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Article

Designing a Novel Multiepitope Vaccine from the Human Papilloma Virus E1 and E2 Proteins for Indonesia with Immunoinformatics and Molecular Dynamics Approaches

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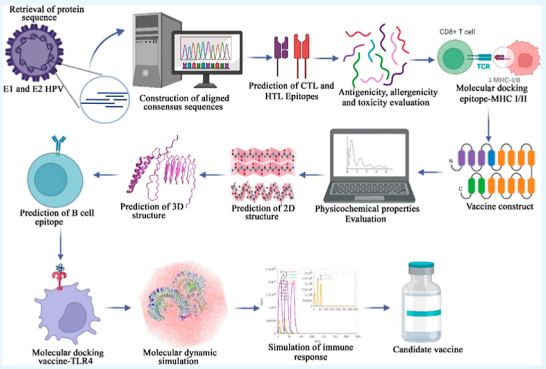
ABSTRACT: One of the deadliest malignant cancer in women globally is cervical cancer. Specifically, cervical cancer is the second most common type of cancer in Indonesia. The main infectious agent of cervical cancer is the human papilloma virus (HPV). Although licensed prophylactic vaccines are available, cervical cancer cases are on the rise. Therapy using multiepitope-based vaccines is a very promising therapy for cervical cancer. This study aimed to develop a multiepitope vaccine based on the E1 and E2 proteins of HPV 16, 18, 45, and 52 using in silico. In this study, we develop a novel multiepitope vaccine candidate using an immunoinformatic approach. We predicted the epitopes of the cytotoxic T lymphocyte (CTL) and helper T lymphocyte (HTL) and evaluated their immunogenic properties. Population coverage analysis of qualified epitopes was conducted to determine the successful use of the vaccine worldwide. The epitopes were constructed into a multiepitope vaccine by using AAY linkers between the CTL epitopes and GPGPG linkers between the HTL epitopes. The tertiary structure of the multiepitope vaccine was modeled with AlphaFold and was evaluated by Prosa-web. The results of vaccine construction were analyzed for B-cell epitope prediction, molecular docking with Toll like receptor-4 (TLR4), and molecular dynamics simulation. The results of epitope prediction obtained 4 CTL epitopes and 7 HTL epitopes that are eligible for construction of multiepitope vaccines. Prediction of the physicochemical properties of multiepitope vaccines obtained good results for recombinant protein production. The interaction showed that the interaction of the multiepitope vaccine-TLR4 complex is stable based on the binding free energy value −106.5 kcal/mol. The results of the immune response simulation show that multiepitope vaccine candidates could activate the adaptive and humoral immune systems and generate long-term B-cell memory. According to these results, the development of a multiepitope vaccine with a reverse vaccinology approach is a breakthrough to develop potential cervical cancer therapeutic vaccines.



1. INTRODUCTION

Cervical cancer cases at 6.5% is ranked as the fourth malignant cancer that causes death in women worldwide.[1] Human papilloma virus (HPV) is associated with 99.7% of cervical cancer infections.[2] The HPV, which is spread through sexual contact, is the most common cause of cervical cancer in women, despite the fact that there are a variety of factors that can put a woman at risk for developing the disease.[3] Based on their carcinogenic properties, they have been classified into two groups: high-risk HPV and low-risk HPV.[4] The high-risk HPV types include 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68, while the low-risk HPV types include 6, 8, 11, 40, 42, 43, 44, 53, 54, 61, 72, and 73.[5] About 70% of cervical cancer cases are caused by HPV types 16 and 18.[6] Data obtained in 2015 showed that the most prevalent types of HPV in Indonesia are 16, 18, 45, and 52.[7] Persistent and untreated high-risk HPV infection can cause cervical intraepithelial neoplasia to develop into carcinoma.[8]

There are several proteins expressed by HPV including the long control region; the early region (E1, E2, E4, E5, E6, and E7 proteins); and the late region (L1 and L2 protein).[9] E1 is the only HPV protein that acts as an ATP-dependent DNA helicase to unwind viral double-stranded DNA. Replication of viral DNA also involves the role of E2 proteins. The double-

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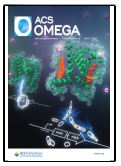

Table 1. Prediction of CTL Epitopes

protein	peptide (**code**)	HLA class I supertype	antigenicity	toxicity	allergenicity	cross-reactivity
E1	ITDDSDMAY (**C1**)	A1, B58, B62	0.5187	no	no	no
	FTFPNAFPF (**C2**)	A1, A24, A26, B58, B62	0.5435	no	no	no
	VLILLLIRY (**C3**)	A3, A26, B62	0.7869	no	no	no
E2	KTGIVTITY (**C4**)	A1, A3, B58, B62	1.207	no	no	no

Table 2. Prediction of HTL Epitopes

protein	peptide (**code**)	HLA class II allele	antigenicity	toxicity	allergenicity	induces IFN-γ	induces IL-4	cross-reactivity
E1	MSFIHFLQGAVISFV (**H1**)	DRB1 0101, DRB1 1201, DQA10501-DQB10301, H2 IEK	0.7214	no	no	yes	yes	no
	KSYFGMSFIHFLQGA (**H2**)	DRB1 0402, DPA10103-DPB10401, DPA10103-DPB10402, DPA10201-DPB10101, DPA10103-DPB10201	0.6719	no	no	yes	yes	no
	YFGMSFIHFLQGAVI (**H3**)	DRB1 0402, DRB1 0901, DRB1 1201	0.7589	no	no	yes	yes	no
	GAVISFVNSKSHFWL (**H4**)	DRB1 0404, DRB1 0405, DRB1 0701, DRB1 0802, DRB1 1501, DRB1 1602, DRB5 0101, DQA10102-DQB10501	0.6942	no	no	yes	yes	no
	AVISFVNSKSHFWLQ (**H5**)	DRB1 0404, DRB1 0405, DRB1 0701, DRB1 0802, DRB1 1501, DRB1 1602, DRB5 0101, DQA10102-DQB10501	0.825	no	no	yes	yes	no
	FLRYQGVEFISFLAL (**H6**)	DPA10103-DPB10401, DPA10201-DPB10101, DPA10301-DPB10402, DPA10103-DPB10201	0.6865	no	no	yes	yes	no
	LRYQGVEFISFLALK (**H7**)	DPA10103-DPB10401, DPA10201-DPB10101, DPA10301-DPB10402, DPA10103-DPB10201	1.1606	no	no	yes	yes	no
E2	KNSLKCLRYRLKKHS (**H8**)	DRB1 0103, DRB1 0801, DRB1 1301, DRB4 0103	0.5537	no	no	yes	yes	no
	SLKCLRYRLKKHSDH (**H9**)	DRB1 0103, DRB1 0801, DRB1 1301, DRB4 0103, H2 IED	0.487	no	no	yes	yes	no

stranded DNA virus cannot replicate without the E1 and E2 proteins, which attach to and unwind the replication origin.[10] For the initiation of viral DNA replication, E1 interacts with E2 to establish the starting point of replication.[11] Moreover, E1 is highly conserved compared to other HPV proteins, making it an attractive vaccine target.[10] E2 is also an attractive target for therapeutic vaccines as it is expressed at several stages of precancerous lesion development.[12] In addition, another study by Ren et al.showed that the E2 protein mediates an alternative pathway to carcinogenesis.[13]

Surgery, chemotherapy, and radiation therapy are some of the therapies available for patients who have been diagnosed with cervical cancer.[14] Additionally, cervical cancer can be prevented through vaccination and periodic screening. Currently, three types of prophylactic vaccines are licensed to prevent HPV infection, namely Cervarix, Gardasil, and Gardasil 9.[15] The three vaccines are based on the L1 gene expression. The complexity of pathogens, immune evasion systems, and mutations in a pathogen that will make the vaccine less effective are some of the challenges that are associated with the development of novel vaccines.[16] The use of E1 and E2 proteins, which have enzymatic properties that make them less prone to mutation, is a potential target in the development of a novel vaccine. HPV has an immune system evasion mechanism, allowing cervical cancer infection to arise. It shows the importance of the adaptive immune response role. Antigen presenting cells (APCs) recognize viral proteins via the pattern recognition receptor (PRR). The protein is then processed by a transporter associated with antigen processing (TAP) into small peptides that are presented to T lymphocytes by HLA class I or II.[17] Therefore, the interaction between peptides and HLA is critical for the stage of antigen identification by T cells. Due to the significant polymorphism of HLA in humans, it is critical to predict HLA alleles in priority areas.

However, these HPV vaccines do not have a therapeutic effect and induce only the production of antibodies to neutralize the virus. Thus far, there have been several developments focused on therapeutic HPV vaccines to induce the cellular immune system as the main target. Therapeutic vaccines mainly function via cytotoxic T-cell activation.[18] In contrast to prophylactic vaccines that induce antibodies, therapeutic vaccines activate cellular immune responses (CD4+ and CD8+ T cells). Antigens that have the best immunogenic properties are presented to the cell surface by HLA class I and HLA class II. The antigens presented by HLA class I form a complex with CD8+ T cells, which then release cytokines to lyze the HPV-infected cells. In addition, HLA class II presents antigens for recognition by CD4+ T cells, activating the T helper cells as well as NK cells.[18]

The development of E1 and E2 vaccines based on the whole genome has been carried out using conventional methods.[19,20] However, conventional vaccines involving the whole genome could potentially increase allergic reactions in patients.[21] Furthermore, conventional vaccines require an extended period of development and have the potential to elicit autoimmune responses.[22] Consequently, the development of multiepitope vaccinations to prevent negative impacts, such as allergies, through the utilization of whole genome vaccines is currently underway. Reverse vaccinology is a solution that reduces the amount of time needed to develop vaccines by using only possible epitopes as candidates for vaccines. In the fight against cancer and other infectious diseases caused by viruses, multiepitope vaccines are an exciting new invention that have great promise. They have the advantage of activating both the cellular immune system and the humoral immune

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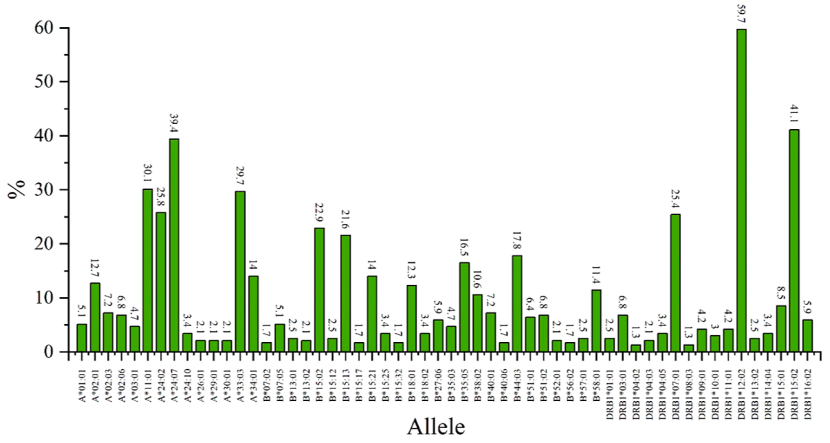

Figure 1. Distribution and frequency of Indonesian HLA alleles.

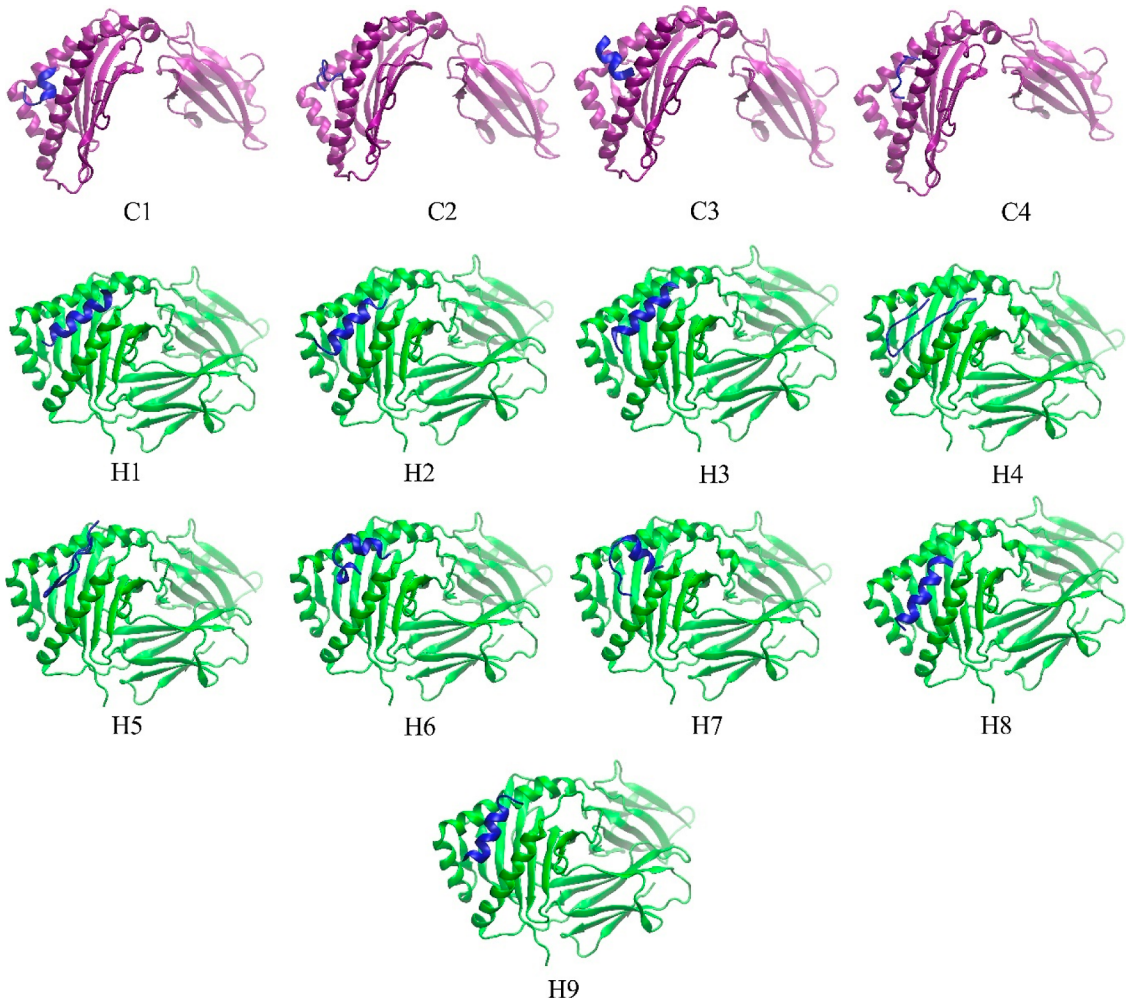

Figure 2. Binding pocket of the epitope (blue) with HLA A*24:07 (purple) and HLA B*DQA10102 (green).

system simultaneously.[23] Therefore, we evaluated the E1 and E2 proteins of HPV types 16, 18, 45, and 52 as potential vaccine candidates in this investigation. Reverse vaccinology was used to predict effective peptides for activating the immune response. Furthermore, several analyses were performed to validate the predicted results, including immune response simulations, the interaction of the TLR4-multiepitope vaccine, and the stability of the interaction for multiepitope vaccine construction.

2. RESULTS AND DISCUSSION

2.1. Prediction of T-Cell Epitopes. The results of the prediction epitope identified 14 cytotoxic T lymphocyte (CTL) E1 epitopes and five CTL E2 epitopes. The allergenicity, antigenicity, and toxicity significance of the epitopes were screened and found three CTL E1 epitopes and one CTL E2 epitope as the suitable candidates (Table 1). Further screening on the epitope that can induce IL-4 and

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Table 3. Binding Affinity Energy of the Complex Epitopes-HLA Class I/II

code	epitope	allele HLA	ΔG (kcal/mol)	K_d (M) at 25.0 °C
		CTL		
C1	ITDDSDMAY	HLA A 2407	−8.2	9.40×10^{-7}
C2	FTFPNAFPF	HLA A 2407	−9.0	2.60×10^{-7}
C3	VLILLLIRY	HLA A 2407	−9.0	2.50×10^{-7}
C4	KTGIVTITY	HLA A 2407	−9.3	1.50×10^{-7}
		HTL		
H1	MSFIHFLQGAVISFV	HLA DQA10102	−11.8	2.40×10^{-9}
H2	KSYFGMSFIHFLQGA	HLA DQA10102	−10.3	2.70×10^{-8}
H3	YFGMSFIHFLQGAVI	HLA DQA10102	−10.1	4.20×10^{-8}
H4	GAVISFVNSKSHFWL	HLA DQA10102	−13.1	2.30×10^{-10}
H5	AVISFVNSKSHFWLQ	HLA DQA10102	−11.1	6.70×10^{-9}
H6	FLRYQGVEFISFLAL	HLA DQA10102	−10.1	3.80×10^{-8}
H7	LRYQGVEFISFLALK	HLA DQA10102	−11.3	5.60×10^{-9}
H8	KNSLKCLRYRLKKHS	HLA DQA10102	−11.9	2.00×10^{-9}
H9	SLKCLRYRLKKHSDH	HLA DQA10102	−10.5	2.00×10^{-8}

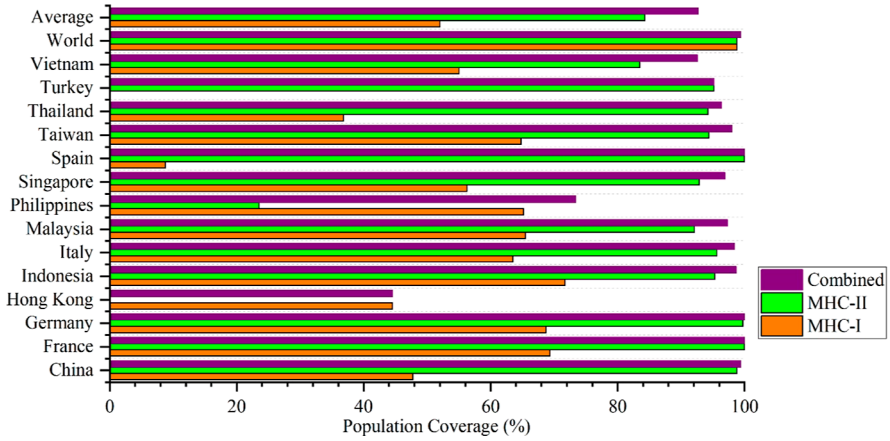

Figure 3. Population coverage of the multiepitope vaccine.

IFN-γ resulted in seven and two epitopes of helper T lymphocyte (HTL) E1 and HTL E2, respectively (Table 2). The results of cross-reactivity prediction indicated that none of the selected epitopes were cross-reactive in humans. Complete epitope prediction results can be found in Tables S1 and S2).

Based on the results of epitope prediction, it was found that E1 and E2 proteins have the potential as antigens to induce the immune system through both CTL and HTL. The cellular immune response plays an important role in eliminating HPV-infected cells. Activation of CTL plays an important role in the recognition of HPV antigens, especially E1 and E2, which are expressed at several stages of viral infection.[24] In addition, CD4+ (HTL) activated by APCs differentiates into Th1, Th2, or Treg cells according to the cytokines produced. The cells also activate B cells, with the secretion of IL-4.[25]

Antigen recognition is mediated by the formation of complexes with the T-cell receptor and the major histocompatibility complex (MHC). Antigen recognition by MHC class I is limited (only 8−11 amino acids). However, MHC class I prefers antigens with a peptide length of 9 amino acids. Meanwhile, MHC class II can recognize antigens with a length of more than 15 amino acids.[26] This correlates with the prediction results of CTL epitopes with a length of 9-mer and HTL epitopes of 15-mer. Another study has also confirmed that the E1 protein has the potential as an antigen. The E1 HPV 18 protein with α-galactosylceramide (α-GalCer) as an adjuvant was tested in vivo in C57BL/6 female C57BL/6 mice. This study reported that the E1-α-GalCer vaccine can generate CD8+ and NK CTL immune responses specific against the E1 protein of HPV in vaccinated mice.[27] HPV vaccines have also been developed using E2. The testing of a modified vaccinia Ankara (MVA) vaccine encoding full length of HPV16 E2 has entered phase III clinical trials with 1176 female and 180 male patients who had intraepithelial lesions. The results confirmed that all patients treated with MVA E2 could produce antibodies against the MVA E2 vaccine and specific cytotoxic responses against cells that transform into papilloma. Viral DNA of HPV was not detected after treatment in 83% of the total treated population. In addition, the MVA E2 vaccine did not cause any noticeable side effects.[20]

2.2. Identification of Indonesian Allele Frequencies. The diversity of HLA alleles affects the vaccine's effectiveness worldwide. Figure 1 shows the frequencies of HLA alleles in Indonesia. The most dominant HLA class I allele in Indonesia was the HLA-A*24:07 allele (39.4%), followed by the HLA-A*11:01 allele (30.1%). Meanwhile, the dominant HLA class II alleles in Indonesia included HLA-DRB1*12:02 (57.7%), HLA-DRB1*15:02 (41.1%), and HLA-DRB1*07:01 (25.4%). The allele diversity data were used in the molecular docking stage.

2.3. Modeling of Epitope Structure and Molecular Docking. The results of the 3D structure prediction of HLA-A*24:07 obtained several models. The structure was used to simulate the interaction of the epitope and HLA A*24:07.

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According to the best epitope prediction results, four CTL epitopes and nine HTL epitopes were obtained to interact with HLA classes I and II. Figure 2 shows the epitopes that were confirmed to interact with HLA class I (HLA A*24:07) and HLA class II (DQA10102_DQB10602, PDB ID: 6DIG). The results of the epitope interaction with HLA showed strong binding free energy and dissociation constant (Table 3). This is confirmed by the binding affinity energy (ΔG), which is very negative, and the dissociation constant (K_d) value is highly negative. The more negative the K_d value, the stronger the complex interaction and the more difficult it will be to release.[28] A strong interaction of epitopes and MHC class I will produce apoptotic signals by releasing cytokines IFNγ and IL-4. Meanwhile, a strong interaction of epitopes and HLA class II will activate NK cells to secrete apoptotic signals against infected cells.[29] Based on the prediction, the epitopes VLILLLIRY and GAVISFVNSKSHFWL were found to highly favor binding with HLA class I or HLA class II receptors.

2.4. Population Coverage of Multiepitope Vaccines. The analysis of population coverage was carried out to establish the percentage of the population that can be protected by the multiepitope vaccine candidate. Figure 3 shows the population coverage of 13 epitopes that can be recognized by HLA class I, HLA class II, and HLA class combined. Based on the prediction results, Indonesian people could respond to the most of all epitopes designed in this research. A total of 71.72% of the population in Indonesia can recognize epitopes as antigens by HLA class I and 95.33% can be recognized by HLA class II. Our epitopes can also generate a good response based on HLA class I in the population either in Indonesia or other countries, such as the Philippines, France, and Germany (65.19, 69.34, and 68.72%, respectively).

2.5. Construction of Multiepitope Vaccines. The prediction of CTL and HTL epitopes obtained four and seven, respectively. The multiepitope vaccine protein sequence was constructed by using the AAY linker for the CTL epitope, while the HTL epitope was linked with the GPGPG linker. The multiepitope vaccine protein has a complete structure consisting of 224 amino acids. The structure includes four CTL epitopes, nine HTL epitopes, and linker (Figure 4). In this study, the AAY linker for CTL epitopes served as a site for cleavage by proteasomes in mammals. In addition, the utilization of AAY linkers has the potential to increase the immunogenicity of a multiepitope vaccine.[30] Another study explained the use of an AAY linker as a proteolytic side marker.[31] The use of a GPGPG linker also served to increase the immunogenicity. Hydrophilic linkers were used in this study to increase the solubility of the vaccine protein. Another research study has shown that the linkers could increase the solubility of vaccine proteins.[32]

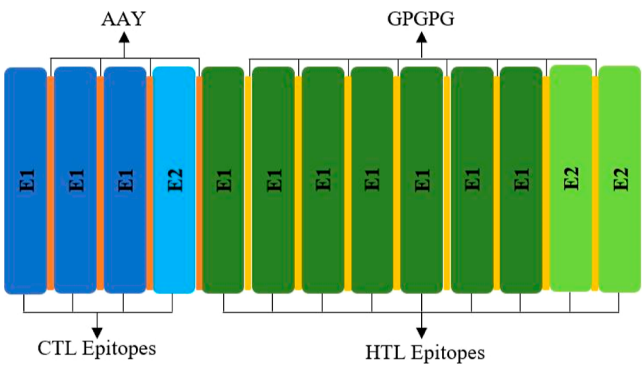

Figure 4. Structural arrangement of the multiepitope vaccine construct.

Currently, vaccine development is performed using an immunoinformatics approach. The development of vaccines by immunoinformatics is faster than the development of conventional vaccines. A multiepitope vaccine has greater advantages than single epitope vaccines and conventional vaccines because it has numerous CTL, HTL, and B-cell epitopes that can activate the robust cellular and humoral immune systems at the same time. Moreover, it contains a variety of epitopes that can broaden the identification of viral antigens or target tumors. Furthermore, to avoid side effects, it is possible to remove redundant components.[23]

2.6. Evaluation and Characterization of Multiepitope Vaccines. Antigenicity prediction was carried out using the VaxiJen v.2.0 server and revealed an antigenicity value of 0.5389, indicating that the multiepitope vaccine was antigenic. The threshold value used in antigenicity prediction was 0.4, with an accuracy rate of 70%.[33] The allergenicity and toxicity of the multiepitope vaccine construct predicted by the AllerTop and ToxIBTL servers confirmed that the vaccine was nonallergenic and nontoxic. Physicochemical properties were predicted with the ProtParam server. The predicted characteristics included molecular weight (MW), stability index, aliphatic index, theoretical isoelectric point (pI), half-life, and grand average of hydropathicity (GRAVY). The MW and theoretical pI were 24938.95 Da and 9.84, respectively. Based on the MW evaluation of the vaccine proteins, proteins with a MW of <110 kDa can easily be purified.[34] The half-life of the vaccine construct was predicted to be 20 h in mammalian reticulocytes, 30 min in yeast, and more than 10 h in *Escherichia coli*. The aliphatic index, stability index, and GRAVY predictive value of the vaccine construct were 80.96, 27.56, and 0.158, respectively. The physicochemical properties of the multiepitope vaccine are given in Table 4. The aliphatic index of a protein/peptide is defined as the relative volume occupied by aliphatic side chains (alanine, valine, isoleucine, and leucine).[35] The high value of the aliphatic index indicates that the protein vaccine has thermal stability. The stability index of the vaccine was calculated to be 27.56, indicating that the vaccine was stable because the stability index was less than

Table 4. Physicochemical Properties of the Vaccine Construct

properties	result
molecular formula	$C_{1145}H_{1695}N_{285}O_{283}S_6$
number of amino acids	224 aa
MW	24173.15 Da
pI	9.84
half-time estimation	30 h (mammalian reticulocytes, in vitro)
	20 h (yeast, in vivo)
	10 h (E. coli, in vivo)
protein instability index (II)	27.57 (stable)
aliphatic index	82.77 (thermostable)
GRAVY	0.25
antigenicity	0.55 (antigenic)
allergenicity	nonallergen
toxicity	nontoxic
solubility	0.14 (poor solubility)

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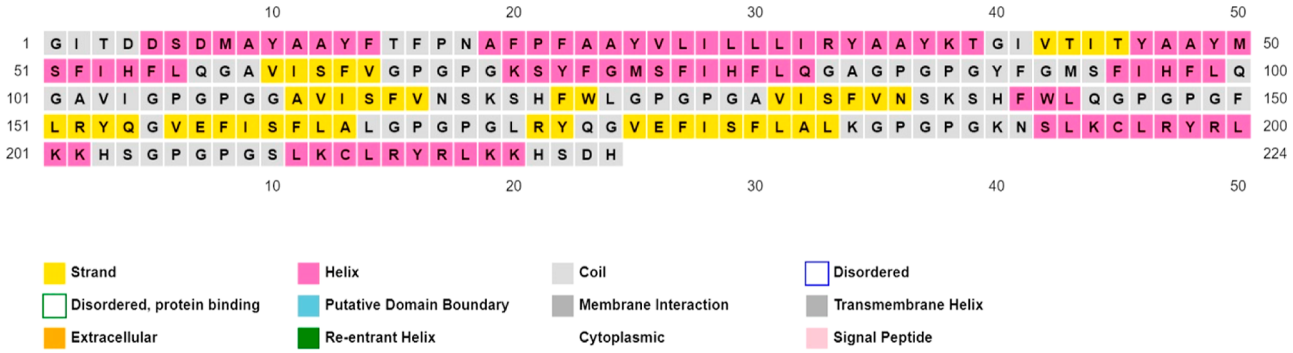

Figure 5. Secondary structure of the final multiepitope vaccine construct predicted using the PSIPRED tool.

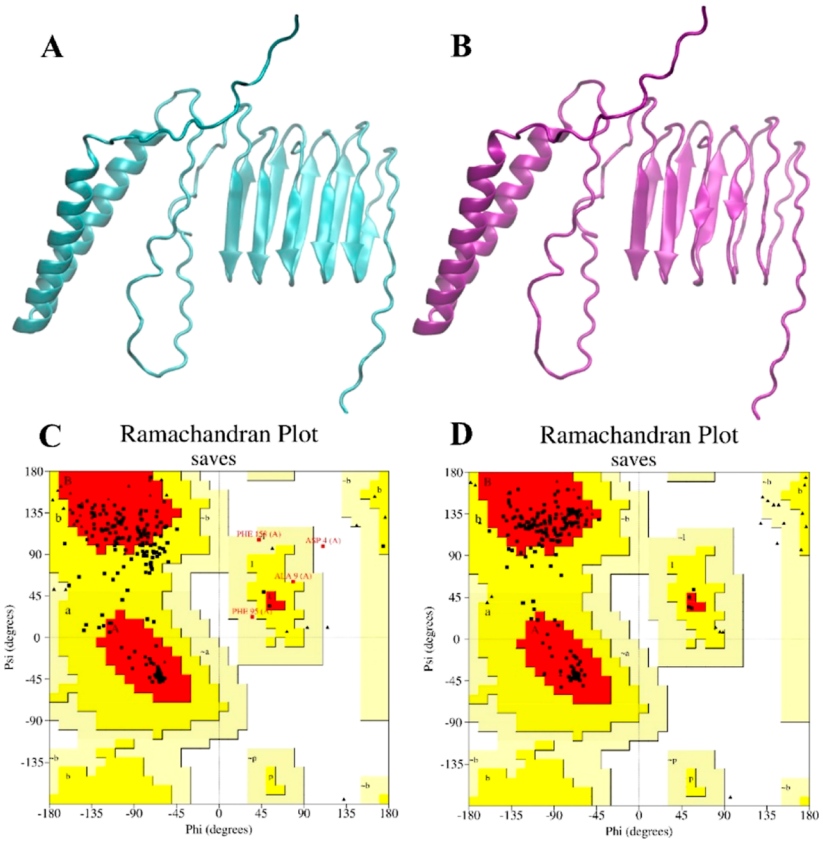

Figure 6. Visualization of the 3D structure of the multiepitope vaccine candidate. (A) 3D structure of the initial structure. (B) 3D structure after refinement. (C,D) Superimposed 3D structure of the initial structure (cyan) and after refinement (purple).

40.[36] Solubility is also a property that should be considered in vaccine candidates. Solubility prediction using the SoluProt server resulted in a solubility value of 0.142. The prediction results indicated that the multiepitope vaccine had poor solubility in *E. coli*. Solubility can be improved by using fusion proteins such as Fh8, glutathione-S-transferase (GST), maltose-binding protein, and other fusion proteins.[37] In another study, the fusion protein was proven to increase the solubility of HIV-1 vaccine proteins.[38]

2.7. Prediction of the Vaccine's Multiepitope Structure. The secondary structure of the multiepitope vaccine predicted using the PSIPRED V4.0 server, i.e., the percentages of α helix, extended strand, and random coil structures were 35.8, 20.96, and 43.23%, respectively (Figure 5). The composition of the secondary structure correlates with the tertiary structure, which has a predominantly coil structure. Tertiary structure prediction using AlphaFold software on the Google Colab server revealed the best 3D structures (Figure 6A). AlphaFold software predicts 3D structures not based on existing protein templates. The prediction results were refined to produce a more stable 3D structure with the GalaxyRefine server (Figure 6B).

Based on the Ramachandran plot generated from the SAVES v6.0 server, the tertiary structure of the initial model was in the favorable region, additionally allowed, generously allowed, and disallowed regions at proportions of 71.9, 25.7, 1.8, and 0.6%, respectively (Figure 6C). After improvement, the number of residues changed sequentially to 90.1, 9.9, 0.0, and 0.0%, respectively (Figure 6D). Ramachandran plot is a form of two-dimensional plot that is created in the space of the angles ϕ and ψ. It is used to characterize types of secondary structure by a single (diffuse) position on the plot.[39] The tertiary structure

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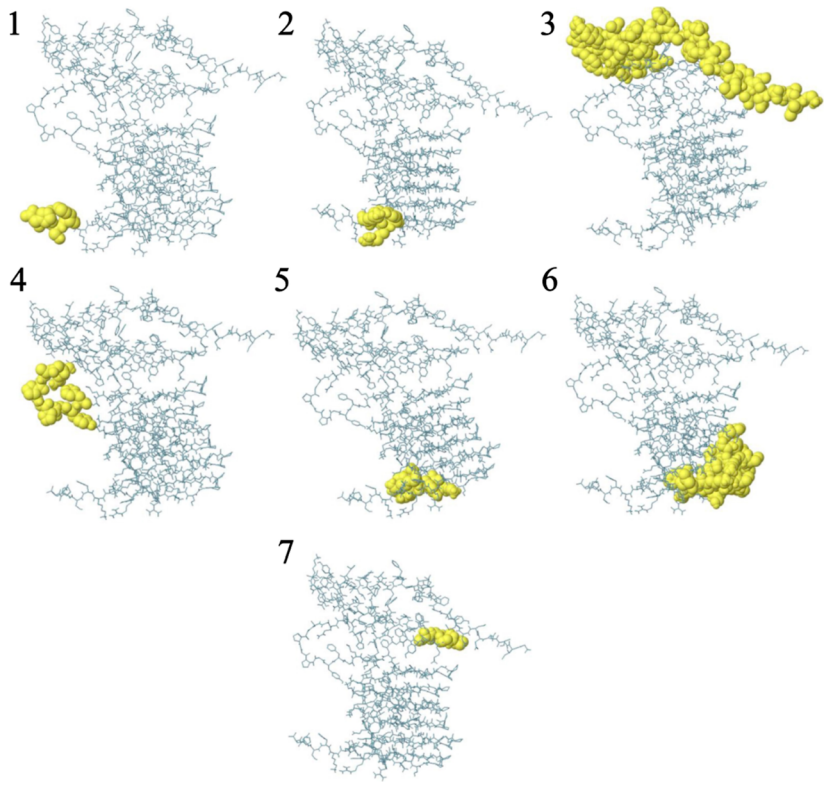

Figure 7. Visualization of the discontinuous B-cell epitopes of the multiepitope vaccine.

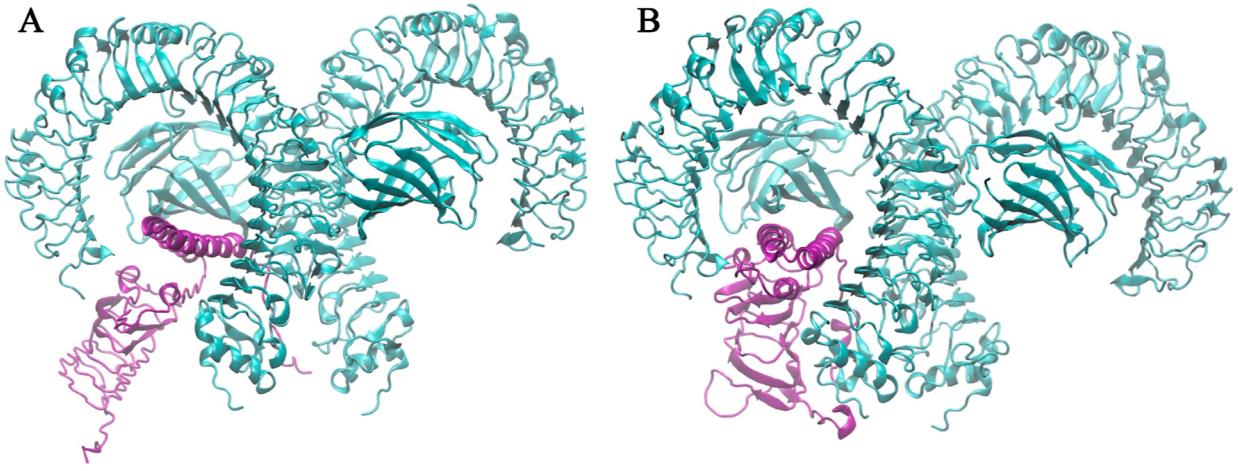

Figure 8. Visualization of the multiepitope vaccine-TLR4 complex obtained via molecular docking (A) and molecular dynamics simulation (B).

of proteins with more than 118 residues can be considered to be of high quality if more than 90% of the residues are in the most favorable region.[40] In addition, the quality of the 3D structure of the multiepitope vaccine protein was also confirmed by the Z-score. The Z-scores of the initial model and the model after improvement were shown to be −1.64 and −2.23, respectively. Consequently, the tertiary structure of the constructed vaccine protein is valid for use in the next MD simulation.

2.8. Prediction of B-Cell Epitopes. The induction of the humoral immune system is important for generating a rapid defense against infections and tumors. B cells are lymphocytes that play an important role in inducing the humoral immune system; therefore, B-cell epitope prediction was needed. The results of prediction of linear B-cell epitopes using BepiPred 2.0 obtained nine of linear B-cell epitopes with the highest score of 1, which indicates that the construction of multiepitope vaccines can be well recognized by B cells (Table S3). The results of the prediction of discontinuous B-cell epitopes using the ELLIPRO server are depicted in Figure 7. There were seven discontinuous B-cell epitopes, with maximum and minimum scores of 0.969 and 0.568, respectively (Table S4). These results corroborate that both CTL and HTL epitopes have recognition regions for B cells. The production of antibodies, presentation of antigens, formation of germinal centers, interaction with other immune cells, and formation of immunological memories are important functions of B cells that contribute in the fight against cancer. However, the immune response to cancer is complex and involves many other cell types and mechanisms in the body. In addition, B

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cells have the ability to prevent the progression of tumors through the production of antibodies that are reactive to tumors, as well as by facilitating the elimination of tumor cells by NK cells, the phagocytosis of tumor cells by macrophages, and the maturation of CD4+ and CD8+ T cells.[41] Wieland et al. showed that E2, E6, and E7 proteins as antigens can be tumor-infiltrating B cells thus used as HPV therapeutic agents.[42] However, further studies are needed to elucidate the role and antigen specificity of tumor-infiltrating cells of the B-cell lineage.

2.9. Interaction and Stability of Vaccine-TLR4 Complex. TLR4 is a transmembrane protein and a member of the PRR group. TLR4 has a role in the pathogen's antigen recognition stage at the APC pairing phase. In this study, the interaction of multiepitope vaccine candidates with TLR4 was analyzed to determine the strength and site of the interaction. Molecular docking of multiepitope vaccine candidates and TLR4 was performed by using the ClusPro 2.0 server. Details of the number of interacting residues, interface area, salt bridges, hydrogen bonds, and nonbonding contacts formed after molecular docking are shown in Table S5. Results of binding free energy prediction conducted with Prodigy servers showed that multiepitope vaccine candidates have strong interaction with TLR4 based on the binding free energy value and dissociation constant of −19.1 kcal/mol and 9.8×10^{-15} M, respectively. This result shows that the abundance of hydrogen bonds and nonbonded contacts contributed to the stability of the multiepitope-TLR4 complex. Figure 8A shows the interaction of the multiepitope vaccine candidate with TLR4 (PDB ID: 4G8A). Visualization of the TLR4-vaccine multiepitope complex interaction is displayed in Figure 9.

Increased HPV infection status is positively correlated with TLR4 gene expression.[43,44] TLR4 is a receptor located on the surface of dendritic cells and macrophages. Activation of APCs through the TLR4 signaling pathway is carried out by recruiting adaptor proteins such as MyD88, which then switches on NF-κB and IL-6. Expression of type 1 IFN plays a crucial part in bridging the gap between innate immunity and adaptive immunity at the signaling pathway's final stage.[45] Therefore, the strong interaction between multiepitope vaccines with TLR4 implies activation of the immune system.

Figure 8B shows the visualization of MD results using the VMD software. The simulation showed that the interaction of the vaccine-TLR4 complex improved, characterized by the increase in hydrogen bond interactions and the formation of the vaccine-TLR4 salt bridge. Table S6 details the number of interacting residues, interface area, salt bridges, hydrogen bonds, and nonbonding contacts formed after molecular dynamics simulation. The binding stability of the vaccine-TLR4 complex was also confirmed by the RMSD data of less than 6 Å and an average RMSF of less than 3 Å during the simulation (Figure 10).

The MMGBSA method was used to calculate the energy difference of the complex of the TLR4-multiepitope vaccine to validate the stability of the complex interaction. The resulting MMGBSA binding free energy was −106.5 kcal/mol. The resulting complex interaction energy was negative, proving that the complex interaction formed was favorable and stable during simulation. Table S7 shows the data on the resulting binding free energy.

2.10. In Silico Cloning. Production of recombinant vaccine proteins is the key stage in the development of multiepitope vaccines. DNA sequence construction of the

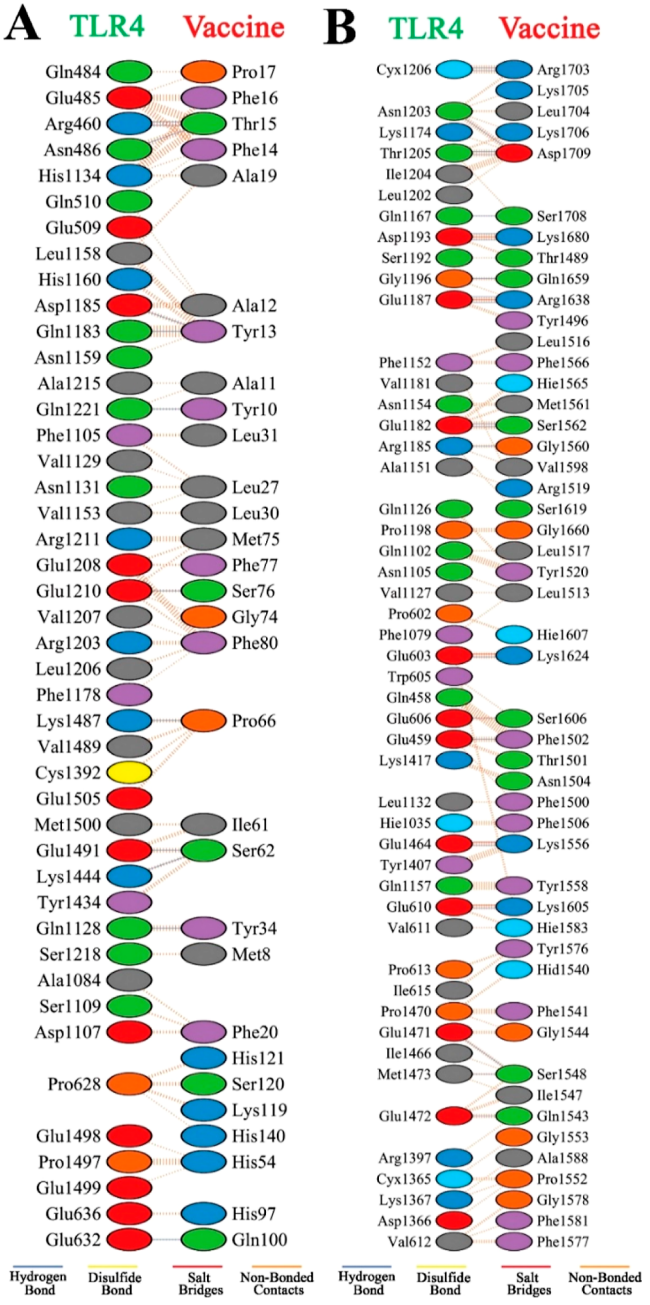

Figure 9. Visualization of the vaccine-TLR4 complex interaction obtained from molecular docking results (A) and molecular dynamics simulation (B).

multiepitope vaccine was reverse translated and calculated using codon adaptation index (CAI) values. Prediction of the CAI using the jCat server was obtained at 0.94. To overexpressed in *E.coli* BL21 (DE3), the favorable CAI and GC content ranged from 0.8 to 1 and 0.35−65%, respectively.[46] The experimental results showed that the % GC was 55.21%, which indicated that the multiepitope vaccine gene could be optimally expressed. For the final analysis, SnapGene software was utilized to carry out the process of in silico cloning of the vaccine design into the pET 30+ vector (Figure 11).

2.11. Simulation of the Immune Response. Predictions of simulated immune responses were generated using the C-IMMSIM server to evaluate the immune response induction of

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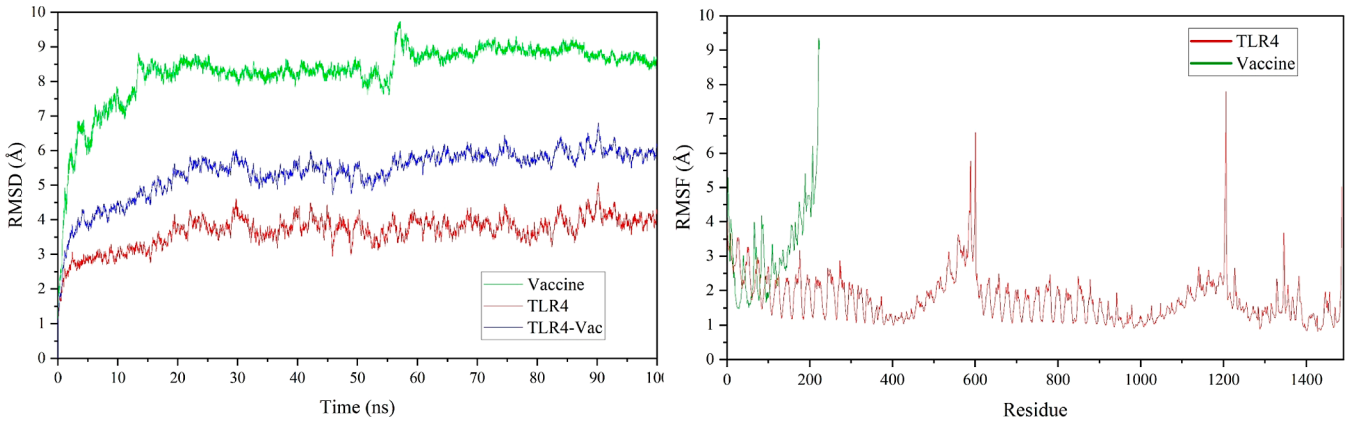

Figure 10. Validation parameter of the multiepitope vaccine-TLR4 complex during simulation. (A) RMSD value. (B) RSMF value.

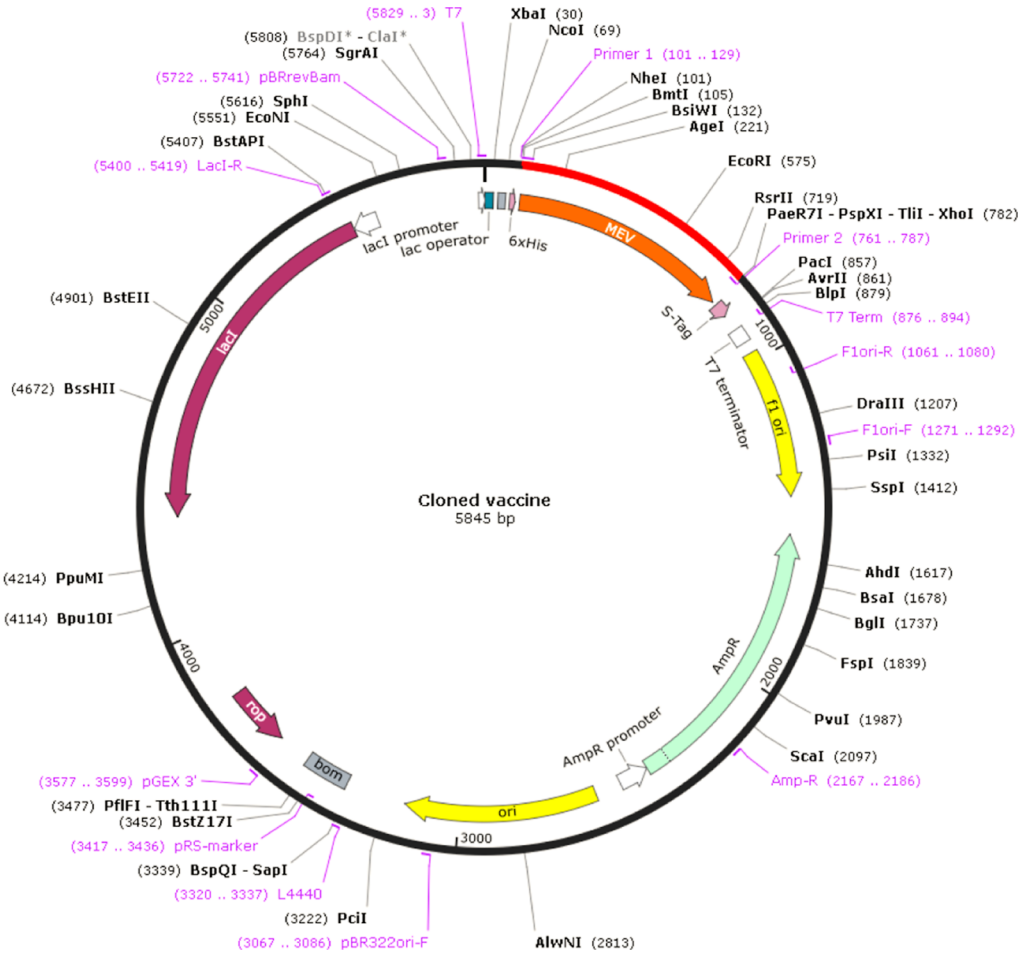

Figure 11. In silico cloning of vaccine sequence into a pET 30+ plasmid vector. The vaccine sequence is represented in red color (101 bp to 787 bp region).

the multiepitope vaccine candidates (Figure 12). The secondary and tertiary responses generated showed a decrease in antigen concentration with a high increase in normal immunoglobulins, namely, IgM, IgM+IgG, and IgG1+IgG2. Generally, the primary immune response is produced by the first exposure to the antigen, which produces IgM. However, the primary response also produces IgG. IgM titers begin to increase on the third day along with IgG. The increase in immunoglobulin is positively correlated with a significant decrease in the antigen. In addition, there were increases in IgM, IgG1+IgG2, IgG1, and IgG2 titers (Figure 12A) that indicated that the antibodies had a good affinity for the multiepitope vaccine. B-cell memory population levels increased to 560 cells/mm^3 after the third dose as seen in Figure 12B. In addition, Figure 12C shows an increase in cytotoxic T-cell activation of 1250 cells/mm^3. Our results suggest that multiepitope vaccines can activate the adaptive immune system, especially cytotoxic T cells, and can function as therapeutic vaccines.

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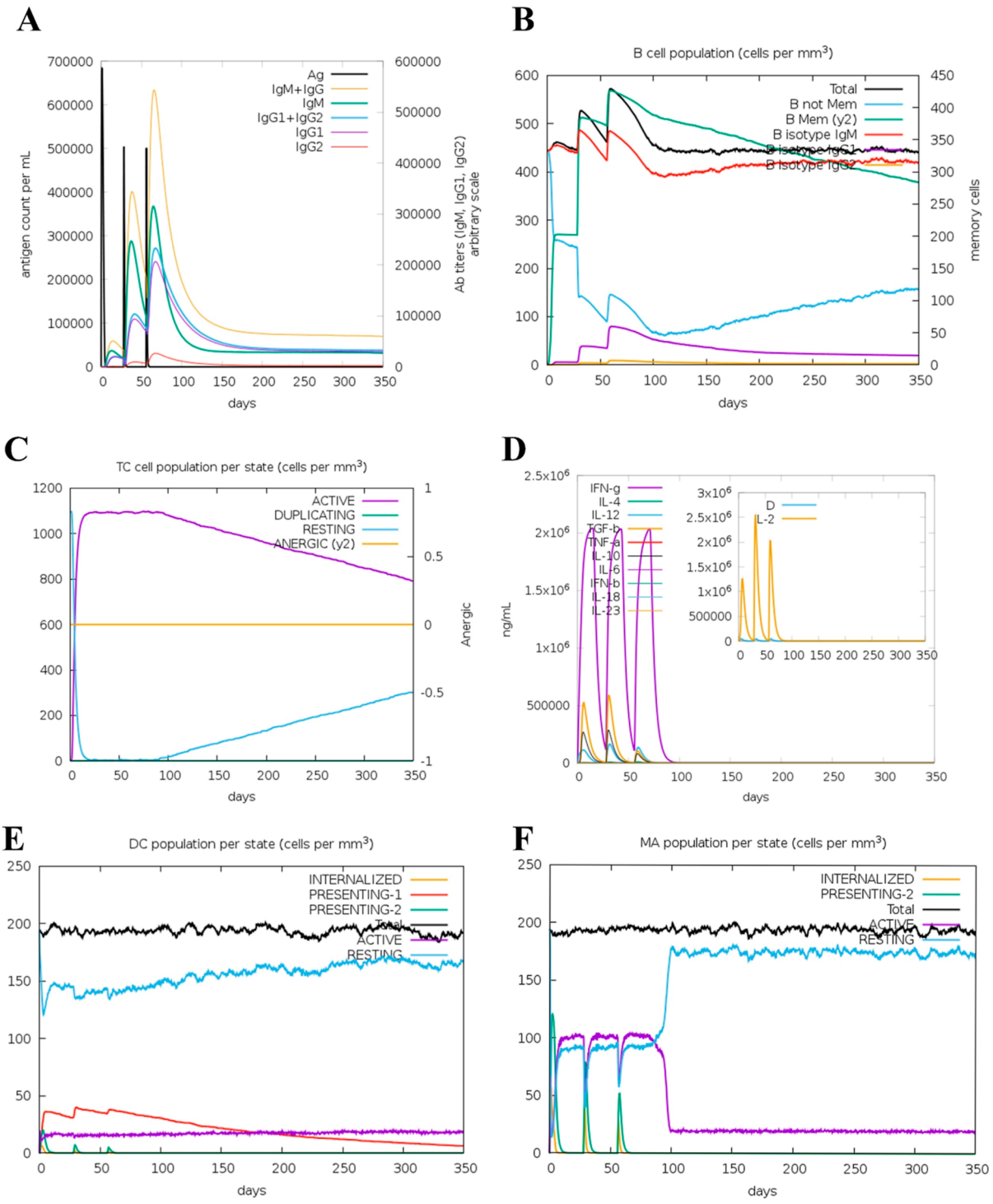

Figure 12. Simulation of immune response of multiepitope vaccine: (A) antigen and immunoglobulins, (B) B-cell population, (C) T-cell population per state, (D) production of cytokine and interleukins, (E) dendritic cell population per state, and (F) macrophage population per state.

In addition, each dose of the multiepitope vaccine could significantly increase the secretion of IFN-γ at each dose by 2 $\times$ 10^6 ng/mL (Figure 12D). The concentration of IFN-γ is higher than those of other cytokines that play an important role in the maturation of APCs. Furthermore, INF- γ is involved in the transformation of T cells into the Th1 subset of the immune system. In addition, IFN-γ stimulates naïve T cells to become effector T cells and inhibits Treg cells.[47] The prediction results also showed an IL-2 secretion of 1.2 $\times$ 10^6 ng/mL with the first dose. The cytokine IL-2 functions as a growth factor for T cells. Thus, the presence of IL-2 indicates that the multiepitope vaccine can activate CTL and suppress Treg cell expression.[48] Meanwhile, after each dose was injected, the Th1 concentration increased. In addition, higher densities of dendritic cells and macrophages indicated that APCs efficiently processed and delivered antigens to CD4+ and CD8+ cells (Figure 12E,F).

The reverse vaccinology is a promising method for vaccine development. Previous research have resulted in the development of vaccines through the process of reverse vaccinology,

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which involves the production of vaccine against Jamestown canyon virus,[49] hepatitis C virus,[50] monkeypox virus,[51] chikungunya virus,[52] Burkholderia pseudomallei,[53] and lumpy skin disease.[54] The immunoinformatics prediction results have also been tested for validity both in vivo and in vitro. The recent research conducted by Namvar et al. has also developed a prophylactic vaccine L1 and L2 using an immunoinformatics approach and validated it in vivo.[31,55] Thus, the reverse vaccinology approach can be used for vaccine development to reduce the time needed to obtain potential vaccine candidates.

3. CONCLUSIONS

The incidence of cervical cancer caused by HPV is currently increasing. However, prophylactic vaccines cannot be used as treatments for cervical cancer. Therefore, therapeutic vaccines are the most effective alternative treatment options for HPV infection. Reverse vaccinology methods can be used to develop immunogenic, safe, and stable vaccines more rapidly. The E1 and E2 proteins of HPV 16, 18, 45, and 52 were targeted for the development of a therapeutic vaccine. The final vaccine construct had four CTL epitopes and nine HTL epitopes, each of which was chosen for its antigenicity, allergenicity, and toxicity after being evaluated. After its construction had been completed with the use of AAY and GPGPG linkers, the vaccine's physicochemical characteristics were analyzed so that improvements could be made. Evaluation of multiepitope vaccine construction showed that the vaccine could cover almost the entire Indonesian population and had potential as a cervical cancer vaccine candidate. In addition, the multiepitope vaccine showed that the multiepitope vaccine has a strong interaction with TLR4, with a binding free energy value of −143.69 kcal/mol. The presence of five salt bridges and the addition of 10 hydrogen bonds confirm the strength of the interaction. Based on the binding interaction, it is concluded that the strength of the interaction is positively correlated with the predicted results and that it can activate a robust immune system. Our research can support further research to produce vaccines and evaluate the effectiveness of vaccine tests in vitro and in vivo.

4. MATERIALS AND METHODS

4.1. Consensus Sequence Construction. The target proteins used in this study were the E1 and E2 proteins of HPV types 16, 18, 45, and 52. The protein sequences were retrieved from the UniProt database (https://www.uniprot.org/). The following reference sequences were used: HPV 16/E1 (P03114), HPV 18/E1 (P06789), HPV 45/E1 (P36728), HPV 52/E1 (P36730), HPV 16/E2 (P03120), HPV 18/E2 (P067890), HPV 45/E2 (P36794), and HPV 52/E2 (P36796). All sample sequences were aligned with the protein sequences using the MAFFT web server.[56,57] The construction of aligned consensus sequences was done using the EMBOSS web server.[58]

4.2. Prediction, Evaluation, and Selection of T-Cell Epitopes. Epitopes of CTL were predicted from the consensus sequence using the server NET CTL 1.2.[59] The predicted CTL epitopes were 9-mers restricted to 12 MHC class I supertypes (A1, A2, A3, A24, A26, B7, B8, B27, B39, B44, B58, and B62), and the default threshold value was set to 0.75. Epitope prediction was performed based on proteasomal TAP transport binding efficiency, *C*-terminal cleavage, and binding affinity for MHC class I. Meanwhile, HTL epitope prediction was performed using the NetMHCII 2.3 server.[60] HTL epitopes with a 15-mer amino acid length were predicted against HLA-DR, HLA-DQ, and HLA-DP. The prediction used strong and weak HLA binding threshold values of 2 and 10%, respectively.

The allergenicity, antigenicity, and toxicity of the predicted CTL and HTL epitopes were investigated further. Prediction of antigenicity was performed using the server VaxiJen v2.0 with a threshold of 0.4, and viruses were chosen as the target organisms. The server can predict bacterial, viral, and tumor antigens with 70 to 89% accuracy.[33] Prediction of allergenicity was also performed for the epitopes using AllerTop.[61] This server classifies epitopes as having "possible allergen" and "possible non-allergen" status. The toxicity of the epitopes was then predicted using the ToxinPred server. The server employs the SVM (Swiss-Prot) algorithm.[62] The result of peptide prediction also predicted the cross-reactivity using the Protein Information Resource server.[63]

The variable ability of HTL epitopes to induce cytokines in the immune system requires the prediction of epitopes that can induce cytokines effectively. The prediction of epitopes that can induce IL-4 and IFN was performed by using the IL4Pred server and IFN epitope. For the prediction of HTL epitopes that can induce IL-4, an SVM-based method was used with a threshold of 0.2,[64] whereas, for the prediction of HTL epitopes that can induce IFN, SVM-based methods and models of IFN versus other cytokines were used,[65]

4.3. Identification of Indonesian Allele Frequencies. The HLA allele frequency distribution of the population in Indonesia was obtained from The Allele Frequency Net Database server.[66] In general, the HLA allele data in Indonesia are dominated by the West Java population as well as the Sundanese and Javanese populations.

4.4. Prediction of Epitope Tertiary Structure and Molecular Docking. PepFold 3.5 server was used to predict epitope tertiary structure.[67−69] Peptide prediction was performed by running 100 conformational change simulations. The best tertiary structure was used based on the highest sOPEP value. Peptides with the highest value were subjected to molecular docking analysis with HLA class I and II using the Haddock 2.4 server.[70,71] The software VMD was used to provide a visualization of molecular docking. Molecular docking results were analyzed using the PDBSum Generate server.[72] HLA-A*24.07 was modeled using the iterative threading assembly refinement server using the HLA-A*24.02 template.[73−75] HLA-A*24.07 has only one residue difference with HLA-A*24.02, which is residue number 70. In HLA-A*24.02 residue number 70 is histidine, while in HLA-A*24.07 it is glutamine. The PDB structure with the highest *C*-score was used for the molecular docking analysis.

4.5. Population Coverage Analysis of Epitopes. The population coverage of the multiepitope vaccine candidates was evaluated using the IEDB server.[76] The server predicted population coverage of T-cell epitope-based vaccines or diagnostics based on MHC binding and/or T-cell restriction data.[76] The analysis was conducted to determine the epitope coverage of CTLs and HTLs that would cover the population in Indonesia based on epitope recognition by HLA.

4.6. Construction of Multiepitope Vaccines. Epitopes that were predicted and qualified as vaccine candidates were constructed as multiepitope vaccines. Multiepitope vaccine construction was performed by combining the predicted epitopes with linkers between epitopes. The AAY linker was

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used to link CTL epitopes, and the GPGPG linker was used to link HTL epitopes.[77] The use of linkers is intended to improve the epitope representation and prevent epitope junctions. In addition, the use of glycine-rich epitopes increases the vaccine solubility.

4.7. Evaluation of the Physicochemical Properties of Multiepitope Vaccines. The multiepitope vaccines were evaluated for their allergenicity, antigenicity, toxicity, and physicochemical properties. The VaxiJen v2.0 server was used to predict antigenicity of the multiepitope vaccines.[33] Meanwhile allergenicity and peptide toxicity were predicted by AllerTop v.2.0 and ToxinPred servers.[61,62] In this study, physicochemical properties were also predicted using the Expasy ProtParam server.[78] The vaccine's solubility in water was predicted using the SoluProt server to prevent protein aggregation.[79]

4.8. Prediction of the Vaccine's Multiepitope Structure. Multiepitope vaccine secondary structure was predicted using the PSIPRED.[80,81] PSIPRED was used to determine the percentage of the secondary structure of the vaccine protein construct with an accuracy of 84.2%. Tertiary structure prediction was performed on the multiepitope vaccines using AlphaFold 2.3 software.[82] AlphaFold software was used on the Google Colab server. The server predicted protein 3D structure using AlphaFold V2.3, which is not based on the existing template.[83] The best prediction model obtained was refined into a 3D structure using the GalaxyRefine server to improve the quality of the 3D structure.[84,85] Structure validation was performed using the ProSa web server and the SAVES v6.0 server.[86−88] The Z-score of the constructed vaccine was calculated using the ProSa server, which provides information on protein quality based on folding. Meanwhile, the SAVES version 6.0 server (PROCHEK tool) evaluated the 3D structure of the protein based on stereochemical quality by examining residue geometry and overall structural geometry.

4.9. Prediction of B-Cell Epitopes. Linear B-cell prediction was performed using the BepiPred 2.0 server.[89] BepiPred-2.0 predicts B-cell epitopes with a random forest algorithm tested on epitopes and nonepitopes of antibody−antigen protein structures. Meanwhile, discontinuous B-cell prediction was performed using the ElliPro server.[90] Compared with other servers, the ElliPro server has better accuracy to predict discontinuous epitopes by modifying Thornton's method and the residue clustering algorithm.

4.10. Interaction and Stability of the Vaccine-TLR4 Complex. Molecular docking is a computer tool that is used to examine complex interactions that occur between ligands and receptors. The tertiary structure of the improved multiepitope vaccine, which was utilized as the ligand, was examined for its ability to interact with the TLR4/MD2 receptor (PDB ID: 4G8A), which was obtained from the PDB database (rcsb.org). The ClusPro 2.0 server was used to perform the protein−protein molecular interaction.[91,92] Critical assessment of predicted interactions has determined that ClusPro 2.0 is the most effective protein−protein docking service since 2004.[93,94] The model with the lowest binding energy was selected as the best model. The best model selected was subjected to binding affinity energy prediction using the PRODIGY server.[95] The PDBSum Generate server was used to visualize the binding interaction between the multiepitope vaccine and TLR4.[72]

Molecular dynamics simulations were performed to investigate the multiepitope vaccine-TLR4 complex stability using AMBER 20 software.[96,97] The ff19SB force field was used to set the multiepitope vaccine parameters with TLR4.[98,99] Model TIP3P water was added to the octahedron system and also was neutralized by the addition of 9 Na^+ ions and 63,869 water residues. The cutoff distance for the calculation used was 10 Å. The simulation began by minimizing the system's energy consumption. The *NVT* ensemble was utilized for 150 ps to raise the temperature from 0 to 300 K. The temperature of the system was kept at 300 K, while the pressure was kept at 1 atm by utilizing a Langevin thermostat in conjunction with an isotropic position scaling method. The construction of the molecular dynamics simulation was performed for a total of 100 ns. Following the completion of the molecular dynamics simulation, the root-mean-square deviation (RMSD) and root-mean-square fluctuation (RMSF) parameters were examined with the use of the CPPTRAJ AMBER 20 tool.[100] The system's binding free energy was determined using the MMPBSA.py module of AMBER 20 software.[101] This calculation was performed to analyze the difference in binding free energy between the two states (solvate phase and gas phase) of the vaccine-TLR4 multiepitope complex using the equation below

$$\Delta G_{\text{binding free energy}} = \Delta G_{\text{bind,vacuum}} + \Delta G_{\text{solv,complex}} - (\Delta G_{\text{solv,ligand}} + \Delta G_{\text{solv,receptor}})$$

4.11. In Silico Cloning. The production of recombinant vaccine proteins was performed by cloning the vaccine construction gene into a plasmid. The final protein sequence of vaccine construction was reverse translated using the EMBOSS backtranseq server to obtain the DNA sequences. The DNA sequences obtained were optimized using the jCat server and selected *E. coli* (K12 strain) as the host that will express the vaccine protein. The server was used to calculate the GC content and CAI values. This serves to characterize the expression of the multiepitope vaccine protein. To the DNA sequence of the multiepitope vaccine, restriction enzymes NheI at the 5′ end and XhoI at the 3′ end were added. The DNA sequence of the multiepitope vaccine was cloned into the pET 30+ vector using SnapGene software.

4.12. Simulation of Immune Responses. The *C*-ImmSim server was used to simulate the immune responses induced by the multiepitope vaccine candidates.[102] Immune response simulations were conducted against the most common HLA types in the Indonesian population, namely, HLA A*11:02, HLA A*24:07, HLA B*15:02, HLA B*15:13, HLA DRB1 12:02, and HLA DRB1 15:02. Simulations were conducted with three injections at four week intervals (days 0, 28, and 56) correlated with parameters 1, 8, and 168 steps in the simulation server. The total amount of vaccine administered was 1000 units of vaccine.[75]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c00425.

Predicted CTL and HTL epitopes for E1 and E2 HPV proteins selected based on antigenicity, toxicity, allergenicity, IL4 induction and IFNγ induction; predicted linear B-cell and discontinuous B-cell epitopes from multiepitope vaccine protein constructs; interaction analysis of multiepitope vaccine with TLR4 from

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molecular docking and molecular dynamics simulations; and MMGBSA-based binding free energies of the multiepitope vaccine-TLR4 complex (PDF)

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Author Contributions

R.: conception, performing experiments, methodology, and writing the original draft. **R.A.:** validation, result validation, and editing the manuscript. **E.A.G.-R.:** investigation, review, and editing the manuscript. **R.H.:** conception, supervision, performing experiments, and editing the manuscript. All authors have read and agreed to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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