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 $\geq$ 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		H-2-Kd	0.07		
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			0.42		
7	IQDEIQQRL	136-144		H-2-Dd	0.51		
8	SGGGGSTLI	305-313			0.54		
9	STANPLASI	323-331					
10	TQFNGVKVL	154-162	9		0.57	≤1	
11	SKFIGITEL	397-405		H-2-Ld	0.66		
12	KVDAVRSSL	337-345			0.67		
13	GGSAPPHAL	418-426		H-2-Dd	0.68		
14	KVDAVRSSL	337-345		H-2-Kd			
15		285-293					
16		265-273					
17	CPPDIIPKV	245-253		H-2-Ld	0.77		**0.8370**
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			
4		285-293					
5		265-273					
6	CPPDIIPKV	245-253		HLA-B*51:01	0.03		**0.8370**
7		54-62					
8		34-42					
9		14-22					
10	ASIDSALSK	329-337		HLA-A*03:01	0.04		
11		269-277					
12		249-257		HLA-A*11:01	0.05		

No.	Peptide	Position	HLA allele	Value	Score
13		38-46			
14	IIPKVQLYK	18-26			0.0392
15		269-277			
16		249-257	HLA-A*03:01	0.07	
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		268-275			
25	DIIPKVQLY	248-256	HLA-B*35:01	0.15	**0.8068**
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		
33	GTNSDSDLK	126-134	HLA-A*11:01	0.25	
34	STANPLASI	323-331	HLA-A*02:06		
35		285-293			
36		265-273	9	≤1	
37	CPPDIIPKV	245-253	HLA-B*35:03	0.29	**0.8370**
38		54-62			
39		34-42			
40		14-22			
41		272-280			
42	KVQLYKTCK	252-260	HLA-A*03:01	0.35	-0.1226
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		0.45	
52		272-280			
53	KVQLYKTCK	252-260	HLA-A*11:01	0.49	-0.1226
54		41-49			
55		21-29			
56	SLGAIQNRF	344-352	HLA-	0.57	

Number	Peptide	Position	Allele	value	Antigenicity
			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		268-276			
63	DIIPKVQLY	248-356	HLA-A*01:01	0.65	**0.8068**
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 $\geq$ 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		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	15		13.432		
9	MGQLYKTCKQAG	1-15					0.3647
10	PPDIIPKVQLYKTCK	269-283			11.483		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					**0.6224**
2	FQLYKTCKQAGTCPP	233-247					**0.6567**
3		227-241					
4	GGSEFQLYKTCKQAG	228-242			0.06		0.2944
5		229-243					
6	GSEFQLYKTCKQAGT	230-244					0.1348
7	SEFQLYKTCKQAGTC	231-245					**0.4693**
8		17-31					
9	DIIPKVQLYKTCKQA	37-51					**0.4131**
10		248-262					
11		268-282					
12		18-32					
13	IIPKVQLYKTCKQAG	38-52					0.3729
14		249-263					
15		269-283					
16		19-33					
17	IPKVQLYKTCKQAGT	39-53					0.1243
18		250-264	15	HLA-DRB1*08:01	0.23	≤1	
19		270-284					
20		21-35					
21	KVQLYKTCKQAGTCP	41-55					0.3654
22		252-266					
23		272-286					
24		16-30					
25	PDIIPKVQLYKTCKQ	36-50					0.3343
26		247-261					
27		267-281					
28		20-34					
29	PKVQLYKTCKQAGTC	40-54					0.3496
30		251-265					
31		271-285					
32		22-36					
33	VQLYKTCKQAGTCPP	42-56					**0.5603**
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		277-291					
39		257-271					
40	QLYKTCKQAGTCPPD	237-251		HLA-DRB1*01:01	15.186	6.69	**0.4742**
41		46-60					
42		26-40					
43		6-20					
44	IIPKVQLYKTCKQAG	272-286			10.576		0.3729
45		273-287					
46	IPKVQLYKTCKQAGT	253-267		HLA-DRB1*02:01	8.879	6.39	0.1243
47		42-56					
48		22-36					
49	TCPPDIIPKVKLGGG	287-301			13.326		**0.6192**
50		267-281					
51	TCPPDIIPKVQLYKT	247-261		HLA-DRB1*03:01	11.491	9.77	0.2364
52		36-50					
53		16-30					
54		279-293					
55		259-273					

No	Peptide	Position
56	YKTCKQAGTCPPDII	239-253
57		48-62
58		28-42
59		8-22
60		272-286
61	IIPKVQLYKTCKQAG	252-266
62		41-55
63		21-35
64	KVQLYKTCKQAGTCP	275-289
65		255-269
66	EFQLYKTCKQAGTCP	235-249
67	KVQLYKTCKQAGTCP	44-58
68		24-38
69	MGQLYKTCKQAGTCP	4-18
70		273-287
71	IPKVQLYKTCKQAGT	253-267
72		42-56
73		22-36
74	QAGTCPPDIIPKVKL	284-298
75	QAGTCPPDIIPKVQL	264-278
76		244-258
77	QAGTCPPDIIPKVGS	53-67
78	QAGTCPPDIIPKVQL	33-47
79		13-27
80	KSTANPLASIDSALS	327-341
81	PPDIIPKVKLGGGGS	291-305
82	PDIIPKVGSGGGGSG	61-75
83	PDIIPKVKLGGGGSG	292-306

HLA allele			Score
HLA-DRB1*04:01	9.755	4.85	**0.5324**
HLA-DRB1*05:01	10.847	8.34	0.3729
HLA-DRB1*07:01	14.046	11.89	0.3654
			0.6224
			0.3654
			0.5022
HLA-DRB1*08:01	18.933	10.80	0.1243
HLA-DRB1*03:01	11.694	11.59	**0.5704**
			0.5081
			0.5011
			0.5081
HLA-DRB1*15:01	10.466	9.80	
HLA-DQB1*03:01	17.02	11.70	**0.9403**
	16.258		**0.6925**
	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

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. 1 l and m)
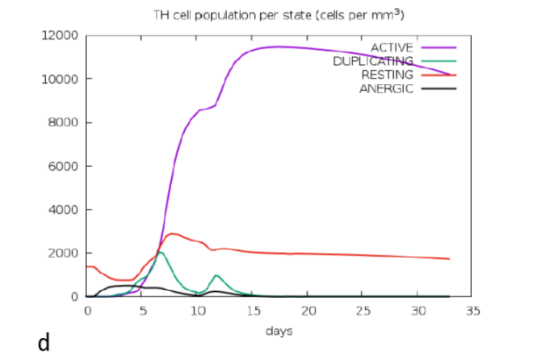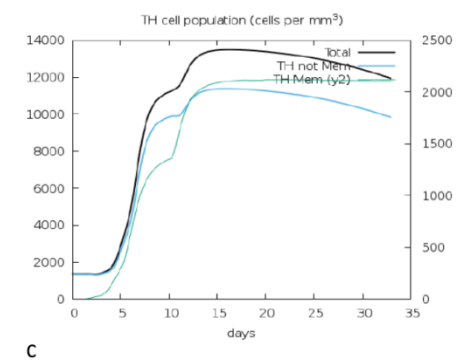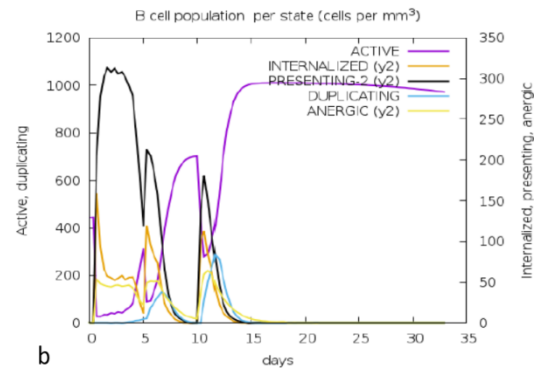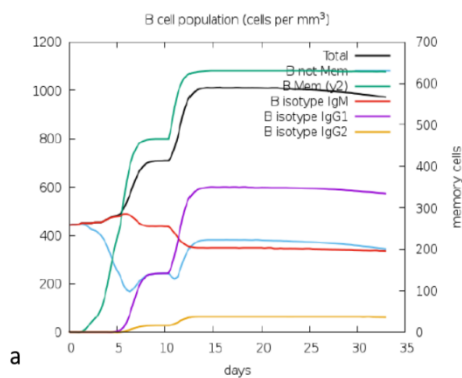
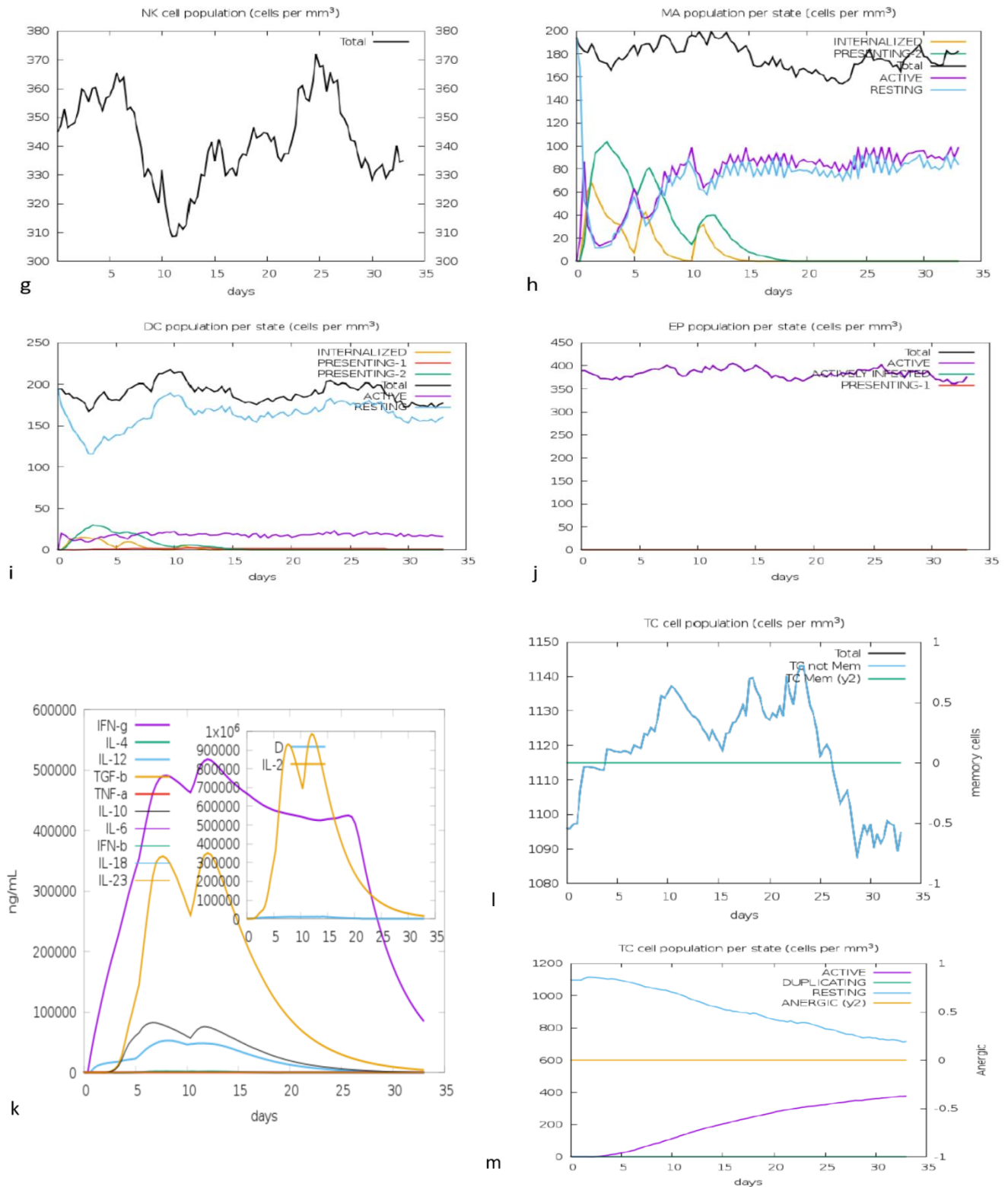increased the population of memory and activated B cells (Fig.
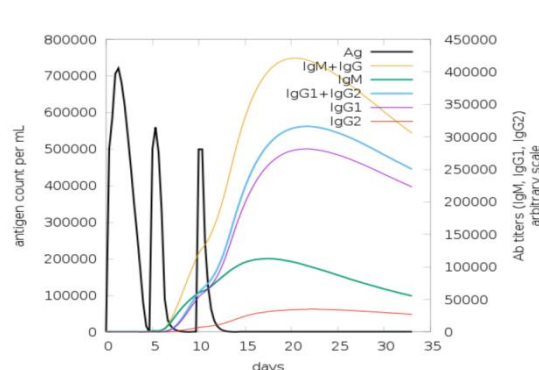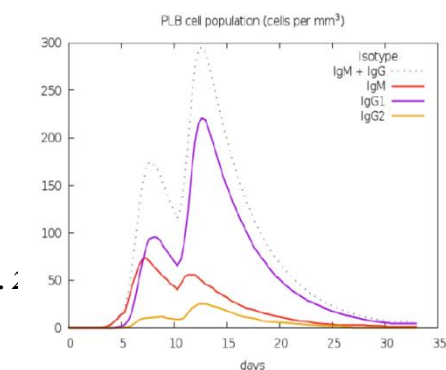
Fig. 1. Simulation of the immune response against the vaccine candidate. **a)** The population of total, memory, and IgM, IgG1 and IgG2 isotypes-bearing B cells. **b)** The population of B lymphocytes per entity-state showing active, internalized the Ag, duplicating and anergic B cells. **c)** Total 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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