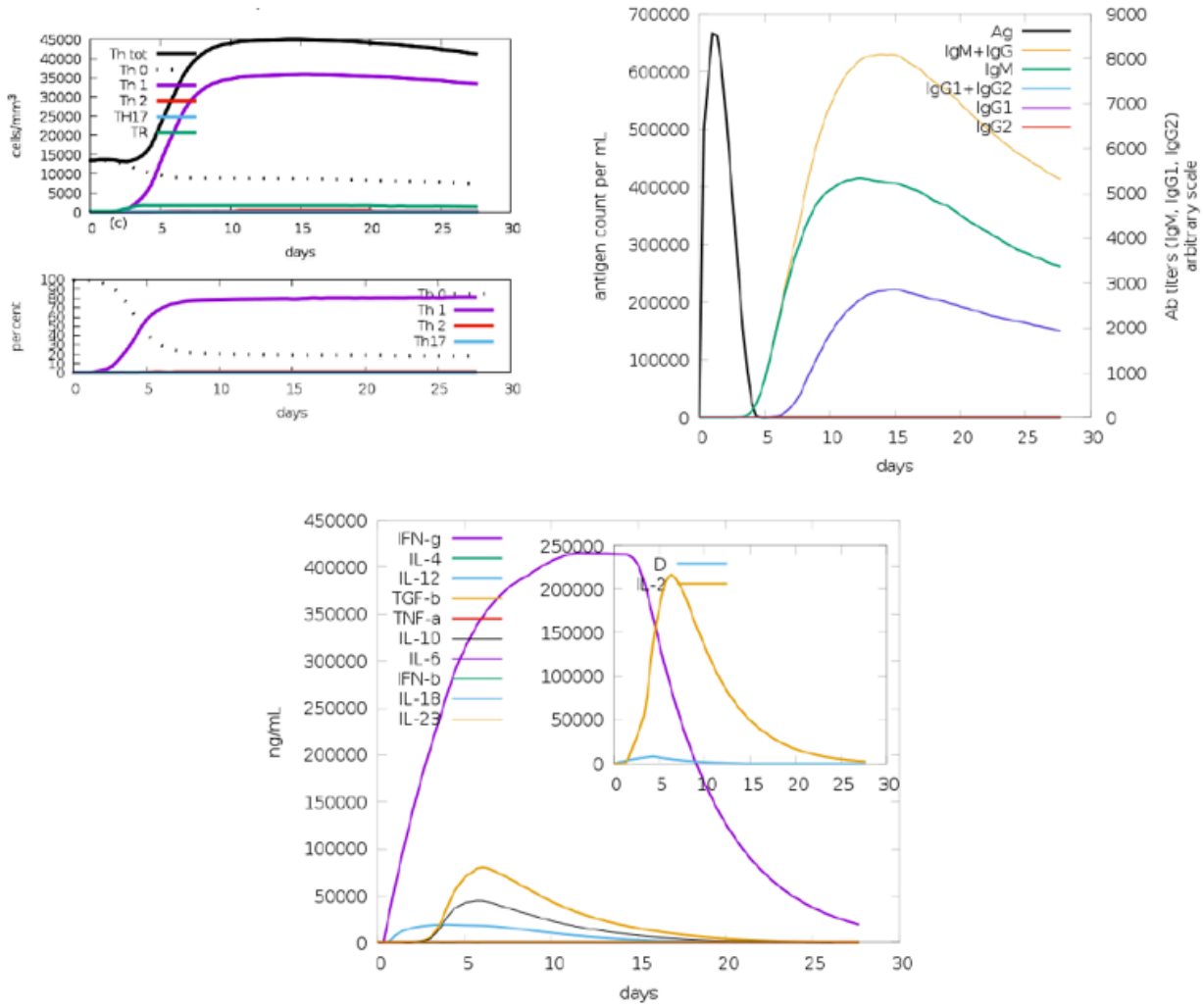

Figure 10. *E. coli* adjuvant vaccine construct. The virus, immunoglobulins, and immune complexes. Concentration of cytokines and interleukins. The inset plot shows danger signal together with the leukocyte growth factor IL-2. The profile of immune responses after primer-boost vaccination by the multi-epitope vaccine. Showing TH phenotypes, Immunoglobulin isotypes and activities, level of cytokines.

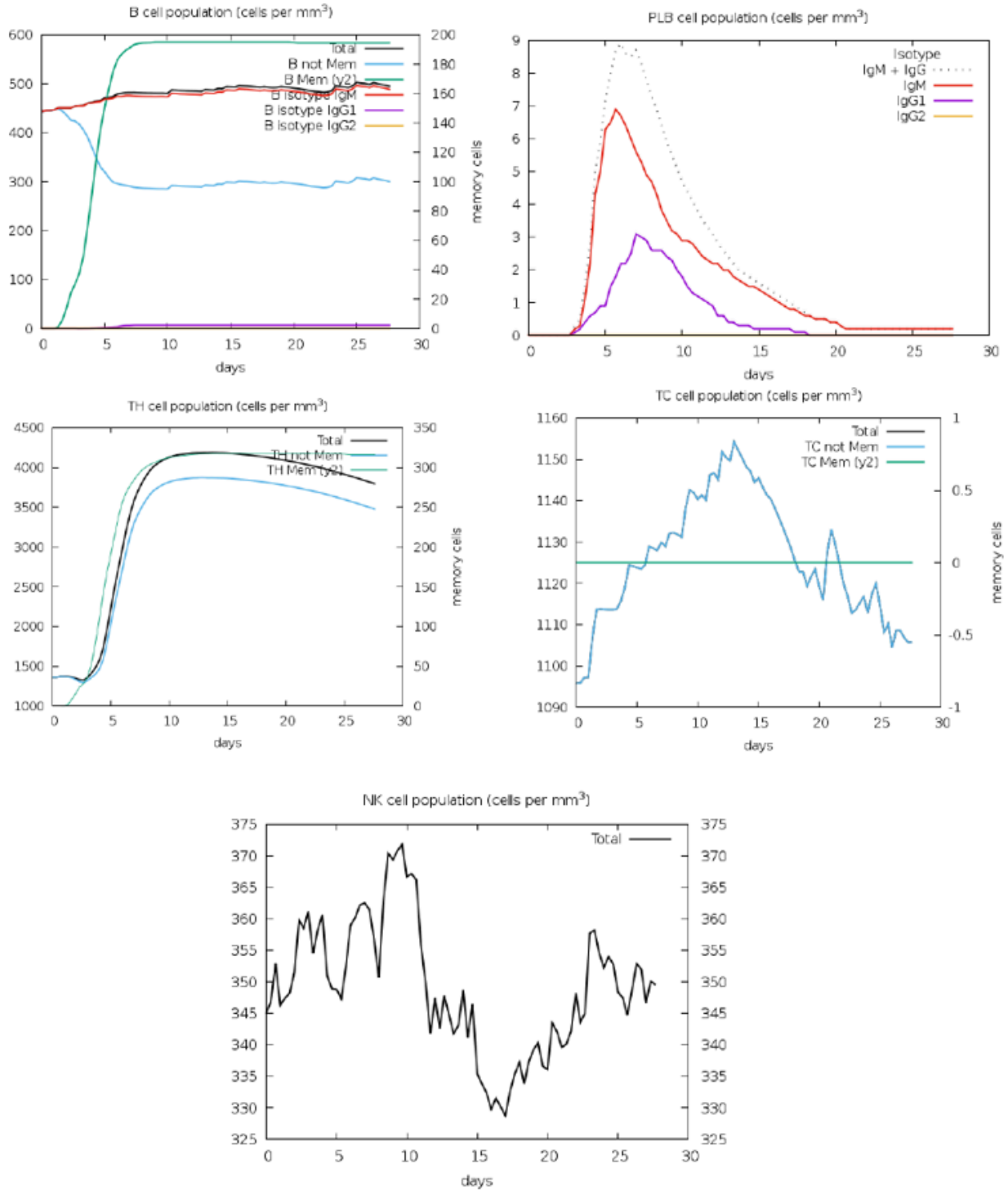

Figure 11. *Bacillus* adjuvant vaccine construct. The profile of immune responses after primer-boost vaccination by the multi-epitope vaccine. Showing the cell counts of the B-cell population, PLB cell population, TH cell population, TC cell Population, and NK cell population.

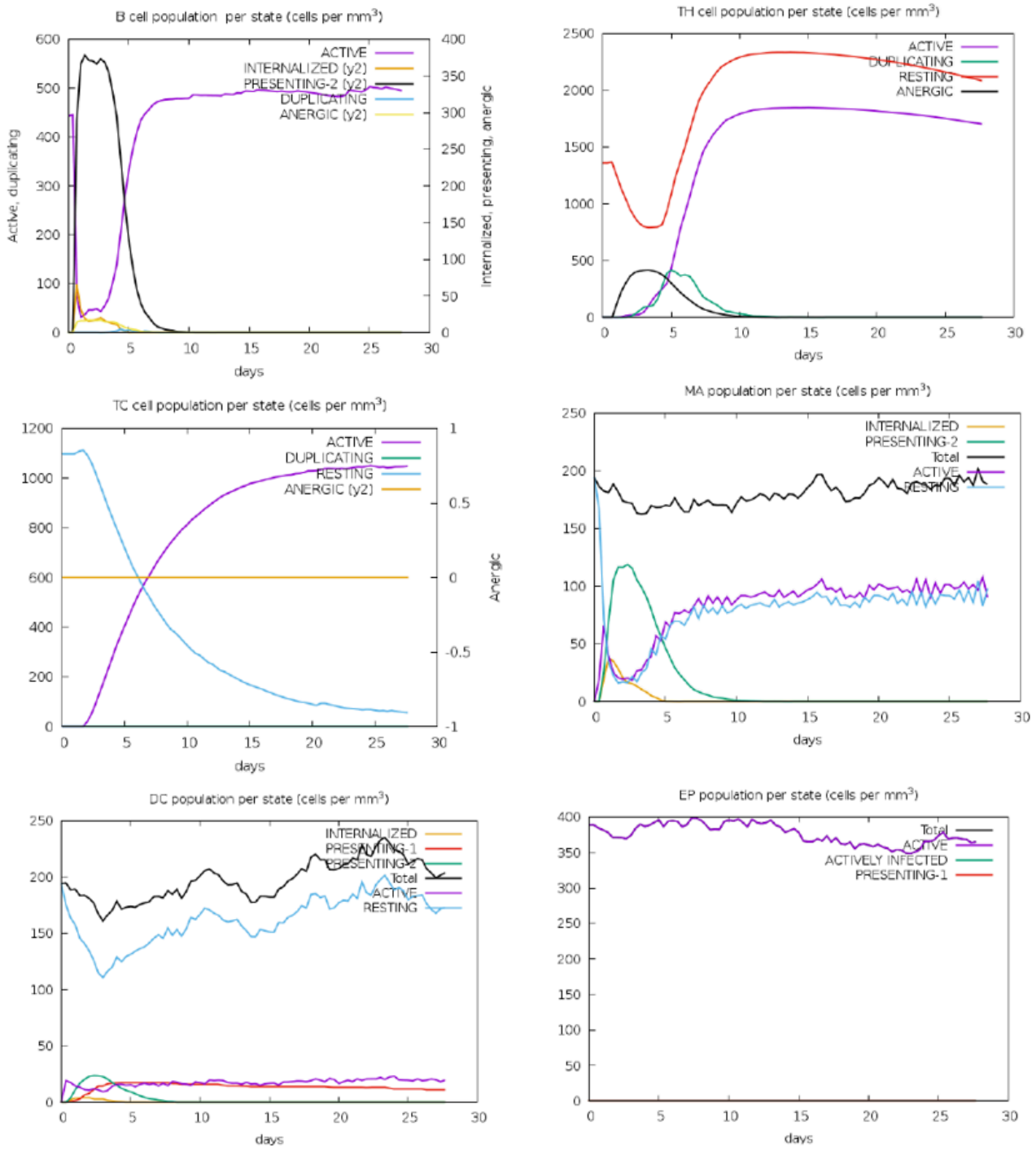

Figure 12. *Bacillus* adjuvant vaccine construct. Cell counts. Act=active, Intern=internalized the Ag, Pres II = presenting to MHC-II, Dup = mitotic cycle, Anergic = anergic, Resting = inactive. The profile of immune responses after primer-boost vaccination by the multi-epitope vaccine. Showing the cell counts of B-cell population per state, TH cell population per state, TC cell population per state, MA population per state, DC population per state, EP population per state.

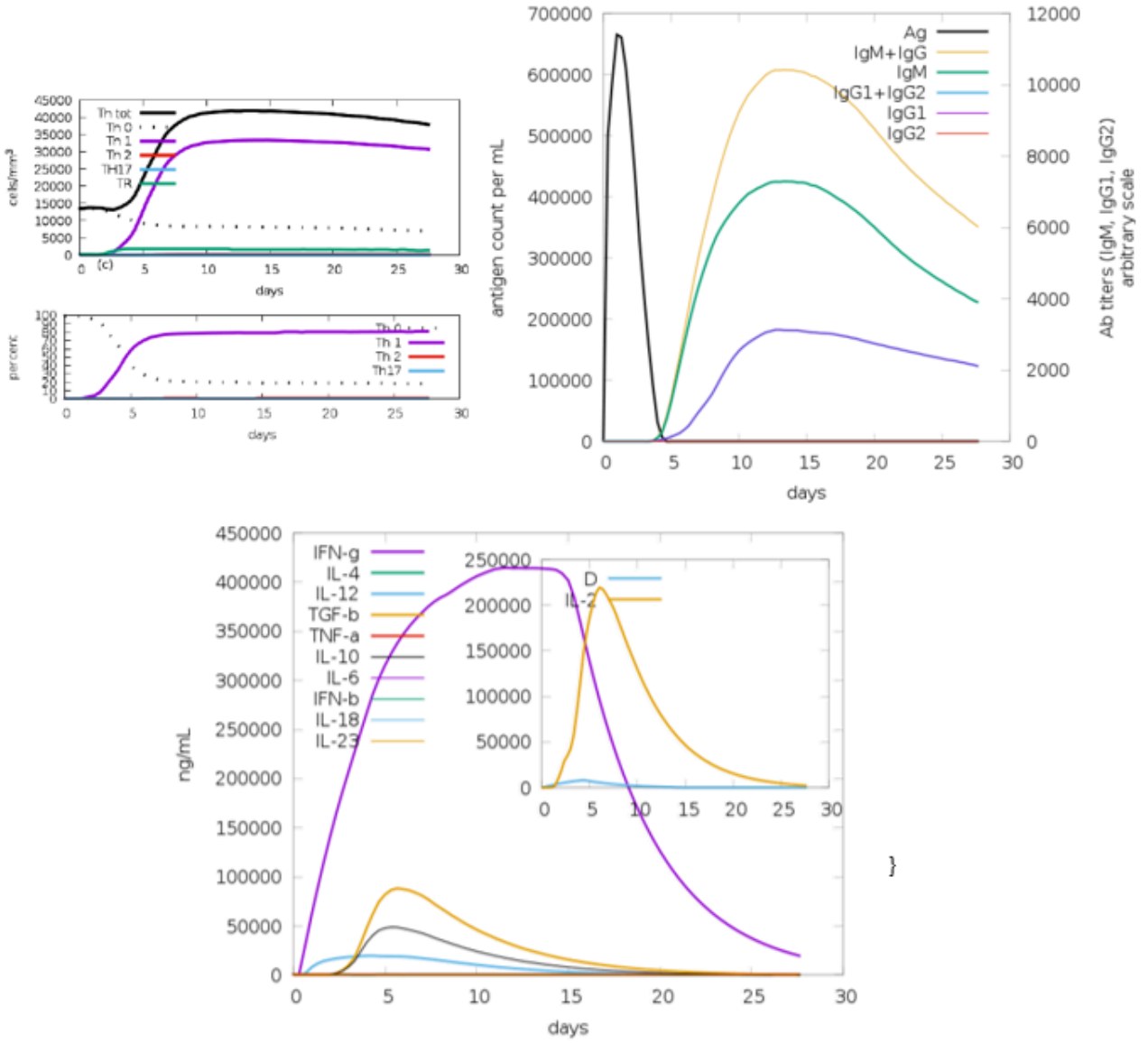

Figure 13. *Bacillus* adjuvant vaccine construct. The virus, immunoglobulins, and immune complexes. Concentration of cytokines and interleukins. The inset plot shows danger signal together with the leukocyte growth factor IL-2. The profile of immune responses after primer-boost vaccination by the multi-epitope vaccine. Showing TH phenotypes, immunoglobulin isotypes and activities, level of cytokines.

Structural modeling, validation, and molecular docking

The SWISS-MODEL server was used to create the vaccine's 3D models. Ramachandran plot analysis was used to validate the structural quality of the predicted model. The SWISS-MODEL server was also utilized to evaluate the modeled structure and to yield the Ramachandran plot. The Ramachandran plot analysis of the vaccine's 3D-model using *E. coli* as the adjuvant, the 3D-model for the vaccine structure shows 95.95% residues in favored regions, 0.48% residues in outlier regions, and 2.42% residues in rotamer outlier regions. With *Bacillus* as an adjuvant, it was revealed that 92.74% of residues were in favored regions, 2.23% in outlier regions, and 2.58% in rotamer outlier regions. The overall quality of the vaccine constructions is confirmed by these scores. Our 3D model's total number of residues in the favored region was within the range of the desired value (more than 90%), indicating its validity.

In order to elicit a strong immunological response, the vaccine must interact with target immune cell receptors. To examine such interactions, TLRs were used in molecular docking studies. TLRs are important in innate immunity because they identify conserved PAMPs on a wide range of pathogens, triggering innate immune activation and orchestrating adaptive immune responses (Carty & Bowie, 2010). TLR4 has been linked to enhanced anti-tumor immunity, which results in the release of inflammatory cytokines (Oblak & Jerala, 2011).

The docking analysis of the vaccine structures with TLR4 was carried out by ClusPro docking server with *E.coli* as an adjuvant showed a center score of -707.2 and a lowest energy score of -773.1 suggests a good binding affinity between the vaccine and the receptor. The ClusPro score for the vaccine construct with *Bacillus* as adjuvant, on the other hand, showed a center score of -996.4 and a lowest energy score of -1051.6.

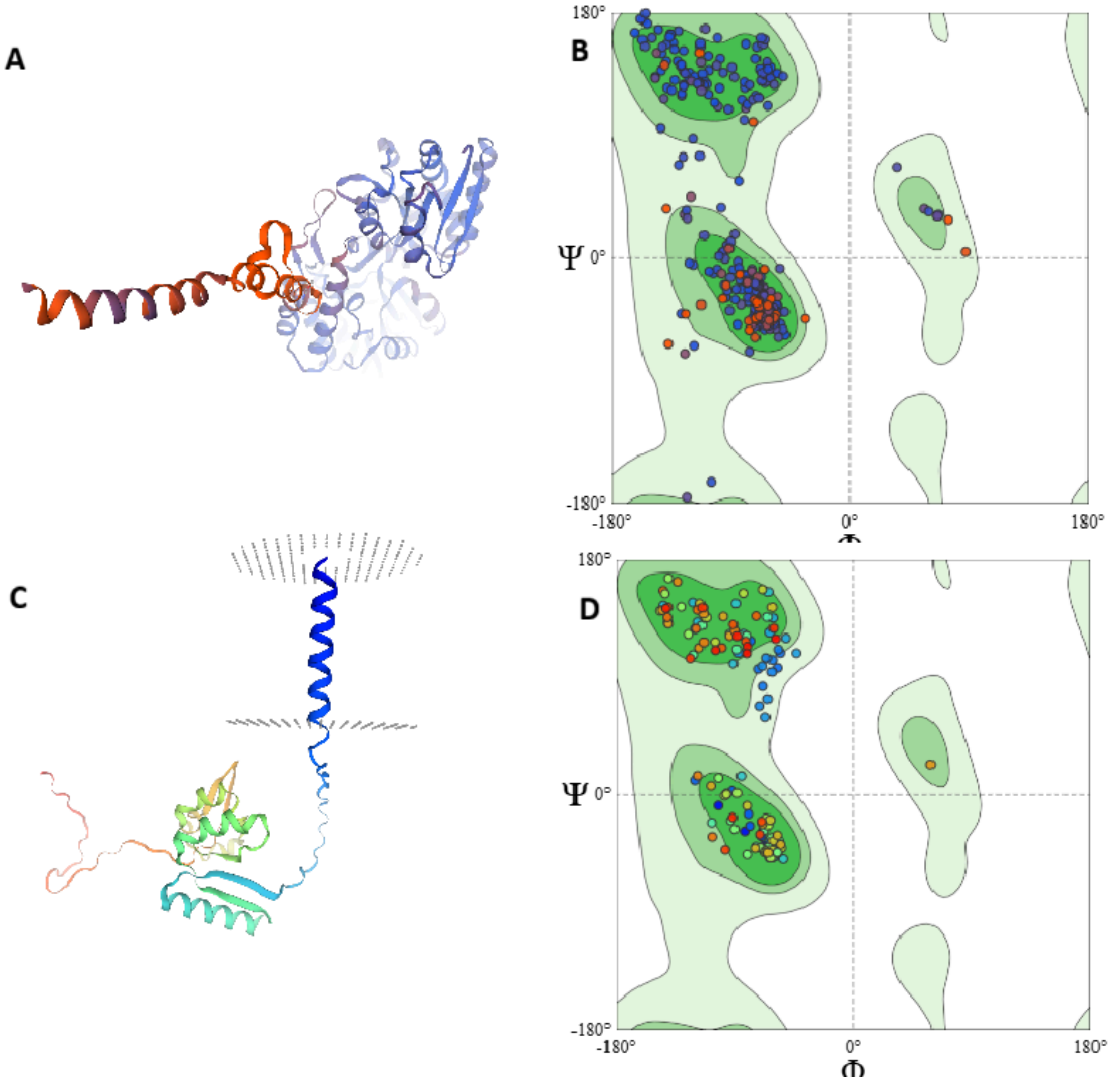

Figure 14. Vaccine structural modeling and validation. (A) Vaccine construct 3D model with *E. coli* as an adjuvant. Red, Limon, and Blue are the helical, sheet, and loop regions, in order. (B) Ramachandran plot analysis using SWISS-MODEL 95.95% of residues were in favored regions, 0.48% in outlier regions, and 2.42% in rotamer outlier regions. (C) Vaccine construct 3D model with *Bacillus* as an adjuvant. Red, Limon, and Blue represent the helical, sheet, and loop region, respectively. (D) Ramachandran plot analysis using SWISS-MODEL 92.74% residues in favored regions, 2.23% residues in outlier regions, and 2.58% residues in rotamer outlier regions.

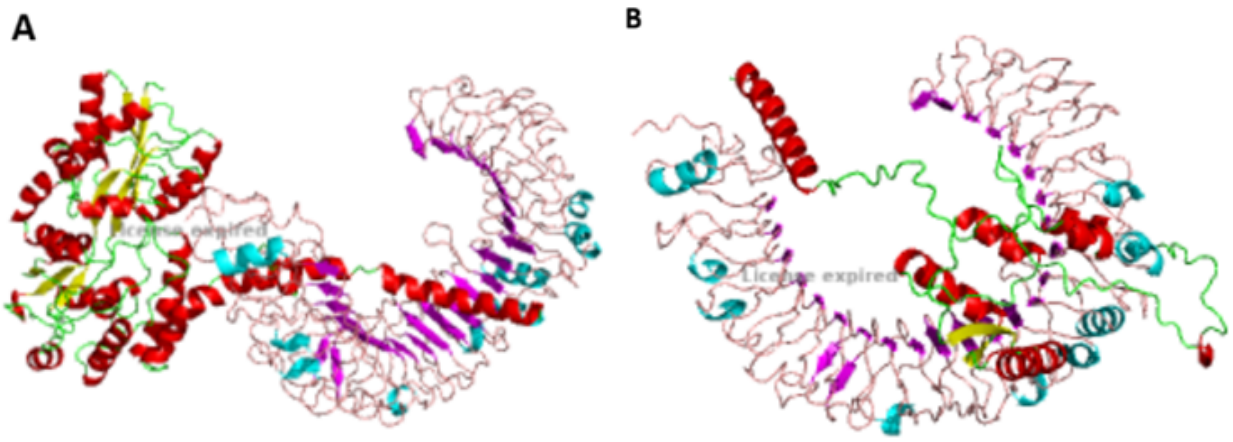

Figure 15. Molecular Docking Figure illustrating the bound TLR4/MD2-vaccine complex following molecular docking. The vaccine design is depicted in red, the TLR4 dimer in blue, and the MD2 co-receptor in green. Within the vaccine-TLR4 complex, a few important hydrogen bonds are concentrated and indicated by purple and green colors. (A) TLR4/MD2-vaccine molecular docking with *E. coli* as adjuvant. (B) TLR4/MD2-vaccine molecular docking with *Bacillus* as adjuvant.

DISCUSSION

NPC is endemic in Southeast Asia with poor prognosis. It is the fourth most prevalent cancer in Indonesia for both sexes, after hematopoietic and reticuloendothelial system malignancies, breast cancer, and cervical cancer. (Gondhowiarjo et al., 2019). NPC diagnosis is based on histological examination, specifically detection of Epstein–Barr virus-encoded small RNA by *in situ* hybridization (EBER-ISH). For treatment, the early-stage cancers are treated with radiation therapy aimed at the tumor. The late stages would usually get chemoradiation. In addition, viral antigen expression in NPC makes this disease an attractive target for immunotherapy strategies.

PIN1 is overexpressed in NPC, which is similar to other types of cancers. The phosphorylated Serine/Threonine-Proline motif is identified and isomerized by this protein. PIN1 is involved in a variety of biological functions, and its dysregulation can lead to cancer (Chen et al., 2018). Several investigations on the role of PIN1 in NPC formation have been undertaken, including one by Xu et al. (2016), who found that PIN1 overexpression promotes tumor cell growth via upregulating cyclin D1. Another gene highly expressed in cancer is ABCC5. It is overexpressed in breast cancer and prostate cancer in addition to NPC, as reported by Du & Yao (2008). ABCC5 overexpression is also reported in NPC cells that are resistant to paclitaxel treatment (Hou et al., 2017).

Thus far, the vaccine for NPC has been targeting EBV. A number of therapeutic EBV vaccines have been tested in clinical trials with promising results (Taylor & Steven, 2016). Developing a multi-epitope-based NPC vaccine might be helpful for patients with more advanced stages of the cancer and who are resistant to treatment. Targeting the antigens that are overexpressed around the tumor area is predicted to evoke an immune response against cancer cells.

This study aims to show that PIN1 and ABCC5 are potential targets for treating NPC patients. A multi-epitope vaccine with short immunogenic sequences that will evoke an immune response is constructed using various computational web servers instead of large proteins or whole genomes. This is done to make sure that the vaccine construct is free of a higher likelihood of allergic responses in the body and needless antigenic load (Chauhan et al., 2019). The development of immunoinformatics studies has overcome the challenges of classical vaccine production. No longer are the expensive and time-consuming

procedures of vaccine production and testing. Immunoinformatics and molecular modeling can be used to investigate the entire range of possible antigens in order to assess potential interaction with host proteins (Tosta et al., 2021). Additionally, multi-epitope vaccines have advantages over conventional as well as single-epitope vaccines. This is due to the fact that: (i) many MHC-I and MHC-II epitopes can be identified by TCRs from various T-cell subsets, (ii) overlapping CTL, HTL, and B-cell epitopes can activate both humoral and cellular immune responses at the same time, and (iii) the vaccine's adjuvant will ensure a long-lasting immune response (Davies & Flower, 2007).

Several studies have examined the role of PIN1 in NPC development, including one by Xu et al. (2016). They concluded that PIN1 overexpression enhances tumor cell growth via the upregulation of cyclin D1. These previous studies are a strong foundation to believe that PIN1 has an important role in NPC tumorigenesis and, therefore, can be a target for treatment. ABCC5, on the other hand, is found to be highly expressed in various cancers; it is overexpressed in breast cancer, prostate cancer, in addition to NPC, as reported by DU & Yao (2008). ABCC5 overexpression is also reported in NPC cells that are resistant to paclitaxel treatment (Hou et al., 2017). Previous studies regarding ABCC5 in NPC also support that it is a potential target for treatment.

The multi-epitope vaccine was created using possible immunogenic epitopes from PIN1 and ABCC5, as well as *E. coli* and *Bacillus* as adjuvants and appropriate linkers. The first step was to obtain the sequence for PIN1 and ABCC5 from the UniProtKB online database in the FASTA format. FASTA format was used in particular because it is widely accepted across various bioinformatics tools and databases, to guarantee a seamless data exchange. The adjuvant was *E. coli*, which has been shown to behave as a possible viral adjuvant and is coupled to the vaccine design at the N-terminus (Eldridge et al., 2021). *Bacillus* was chosen because it has a low manufacturing cost, is easy to administer because it is safe for human ingestion, and produces spores that have adjuvant effects (Rosales-Mendoza & Angulo, 2015). Glycine-rich linkers (GPGPG) were chosen to link the screened epitopes because they improve solubility and allow adjacent domains to interact freely (Kavoosi et al., 2007). AAY as well as EAAK linkers were used in concordance with previous studies, such as the one done by Naveed et al. (2022). The predicted CTL and HTL epitopes were screened using several immunological filters: they had to be antigenic and immunogenic, linked with numerous MHC class I and MHC class II alleles (promiscuous), and contain overlapping CTL and HTL epitopes.

This study shows six peptides from PIN1, as well as twenty peptides from ABCC5, were discovered to be ideal targets for B-cell interaction using the Bepipred linear prediction. B-cell linear epitope is important in developing vaccines because the more linear the epitope, it means that it can be highly recognized by an antibody (Chandra & Singh, 2012). The presence of these epitopes in the constructed vaccine is critical for a successful immune response. Hence, in this study, the linear B-cell epitopes were predicted using the Bepipred server. The peptides that consist of fewer than seven amino acids were eliminated, hence only those are included (**Tables 2 & 3**). The default threshold was 0.500 for both PIN1 and ABCC5, and all the values above the threshold were determined as potential linear epitopes. In essence, a threshold value of 0.500 is deemed to be adequate for the method's accuracy; this increases its specificity. A threshold value too high can make the classifier too selective when predicting for B-cell epitopes, which leads to a lot of potential epitopes being missed without an associated performance boost (Galanis et al., 2021). The average value was 0.533 and 0.457 for PIN1 and ABCC5, respectively. The highest and lowest values for PIN1 are 0.688 and 0.260, respectively. Whereas for ABCC5, the highest and lowest values are 0.681 and 0.240, respectively (**Figures 1 & 2**).

The Emini surface accessibility prediction method was used to check the epitopes for surface accessibility. This method aims to identify the region of PIN1 as well as ABCC5 proteins that are located on

the surface since the active sites of the protein are on the surface (Lins et al., 2003). Four peptides were identified to be located on the surface of PIN1 by Emini surface accessibility prediction. However, ABCC5 shows more peptides located on the surface, the number being nineteen. The default threshold as well as average value was 1.000 for both PIN1 and ABCC5, and all the values above the threshold were the region that was on the surface. The highest and lowest values for PIN1 are 4.097 and 0.064, respectively. Whereas for ABCC5, the highest and lowest values are 9.915 and 0.038, respectively. The location and length of the predicted peptide are shown in **Tables 4 & 5** and **Figures 3 & 4**. ABCC5 is a surface protein, which means that ABCC5 has more potential to be recognized by antibodies that recognize accessible surface epitopes; T cells recognize peptides presented by MHC, including those derived from intracellular proteins.

Other than that, antigenicity prediction was also done using Kolaskar and Tongaonkar predictions. From this prediction, it was discovered that four regions in PIN1 are antigenic. However, forty-one regions from ABCC5 were discovered to be antigenic. The default threshold for PIN1 and ABCC5 was 0.995 and 1.038, respectively, and all the values above the threshold were determined as potential regions that were antigenic. The average value was 0.995 with a lowest value of 0.885 and a highest value of 1.192 for PIN1. On the other hand, ABCC5 had an average value of 1.038, with the lowest value of 0.866 and the highest score of 1.259. The yellow color indicates a potential antigenic site in PIN1 and ABCC5 above the threshold, while the green color does not (**Figures 5 & 6**). In the end, four predicted B-cell epitopes that passed the linear B-cell prediction and have good antigenicity are included in this vaccine development (**Figure 4**). For T-cell interaction, we know that CTL can only recognize antigens presented on MHC-I. In this study, it was found that there were five peptides identified from PIN1 to have binding with MHC-I predicted using NetMHCPan. ABCC5 on the other hand, has eighty-five peptides that have binding with MHC-I predicted using NetMHCPan. These epitopes were selected as they passed the criteria of a good epitope based on NetMHCPan. MHC-I binding affinity, ligand combined score, Proteasomal C-terminal cleavage, TAP transport efficiency, and percentage rank are all predicted by NetMHCPan, with a percentage rank of less than 1.0 indicating a good epitope score (Ayyagari et al., 2022). Epitopes that have a percentage rank lower than 1.0 were then listed in **Tables 10 & 11**. Strong binders are defined as having a %Rank<0.5 ranking of the predicted affinity compared to a set of 400.000 random natural peptides, and weak binders with a %rank<2. In the NetMHCpan-4.1 software, the %Rank<0.5 measures the predicted affinity without regard to the inherent biases of the molecules. The web server advised users to select candidate binders based on %Rank rather than nM Affinity, which predicted binding affinity in nanomolar units. There are strong and weak binders described in the software. If the peptide has a %Rank of less than the default threshold for strong binders, which is 0.5%, the peptide is considered to be a strong binder. The peptide is classified as a weak binder; on the other hand, if the %Rank is over the strong binder threshold but still less than the weak binder barrier, which is set at 2% by default from the server (Andreatta & Nielsen, 2016; Nielsen et al., 2003). Three epitopes from ABCC5 were found to interact with the most alleles (eight). The epitopes and alleles are MTFSWLSSL (HLA-A*02:01, HLA-A*02:03, HLA-A*33:03, HLA-A*34:01, HLA-B*15:02, HLA-B*15:13, HLA-B*15:21, HLA-B*58:01); FMVKHLLEY (HLA-A*34:01, HLA-B*15:02, HLA-B*15:13, HLA-B*15:21, HLA-B*18:01, HLA-B*35:05, HLA-B*38:02, HLA-B*44:03); and IAISGTFAY (HLA-A*34:01, HLA-B*15:02, HLA-B*15:13, HLAB*15:21, HLA-B*18:01, HLA-B*35:05, HLA-B*44:03, HLA-B*58:01) (**Table 11**).

T-helper cells, on the other hand, can only recognize antigens on MHC-II. From the prediction, eight peptides were identified as having strong or weak binding to MHC-II from PIN1. ABCC5, however, has 54. 30 peptides that have strong binding were chosen for further analysis. Similar to MHC-I prediction, these 30 peptides of MHC class II were chosen because they have a percentage rank lower than 2.0, which indicates

a strong peptide. The percentage rank is based on the IC50 value and further comparison with a group of 100,000 natural peptides at random (Reynisson et al., 2020). This prediction was carried out using the NetMHCIIpan-4.0 software, and, as with the CTL binding to MHC-I prediction software counterpart NetMHCpan 4.1, the peptide will be deemed a strong binder if the %Rank is below 0.5% and a weak binder if it is above the threshold but below 2%.

From several identified epitopes, nine CTL-predicted epitopes and five HTL-predicted epitopes were chosen for the vaccine construct (**Tables 12 & 13**). The CTL epitopes were chosen based on their immunogenicity score because a high score means the epitope is immunogenic and might provoke an immune response. A positive score indicates the ability of epitopes to be recognized by CTL, while a negative score indicates a poor recognition of CTL (Fleri et al., 2017). Moreover, since 35 CTL epitopes from ABCC5 and PIN1 were identified as good epitopes based on the percentage rank, the allele that those epitopes interact with is also considered in deciding the nine CTL epitopes. The alleles that are considered major alleles in Indonesia are listed in **Table 9**, and the epitopes chosen are those that can interact with those alleles. Besides that, five HTL epitopes are chosen for vaccine construction. The HTL epitopes are chosen based on their ability to elicit IFN-γ and to interact with major MHC-II alleles in Indonesia (**Table 13**). The sequence VHMIKNKPA was found to have the highest IFN-γ epitope prediction score, meaning it has a substantial effect on the production of IFN-γ. The IFN- epitopes server can predict epitopes with a maximum accuracy of 81.39% using a variety of methods, including a machine-learning strategy, motif-based analysis, and a hybrid accuracy approach (Dhanda et al., 2013). Because HLA is known to be naturally polymorphic, the epitopes' population coverage as well as their matching HLA alleles is significant. This vaccine is aimed to benefit patients in Indonesia. As a result, we examined Indonesia's population coverage for the selected predicted peptides. The population coverage analysis was found to be 99.27% for MHC-I and 95.26% for MHC-II (**Table 14**). We can extrapolate from this that our vaccine will elicit a powerful anti-cancer immune response in the great majority of those who receive it.

When selecting a target for vaccine development, antigenicity is also taken into account; this is supported by its definition, antigenicity is the capability of an antigen to specifically bind with T-cell or B-cell receptors. The antigenicity was analyzed using VaxiJen software, and it was found that the overall score prediction for the antigen was 0.4953, which is a probable non-antigen, and the AntigenPro score was 0.786053, which indicated a higher antigenicity vaccine for the vaccine construct with *E. coli* as the vaccine adjuvant. For *Bacillus,* on the other hand, the Vaxijen score was 0.5343 (a score > 0.4 is considered to be antigenic), which is a probable antigen, and the AntigenPro score was 0.479917, which indicated a lower antigenicity vaccine, which means that this vaccine is moderately antigenic (Flower et al., 2017).

Epitope similarity with the other human proteins needs to be taken into account when choosing the peptides for a cancer vaccine, since the peptide administered might induce autoimmune responses. The peptides chosen have to generate good immune responses against cancer cells, but the peptides also show homology to other proteins in the body. This raises concern that the vaccine might evoke an immune response against non-cancer cells.

The structural validation of our vaccine construct performed by Ramachandran plot analysis using SWISSMODEL showed that the Ramachandran plot analysis of the vaccine's 3D-model using The Ramachandran plot analysis of the vaccine's 3D-model using *E. coli* as the adjuvant, the 3D-model for the vaccine structure shows 95.95% residues in favored regions, 0.48% residues in outlier regions, and 2.42% residues in rotamer outlier regions. With *Bacillus* as an adjuvant, it was revealed that 92.74% of residues were in favored regions, 2.23% in outlier regions, and 2.58% in rotamer outlier regions (**Figure 14**). The high percentage of residues in the favored regions thereby validates both the tertiary structure of the vaccines.

TLR4 is found in monocytes, macrophages, granulocytes, and immature DCs, among other immune cells (Vaure & Liu, 2014). Direct interaction between TLR4 and the pathogen is responsible for TLR4 activation. In the case of vaccines, the adjuvant is responsible for this. *Bacillus* activates TLR4, as reported by Akazawa et al. (2004). Accordingly*, E. coli* has been shown to activate TLR4, as reported by Lonez et al. (2015). The docking analysis of the vaccine structures with TLR4 was carried out by ClusPro docking software. Docking enables scientists to virtually examine a library of compounds and identify the strongest binders using a variety of score algorithms. It investigates how two molecules, such as a vaccine and a receptor, fit together and dock well. The ClusPro score for the vaccine construct with *E.coli* as an adjuvant showed a center score of -707.2 and a lowest energy score of -773.1, which suggests a good binding affinity between the vaccine and the receptor. A negative score indicates better docking, as it means less energy is needed for both proteins to interact. The ClusPro score for the vaccine construct with *Bacillus* as adjuvant, on the other hand, showed a center score of -996.4 and the lowest energy score of -1051.6 (**Figure 15**).

CONCLUSION

The immunoinformatics study prioritized PIN1 and ABCC5-derived CTL, HTL, and IFN-γ–linked epitopes for a population-tailored, multi-epitope vaccine candidate against nasopharyngeal carcinoma. In silico analyses predicted antigenicity and broad HLA coverage in Indonesia, and suggested favorable receptor docking for the adjuvant-bearing construct. These findings nominate a tractable set of epitopes for experimental triage. However, all claims remain predictive: natural antigen processing, HLA binding, T-cell activation, adjuvant mechanism (e.g., TLR4 engagement), structural stability, and safety require empirical validation. Immediate priorities are peptide/HLA binding assays, T-cell immunogenicity in donor PBMCs, immunopeptidomics to confirm processing, and receptor-reporter assays for the adjuvant. If corroborated, this host- antigen strategy could complement EBV-directed approaches in NPC. All findings are computational predictions subject to model bias and parameter choices. We did not experimentally verify antigen processing, HLA binding, T-cell activation, or TLR engagement. MD, docking, and immune simulations provide hypothesis-generating insights but not efficacy evidence. Future work will include *in vitro* HLA binding and T-cell assays, immunopeptidomics to confirm natural processing, receptor-reporter tests for the adjuvant, and *in vivo* safety and immunogenicity studies.

AUTHOR CONTRIBUTIONS

PA: Conceptualization, Software, Formal analysis, Investigation, Data curation, Writing – Original draft, Funding acquisition. **MG:** Methodology, Writing – Review & Editing, Supervision.

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COMPETING INTERESTS

The authors declare no conflict of interest.

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ADDITIONAL INFORMATION

None.

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