Current HIV Research, 2018, *16*, 322-337

RESEARCH ARTICLE

In Silico Design and Immunologic Evaluation of HIV-1 p24-Nef Fusion Protein to Approach a Therapeutic Vaccine Candidate

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Abstract: ***Background*:** Acquired immune deficiency syndrome (HIV/AIDS) has been a major global health concern for over 38 years. No safe and effective preventive or therapeutic vaccine has been developed although many products have been investigated. Computational methods have facilitated vaccine developments in recent decades. Among HIV-1 proteins, p24 and Nef are two suitable targets to provoke the cellular immune response. However, the fusion form of these two proteins has not been analyzed in silico yet.

Objective: This study aimed at the evaluation of possible fusion forms of p24 and Nef in order to achieve a potential therapeutic subunit vaccine against HIV-1.

Method: In this study, various computational approaches have been applied to predict the most effective fusion form of p24-Nef including CTL (Cytotoxic T lymphocytes) response, immunogenicity, conservation and population coverage. Moreover, binding to MHC (Major histocompatibility complex) molecules was assessed in both human and BALB/c.

Results: After analyzing six possible fusion protein forms using AAY linker, we came up with the most practical form of p24 from 80 to 231 and Nef from 120 to 150 regions (according to their reference sequence of HXB2 strain) using an AAY linker, based on their peptides affinity to MHC molecules which are located in a conserved region among different virus clades. The selected fusion protein contains seventeen MHC I antigenic epitopes, among them KRWIILGLN, YKRWIILGL, DIAGTTSTL and FPDWQNYTP are fully conserved between the virus clades. Furthermore, analyzed class I CTL epitopes showed greater affinity binding to HLA-B 57*01, HLA-B*51:01 and HLA-B 27*02 molecules. The population coverage with the rate of >70% coverage in the Persian population supports this truncated form as an appropriate candidate against HIV-I virus.

Conclusion: The predicted fusion protein, p24-AAY-Nef in a truncated form with a high rate of T cell epitopes and high conservancy rate among different clades, provides a helpful model for developing a therapeutic vaccine candidate against HIV-1.

ARTICLE HISTORY

Received: August 26, 2018
Revised: December 04, 2018
Accepted: December 27, 2018

DOI:
10.2174/1570162X17666190102151717

Keywords: HIV-1 vaccine, p24-Nef-fusion protein, truncated protein, cytotoxic T lymphocytes, combination antiretroviral therapy, major histocompatibility complex.

1. INTRODUCTION

Human Immunodeficiency virus type 1 (HIV-1) remains a considerable global health challenge, with almost 36.7 million HIV infected individuals and prevalence of 0.8% among adults by 2017 [1, 2]. The development of combination antiretroviral therapy (cART) has changed the mortality rate from this virus to chronic manageable disease [3]. However, it cannot eliminate the virus, only helps infected individuals with HIV live longer beside its side effects and costs [4, 5]. Vaccination has been widely considered as the most effective medical strategy to control infectious disease. Conventional vaccines stimulate the humoral immune response which acts through the induction of antibodies (Ab) with neutralizing power [6, 7]. Nonetheless, serious hardships stand in the way of a successful HIV vaccine. This stems from largely inaccessible neutralizing epitopes that are located beneath a glycan shield, the enormous genetic diversity within and between HIV subtypes and generation of sufficiently immunogenic antigens [8, 9].

Besides the efforts on eliciting an effective humoral response, HIV vaccine will also require inducing cellular im-

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1873-4251/18 $58.00+.00

munity to eliminate virally infected cells [10]. There has been substantial progress in generating recombinant DNA technology which has resulted in the development of fusion proteins in recent past decades [11, 12]. Indeed, fusion proteins are new biomolecules got by genetically fused two or more genes that originally code for different proteins. Consequently, fusion proteins have different functions derived from their domains [13, 14].

In comparison to traditional vaccines, synthetic fusion protein-based vaccines are more advanced and practical especially for such pathogenic virus with a great amount of mutation and limited possibility of culture in cell lines [15, 16]. In fact, this kind of vaccine provides opportunities for specific purposes and makes it possible to exclude deleterious sequences from full-length genome [17, 18]. Since most of the biological functions of proteins are based on their 3D structure; stability, interaction between domains and also proper folding, these features must be considered in vaccine design [19, 20]. Moreover, Fusion proteins are often more susceptible to misfolding than single-domain proteins because of the interaction between different peptide domains. Therefore, in silico analysis of multi-domains proteins is an essential estimation in fusion protein generating projects. In other words, it is based on computational approaches in order to clarify immunogenic residues [19, 21].

HIV-1 genome in form of single-strand RNA (ssRNA) with 9181 nucleotides encodes three main proteins (Gag, Pol, Env) and some accessory proteins like Nef in an open reading frame (ORF) with two long terminal repeat (LTR) at the two ends [8]. Two of the most investigated HIV-1 proteins in vaccine studies are Gag and Nef due to their significant potency to call cellular immune response [22-24].

Capsid or p24; the 231 aa (amino acids) product of Gag precursor; plays crucial roles in particle assembly and entry into a new target cell. This protein forms the shell of the core with two different domains linked by a flexible loop. The two CA (p24) domains play different roles in virus morphogenesis [25]. The N-terminal domain is essential for the formation of the mature core, whereas the C-terminal CA domain is crucial for core and particle assembly, too [26, 27].

A stretch of twenty residues is located at the C-terminal p24 domain which is called the major homology region (MHR) due to its conservation among unrelated retroviruses. This uniquely conserved region suggests a critical role in the retroviral life cycle. It has been shown that MHR is essential for virus replication by genetic analysis and has vital roles in assembly and at post-assembly stages both [27-30].

One of the earliest HIV-1 translation products after entry is Nef protein which yields 200 to 215 aa but in the form of 206 aa is the most common. It is composed of an N terminal domain (anchor), a core domain, and a flexible C-terminal domain [31]. In many studied infected people with Nef-deleted HIV-1s, lack of disease progression was observed [31, 32]. This fact defines Nef as a pathogenic factor with pleiotropic properties and a great number of interaction partners [33]. The well-studied Nef activities which assess possible roles for each function in pathogenesis are down-regulation of CD4, CD8, CD3 and major histocompatibility complex I (MHCI), modulation of cellular activation and signaling pathways, and giving rise to virion infectivity [34, 35].

Each of the mentioned Nef functions could serve as contributors to its elusive role in pathogenesis and replication. Nef contains variable regions among different HIV-1 isolated and it goes through many mutations to escape the immune system [36, 37].

p24 and Nef proteins are particularly important for vaccine development as they mediate virus assembly, entry, pathogenesis and replication. They are also the primary targets of a cellular immune response. Well-characterized protective effects of these proteins based on T-cell epitopes can be a great effort for effective therapeutics over the current treatment [36, 38, 39]. Computational approaches have been applied to facilitate immunological characterizing of residues in order to achieve hypothetical vaccine candidates [40, 41].

To our best of knowledge, the fusion form of p24 and Nef as a truncated protein has not been studied in silico or *in vitro* yet. So, in the present study we aimed at immunoinformatic analysis of conserved and immunogenic p24-Nef-fusion protein with a flexible linker (AAY) using bioinformatics tools, to evaluate and compare T-cell epitopes in human and BALB/c, Conservancy of possible fusion forms and determine the vaccine antigenic constructs to include short protein sequences present at high frequencies in natural virus serotypes for further experimental study.

2. MATERIALS AND METHODS

The outline of applied methodology has been illustrated in Fig. (**1**).

2.1. Amino Acid Sequence Retrieval

Totally, 29 protein sequences of p24 and 22 protein sequences of Nef were selected (accession number of p24 of different clades: NP_579880 (reference sequence), BAM37368, BAM37377, BAM37395, BAM37404, BAM37413, BAM37422, BAM37431, BAM37440, CAB86989, CAB87170, CAB87070, CAT99391, CAT99401, CAT99384, CAB87008, CAB87186, CAT99436, CAB87015, CAB87183, CAB87119, CAB87017, CAT99442, CAB87090, CAB86999, CAB87037, CAB87085, CAB87102, CAB87065, accession number of Nef of different clades: NP_057857 (reference sequence), BAM37376, BAM37385, BAM37394, CAA13437, CAA13440, CAA13460, CAA13483, CAA13494, CAA13497, AAL06124, AAL06122, AAL06127, CAA13463, CAA13464, CAA13465, CAA13466, CAA13467, CAA13468, CAA13473, CAA13472, CAA13471) in FASTA format were obtained from GenBank of National Center for Biotechnology Information (NCBI) including different HIV-1 clades and also CRF 35, the circulating form among Iranian population. We considered at least three sequences for each obtained subtypes.

2.2. Conserved Regions Determination

To align the retrieved sequences and obtain conserved regions we applied MEGA6.0 using ClustalW by comparing

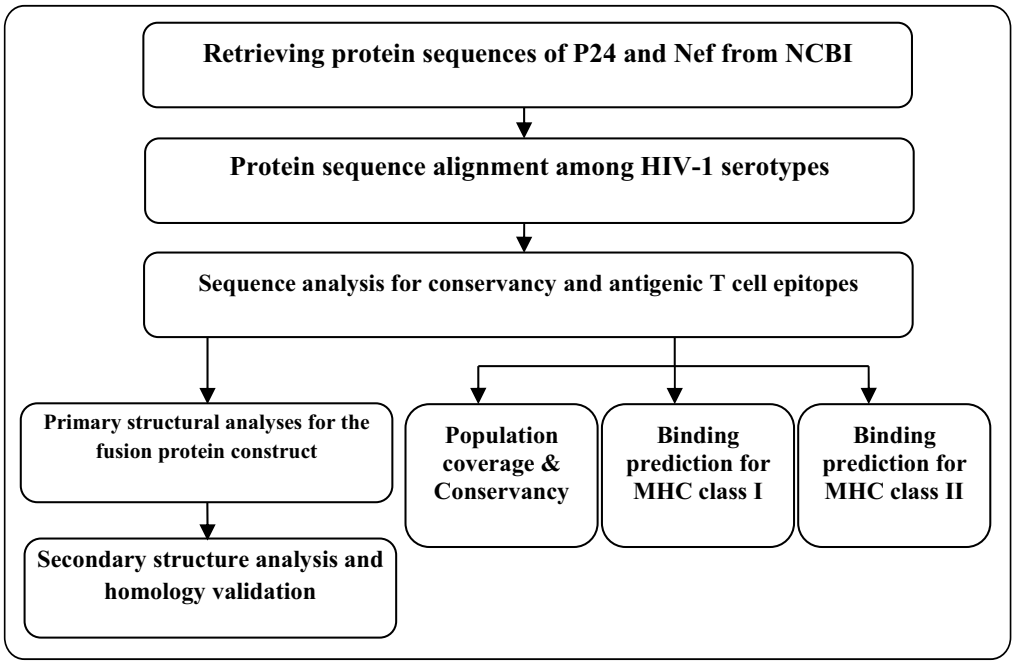

Fig. (1). Flowchart representing the immune informatics prediction of potential T lymphocyte epitopes and screening procedure of modeled structures for the development of fusion protein p24-Nef.

the whole length amino acid of p24 and Nef against reference sequences [42]. These two proteins were analyzed for conserved domains in NCBI-Conserved Domains (http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?uid=111760).

2.3. T -cell Epitope Prediction in BALB/c and Human

In order to predict binding of peptides to MHC class I and II molecules; the reference sequence of p24 and Nef in the form of fusion protein by a flexible linker with a proteasome cutting site (AAY) were submitted in MHC I and MHC II binding prediction tool (http://tools.iedb.org/ mhc/n) in IEDB. Prediction methods include Stabilized Matrix Method (SMM), Artificial Neural Network (ANN), or Scoring Matrices derived from Combinatorial Peptide Libraries (Comblib_Sidney2008) method. We also used MHC-NP (http://tools.immuneepitopes.org/mhcnp), net CTLpan1.1 server (http://www.cbs.dtu.dk/services/NetCTLpan/) [43-45] and RankPEP server (http://imed.med.ucm.es/ tools/ rankpep.html). The obtained results from all different tools were in a similar range, so here we report IEDB output. Epitopes lengths were set as 9-mer for MHC class I and 15-mer for MHC class II separately for mice and human. BALB/c MHC class I alleles including H2-Dd, H2-Kd and H2-Ld and MHC class II alleles including H2-IAd and H2-IEd were investigated. Due to the fact that diversity of antigens among different strains and the extent of recognition by the variable HLA molecules in the recipient population may affect on subunit vaccine and in order to include the most popular HLA in Persian population based on the available report (http://www.allelefrequencies.net) [46-48], we selected HLA-A*01, 02, 03, 11, 24, 26, 32, HLA-B*35, 51, 50, 27, 57 for MHC class I and HLA-DRB1*15, 11, 13, 03, 04, 07 for MHC class II. The conserved peptides which bind to MHC class I and II molecules were sorted by their percentile rank and those ≤1 were selected. Subsequently, we chose the top model of predicted fusion proteins to evaluate HLA binding potency.

2.4. Prediction of p24-Nef Antigenicity

The antigenicity scores of all the predicted binders were achieved by VaxiJen v2.0 online antigen prediction tool (http://www.ddg-pharmfac.net/vaxijen/) [49, 50]. This tool provides antigen sorting based on the protein physicochemical qualities without the usage of sequence alignment. Epitopes with antigenic score > 0.5 were considered antigenic.

2.5. Population Coverage and Epitope Conservancy

MHC I and MHC II potential binders from the selected fusion form of p24-AAY-Nef were assessed for population coverage analysis against the whole world population, especially Persian population, with the selected human MHC I and MHC II interacting molecules using IEDB population coverage calculation tool (http://tools.iedb.org/ tools/ population/iedb_input). Population coverage calculation is based on total HLA hits score which is obtained from IEDB, these data derived from the relative frequency of an allele at a particular locus in a population (Sequence identity threshold=>100). Moreover, we evaluated the conservancy level of each potential epitope by searching identities in 29 amino acid sequences of p24 and 22 amino acid sequences of Nef from different clades retrieved from the database (http://tools.iedb.org/conservancy/iedb_input) [51].

2.6. Toxicity Analysis

At the final step, we examined the selected model of p24-AAY-Nef for toxicity using ToxinPred (http://crdd.osdd.net/raghava/toxinpred/) [52]. This website provides the confirmation of non-toxicity of epitopes for the host according to all physic-chemical parameters (http://crdd.osdd.net/raghava/toxinpred/).

2.7. Primary Structure Analysis

Expasy's ProtParam server (http://web.expasy.org/ protparam/) was used [53] to analyze physicochemical properties of fragments including molecular weight, theoretical pI, atomic composition, extinction coefficient, estimated half-life, aliphatic index and Grand average of hydropathicity (GRAVY) with a flexible linker, which includes proteasome cleavable site.

2.8. Secondary Structure Analysis and Disulfide Connectivity

Self-Optimized Prediction Method with Alignment (SOPMA) (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html) and Jpred (http://www.compbio.dundee.ac.uk/jpred/) tools was used to predict secondary structure and calculation of the number of a-helix, b sheets, random coils, and extended strands of p24-AAY-Nef protein [54, 55]. Disulfide connectivity of p24-AAY-Nef-fusion protein was checked using DiANNA tool which is a neutral network application to predict cysteine states of protein. Cys-cys linkages are the other crucial factor in understanding the secondary and tertiary structure of protein due to its important role in fold stabilization [56].

2.9. Homology Modeling Validation

The 3D model was obtained using the Iterative Threading ASSEmbly Refinement (I-TASSER) online server program which generates 3D models along with their confidence score (C-Score). C-Score is calculated based on the importance of threading template alignments and the proximity parameters of the structure assembly reproductions [57]. The further analysis of pattern was done by three indicators: C-score, DFIRE2 energy profile and Stereochemical qualities [58]. The Stereochemical analysis of obtained 3D model was done by PROCHECK, ERRAT, VERIFY 3D and was proved by Structural Analysis and Verification Server (SAVES;http://nihserver.mbi.ucla) and Ramachandran plot assessment (RAM-PAGE; mordred.bioc.cam.ac.uk/~rapper/rampage.php) [59-61].

2.10. Statistics Analysis

We applied Student's T-test using SPSS 19 to evaluate variable parameters and a p-value < 0.05 was considered significant.

3. RESULTS

3.1. Retrieval of Protein Sequences, Identification and Selection of Conserved Domains

At this step, we aimed at choosing the highly conserved regions among all clades. Consequently, for generating proper truncated forms the first 79 amino acids of p24 were excluded and 70-150 region of Nef was investigated (Fig. **2a**, **b**). Based on the sequence alignment, the most conserved regions are located between 80-231 amino acid sequence of p24 and 70-90 and 120-150 amino acid sequences of Nef protein. According to this analysis, six possible arrangements of p24-AAY-Nef protein by AAY (a flexible linker) were selected for further assessment (Fig. **3a**, **b**).

3.2. MHC class I and II Binding Prediction in BALB/c

In order to design a preferable vaccine model, we looked for a premium length with 9-mer coverage of T-cell epitopes. To achieve that, p24 and Nef-fusion protein forms were subjected to IEDB MHC I binding prediction tool. The IEDB recommended, MHC-NP, net CTLpan1.1, RANKPEP and netMHCpan3.0 server which were used to predict the potent epitopes from HIV-1 p24 and Nef. T-cell epitopes which were evaluated by at least four tools were selected to improve the accuracy of prediction. In contrast with Model 1, the two truncated Model 5 and Model 6 have fewer MHC binders with high antigenicity score which are placed in the most conserved regions. High affinity (percentile rank ≤ 1) peptides with the antigenic property are listed in Table **1**. The six predicted models of fusion protein were subjected to MHC II binding prediction tool. In the full-length form of p24-Nef-fusion protein, 5 predicted epitopes were found to interact with MHC II different molecules. High affinity (percentile rank ≤ 1) peptides interacting with MHC I and II with antigenic property are listed in Tables **1** and **2**. Models 2, 3 and 4 showed less affinity than Model 5 and 6. (For other predicted models see Supplementary Tables **1** and **2**)

In a comparison of Model 1 and other predicted models, the two truncated Models 5 and Model 6 indicate more conserved regions. Furthermore, they include MHC I and II binder peptides with high antigenicity score. As a result, these two truncated models provide better immunogenic conserved peptides than other predicted models. In addition to all above, the truncated Models 5 and 6 contain similar MHC class I. As we look for a conserved model in comparison to the full-length form, these two models both contain antigenic epitopes GGPGHKARV and PGPGVRYPL. For MHC class II, Model 5 includes 5 epitopes like the full-length form from which YKTLRAEQASQEVKN and FYKTLRAEQASQEVK are antigenic. Model 6 has three epitopes which include the same antigenic peptides with Model 5.

3.3. MHC Class I and II Binding Prediction in Human

At the next step and to come up with the best fusion model, we submitted these two selected fusion protein sequences using IEDB MHC prediction tool against human MHC class I and II with more frequency among Persian population. The differences in MHC class I binding peptide frequency in the two mentioned truncated proteins are shown in Tables **3** and **4** (for more details see supplementary Tables **3** and **4**). Model 6 includes 25 epitopes from those 17 peptides are antigenic. Model 5 contains 11 anitgenic epitopes of total 19 peptides interacting with MHC class I. It seems that Model 6 with more antigenic regions has greater chance to call cellular immune response although it is not statistically significant (p-value > 0.05). However, there was no difference in MHC class II binding power between them for the reason that the part of the fusion protein which interacts with MHC II molecule (≤ 1 at percentile rank) is only p24 which is the same in the both of predicted models. For MHC class II there is a great number (>60 epitopes) of binders related to the selected HLAs (See Supplementary Tables **5** and **6**). The summary of the predicted epitopes is in Table **5**.

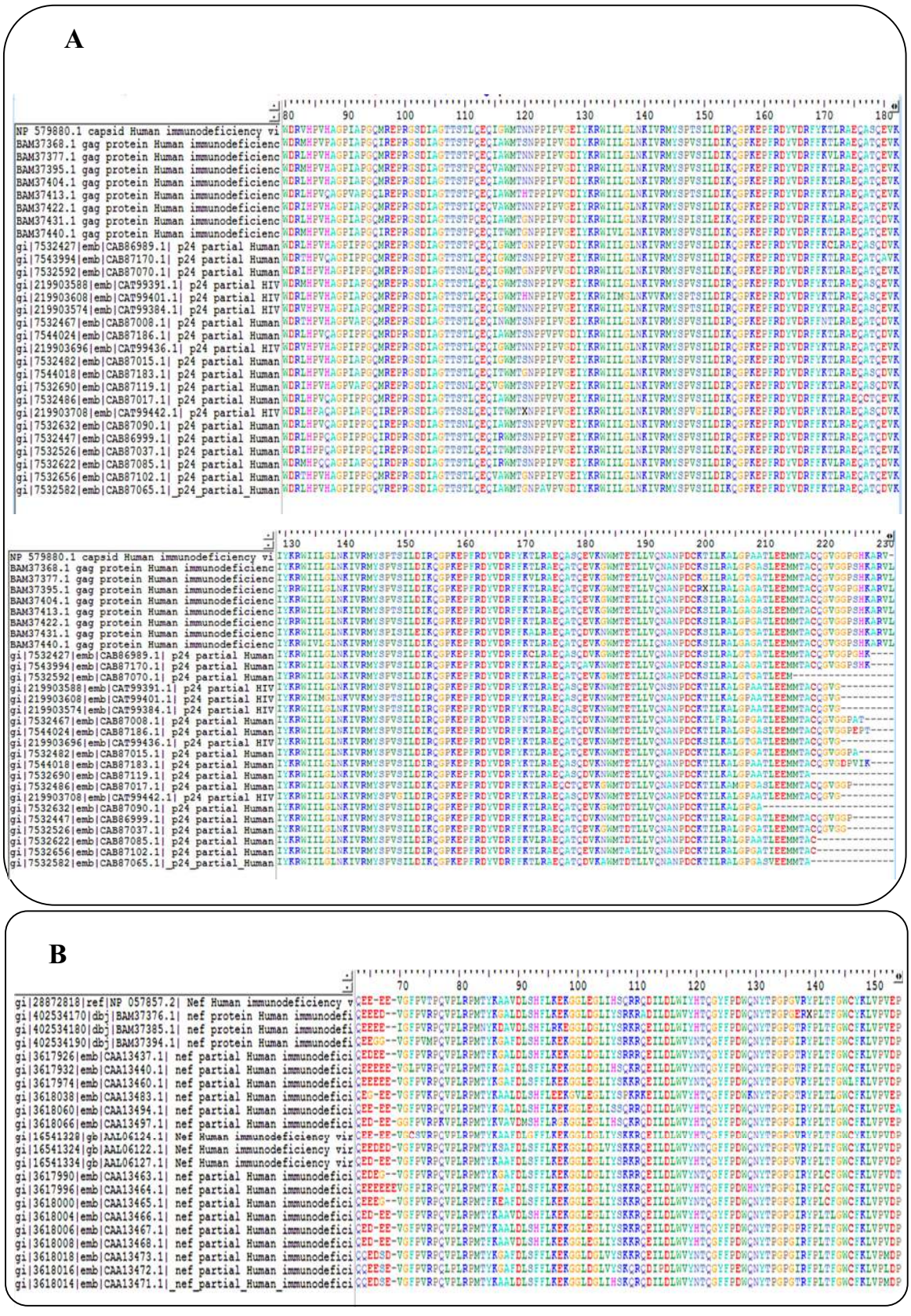

Fig. (2). Representative multiple sequences alignment of HIV-1 p24 (**A**) and Nef (**B**) regions among different subtypes. As it is clear, p24 from 80 to 231 aa has acceptable conservancy which suggests this region has a crucial role in the virus life cycle. Nef shows more variety as it is a key factor for viral immune evasion. There are two regions from 70-90 and 120-150 aa which seem to be more conserved between the clades.

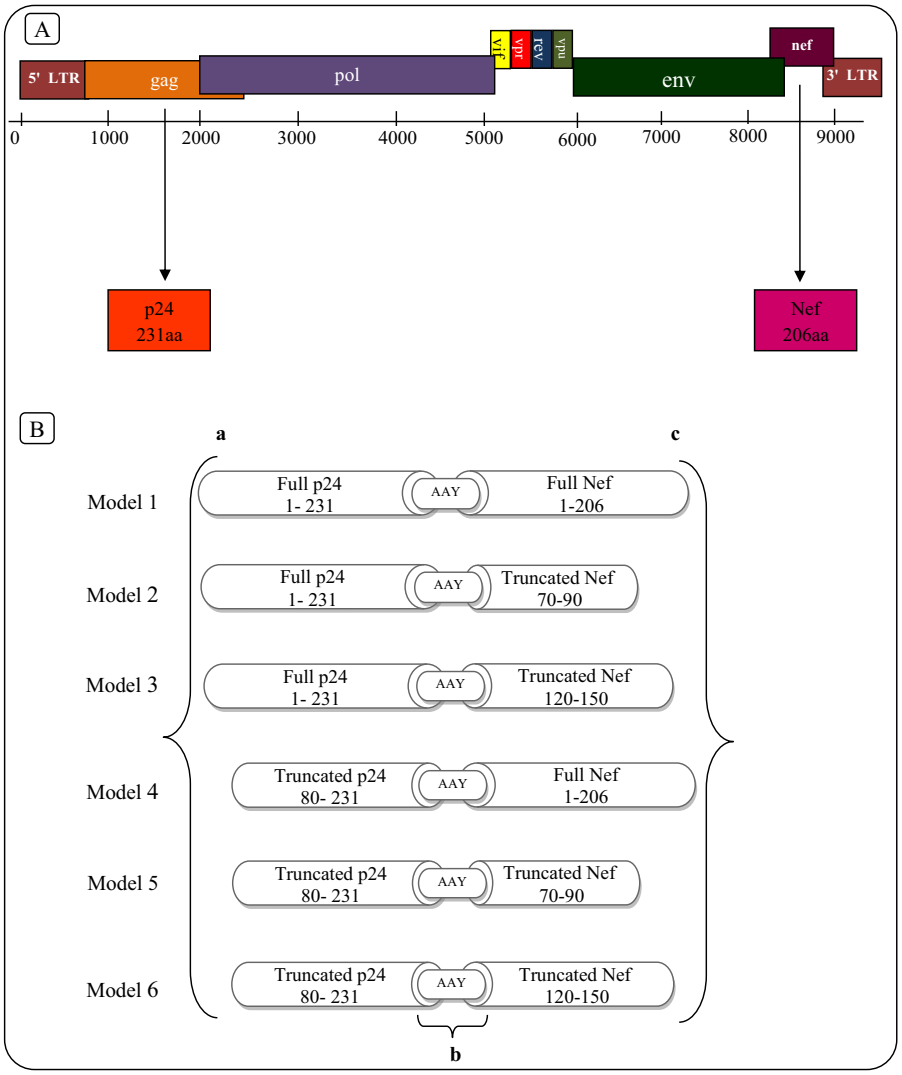

Fig. (3). Schematic representation of HIV genome arrangement indicating p24 and Nef origins (**A**). Schematic representation of six predicted models from HIV-1 p24 and Nef sequences (**B**), The amino acids from p24 (**a**), The flexible spacer (**b**), The amino acids from Nef (**c**).

Table 1. In silico analysis of predicted BALB/c MHC class I epitopes.

Antigenicity Score	Percentile Rank	Allele	Length	*Position	Peptide
Predicted Model 1					
0.400	0.4	H-2-Dd	9	15-23	ISPRTLNAW
0.658	0.4	H-2-Dd	9	32-40	FSPEVIPMF
1.763	0.4	H-2-Dd	9	363-371	PGPGVRYPL
-0.099	0.5	H-2-Kd	9	142-150	VRMYSPTSI
0.450	0.6	H-2-Kd	9	233-241	AYMGGKWSK
0.228	0.7	H-2-Kd	9	7-15	QGQMVHQAI
0.324	0.7	H-2-Dd	9	83-91	VHPVHAGPI
1.880	0.7	H-2-Dd	9	222-230	GGPGHKARV
0.786	1	H-2-Ld	9	2-10	IVQNIQGQM
-0.317	1	H-2-Dd	9	88-96	AGPIAPGQM

(Table 1) contd….

Antigenicity Score	Percentile Rank	Allele	Length	*Position	Peptide
Predicted Model 5					
-0.099	0.5	H-2-Kd	9	63-71	VRMYSPTSI
0.324	0.7	H-2-Dd	9	4-12	VHPVHAGPI
1.880	0.7	H-2-Dd	9	143-151	GGPGHKARV
-0.317	1	H-2-Dd	9	9-17	AGPIAPGQM
1.763	0.4	H-2-Dd	9	165-173	PGPGVRYPL
Predicted Model 6					
1.763	0.4	H-2-Dd	9	165-173	PGPGVRYPL
-0.099	0.5	H-2-Dd	9	63-71	VRMYSPTSI
0.324	0.7	H-2-Dd	9	4-12	VHPVHAGPI
1.880	0.7	H-2-Dd	9	143-151	GGPGHKARV
-0.317	1	H-2-Dd	9	9-17	AGPIAPGQM

BALB/c MHC I interacting with 9-mer peptides of predicted models were sorted based on percentile rank calculated by IEDB tools. Model 1: Full p24-AAY-Nef, Model 5: p24 (80-231)-AAY-Nef (70-90), Model 6: p24 (80-231)-AAY-Nef (120-150).
*Positions for peptides are as: Model 1:1-231 aa of p24, 232-234 aa of linker, 234-440 aa of Nef.
Model 5: 1-152 aa of p24, 153-155 aa of linker, 156-176 aa of Nef.
Model 6: 1-152 aa of p24, 153-155 aa of linker, 156-186 aa of Nef
Antigenicity scores ≥ 0.5 are shown in bold.

Table 2. In silico analysis of predicted BALB/c MHC class II.

Antigenicity Score	Percentile Rank	Allele	Length	*Position	Peptide
Predicted Model 1					
0.170	0.28	H2-IAd1	15	310-324	LRPMTYKAAVDLSHF
0.216	0.62	H2-IAd1	15	167-181	RFYKTLRAEQASQEV
0.397	0.62	H2-IAd1	15	309-323	PLRPMTYKAAVDLSH
0.583	0.64	H2-IAd1	15	169-183	YKTLRAEQASQEVKN
0.548	0.96	H2-IAd1	15	168-182	FYKTLRAEQASQEVK
Predicted Model 5					
0.170	0.28	H2-IAd1	15	162-176	LRPMTYKAAVDLSHF
0.397	0.61	H2-IAd1	15	161-175	PLRPMTYKAAVDLSH
0.216	0.62	H2-IAd1	15	88-102	RFYKTLRAEQASQEV
0.583	0.64	H2-IAd1	15	90-104	YKTLRAEQASQEVKN
0.548	0.95	H2-IAd1	15	89-103	FYKTLRAEQASQEVK
Predicted Model 6					
0.216	0.62	H2-IAd1	15	88-102	RFYKTLRAEQASQEV
0.583	0.64	H2-IAd1	15	90-104	YKTLRAEQASQEVKN
0.548	0.95	H2-IAd1	15	89-103	FYKTLRAEQASQEVK

BALB/c MHC II interacting with 15-mer peptides of predicted models were sorted based on percentile rank calculated by IEDB tools. Model 1: Full p24-AAY-Nef, Model 5: p24 (80-231)-AAY-Nef (70-90), Model 6: p24 (80-231)-AAY-Nef (120-150). Antigenicity scores >0.5 are shown in bold.
*Positions of peptides are as: Model 1:1-231 aa of p24, 232-234 aa of linker, 234-440 aa of Nef.
Model 5: 1-152 aa of p24, 153-155 aa of linker, 156-176 aa of Nef.
Model 6: 1-152 aa of p24, 153-155 aa of linker, 156-186 aa of Nef
Peptides with antigenicity score ≥ 0.5 are bold.

Table 3. Predicted epitopes of Model 5 interacting with human MHC class I.

Toxicity	Antigenicity	Percentile Rank	HLA class I	Length	*Position	Peptide
Non-Toxin	**0.9776**	0.3	HLA-B*57:01	9	30-38	STLQEQIGW
Non-Toxin	0.1162	0.4	HLA-A*32:01	9	64-72	RMYSPTSIL
Non-Toxin	0.1544	0.4	HLA-B*35:03	9	154-162	AYVTPQVPL
Non-Toxin	**1.0925**	0.4	HLA-B*51:01	9	42-50	NPPIPVGEI
Non-Toxin	0.3978	0.45	HLA-B*57:01	9	97-105	QASQEVKNW
Non-Toxin	0.1389	0.5	HLA-A*32:01	9	165-173	MTYKAAVDL
Non-Toxin	**2.0840**	0.5	HLA-B*27:05	9	52-60	KRWIILGLN
Non-Toxin	-0.6939	0.55	HLA-A*11:01	9	83-91	RDYVDRFYK
Non-Toxin	-0.6939	0.6	HLA-A*03:01	9	83-91	RDYVDRFYK
Non-Toxin	**1.6770**	0.6	HLA-B*27:05	9	51-59	YKRWIILGL
Non-Toxin	0.2740	0.6	HLA-B*27:02	9	63-71	VRMYSPTSI
Non-Toxin	**0.7651**	0.6	HLA-B*51:01	9	152-160	LAAYVTPQV
Non-Toxin	-2.0476	0.6	HLA-A*01:01	9	82-90	FRDYVDRFY
Non-Toxin	**2.3974**	0.65	HLA-A*03:01	9	71-79	ILDIRQGPK
Non-Toxin	**0.9826**	0.65	HLA-B*57:01	9	168-176	KAAVDLSHF
Non-Toxin	**0.5307**	0.75	HLA-A*26:01	9	24-32	DIAGTTSTL
Non-Toxin	**1.5757**	0.8	HLA-B*35:01	9	5-13	HPVHAGPIA
Non-Toxin	-0.2336	0.8	HLA-A*11:01	9	160-168	VPLRPMTYK
Non-Toxin	**0.6413**	0.8	HLA-B*57:01	9	124-132	KALGPAATL
Non-Toxin	0.1162	1	HLA-A*03:01	9	64-72	RMYSPTSIL
Non-Toxin	-0.2336	1	HLA-A*03:01	9	160-168	VPLRPMTYK
Non-Toxin	**1.1492**	1	HLA-B*51:01	9	114-122	NANPDCKTI

HLA: Human Leucocyte Antigen. Most probable predicted epitopes interacting with different MHC class I alleles of Model 5[p24(80-231)-AAY-Nef(70-90)]. The toxicity of each peptide is also predicted.
*Positions of peptides are as: 1-152 aa of p24, 153-155 aa of linker, 156-176 aa of Nef.
Peptides with antigenicity score ≥ 0.5 are bold.

3.4. Population Coverage and Conservancy Analysis

Peptides that are predicted to interact with MHC I and II molecules in the selected Model 6 were tested for population coverage analysis using the IEDB population coverage tool to cover most HIV chronic infected individuals specifically Persian population. In addition, we selected Southwest Asia, South Asia, Europe, North America, South America and Africa continent. (http://tools.immuneepitope.org/tools/population/iedb_input. The results of total population coverage in Persian and the other populations are listed in Table **6**. The selected Model 6 has an acceptable coverage of 73.67% for MHC class I and 85.21% for MHC class II in Persian population (For predicted model 5 see supplementary Table **7**).

In order to testify the Conservancy of predicted peptides of Model 6, we used IEDB tool (http://tools.iedb.org/ conservancy/). Thus all peptides (with an antigenic score >0.5) were submitted against related p24 and Nef sequences at a high threshold. Subsequently, we identified fully conserved (100%) epitopes, KRWIILGLN, YKRWIILGL, FPDWQ NYTP and DIAGTTSTL, which suggest important roles in the virus life cycle and infectivity. Moreover, ILDIRQGPK (96.3%) and RYPLTFGWC (95%) and NPPIPVGEI (85.19%) were greatly preserved among all clades.

3.5. Toxicity Analysis

An important feature of a vaccine is the ability to induce a particular immune response which only targets the virus, not the host tissue. To achieve that, Model 6 at the final step was examined for toxicity using ToxinPred tool. As shown in Table **5**, it is evaluated non-toxic in all 15-mer peptides and also it is non-toxic as a protein in form of the full-length sequence.

Table 4. Predicted epitopes of Model 6 interacting with human MHC class I.

Toxicity	Antigenicity	World PPC%	Percentile Rank	HLA class I	Length	*Position	Peptide
Non-Toxin	**1.9654**	1.49	0.2	HLA-B*27:02	9	169-177	VRYPLTFGW
Toxin	**1.4541**	19.0	0.2	HLA-B*35:01	9	171-179	YPLTFGWCY
Non-Toxin	**0.5029**	17.72	0.3	HLA-A*26:01	9	163-171	YTPGPGVRY
Non-Toxin	**0.9776**	3.47	0.3	HLA-B*57:01	9	30-38	STLQEQIGW
Non-Toxin	**0.5922**	3.47	0.35	HLA-B*57:01	9	152-160	LAAYYFPDW
Non-Toxin	0.1162	16.06	0.4	HLA-A*32:01	9	64-72	RMYSPTSIL
Non-Toxin	**1.0905**	9.96	0.4	HLA-B*51:01	9	42-50	NPPIPVGEI
Non-Toxin	0.3978	3.47	0.45	HLA-B*57:01	9	97-105	QASQEVKNW
Non-Toxin	**2.0840**	3.59	0.5	HLA-B*27:05	9	52-60	KRWIILGLN
Non-Toxin	-0.0640	3.47	0.5	HLA-B*57:01	9	148-156	KARVLAAYY
Non-Toxin	-0.6939	26.29	0.55	HLA-A*11:01	9	83-91	RDYVDRFYK
Non-Toxin	-0.6939	26.29	0.6	HLA-A*03:01	9	83-91	RDYVDRFYK
Non-Toxin	**1.6770**	3.59	0.6	HLA-B*27:05	9	51-59	YKRWIILGL
Non-Toxin	-0.1074	3.59	0.6	HLA-B*27:05	9	149-157	ARVLAAYYF
Non-Toxin	**1.4425**	3.36	0.6	HLA-B*35:03	9	157-165	FPDWQNYTP
Non-Toxin	0.2740	1.49	0.6	HLA-B*27:02	9	63-71	VRMYSPTSI
Non-Toxin	**1.4541**	9.96	0.6	HLA-B*51:01	9	171-179	YPLTFGWCY
Non-Toxin	-2.0476	13.77	0.6	HLA-A*01:01	9	82-90	FRDYVDRFY
Non-Toxin	**2.3974**	26.29	0.65	HLA-A*03:01	9	71-79	ILDIRQGPK
Non-Toxin	**0.5029**	13.77	0.65	HLA-A*01:01	9	163-171	YTPGPGVRY
Non-Toxin	**0.5307**	17.72	0.75	HLA-A*26:01	9	24-32	DIAGTTSTL
Non-Toxin	-0.1074	1.49	0.8	HLA-B*27:02	9	149-157	ARVLAAYYF
Non-Toxin	**1.5757**	19.0	0.8	HLA-B*35:01	9	5-13	HPVHAGPIA
Non-Toxin	**0.6413**	3.47	0.8	HLA-B*57:01	9	124-132	KALGPAATL
Toxin	**0.8826**	3.47	0.8	HLA-B*57:01	9	173-181	LTFGWCYKL
Non-Toxin	0.1162	26.29	1	HLA-A*03:01	9	64-72	RMYSPTSIL
Non-Toxin	**0.7327**	22.38	1	HLA-A*24:02	9	155-163	YYFPDWQNY
Non-Toxin	**1.4649**	22.38	1	HLA-A*24:02	9	170-178	RYPLTFGWC
Non-Toxin	**1.1841**	9.96	1	HLA-B*51:01	9	114-122	NANPDCKTI

HLA: Human Leucocyte Antigen. Most probable predicted epitopes interacting with different MHC class I alleles of Model 6 [p24 (80-231)-AAY-Nef (120-150)].
*Positions of peptides are as:Model 6: 1-152 aa of p24, 153-155 aa of linker, 156-186 aa of Nef
The toxicity of each peptide is also predicted.
Peptides with antigenicity score ≥0.5 are bold.

Table 5. List of epitopes with high affinity to human MHC class II alleles of selected Model 6.

Toxicity	Antigenicity	World PPC%	Percentile Rank	*Position	HLA class II	Peptide
Non-Toxin	0.5393	56.84	0.07	61-75	HLA-DRB1*15:02	KIVRMYSPTSILDIR
			0.11		HLA-DRB1*04:09	
			0.12		HLA-DRB1*04:08	
			0.13		HLA-DRB1*04:06	
			0.13		HLA-DRB1*04:03	
			0.14		HLA-DRB1*04:10	
			0.2		HLA-DRB1*11:04	
			0.2		HLA-DRB1*11:06	
			0.27		HLA-DRB1*04:02	
			0.32		HLA-DRB1*04:07	
			0.33		HLA-DRB1*11:07	
			0.33		HLA-DRB1*11:02	
			0.37		HLA-DRB1*04:01	
			0.39		HLA-DRB1*04:04	
			0.44		HLA-DRB1*13:01	
			0.44		HLA-DRB1*07:03	
			0.52		HLA-DRB1*11:01	
			0.59		HLA-DRB1*13:07	
			0.62		HLA-DRB1*13:05	
			0.69		HLA-DRB1*13:10	
			0.74		HLA-DRB1*13:09	
			0.81		HLA-DRB1*13:03	
			0.89		HLA-DRB1*04:05	
Non-Toxin	0.6369	48	0.07	62-76	HLA-DRB1*15:02	IVRMYSPTSILDIRQ
			0.12		HLA-DRB1*04:08	
			0.14		HLA-DRB1*04:10	
			0.2		HLA-DRB1*11:04	
			0.2		HLA-DRB1*11:06	
			0.27		HLA-DRB1*04:02	
			0.33		HLA-DRB1*11:07	
			0.33		HLA-DRB1*11:02	
			0.41		HLA-DRB1*04:04	
			0.44		HLA-DRB1*13:01	
			0.44		HLA-DRB1*07:03	
			0.55		HLA-DRB1*11:01	
			0.57		HLA-DRB1*04:01	
			0.59		HLA-DRB1*13:07	
			0.62		HLA-DRB1*13:05	

(Table 5) contd….

Toxicity	Antigenicity	World PPC%	Percentile Rank	*Position	HLA class II	Peptide
Non-Toxin	1.0474	20.9	0.07	63-77	HLA-DRB1*15:02	VRMYSPTSILDIRQG
			0.44		HLA-DRB1*13:01	
			0.44		HLA-DRB1*07:03	
			0.53		HLA-DRB1*11:02	
			0.95		HLA-DRB1*04:02	
Non-Toxin	0.7361	11.3	0.4	47-61	HLA-DRB1*15:02	VGEIYKRWIILGLNK
			0.55		HLA-DRB1*13:05	
			0.77		HLA-DRB1*07:03	
Non-Toxin	0.5159	11.59	0.4	48-62	HLA-DRB1*15:02	GEIYKRWIILGLNKI
			0.45		HLA-DRB1*13:07	
			0.55		HLA-DRB1*13:05	
			0.6		HLA-DRB1*04:08	
			0.77		HLA-DRB1*07:03	

The predicted epitopes interacting with different MHC class II alleles of Model 6 [p24(80-231)-AAY-Nef(120-150)]. The toxicity of each peptide is also predicted.
*Positions of peptides are as: 1-152 aa of p24, 153-155 aa of linker, 156-186 aa of Nef.
Peptides with antigenicity score ≥0.5 are bold.

Table 6. Population coverage of selected Model 6.

Population	MHC I PPC	Average of Epitope Hits	MHC II PPC	Average of Epitope Hits
IRAN	73.67%	2.08	85.21%	12.05
Southwest Asia	64.89%	1.08	60.48%	7.94
South Asia	75.96%	2.25	66.14%	8.06
Europe	84.15%	3.19	72.28%	8.65
North America	71.44%	2.25	72.08%	8.49
South America	50.67%	1.3	53.48%	5.93
Africa	43.95	1.22	36.65%	4.48

PPC: Percent of Population Coverage. Model 6 evaluation of population coverage for MHC I and MHC II using IEDB tool shows an acceptable average of coverage among different population worldwide.

3.6. Primary Structure Analysis

Physiochemical characterization of selected Model 6 fusion protein was obtained using Expasy's ProtParam server which estimated molecular weight, theoretical isoelectric point and average hydropathicity that indicates the solubility and hydrophobicity of protein. The fusion Model 6 with 186 amino acids and 20.958 Da with pI: 8.75 and 17 positively charged residues (Arg+ Lys) in the polypeptide and 20 negatively charged residues (Asp+Glu). This Model is also predicted to be soluble and hydrophilic (Grand average of hydropathicity (GRAVY)**:** -0.397).

3.7. Secondary Structure Analysis and Disulfide Connectivity

We applied SOPMA tool to predict secondary structure of Model 6 features including beta turns, helices, random coils contribution and C-score. The greater ratio of random coils and extended strands is correlated with an increase in forming of protein antigenic epitope. As the result, it is composed of 35.48% α-helix and 6.99% β-sheet which besides the highest level of random coils (41.40%) can form higher antigenic epitopes. Fusion protein Model 6 has 1 predicted Disulfide Bridge at 139 – 178 (p24: EMMTACQGVGG – Nef: LTFGWCYKLVP) which renders extracellular stability.

3.8. Tertiary Structure Prediction and Validation

Three-dimensional structure of Model 6 was predicted using the I-TASSER online server which generates five top models with C-scores ranging from-5 to 2. The one with the highest C-score represents the best model. This value of selected Model 6 was in the acceptable confidence (–1.93). Chimera version v1.2 was applied to generate the protein image [62]. Moreover, Ramachandran plot analysis through RAMPAGE of the predicted Model 6 resulted in 81% in the favored region and showed that most of the residues are in the allowed regions (Fig. **4**).

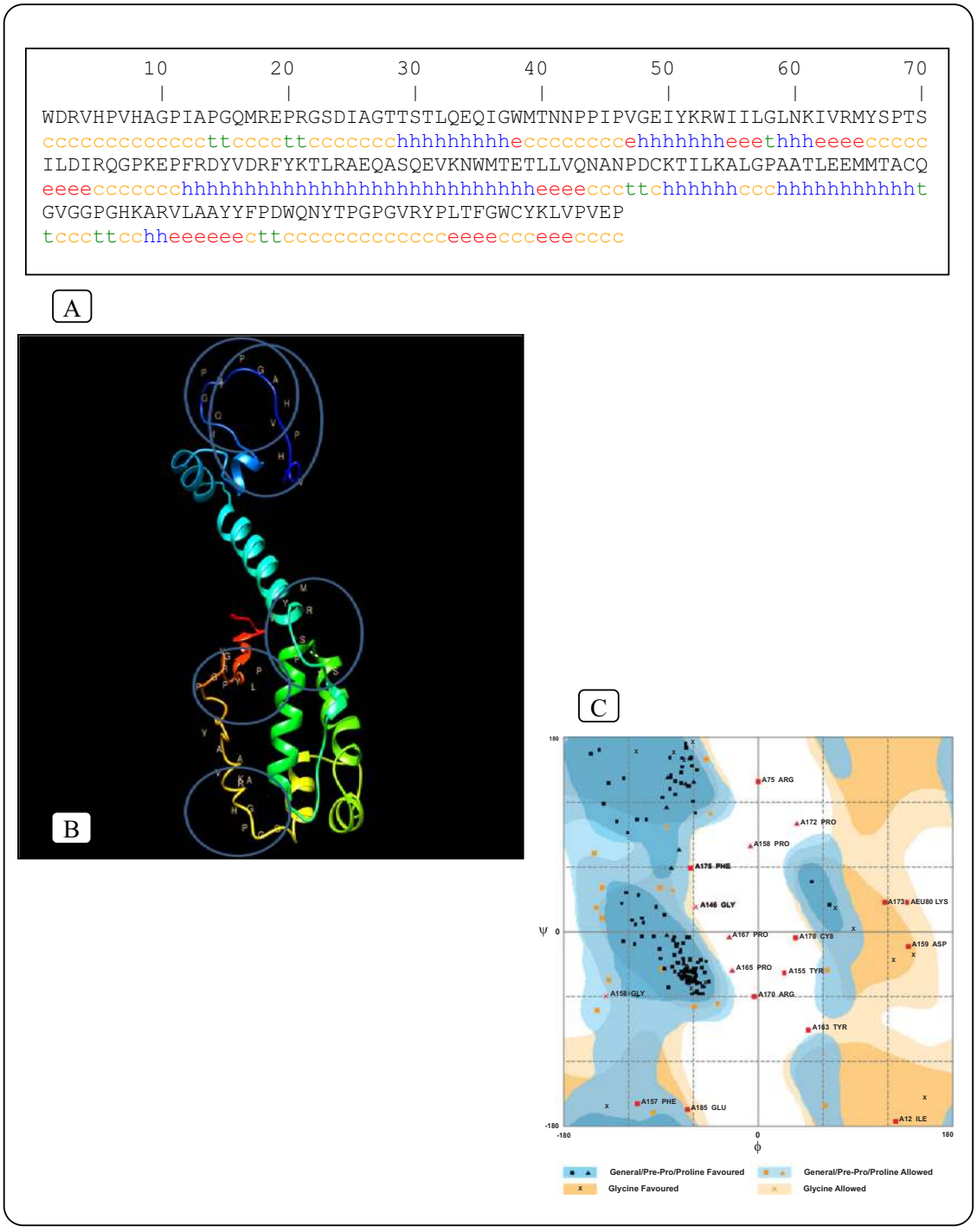

Fig. (4). Sequence and structural analysis of Model 6. Secondary structure of the protein with respect to protein sequence (**A**) and Predicted 3D model of selected P24-AAY_Nef protein obtained from I-TASSER with the epitopic regions indicated in circles (**B**). Image was produced using the Swiss PDB viewer and chimera version v1.2. (**C**) Ramachandran plot is showing validation of protein structure using the phi and psi angles distribution in the protein. The Ramachandran plot shows that 81 % of amino acid residues of modeled structure were incorporated in the favored regions of the plot. Ninety-two percent of the residues were in allowed regions of the plot and 9.8 % of residues were in outlier regions.

4. DISCUSSION

The ability of HIV-1 virus to develop high levels of genetic diversity, and therefore acquiring mutations to escape immune pressures, leads to the difficulties in producing a vaccine [63].

The other main cause of this failure may stem from the fact that in infected patients, continuous antigenic stimulation leads to defect in cytotoxic T lymphocytes (CTLs) activation [27, 63]. Therefore, the immunogenic parts of the virus with the ability to recall the cellular immune response

would be a great approach in a therapeutic vaccine model. In silico study lets us have a pre-experimental vision and the opportunity to evaluate these parameters based on the pool of submitted data from all over the world in order to carry out the more accurate experiment [64]. Compared to safety and cost limited experimental procedures in vaccine development, bioinformatics analysis provides us with a significant possibility to design vaccines on the basis of numerous tools, methods and databases [65, 66].

In the current study, we have predicted a fusion protein of two highly immunogenic HIV-1 proteins, p24 and Nef for designing a subunit therapeutic vaccine. Subunit vaccine which targets a specific immunogenic protein would be helpful in generating a strong immune response in the host [67, 68]. These two proteins first were studied by Mahdavi et al, as a fusion peptide vaccine p24 (aa159–173) and Nef (aa102–117). They applied this fusion peptide with complete Freund's adjuvant on BALB/c mice. As they reported, the adjuvanted fusion peptide induced lymphocyte proliferation, CTL response, and cytokines and IL-4 and IFN-γ in the Th1 pathway. They also stated that humoral immune response to the adjuvanted fusion peptide led to an increase in IgG2a more than IgG1 subtype which indicates that the applied HIV-1 peptide construct can elicit both cellular and humoral immune responses in an animal model [69].

Gonzalez *et al.* investigated the immunogenicity of p24-Nef in mice, using cholera toxin B subunit as an adjuvant. They used purified chloroplast-derived p24 and immunize BALB/c mice and claimed that this purification method elicited a strong antigen-specific serum IgG response in comparison with that produced by Escherichia coli-derived p24. They also reported that oral administration of a partially purified preparation of chloroplast-derived p24-Nef-fusion protein as a booster either Nef or p24, resulted in strong antigen-specific IgG1 and IgG2a associated responses and concluded that HIV-1 p24-Nef is a promising strategy as a component of a subunit vaccine [70]. These two studies suggest that the fusion form of p24-nef as a peptide and protein vaccine have resulted in acceptable cellular and humoral responses in BALB/c.

The present study aimed at a full investigation of in silico design to choose the best region of these proteins.

There is no doubt that in such variable and highly mutated virus, designing a subunit vaccine based on the most conserved area with the affinity to MHC class I and also class II molecules is highly reasonable [71, 72].

We applied Nef because it is highly variable and immunogenic among HIV-1 serotypes [73]. What is more, Nef is the most important viral proteins in immune evasion [74, 75]. In addition, p24 is immunologically important and is the key factor for viral maturation and assembly [76]. Moreover, it is highly conserved at a stretch of 20 residues (from 152 to173 amino acid). This uniquely conserved region at the C-terminal of p24 suggests an important role in the retroviral life cycle [29, 77, 78]. In our study, we focused on the region from 80-231 with 152 amino acids with a high rate of conservancy containing MHR, too.

Available submitted sequences of HIV-1 in NCBI database including those from Persian isolates were aligned and compared to the reference sequences of both protein. At the next step, we evaluated six possible fusion forms of these two HIV-1 proteins in a full length and truncated forms, too. Generally, the full lengths of both proteins include more MHC molecule binding regions. Nevertheless, they are not all conserved nor epitopic. According to our result, we focused on the two truncated possible forms, Model 5 and Model 6 based on the T -cell epitopes localization in conserved regions. Then, we compared them on the basis of their physiochemical properties, ability to human HLA binding for both classes I and II, antigenicity and toxicity score. Although the estimated scores in physiological and structural parameters were almost similar between the two forms, their ability for CTL binding was different.

Fusion protein Model 6 contains five regions which interact with BALB/c MHC class I molecules. Among them, PGPGVRYPL and GGPGHKARV show the high antigenicity score, too. In comparison with the full-length protein, Model 1, which contains 10 peptides with affinity to MHCI, only 4 peptides have high antigenicity scores. It is to say that, the frequency of binders are higher in the full-length fusion form of p24 and Nef, however, only less than half of them are epitopic regions.

To insure that this is the more preferable form, we also examined human MHC class I and class II for both truncated models. According to the summarized results (Tab.4), the truncated Model 6 contains 25 peptides which have an affinity to MHC class I molecules, from which seventeen epitopes with high antigenicity score were determined. The number of peptides reduces to 19 with 11 epitopic regions in Model 5 for selected types of HLAs with a higher rate of prevalence in the Persian population.

Among all the predicted MHC I epitopes, ILDIRQGPK and KRWIILGLN have higher Vaxijen scores which derived from p24 protein (2.3974, 2.0840). In addition to antigenic nature, they are highly conserved among different subtypes with 96.3% and 100% conservancy. What is more, YKRWIILGL, DIAGTTSTL and FPDWQNYTP epitopes show 100% conservancy between the clades. There are also two epitopes, VRYPLTFGW and RYPLTFGWC, with high antigenicity score which stem from Nef. On the other words, both parts of the fusion protein contain immune dominant T -cell epitopes. Taken together, this fusion protein includes highly conserved antigenic T -cell epitopes from both p24 and Nef fragments. From another point of view, YPLTFGWCY interacts with HLA-B*35:01 and HLA-B*51:01 and NANPDCKTI interacts with HLA-B*51:01 which are common in the Persian population. Moreover, ILDIRQGPK interacts with HLA-A*03:01 which is popular among most of the world population.

The predicted peptides of the chosen Model 6 with affinity to human MHC I, showed 73.67% Persian population coverage and nearly 63% whole world coverage, too. There are also 63 peptides that have an affinity to MHC class II with 85.21% Persian population coverage. It also has high population coverage in Europe, America and Southwest Asia for both MHC classes (Table **6**). The toxicity examine showed that this fusion protein is totally non-toxic. In other words, the fusion Model 6, has acceptable population coverage and preferably is non-toxic.

Finally, we came up with the most suitable fusion model 6 based on containing more HLA class I binders which are conserved and epitopic with acceptable population coverage. Therefore this protein would be capable to trigger the cellular immune response in BALB/c.

Eventually, a new fused construct of HIV-1 proteins including p24 (80-231) and Nef (120-150) [NP_579880 and NP_057857] was selected among six possible fusion forms for further evaluation of cellular immune response as a therapeutic vaccine in BALB/c.

CONCLUSION

The procedure of this study provides us with an analysis of possible fusion forms of two immunogenetic HIV-1 proteins to design the lab experiment. In silico study of p24 and Nef as a newly designed construct with a truncated form of both fragments showed strong MHC binders and CTL epitopes which are supposed to provoke a cellular immune response with a high rate of Persian population coverage. Therefore, this data as the first step supports p24 (80-231)-AAY-Nef (120-150) as a therapeutic vaccine formulation which may help us to achieve a novel strategy against HIV-1.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

M. Sadat Larijani was supported by Pasteur Institute of Iran to pursue her study in the Ph.D. thesis.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publishers web site along with the published article.

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