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Immunoinformatics Approach for Designing an HPV Epitope-Based Vaccine Candidate Harboring Built-in Adjuvants

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ABSTRACT

Introduction: Cervical cancer is one of main causes of cancer death in women, especially in developing countries. Therefore, a low-cost broad-spectrum preventive vaccine is immediately needed. The RG-1 epitope of L2 protein is a major cross-neutralizing epitope but has low immunogenicity. This defect can be overcome by using built-in adjuvants such as TLR agonists and bacterial toxoid epitopes. To address this issue, we designed an epitope-based vaccine against HPV16 using immunoinformatic tools. **Methods:** The HPV16 RG-1 epitope was linked to built-in adjuvants including the D1 domain of flagellin and RS09 epitope as TLR agonists, and a tetanus toxoid epitope for induction of immune responses. Using immunoinformatic tools, the immunological characteristics of the construct were evaluated. In the first step, MHC-I and II binding, CD4$^+$ T cell immunogenicity prediction, and in the second step, immunogenicity simulation of the construct were investigated. **Results:** MHC-I and II predicted epitopes showed a high potentiality to bind to mice and human MHC alleles. The results of the binding of the RG-1 epitope to MHC-I and MHC-II showed that RG-1 could induce humoral and cellular immune response while fused to three built-in adjuvants. Also, the CD4$^+$ immunogenicity assessment results predicted that several epitopes in the designed construct, including epitopes of D1 domain and tetanus toxoid P2 epitope, behaved as potentially strong Th inducers. The immunogenicity simulation results showed that the construct could potentially provide sufficient antigen, and induce suitable humoral and cellular immune responses. **Conclusion:** The development of new vaccine strategies has been the focus of several studies. The results showed that the designed construct can potentially provide an effective model for developing a preventive vaccine candidate against a variety of HPV genotypes.

INTRODUCTION

Currently, human papillomavirus (HPV) infections are the most common sexually transmitted infections in the world. The main diseases of HPV include cervical intraepithelial neoplasia (CIN), condyloma, and also anogenital and oropharyngeal cancers. These diseases are caused by different types of HPV viruses [1]. The current HPV vaccines are Cervarix® and Gardasil® that are based on L1 virus like particle (VLP), and induce HPV type-specific protective immune response. Another limitation of these vaccines is the costly production in eukaryotic hosts [2].

The N-terminal region of the L2 capsid protein contains conserved protective epitopes capable of inducing neutralizing antibodies [3]. The RG-1 epitope of L2 protein (17-36 amino acids; aa) is the candidate immunogen for inducing cross-neutralizing antibodies [4]. The neutralizing antibodies against L2 protein are significantly less protective than L1 VLP, and different strategies have been employed to potentiate the immunogenicity of L2 protein [5]. Among various strategies, built-in adjuvants have gained considerable attention due to several advantages [6, 7], and one group of the promising built-in adjuvants is the Toll-like receptor (TLR) agonists that can potentially induce strong immune responses. TLRs are a type of pattern recognition receptors (PRRs) that, by activating pathogen-associated molecular pattern (PAMP), induce innate and adaptive immune responses [8]. Of this group, the bacterial flagellin from the Salmonella enterica plays role as a TLR5 ligand [9], and RS09 is a synthetic short TLR4 ligand peptide [10] that activates innate and adaptive immunity by NF-kB (Nuclear Factor kappa B) signaling pathway. Other studies have shown that both lipidation, as TLR3 agonist, and Fcγ-receptor 1 targeting help improve the immunity of RG-1 epitopes, and produce potent neutralizing antibodies against mucosal and cutaneous HPV types [7]. TLR4 agonists as adjuvants are being used in several commercial vaccines such as Fendrix® and Cervarix® [11]. Alongside the TLR agonists, adjuvants that can stimulate T helper (Th) cells are important in the development of high-affinity matured antibodies and long-lived memory B cells [6]. Accordingly, several studies have shown that the bacterial toxoids, in whole or in part, can act as universal Th epitopes in the form of built-in adjuvants [12]. Therefore, built-in adjuvants have been used effectively in numerous vaccine studies [6].

Nowadays, bioinformatics tools have greatly helped to make predictions in various biological fields. Immunoinformatics servers are especially valuable in vaccine development for the identification of potential B and T cell epitopes and analyzing different aspects of immunogenicity potentiality [13]. In this study, we aimed to design a prophylactic vaccine by genetically fusing different fragments containing repeats of HPV16 RG-1 epitope and built-in adjuvants, including the D1 domain of Salmonella flagellin, RS09 epitope, and P2 universal T helper epitope of tetanus toxoid (TT-P2), to induce antibodies against HPV. Prediction of binding of construct peptides to MHC I and MHC II (Major Histocompatibility Complex), and immune response simulation were carried out to achieve the best positioning of the fragments to induce potent immune response.

MATERIALS AND METHODS

Peptide Sequence Retrieval

The sequences of HPV16 RG-1 (Accession No. P03107) and the tetanus toxoid P2 epitope (Accession No. P04958.2) were obtained from the National Center for Biotechnology Information (NCBI) database. RS09 as a synthetic short peptide was also included [10]. The D1 domain of Salmonella enterica serovar Dublin flagellin was obtained from KEGG database (http://www.genome.jp) (Accession No. KEGG DRUG: D10368). The abovementioned segments were joined by linkers to make a construct containing antigenic epitopes along with built-in adjuvants.

Disulfide Connectivity Prediction

For predicting the disulfide bond and Cys-Cys interactions that play an important role in folding stabilization [14], the DiANNA server (http://clavius.bc.edu/~clotelab/DiANNA/) was employed.

CTL Epitope Prediction

The H-2Dd, H-2Kd, and H-2Ld mouse MHC class I alleles and human HLA-A and HLA-B alleles were selected for predicting peptide binding to MHC-I using the IEDB (http://tools.iedb.org/mhci/) [15] and Rankpep (http://imed.med.ucm.es/Tools/rankpep.html) [16] servers. Epitope lengths were set as 9-mer separately for mice and humans.

T helper Cell Epitope Prediction

The IEDB (http://tools.iedb.org/mhcii/) [17, 18] and Rankpep (http://imed.med.ucm.es/Tools/rankpep.html) [19] servers were used for predicting binding to mice MHC-II molecules covering mouse H-2IAb, H-2IAd, H-2IEd, H-2IEk, and H-2IEs, and human HLA-DR and HLA-DQ alleles. The IEDB resource was used to predict MHC-II binding epitopes through 15-mer epitope length and the IEDB-recommended method.

The $CD4^+$ T-cell immunogenicity server at http://www.iedb.org/CD4episcore/ was also applied to predict the allele-independent $CD4^+$ T-cell immunogenicity at the population level. The user can predict the T cell immunogenicity using the seven-allele method [20].

Prediction of RG-1 Antigenicity

RG-1 antigenicity prediction was performed by VaxiJen v2.0 (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) server [21]. This server performs the epitope antigen prediction based on the physicochemical property of the protein without using sequence alignment. Epitopes with antigenic score ≥ 0.4 were considered antigenic.

Prediction of Population Coverage

IEDB server population coverage (http://tools.iedb.org/population) was used to analyze the binding of recombinant protein peptides to MHCI and MHCII in population coverage against the total world population and the population of Iran [22].

Immunogenicity Simulation

The immunogenic behavior of the construct was simulated using the C-ImmSim server at http://150.146.2.1/C-IMMSIM/index.php. The C-ImmSim is an agent-based computer model that shows various cells representing innate and adaptive immunity. By following the set of rules obtained at the experimental level, interactions with the vaccine candidate can simulate behaviors that may indicate the possible production of immune memory. This is achieved by combining the immune system using three chambers: the bone marrow, the thymus, and the lymphatic organs. By the TOVA approach for the fusion protein of the prophylactic vaccine, three injections were given at the interval of two weeks [23], so the simulation was adjusted to three injections at days 0, 28, and 35.

RESULTS

Retrieval of the Peptide Sequence of the Fusion Construct

Dual 3x repeats of the selected HPV16 RG-1 epitope along with the two different TLR built-in agonists including part of flagellin and RS09 were employed in the construct. From the flagellin protein of Salmonella enterica serovar Dublin that is recognized by TLR5, the D1 domain was selected, in addition to the RS09 synthetic short peptide as a TLR4 agonist. Also, the tetanus toxoid epitope P2 as the universal T helper epitope was included. The six different parts in the vaccine construct were fused with linkers for better antigen and adjuvant presentation and availability.

Disulfide Connectivity Analysis

Three disulfide bonds at RG-1 epitopes were predicted with the DiANNA tool, which implies to the folding stabilization of the construct (Table 1).

Table 1. DiANNA-predicted disulfide bonds at RG-1 epitopes.

No.	Cysteine sequence	Distance	Bond	Score
1	28-34	6	QLYKTCKQAGT-KQAGTCPPDII	0.87253
2	48-245	197	QLYKTCKQAGT-KQAGTCPPDII	0.72505
3	54-259	205	KQAGTCPPDII-QLYKTCKQAGT	0.84666

CTL Epitope Analysis

The 9-mer epitopes interaction with MHC-I in the context of the fusion protein was analyzed using IEDB and Rankpep severs to predict the potential epitopes of the construct. The results showed that epitopes derived from RG-1 were able to bind to MHC-I with high antigenicity score, as obtained through analysis by VaxiJen v2.0. The RG-1 epitopes with the antigenic property that were specific for murine and human MHC-I and MHC-II alleles, are shown in Tables 2 to 5. In the fusion protein, 23 peptides in mice (Table 2) and 71 peptides in human (Table 3) were predicted to interact with MHC-1 molecules. As seen in Tables 2 and 3, prediction of mice and human 9-mer peptides interaction with MHC I using IEDB server based on percentile rank, and also using Rankpep server were sorted by type of allele. RG-1 peptides with antigenicity score are shown, and antigenicity score ≥ 0.4 are bold. Position of peptide are as RG-1 (5-64 aa and 236-295 aa), D1 flagellin (82-121 aa and 313-372 aa), tetanus toxoid-P2 (393-407 aa), RS09 (423-429 aa), and flexible linker (65-81 aa, 122-235 aa, 296-312 aa, 373-392 aa and 408-422 aa).

Table 2. Murine MHC-I binding epitope prediction.

Number	Peptide	Position	Length	Allele	Percentile rank	Binding threshold	Antigenicity*
1	RFDSAITNL	351-359	9	H-2-Kd	0.07	≤1	
2	QYIKANSKF	391-399			0.1		
3	RFDSAITNL	351-359		H-2-Dd	0.37		
4	KANSKFIGI	394-402			0.37		
5	GFNVNSPGI	196-204		H-2-Kd	0.38		
6	VGANDGETI	172-180		H-2-Dd	0.42		
7	IQDEIQQRL	136-144			0.51		
8	SGGGGSTLI	305-313			0.54		
9	STANPLASI	323-331					
10	TQFNGVKVL	154-162		H-2-Ld	0.57		
11	SKFIGITEL	397-405			0.66		
12	KVDAVRSSL	337-345			0.67		
13	GGSAPPHAL	418-426		H-2-Dd	0.68		
14	KVDAVRSSL	337-345		H-2-Kd	0.77		
15	CPPDIIPKV	285-293		H-2-Ld			**0.8370**
16		265-273					
17		245-253					
18		54-62					
19		34-42					
20		14-22					
21	ALNEINNNL	105-113		H-2-Kd	0.78		
22	NNLQRVREL	111-119		H-2-Ld	0.87		
23	TQFNGVKVL	154-162		H-2-Kd	0.92		

*Bold value is acceptable

Table 3. Human MHC-I binding epitope prediction.

Number	Peptide	Position	Length	Allele	Percentile rank	Binding threshold	Antigenicity*
1	ASIDSALSK	329-337		HLA-A*11:01	0.01		
2	QYIKANSKF	391-399		HLA-A*24:02	0.02		
3	ALNEINNNL	105-113		HLA-A*02:01	0.03		
4	CPPDIIPKV	285-293		HLA-B*51:01			**0.8370**
5		265-273					
6		245-253					
7		54-62					
8		34-42					
9		14-22					
10	ASIDSALSK	329-337		HLA-A*03:01	0.04		
11		269-277		HLA-A*11:01	0.05		
12		249-257					

13	IIPKVQLYK	38-46	9			≤1	0.0392
14		18-26					
15		269-277		HLA-A*03:01	0.07		
16		249-257					
17		38-46					
18		18-26					
19	GSDDDDKQY	384-392		HLA-A*01:01	0.08		
20	ALNEINNNL	105-113		HLA-A*02:06			
21	KQYIKANSK	390-398		HLA-A*03:01	0.09		
22	IQDEIQQRL	136-144		HLA-A*02:06	0.11		
23	GGSEFQLYK	229-237		HLA-A*11:01	0.13		-0.0848
24	DIIPKVQLY	268-275		HLA-B*35:01	0.15		**0.8068**
25		248-256					
26		37-45					
27		17-25					
28	SLGLDGFNV	191-199		HLA-A*02:01	0.16		
29	SIDSALSKV	330-338		HLA-A*02:06	0.2		
30	SSLGAIQNR	343-351		HLA-A*11:01	0.23		
31	SLGLDGFNV	191-199		HLA-A*02:06	0.24		
32	RFDSAITNL	351-369		HLA-A*24:02	0.25		
33	GTNSDSDLK	126-134		HLA-A*11:01			
34	STANPLASI	323-331		HLA-A*02:06			
35	CPPDIIPKV	285-293		HLA-B*35:03	0.29		**0.8370**
36		265-273					
37		245-253					
38		54-62					
39		34-42					
40		14-22					
41	KVQLYKTCK	272-280		HLA-A*03:01	0.35		-0.1226
42		252-260					
43		41-49					
44		21-29					
45	IQDEIQQRL	136-144		HLA-A*02:01	0.39		
46	SIDSALSKV	330-338					
47	ETITIDLQK	178-186		HLA-A*11:01	0.43		
48	SALSKVDAV	333-341		HLA-B*51:01			
49	GGSEFQLYK	229-237		HLA-A*03:01	0.44		
50	SALSKVDAV	333-341		HLA-A*02:06			
51	KQYIKANSK	390-398		HLA-A*11:01	0.45		
52	KVQLYKTCK	272-280			0.49		-0.1226
53		252-260					
54		41-49					
55		21-29					
56	SLGAIQNRF	344-352		HLA-	0.57		

				A*24:02			
57	TLINEDAAA	311-319		HLA-A*02:06	0.58		
58	NLGNTVTNL	458-366		HLA-A*02:01	0.62		
59	TLINEDAAA	311-319			0.63		
60	VLSQDNQMK	161-169		HLA-A*03:01	0.64		
61	NPLASIDSA	326-334		HLA-B*35:01			
62	DIIPKVQLY	268-276		HLA-A*01:01	0.65		**0.8068**
63		248-356					
64		37-45					
65		17-25					
66	AITNLGNTV	355-363		HLA-A*02:06	0.69		
67	NQMKIQVGA	166-174			0.71		
68	SIQDEIQQR	135-143		HLA-A*11:01	0.87		
69	KVDAVRSSL	337-345		HLA-A*02:06	0.88		
70	VLSQDNQMK	161-169		HLA-A*11:01	0.92		
71	ALSKVDAVR	334-342		HLA-A*03:01	0.96		

*Bold values are acceptable

T helper Cell Epitope Analysis

The 15-mer high-binding MHC-II epitopes for mouse and human alleles were predicted with the IEDB and Rankpep web servers. The results showed that RG-1 could act as a suitable T cell epitope that was able to bind to various alleles of mice MHC-II (Table 4), and human HLA-DR and HLA-DQ (Table 5). Among predicted epitopes in fusion protein, RG-1 had the highest scores of binding affinity to MHC-II. As seen in Tables 4 and, prediction of mice 15-mer peptides interaction with MHC II using IEDB server based on percentile rank, and with Rankpep server sorted by type of allele. RG-1 peptides with antigenicity score are shown and antigenicity score ≥ 0.4 are bold. Position of peptides are as RG-1 (5-64 aa and 236-295 aa), D1 flagellin (82-121 aa and 313-372 aa), tetanus toxoid-P2 (393-407 aa), RS09 (423-429 aa) and flexible linker (65-81 aa, 122-235 aa, 296-312 aa, 373-392 aa and 408-422 aa).

Additionally, allele-independent CD4$^+$ T-cell immunogenicity was predicted by the IEDB server, and the results showed that D1 flagellin and TT-P2 epitopes were potentially highly immunogenic Th cell epitopes, and D1 flagellin has acquired this property due to the order of positioning in the construct (Table 6).

Table 4. Murine MHC-II binding epitope prediction.

Number	Peptide	Position	Length	Allele	Percentile rank	Binding threshold	Antigenicity*
1	IPPKVQLYKTCKQAG	272-286	15	I-Ed	19.108	12.73	**0.5202**
2		252-266					
3		41-55					
4		21-35					
5	RSSLGAIQNRFDSAI	345-359			15.959		
6	GGSEFQLYKTCKQAG	232-246			11.48		0.2944
7	DDDKQYIKANSKFIG	390-404		I-Ek	13.71	10.02	
8	STLINEDAAAAKKST	313-327			13.432		
9	MGQLYKTCKQAG	1-15			11.483		0.3647
10	PPDIIPKVQLYKTCK	269-283					0.3707
11		249-263					
12		38-52					
13		18-32					
14	IIPKVQLYKTCKQAG	272-286			10.551		0.3729
15	PDIIPKVKLGGGGSG	290-304		I-Es	21.586	16.18	**0.8718**
16	PDIIPKVQLYKTCKQ	270-284			15.218		0.3343
17		250-264					
18		39-53					
19		19-33					

*Bold values are acceptable

Table 5. Human MHC-II binding epitope prediction.

Number	Peptide	Position	Length	Allele	Percentile rank	Binding threshold	Antigenicity*
1	EFQLYKTCKQAGTCP	232-246	15	HLA-DRB1*08:01	0.06	≤1	**0.6224**
2	FQLYKTCKQAGTCPP	233-247					**0.6567**
3	GGSEFQLYKTCKQAG	227-241					0.2944
4		228-242					
5		229-243					
6	GSEFQLYKTCKQAGT	230-244					0.1348
7	SEFQLYKTCKQAGTC	231-245					**0.4693**
8	DIIPKVQLYKTCKQA	17-31			0.23		**0.4131**
9		37-51					
10		248-262					
11		268-282					
12	IIPKVQLYKTCKQAG	18-32					0.3729
13		38-52					
14		249-263					
15		269-283					
16	IPKVQLYKTCKQAGT	19-33					0.1243
17		39-53					
18		250-264					
19		270-284					
20	KVQLYKTCKQAGTCP	21-35					0.3654
21		41-55					
22		252-266					
23		272-286					
24	PDIIPKVQLYKTCKQ	16-30					0.3343
25		36-50					
26		247-261					
27		267-281					
28	PKVQLYKTCKQAGTC	20-34					0.3496
29		40-54					
30		251-265					
31		271-285					
32	VQLYKTCKQAGTCPP	22-36					**0.5603**
33		42-56					
34		253-267					
35		273-287					
36	KQYIKANSKFIGITE	390-404		HLA-DRB1*01:15	0.88		**0.6303**
37		390-404		HLA-DRB1*01:03	0.96		
38	QLYKTCKQAGTCPPD	277-291		HLA-DRB1*01:01	15.186	6.69	**0.4742**
39		257-271					
40		237-251					
41		46-60					
42		26-40					
43		6-20					
44	IIPKVQLYKTCKQAG	272-286			10.576		0.3729
45	IPKVQLYKTCKQAGT	273-287		HLA-DRB1*02:01	8.879	6.39	0.1243
46		253-267					
47		42-56					
48		22-36					
49	TCPPDIIPKVKLGGG	287-301		HLA-DRB1*03:01	13.326	9.77	**0.6192**
50	TCPPDIIPKVQLYKT	267-281			11.491		0.2364
51		247-261					
52		36-50					
53		16-30					
54		279-293					
55		259-273					

56	YKTCKQAGTCPPDII	239-253		HLA-DRB1*04:01	9.755	4.85	**0.5324**
57		48-62					
58		28-42					
59		8-22					
60	IIPKVQLYKTCKQAG	272-286		HLA-DRB1*05:01	10.847	8.34	0.3729
61		252-266					
62		41-55					
63		21-35					
64	KVQLYKTCKQAGTCP	275-289		HLA-DRB1*07:01	14.046	11.89	0.3654
65		255-269					
66	EFQLYKTCKQAGTCP	235-249					**0.6224**
67	KVQLYKTCKQAGTCP	44-58					0.3654
68		24-38					
69	MGQLYKTCKQAGTCP	4-18					**0.5022**
70	IPKVQLYKTCKQAGT	273-287		HLA-DRB1*08:01	18.933	10.80	0.1243
71		253-267					
72		42-56					
73		22-36					
74	QAGTCPPDIIPKVKL	284-298		HLA-DRB1*03:01	11.694	11.59	**0.5704**
75	QAGTCPPDIIPKVQL	264-278					**0.5081**
76		244-258					
77	QAGTCPPDIIPKVGS	53-67					**0.5011**
78	QAGTCPPDIIPKVQL	33-47					**0.5081**
79		13-27					
80	KSTANPLASIDSALS	327-341		HLA-DRB1*15:01	10.466	9.80	
81	PPDIIPKVKLGGGGS	291-305		HLA-DQB1*03:01	17.02	11.70	**0.9403**
82	PDIIPKVGSGGGGSG	61-75			16.258		**0.6925**
83	PDIIPKVKLGGGGSG	292-306			15.962		**0.8718**

Table 6. The CD4$^+$ T cell immunogenicity prediction results for seven human alleles.

Peptide	FNGVKVLSQDNQMKI	DDDDKQYIKANSKFI	QYIKANSKFIGITEL
Start	156	386	391
End	170	400	405
Immunogenicity score	91.7904	70.4582	59.0289
Median percentile rank (7-allele)	20	9.8	12
HLA-DRB 5:01:01 Percentile rank	20	3.2	4.8
HLA-DRB 1:03:01 Percentile rank	8.5	55	12
HLA-DRB 3:02:02 Percentile rank	40	1.7	5.9
HLA-DRB 3:01:01 Percentile rank	45	54	52
HLA-DRB 1:15:01 Percentile rank	7.6	9.8	27
HLA-DRB 1:07:01 Percentile rank	29	7.8	3
HLA-DRB 1:07:02 Percentile rank	8.7	75	26

Population Coverage Analysis

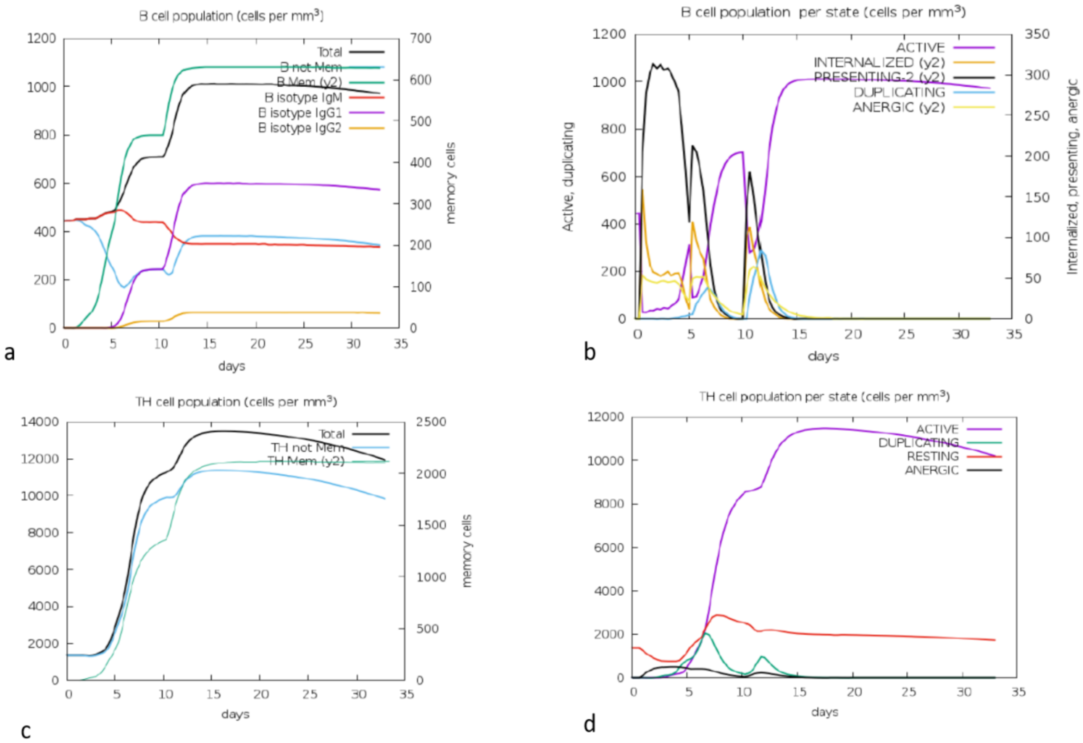
The distribution of HLA varies across different geographical areas around the world. Therefore, when designing an effective vaccine, population coverage should be considered to cover the maximum possible population. Population coverage for both MHC I and MHC II was estimated in nine designated geographical regions of the world (Table 7). Evaluation of recombinant protein population coverage for MHC I and MHC II using the IEDB tool showed that the average acceptable coverage among different populations around the world (Table 7).

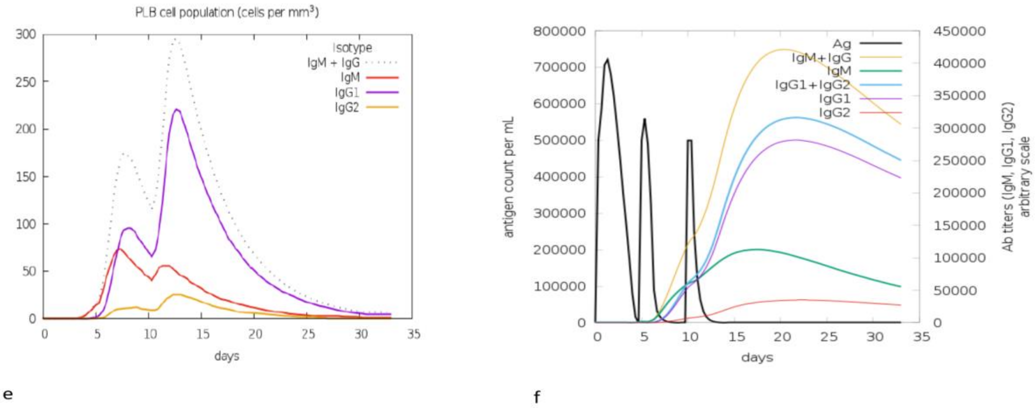
Table 7. Population coverage of recombinant protein.

Number	Population/area	MHC Class Combined PPC	Average of Epitope Hits
1	Iran	81.44%	1.36
2	South Asia	72.06%	1.13
3	Southwest Asia	70.99	1.07
4	Southeast Asia	83.59%	1.26
5	East Africa	51.48%	0.71
6	West Africa	58.95%	0.87
7	Europe	92.93%	1.78
8	North America	86.02%	1.47
9	South America	59.68%	0.8

Immune Response Simulation

The C-IMMSIM server was used to simulate the immune response against the vaccine candidate. Booster immunization increased the population of memory and activated B cells (Fig. 1a and b). Potential increase in memory and active Th cell population that could stimulate B cells proliferation was also depicted (Fig.1 c and d). Simulation of the antibody induction showed considerable IgM and IgG levels, and a significantly higher IgG1 subtype response (Fig. 1e). The IgM+IgG production was induced following the first injection, and the IgG1 titer was predicted to be more than IgG2 (Fig. 1f). The simulation results also showed that the population of professional APCs including NK cells, DCs, and macrophages were increased following administration of the construct (Fig. 1g, h, and i). In addition, epithelial cells remained active from day one to day 30 (Fig. 1j). The results also showed considerably increased IFN-γ, TGF-β, and IL-2 levels which could correlate with the general activation of T cells (Fig. 1k) that may also help in inducing potent humoral immune response. Furthermore, simulation of immune responses indicated that the administration of the designed fusion protein increased the number of $CD8^+$ T cells after the first and second injections, while their number decreased after the third immunization (Fig. l l and m)





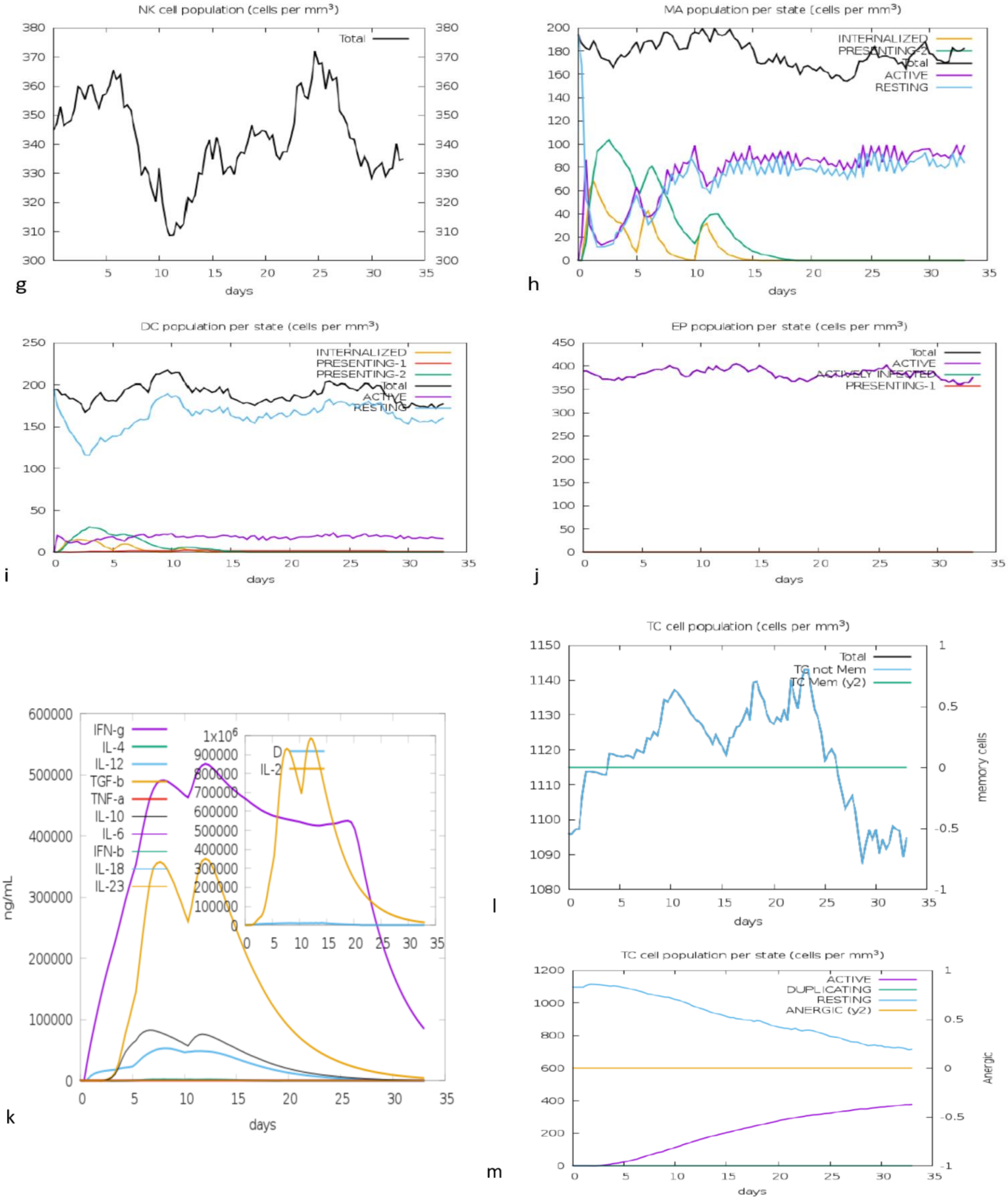


Fig. 1. Simulation of the immune response against the vaccine candidate. **a)** The population of total, memory, and IgM, IgG1 and IgG2 isotypes-bearing B cells. **b)** The population of B lymphocytes per entity-state showing active, internalized the Ag, duplicating and anergic B cells. **c)** Total and memory CD4$^+$ T-helper lymphocytes population. **d**) CD4$^+$ T-helper lymphocytes count sub-divided per entity-state showing active, resting, anergic and duplicating cells. **e)** Antibody-secreting plasma B cells (PLB) count sub-divided per isotype (IgM, IgG1 and IgG2). **f)** The antigen, the immunoglobulins and the immunocomplexes. **g)** Total number of natural killer cells. **h)** Total number of active, resting, Ag internalized, and Ag presenting dendritic cells. **i)** Total count, Ag internalized, active and resting macrophages. **j)** Total count of epithelial cells broken down to active. **k)** The concentration of cytokines and interleukins (D) in the insert plot is the danger signal). **l)** Total and memory CD8$^+$ T-cytotoxic lymphocytes (TC) count. **m)** The population of active, duplicating, resting and anergic CD8$^+$ T-cytotoxic lymphocytes.

DISCUSSION

Sexually transmitted HPVs, of them 15 genotypes are categorized as high-risk, can cause cervical cancer and other subsets of anogenital and pharyngeal carcinomas, which together account for 5% of all cancers worldwide [24]. Current L1 VLP-based vaccines do not protect against all carcinogenic HPV types, and are expensive to be included in the public vaccination program of the developing countries [25]. In contrast, the L2 protein harbors an immunogenic epitopes called RG-1, that can induce cross-neutralizing antibodies against different HPV types; however, the titer of antibodies is usually below the level needed for protection [5]. In one study, HPV16 RG-1 induced cross-neutralizing antibodies in rabbits and mice [3]. Motevali et al. reported that using two RG-1 epitopes could increase neutralizing antibodies against HPV to an appropriate level [26]. In the present study, the three consecutive RG-1 epitopes were applied twice in the construct, and the immunogenicity potential was investigated through immunoinformatic analyses, and the results were similar to the experimental results by Motevali et al (22).

Built-in adjuvants are effective in helping immunogenicity of epitope-based vaccine candidates [6]. Based on the previous studies, we used built-in adjuvant including TLR agonists to improve the immunogenicity of epitopes. Conjugation of L2 RG-1 epitope to TLR agonists showed great potential for enhancing the immunogenicity of the L2-based vaccines. The fusion of L2 peptides with bacterial flagellin, as TLR5 agonist, also can protect against different types of HPV [9]. Bacterial flagellin can be used as a built-in adjuvant that is able to induce an enhanced immune response via NF-κB signaling, especially in the form of a flagellin vaccine model [27]. Entolimod is a salmonella flagellin-derived drug that has been optimized pharmacologically, and contains only the full N and C-terminal domains of the parental protein isolated by a flexible linker, and fully preserves the NF-κB induction activity of flagellin. The FDA (Food and Drug Administration) has approved Entolimod for cancer treatment and radio-protective activity [28]. Another built-in adjuvant that was used in this study, was RS09 that is a synthetic seven amino acid peptide which functionally mimics bacterial LPS (lipopolysaccharide) and activates NF-KB signaling pathway via TLR4, and induces antibody production [10].

In addition to the TLR agonists described above, the universal T-helper tetanus toxoid P2 epitope was added to the designed construct to help increase the Th cell responses [29]. The TT-P2 epitope is a universal CD4$^+$ T-cell recall chimeric peptide with the sequence of “QYIKANSKFIGITEL”, which causes extensive MHC-II coverage in mice and humans [30]. Cui B. et.al found that the universal activation of Th cells that was mediated by the P2 epitope could induce more efficient affinity maturation of antibodies [30]. The CD4 immunogenicity evaluation results also predicted that a flagellin-derived epitope in the construct (FNGVKVLSQDNQMKI) is potentially a strong universal Th epitope.

Epitope selection was done with various servers to increase the probability of finding the most potent epitopes. The segments of the designed construct were chosen in a way to cover the MHC-I and MHC-II alleles due to the need for both cellular and humoral immune responses to induce a potent protective immunity against HPV [31]. Detection of viral proteins by the immune system activates T cells and produces cytokines that cause B cells to grow and mature. B cells located in the lymphoid tissue of the genitals can be activated by T cells, and produce antibodies, and the neutralizing antibodies can recognize the HPV-L1 and L2 proteins to protect against infection [32].

The results of evaluating the binding of the epitopes to mouse and human MHC-I and MHC-II alleles revealed that the 9-mer CPPDIIPKV epitope (12-20) form RG-1 was the suitable B cell epitope. Interestingly, our results also indicated that the three repeats of HPV16 RG-1 epitope interact with MHC-I as well as MHC-II molecules. Additionally, the 15-mer P2 epitope (KQYIKANSKFIGITE) binds, with the highest score, to HLA-DRB1 alleles.

In bioinformatics studies to discover the optimal immunogenicity of the RG-1 epitope, it has been shown that the epitope fusion with flagellin has led to a better exposure and increased immunogenicity [33]. Chen et al. designed a construct encoding three repeats of HPV16 RG-1 epitope in fusion with modified IgG1 Fc, and investigated its efficacy in a mice model. They showed that the designed construct co-administered with Freund's adjuvant strongly induced cross-neutralizing antibodies, and protected mice from HPV infection for more than eleven months [32]. In another study, Kalnin et al. showed that vaccination with the RG-1 epitope fused to the flagellin provided stable immunity without the need for other external adjuvants, and broad-spectrum and stable neutralizing antibodies were produced against a variety of HPV serotypes [34].

Using C-IMMSIM for immune stimulation by HLA heterozygous alleles, we assessed the nature of the immunogenicity of the fusion protein. Without additional external adjuvant, the construct with built-in adjuvants created an immune profile with adequate antigen presentation and subsequent production of immune memory in T and B cell groups. In addition, there was a polarization towards Th1 cells and induction of several immunoglobulins including IgG1 + IgG2, IgM, and IgG + IgM after the first injection (Fig. 2). Comparable simulations have been previously performed using the C-IMMSIM algorithm with validated experimental peptides and correlations with *in vitro* studies [35].

As known, the use of a protein vaccine containing built-in adjuvants is a noteworthy strategy for developing a preventive vaccine against HPV infections. In the present study, an antigenic peptide-based vaccine candidate harboring three built-in adjuvants was designed by employing various immunoinformatics tools. The designed construct, consisting of two replicas of the RG-1 triple repeats along with the D1 domain of flagellin and RS09 as TLR5 and TLR4 agonists, respectively, and tetanus toxoid P2 epitope, had acceptable immunological properties in inducing immune responses for protection against HPV infection. However, further *in vitro* and *in vivo* immunoassays are needed to assess the construct’s immunogenicity, which are currently under process.

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CONFLICT OF INTEREST

The authors declare they have no conflict of interests.

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