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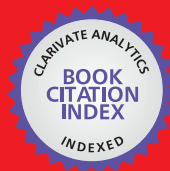
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Artificial Epitope-Based Immunogens in HIV-Vaccine Design

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Abstract

One of the promising approaches for designing HIV vaccines is construction of synthetic polypeptide HIV-1 immunogen using a wide range of conservative T- and B-cell epitopes of the main virus antigens. In theory this approach helps cope with HIV-1 antigenic variability, focuses immune responses on protective determinants and enables to exclude from the vaccine compound adverse regions of viral proteins that can induce autoantibodies or antibodies enhancing infectivity of virus. The paper presents the experience of our team in development of artificial polypeptide HIV-1 immunogens, which can induce both a humoral response, and responses of cytotoxic (CD8 + CTL) and helpers (CD4 + Th) T-cells. The design of HIV-immunogens has been done using our original software, TEpredict and PolyCTLDesigner. We describe development of the candidate HIV-1/AIDS vaccine – CombiHIVvac, which included two artificial polypeptide immunogens TBI and TCI for stimulating humoral and cellular responses. The results of the specific activity and safety of CombiHIVvac vaccine, obtained during preclinical and clinical trials, are presented.

Keywords: HIV-1 vaccine, artificial polypeptide T- and B-cell immunogens, rational design, preclinical and clinical trials

1. Introduction

For over 200 years a vaccine development effort was to isolate microbes or viruses and prepare a killed or attenuated pathogen vaccine. For many self-limiting bacterial and viral infections

the vaccine strategy is to mimic the infectious process and natural immunity against a particular pathogen. However, for HIV-1 and for many other chronic viral, bacterial, fungal and parasitic infections, and cancer natural immunity is insufficient for protection [1, 2]. Novel and effective approaches in polyepitope HIV-vaccine development are needed today [3, 4].

Problems that retard development of HIV-1 vaccine are well-known. Firstly, HIV-1 has a high rate of escape mutations with the result that the virus can change the antigenic structure quicker than the immune system is switched to new antigenic variants. Secondly, it is still unclear which type of immune response is more significant when preventing infection: induction of HIV neutralizing antibodies (in systemic vs. mucosal compartments), CD4⁺ T-helper cells, cytotoxic CD8⁺ T-cells (both potent high avidity CD8⁺ T-effector/memory responses and central memory responses), innate immunity, or all factors together. However, recent publications demonstrate that a humoral response to vaccine may be critical to prevent acquisition of HIV, while CD8⁺ T-cells may be required to control viral replication in vaccinated individual. Thirdly, virus proteins include regions with pathogenic properties due to molecular mimicry of physiologically significant functions or induction of autoimmune responses that might contribute to immunodeficiency. Finally, when studying HIV-infection, experimental models are very limited [1, 5–7].

Well-known HIV-1 vaccine design strategies are based on the use of different forms of viral antigen including inactivated virus, modified or attenuated virus, native and genetically engineered proteins, and peptides [5]. The first generation candidate vaccines (such as AIDSVAX B/B and AIDSVAX B/E) were constructed for inducing humoral immunity, to elicit virus-neutralizing antibodies. Development of such vaccines was based on the use of full-length proteins of HIV envelope or their fragments [8]. The second generation vaccines (e.g. Merck Ad5 gag/pol/nef of B subtype) were aimed to mediate protection by inducing HIV-specific cytotoxic T-lymphocytes (CTLs) capable to recognize and eliminate HIV-infected cells [9]. Many candidates were tested in human or animals; however, none of them has demonstrated efficacy in phase II-III trials [10].

The first promising and statistically significant results were obtained in clinical trials of RV144 vaccine stimulating both humoral and cellular immunity. It is a combination of two previously developed vaccines ALVAC-HIV (Sanofi Pasteur) and AIDSVAX B/E gp120 (VAXGEN) [11]. Despite rather low protective efficacy (31.2%), RV144 clinical trials made it possible to draw several weighty conclusions, i.e. (1) HIV-1 vaccine is not a myth but a reality; (2) efficient vaccine should induce both humoral and cell immune responses against HIV-1, and (3) new approaches are needed to increase vaccine efficacy [2, 3, 12].

One of them includes construction of completely artificial polyepitope (mosaic) anti-HIV-1 immunogens comprising a broad range of protective T- and B-cell epitopes based on the main viral antigens capable of inducing production of neutralizing antibodies and responses of cytotoxic (CD8⁺ CTL) and helper (CD4⁺ Th) T-lymphocytes. This approach seems to be rather promising when developing new generation HIV-vaccines. In theory, it makes it possible to overcome HIV-1 antigenic variability, focus immune responses on protective epitopes and allows to exclude undesirable determinants from a vaccine compound capable of inducing autoantibodies or antibodies increasing virus infectivity [3, 4, 13]. This paper discusses our experience in designing artificial polyepitope antigens – HIV-1 candidate vaccines.

2. Artificial TBI and TCI immunogens

The first immunogen designed in our project, short for T- and B-cell epitopes containing Immunogen (TBI), was constructed with the use of conservative epitopes from Env and Gag HIV-1 based on a well-known protein space motif, i.e. four helix bundle (**Figure 1**). When designing immunogen, four Th-cell epitopes (amphipathic α -helix) and five B-cell epitopes (regions with flexible hydrophilic loops) were used as blocks [14, 15]. The rationale for TBI design was that combining T- and B-cell epitopes in one construct will stimulate both proper B-cell and T-cell responses and the necessary interplay between B- and T-cells. Recombinant protein TBI has a CD spectra similar to ones in α -helical proteins and was able to form crystals - that was demonstrated for artificial protein with a predicted tertiary structure for the first time [16]. Based on its ability to crystallize we assumed that TBI protein structure is similar to that of the natural proteins.

Mice and Macaque rhesus immunized with TBI formed both cell and humoral responses to HIV-1. TBI-induced antibodies showed virus-neutralizing activity to HIV-1 [17].

The second artificial polypeptide immunogen we developed was TCI (short for T-Cell Immunogen) aimed at stimulation of T-cell immunity [18]. When constructing immunogen, we selected highly conservative T-cell epitopes among three main HIV-1 subtypes (A, B, and C) (**Figure 2**). TCI comprises more than 80 T-cell epitopes (both CD8+ CTL and CD4+ Th) from Env, Gag, Pol, and Nef proteins [18]. We analyzed CTL-epitopes that were together restricted with 10 different optimally selected alleles of human MHC class I. As known, it is sufficient to cover genetic diversity of MHC class I antigens in population from almost all geographic regions. Since antigen processing and presentation through MHC class I pathway were found to be the most efficient for proteins synthesized inside the cell, the target vaccine construct was designed in the form of DNA-vaccine via cloning a gene encoding TCI protein into vector plasmid pcDNA3.1 [18].

The obtained DNA-vaccine pcDNA-TCI was used for genetic immunization; we showed that the vaccine is capable of inducing both specific T-cell responses and specific antibodies in immunized BALB/c mice [18–20].

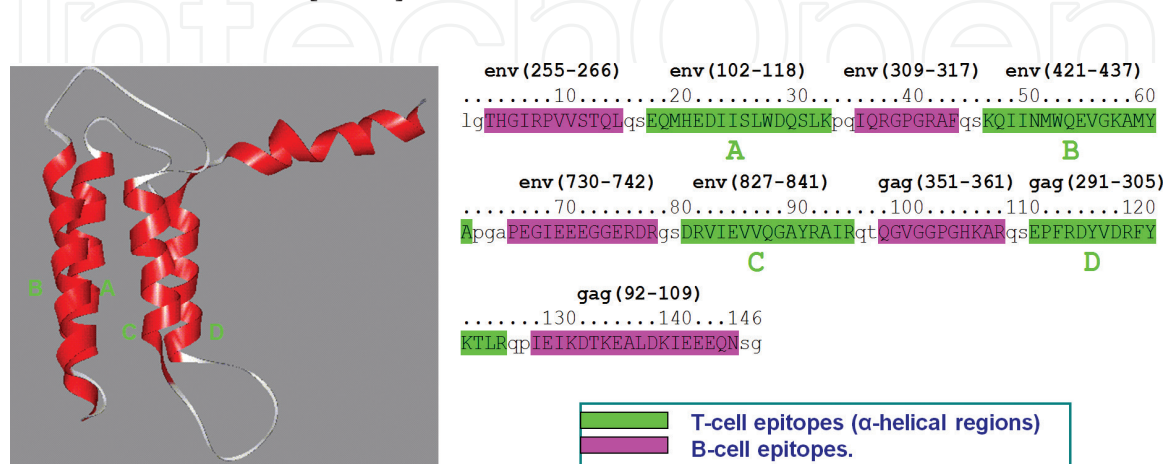


Figure 1. A model of TBI protein tertiary structure. T-cell epitopes are located in the region of α -helices, B-cell epitopes are located at loop sites and N- and C-terminuses.

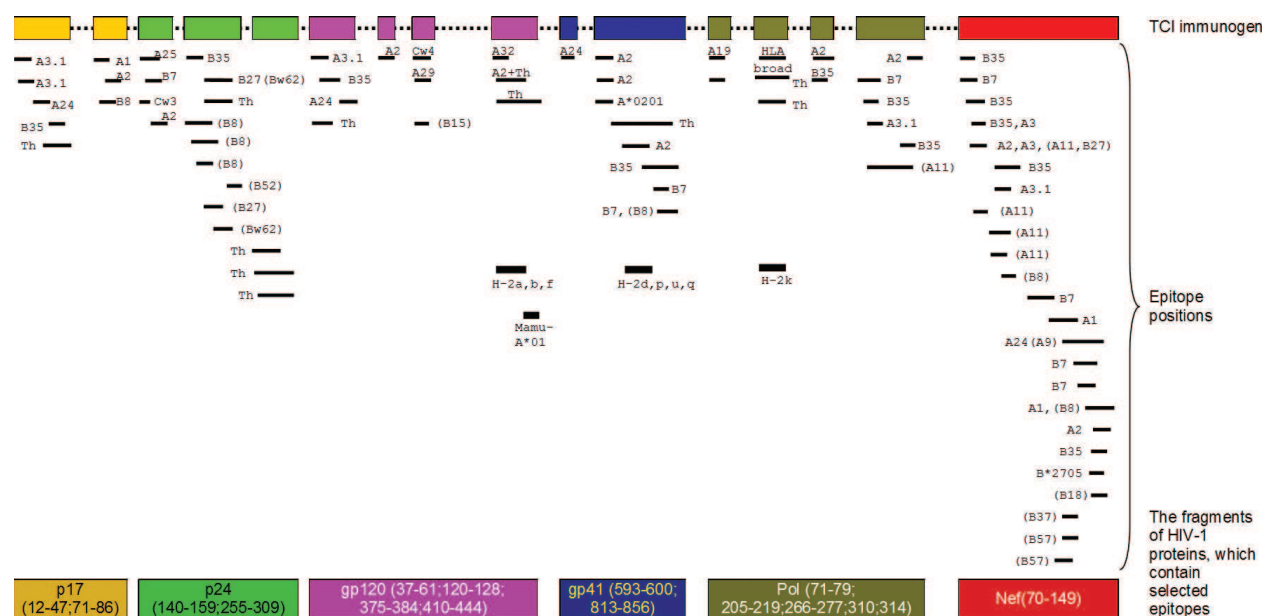


Figure 2. Design of the CTL immunogen, a candidate for the in HIV-1 vaccine: a general schematic. The bar patterns indicate the polyepitope CTL immunogen and the origin of the sequences. The positions of individual epitopes and their MHC restrictions (HLA-A, B, Cw – human; H-2a, b, d, f, k, p, u, q – mouse; Mamu-A*01 – *Macaca mulatta*) are depicted as lines below the CTL immunogen. Th stands for helper epitopes.

In the following, recombinant protein TBI and plasmid pcDNA-TCI were used for the development of CombiHIVvac vaccine [21].

3. CombiHIVvac: a combined vaccine containing two immunogens in a single construct. Preclinical and clinical trials

Since an effective immunoprophylactic vaccine against HIV-infection must induce specific humoral and T-cell immune responses [2, 3, 12], we constructed CombiHIVvac vaccine comprising both above mentioned immunogens, i.e. TBI and TCI [21].

CombiHIVvac was constructed in the form of micelle-like particles based on the original technique combining two different immunogens in a single construct, i.e. polyepitope TBI protein and DNA-vaccine pcDNA-TCI encoding polyepitope protein TCI [19, 21] (Figure 3).

TBI protein is conjugated to dextran and mixed with DNA, which leads to formation of microparticles presenting TBI on the surface and containing the DNA inside. Positively charged spermidine provides the binding of the conjugate dextran/protein TBI with negatively charged DNA-vaccine promoting formation of particles on the self-assembly principle (50–250 nm in diameter) [22].

We have previously shown that by combining two immunogens (TBI and TCI) in one construct significant enhancement HIV-specific B cell response was observed [23]. In our opinion, the formation of such particles plays a critical role in the registered effect. CombiHIVvac particles enable more effective absorption by antigen-presenting cells (APCs) compared to individual immunogens. Since TBI protein is fixed on the particle surface and is represented in multiple copies, this

provides multiple enhancement of vaccine antigenicity. Besides, pcDNA-TCI enclosed in the vaccine structure is more protected against degradation by DNase I than free pcDNA-TCI, as it was previously demonstrated, resulting in prolongation of DNA-vaccine presence in an organism. Finally, the presence of CD4+ T-helper epitopes in the protein TCI may be the main reason underlying the increased synthesis of antibodies to TBI protein due to a CD4-mediated stimulation of B-cell proliferation and differentiation.

To carry out CombiHIVvac preclinical and clinical trials, we produced experimental series of vaccine of the standard quality according to WHO recommendations. Preclinical studies indicating the safety of the vaccine in tests with animals have been performed, namely, the acute and chronic toxicity has been studied in mice and guinea pigs and the absence of deviations in the vital organs of animals, as well as no changes in hematological and morphological parameters and no immunotoxicity and allergenic activity, have been shown for both single and tenfold administration of vaccine. Specific activity was evaluated based on the parameters of humoral and cellular immunity in BALB/c mice after their twofold immunization. The CombiHIVvac vaccine has been shown to induce formation of HIV-specific antibodies and CTLs [19, 21, 24, 25]. The vaccine did not cause any pyrogenic reaction in rabbits and did not affect the central nervous system and the detoxification liver function in mice. The duration of vaccine persistence in the organisms of laboratory animals has also been estimated and it has been shown that such vaccine component as the plasmid DNA completely eliminated from the organs and tissues of mice for 2 months after vaccination [21]. Thus, preclinical studies showed that CombiHIVvac is safe in animal trials.

Phase I clinical trials were carried out in healthy volunteers to study reactogenicity, safety, and immune activity of CombiHIVvac. The results of clinical trials published in [26]

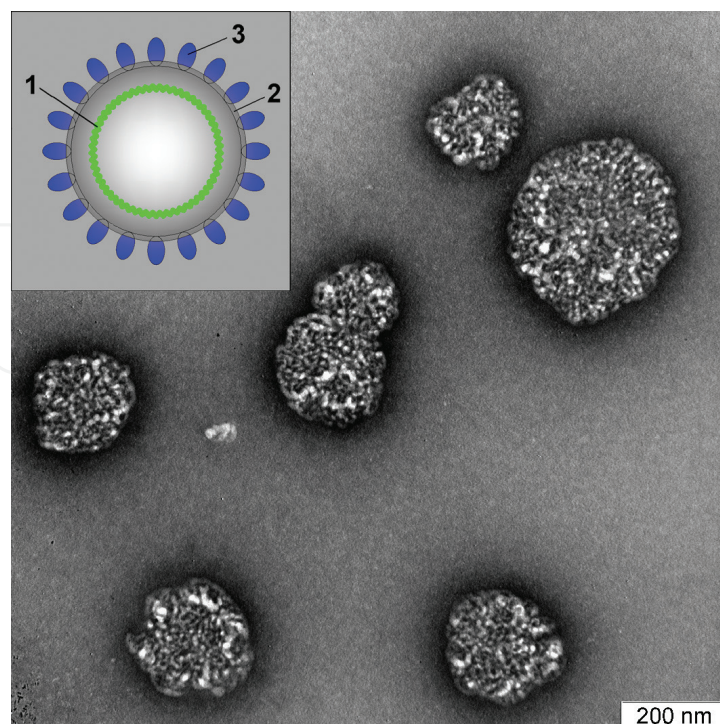


Figure 3. TEM images of CombiHIVvac microparticles with different magnification. A – scale bar 1000 nm, the insert in the left upper corner is a scheme of a CombiHIVvac particle (1 – pcDNA-TCI, 2 – spermidine/dextran, 3 – TBI).

demonstrated that CombiHIVvac is well-tolerated and safe. Neither single nor twofold (with 28-days interval) intramuscular vaccine administration induced significant changes in biochemical and physiological indicators as compared to the baseline values. Local reactions to vaccine administration were absent. There were no pathological changes in volunteers for all observation time. The mean values of the examined biochemical and clinical parameters were within the physiological ranges or near their limits. We failed to detect any regularity in changes of indicators depending on the time from the date of immunization. All studied indicators of immune status came back to the baseline level registered before the vaccination [26].

When carrying out Phase I clinical trials, we assessed CombiHIVvac specific activity in addition to its safety. The obtained results revealed that CombiHIVvac induces both humoral and cell HIV-specific immune responses. It was confirmed by several methods including immunoblotting, ELISA, env-pseudotyped virus neutralization assay, IFN- γ ELISpot and peptide-MHC-pentamers.

The specific immune response was detected via ELISA 14 days after the first immunization; the second immunization led to enhance the immune response. The maximum immune response is observed by ELISA on the 14th day after the second vaccine administration. Up to the end of observation time (1 year) we detected antibodies in 29% of volunteers.

To confirm antibody production capable of recognizing native HIV-1 during CombiHIVvac administration, we used a kit “New Lav blot” (Bio Rad) on which strips of separate proteins

Analysis		Percentage of vaccinated volunteers with positive responses							
		Time after		Time after the second vaccination, days					
		the first vaccination, days							
		14	28	14	28	90	180	270	360
Neutralization of virus ^a	B/SF162	—	—	71	71	71	64	57	0
	B/PVO4	—	—	36	36	29	29	15	0
	A/392	—	—	86	86	86	71	57	0
	A/SP2010	—	—	79	79	79	64	7	0
IFN- γ ELISpot ^b		71	79	100	93	86	79	50	43
MHC pentamers ^c		100	100	100	100	80	100	80	60

^aThe neutralization of virus was evaluated as IC₅₀ value obtained by neutralizing the clones of pseudoviruses of subtypes A (SP-2010 and SP-392) and B (SF162 and PVO4) with blood sera of volunteers vaccinated with CombiHIVvac. The reaction was considered as positive if the neutralization titer was greater than or equal to 1: 100. The neutralizing activities of sera on the 14th and 28th days after immunization were lower than 1: 100.

^bThe ELISpot responses were considered as positive if the number of IFN- γ -producing cells in the vaccinated volunteers was two times larger than the control value.

^cThe results of determination of HIV-specific CD8⁺ T-lymphocytes in HLA-A*0201-positive volunteers repeatedly vaccinated with CombiHIVvac obtained with the use of MHC pentamers in a complex with Env peptide (KLTPLCVTI) of HIV-1 are given.

Table 1. Evaluation of the response of HIV-specific T-lymphocytes and the activity of virus-neutralizing antibodies in repeatedly vaccinated volunteers.

of virus lysate were sorbed. Using immunoblot analysis, we demonstrated the presence of antibodies to HIV-1 proteins p17, p24, p55, p68, and gp120, i.e. to those proteins which epitopes compose B-cell vaccine component – TBI protein. The response rates differed among volunteers within the same group. Furthermore, during 1 year after the second immunization we registered antibodies at least to one of those proteins in 100% of volunteers.

The results of the study of T-cell response via the IFN- γ ELISpot in repeatedly vaccinated volunteers (**Table 1**) show the HIV-specific response of T-lymphocytes in all volunteers (100%) on the 14th day after the first vaccination, remaining sufficiently strong for 6 months after the second vaccination. Using MHC pentamers in a complex with Env peptide (KLTPLCVTL, gp120 aa 120–128) of HIV-1, it was demonstrated that KLTPLCVTL CD8 T lymphocytes occur in all volunteers (100%) up to the sixth month after the second vaccination (**Table 1**).

Thus, the performed clinical trials showed that the CombiHIVvac vaccine is well tolerated and safe (does not induce any significant changes in biochemical and physiological parameters in comparison to the background values), characterized by low reactogenicity (local reactions to the vaccine are absent) and most importantly capable of inducing the specific humoral and cellular immunity.

Based on the obtained results, the Ministry of Health and Social Development of the Russian Federation has recommended the vaccine for advanced (Phase II) clinical trials.

4. Possible development of CombiHIVvac vaccine platform

Preclinical and clinical trials of CombiHIVvac demonstrated that a combination of two completely artificial polyepitope T- and B-cell antigens is capable of inducing HIV-specific CTLs and antibodies in laboratory animals and human. Furthermore, TCI protein expressed in cells as part of pcDNA-TCI plasmid fulfills a double function: (1) induces specific CD8⁺ CTL responses and (2) acts as an adjuvant synergistically effecting on synthesis of antibodies to TBI protein with virus-neutralizing activity at least to two HIV-1 subtypes (A and B) [23, 26].

The obtained results imply that CombiHIVvac is actually an original platform for the development and further improvement of combined DNA-protein HIV-vaccines using a broad range of conservative T- and B-cell epitopes based on virus antigens. Providing that TBI and TCI immunogens in CombiHIVvac composition were developed more than 15 year ago concurrently with clinical trials of CombiHIVvac, we carried out works on enhancement of immunogenic and protective properties of artificial polyepitope antigens utilizing new data on the structural-functional organization and immunology of HIV-1.

4.1. B-cell epitopes to HIV-1 generating broadly neutralizing antibodies (bNAbs)

At present when developing efficient B-cell immunogens, researchers mainly rely on epitopes recognized by antibodies neutralizing a broad spectrum of HIV-1 strains (bNAbs). In recent years dozens of B-cell HIV epitopes recognized by bNAbs have been detected [27].

It was shown that many of these antibodies can prevent infection, and some can suppress active infection in hu-mice or macaques [28–32]. Recently results of Phase I clinical trials of

mAbs VRC01 were published [33]. It is shown that they are safe and well tolerated after multiple intravenous or subcutaneous administrations in humans, in addition VRC01 from participants' sera were found to avidly capture HIV virions and to mediate antibody-dependent cellular phagocytosis [33].

Exceptional features of bNAbs inspire many researches to develop immunogen capable of their producing (induction). One of the evolving research areas focusing on the design of such immunogens is based on the development of HIV-1 envelope (Env) trimers [6, 34–36]. Despite substantial progress in this area, (a number of questions must be addressed). Firstly, although trimers are rather stable in solution, they produce conformational conditions that fail to provide binding and induction of bNAbs. Secondly, trimers expose undesired immunodominant non-protective HIV epitopes that could prevent adaptive immune response from recognizing neutralizing epitopes, block protective immunity and/or induce increased HIV-infection [4, 36].

An alternative approach to solving this problem includes constructing completely artificial polypeptide anti-HIV-1 immunogens comprising a set of protective epitopes assembled in a single mosaic (polypeptide) construct. Unfortunately, the most bNAbs recognize conformational epitopes and considerably more rarely linear epitopes [37–39]. Furthermore, conformational B-cell epitopes are frequently formed in HIV by lipids and glycans or their combinations [37–40]. It complicates the design of immunogens capable of inducing sufficient B-cell response. Phage peptide libraries offer the unique possibility to obtain mimics of such epitopes [41–46].

Using phage peptide library we can select peptides mimicking epitopes recognized by bNAbs, that make it possible to construct mosaic immunogen on their base to simultaneously induce several neutralizing antibodies [43, 47–52]. **Figure 4** depicts general working scheme.

In our study we used a number of bNAbs against HIV-1, i.e. 2G12, 2F5, IgG1b12, Z13e1, VRC-01, VRC-03, and 697-30D to obtain peptide-mimics. The last five bNAbs were kindly furnished upon NIH AIDS Reagent Program, USA. Each monoclonal antibody was used to perform biopanning using phage peptide libraries (New England Biolabs, USA) [20, 47, 48, 53].

After biopanning of phage libraries using monoclonal antibody 2G12 (recognizes conformational epitope) and 2F5 (recognizes linear epitope), we isolated peptide-mimics that have another amino acid sequences compared to natural epitopes, but able to elicit antibodies in laboratory animals capable to compete with initial bNAbs and neutralizing the virus.

As a result we obtained a collection of phagotops carrying on their surface peptide-mimics of epitopes recognized by above mentioned bNAbs. Specific activity of selected peptides was studied both free and in the compound of phage particles. We carried out chemical synthesis of 134 free peptides. Evaluation of their capacity to compete with HIV-1 epitope for binding to monoclonal antibodies VRC-01, VRC-03, and IgG1b12 was carried out using pseudovirus particles in virus-neutralization assay. To study peptides immunogenicity in the compound of phage particles, the latter were produced in preparative amount using bacterial cells. We used the obtained samples to immunize laboratory animals from which we sampled sera to study their virus-neutralizing activity. It was shown that sera of rabbits immunized with a mix of bacteriophages are able to neutralize pseudotyped viruses obtained on the base of

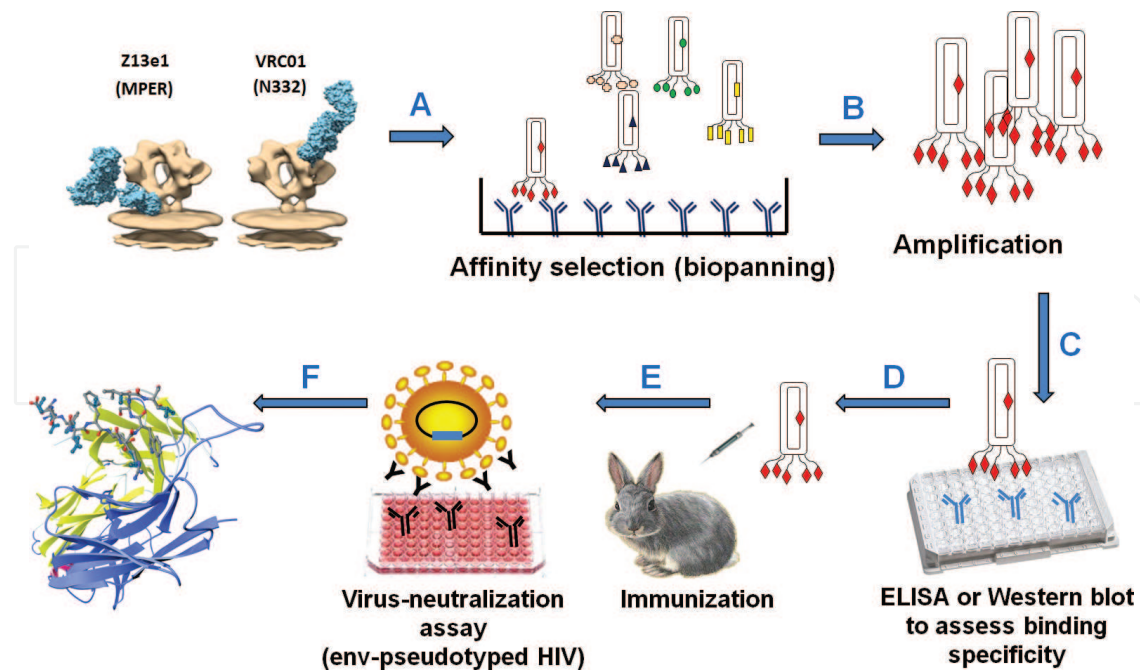


Figure 4. Phage display applications for artificial immunogen design. (A) Randomized peptide library are used to map the residues forming the epitope(s) recognized by monoclonal antibodies immobilized on a solid support; (B) amplification of selected phages; (C) ELISA assay or western blot to determine specificity of selected phages binding; (D) phage particles are used to presentation isolated peptides-mimotopes to the immune system; (E) virus-neutralization assay of immune serum, selection of the most promising mimotope; (F) artificial immunogen design.

HIV-1 subtypes A, B, and AG [20, 47, 48, 53]. Consequently, we succeeded to demonstrate immunologic imitation of conformational antigenic determinants, i.e. HIV-1 epitopes, by linear peptides. Obtained peptide-mimics are material that can serve as a basis for the development of immunoprophylactic HIV-1 vaccine. Besides, peptides can be used when designing diagnostic systems for the detection of antibodies to HIV-1.

4.2. Design of polyepitope T-cell antigens

The progress in identification of T-cell epitopes as well as understanding mechanisms of processing and presentation of antigens through MHC class I and II pathway make it possible to rational design artificial polyepitope vaccines [13, 54].

It is known that CTL recognizes viral protein-antigens synthesized inside the cell not as full-length molecules but as short peptides (8–10 amino acid residues) in complex with MHC class I molecules. These short antigenic epitopes emerge from endogenously synthesized proteins due to proteasome-mediated processing and then are transported to the lumen of endoplasmic reticulum (ER) using transport proteins TAP (transporter associated with antigen processing) where they bind to emerging MHC class I molecules [55, 56]. Since antigen must be synthesized in a cell to induce response of CTL, target T-cell vaccine should be designed as DNA-vaccine because it is the most natural way of presenting CTL-epitopes to CD8⁺ T-lymphocytes through MHC class I pathway [57].

As opposed to stimulation of CTL, when inducing CD4⁺ T-lymphocyte-helpers response, antigen should be presented to these cells in a complex with MHC class II molecules. Usually

processing and presentation of antigen take place for extracellular antigens which are delivered in cells via endocytosis and phagocytosis. In this case antigen processing occurs in lysosome.

Thus, when designing polyepitope T-cell immunogens capable of inducing high levels of CD4+ and CD8+ T-lymphocyte responses to all epitopes in its compound, one should provide efficient proteasome- and/or lysosome-mediated processing of expression product of target gene through MHC class I and II pathway. For the purpose the following strategies are appropriate:

1. To design poly-CTL-epitope construct one may use spacer sequences dividing epitopes that comprise sites of proteasomal cleavage [58–60] and/or motif for binding to TAP [61–63] to provide polyepitope processing and transport of released peptides (epitopes) into ER.
2. To induce T helper lymphocytes response fragments with T-helper epitopes can be combined with the use of motif [KR][KR] which is a cleavage site for a number of lysosomal cathepsins participating in antigen processing [64, 65].
3. To target polyepitope immunogen into proteasome and presentation of CTL-epitopes to CD8+ T-lymphocytes through MHC class I pathway, researchers typically use genetic attachment of ubiquitin sequence to its N- or C-termini [66].
4. To degrade polyepitope immunogen and present released Th-epitopes to CD4+ T-lymphocytes through MHC class II pathway, researchers typically use a genetic attachment of the sequence of LAMP-1 protein tyrosine motif (Lysosomal-associated membrane protein 1) to its C-terminus to direct the polyepitope immunogen from the secretory pathway to the lysosome [67–70].

To evaluate which of these strategies provide a rational approach to constructing T-cell antigens, we designed a set of polyepitope constructs covering a range of possible structural variants.

To assess the influence of ubiquitin and spacer sequences flanking epitopes on immunogenicity of the polyepitope construct, we designed a set of polyepitope immunogens considering different strategies of processing and presentation of the target antigens. The designed constructs comprised similar set from 10 HLA-A2-restricted CTL-epitopes of the main HIV-1 antigens Env, Gag, Pol, Nef, and Vpr, but differed in a number of structural properties, namely (i) the presence or absence of spacers; (ii) the structure of spacer sequences, and (iii) the presence of N- or C-terminal sequence of ubiquitin. Genes encoding the designed antigens were cloned into plasmid vector and vaccinia virus.

Immunogenicity of the designed immunogens were evaluated after 3-fold prime-boost immunization of HLA-A2 transgenic mice with the obtained recombinant plasmids and recombinant vaccinia virus (rVV). It was demonstrated that the vaccine construct inducing the majority of complexes [peptide/MHC class I] *in vitro* was also the most immunogenic during animal vaccination. This construct comprises N-terminal ubiquitin to target the polyepitope on proteasome. Besides, in the compound of this construct epitopes are divided by spacer sequences comprising sites of proteasomal cleavage of the polyepitope and motifs for TAP-dependent transport of the released peptides into ER where they bind to MHC class I molecules [54].

The obtained results became the basis for the development of original software TEpredict and PolyCTLDesigner that we consider as a universal platform for rational design of polypeptide immunogens – candidate DNA vaccines for induction of T-cell immunity both against infectious and oncological diseases [71, 72] (**Figure 5**).

PolyCTLDesigner enables the user to select a minimal set of epitopes with known or predicted specificity to different allelic variants of MHC class I molecules. This set covers selected repertoire of HLA alleles with the given degree of redundancy. After that PolyCTLDesigner uses the model by Peters et al. [73] to predict binding affinity to TAP for the selected set of known or predicted epitopes. According to this model, the main contribution into peptide binding to TAP is provided by the first three N-terminal amino acid peptide residues and the last C-terminal residue. Considering that epitope C-terminus must be unchanged since C-terminus should contain the site of proteasomal cleavage [74], only N-terminus of antigenic peptide can be extended (if necessary) for optimization of interaction with heterodimer TAP1/TAP2.

Then PolyCTLDesigner analyzes all possible matching of the selected peptides and detects an optimal spacer sequence for each pair providing adequate cleavage of epitopes with release of C-terminus of proximal peptide. To predict proteasomal and/or immunoproteasomal cleavage, PolyCTLDesigner uses models developed by Toes et al. [75].

When analyzing epitopes matching, PolyCTLDesigner creates a directed graph with nodes corresponding to epitopes and edges corresponding to acceptable matching. Each edge has relevant weight vector characterized by the efficiency of proteasomal cleavage, spacer length

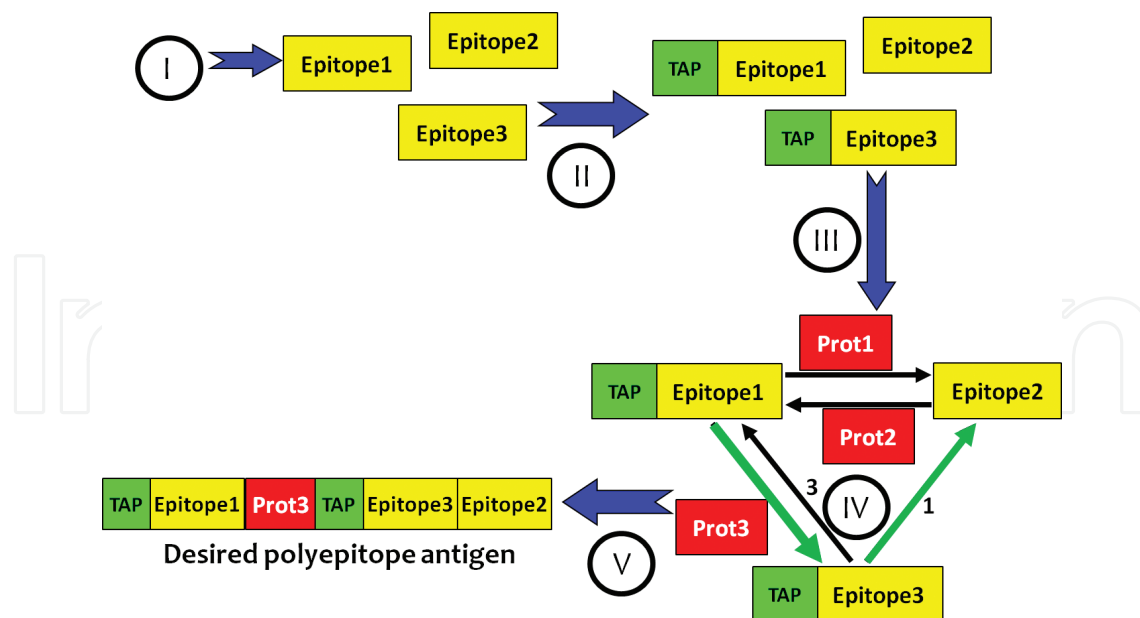


Figure 5. PolyCTLDesigner operation algorithm. I – Selection of minimal set of CD8+ T cell epitopes with the known or predicted specificity towards various allelic variants of MHC class I molecules; II – Prediction of binding affinity of peptides to TAP and, if necessary, addition of N-terminal flanking residues to optimize this binding; III – Prediction of optimal spacer sequences for each pair of peptides; IV – Creating weighted graph, where the nodes are the target epitopes, and the edges are possible variants of their association. Each edge is a weight vector whose attributes are: efficiency of proteasomal cleavage, length of the spacer, and number of predicted non-target epitopes at junction; V – Designing polyepitope immunogen sequence. Resulting sequence is defined as the longest simple path in the graph that has the lowest weight; Prot1, Prot2, Prot3 – proteasomal cleavage sites.

and number of predicted non-target epitopes at the junction. Finally, the software designs an optimal polyepitope immunogen sequence that is calculated as a complete simple way in the constructed graph with the least length (weight).

Besides, PolyCTLDesigner makes it possible to construct a sequence of the epitope fragment comprising T-helper epitopes. In the compound of the selected antigens software predicts peptide fragments with the length of 20–40 amino acid residues with the majority of overlapping T-helper epitopes restricted by the widest possible repertoire of HLA class II allomorphs. Then five C- and N-terminal amino acid residues from the initial antigen sequence are added to each of the selected fragments since it was shown that they can play significant role in binding to T-cell receptors of CD4+ T-lymphocytes [76, 77]. Fragments with T-helper epitopes are combined using [KR][KR] motif which is a cleavage site for a number of lysosomal cathepsins involved in antigen processing.

More detailed information on PolyCTLDesigner software is available at <http://tepredict.sourceforge.net/PolyCTLDesigner.html>.

We used the developed software when designing new polyepitope constructs – candidate DNA-vaccines against HIV-1. Particularly, when evaluating the influence of proteasome-dependent and lysosome-dependent degradation of polyepitopes on immunogenicity of the target polyepitope construct, we designed three polyepitope HIV-1 immunogens, i.e. TCI-N1, TCI-N2, and TCI-N3 using cytotoxic and helper T-cell epitopes of HIV-1 [78].

All three polyepitope immunogens are based on the same core sequence of polyE, while differences between immunogens lie in the use of different terminal signal sequences (**Figure 6**). Immunogen TCI-N1 comprises only the core sequence polyE. Sequence polyE of TCI-N2 immunogen includes

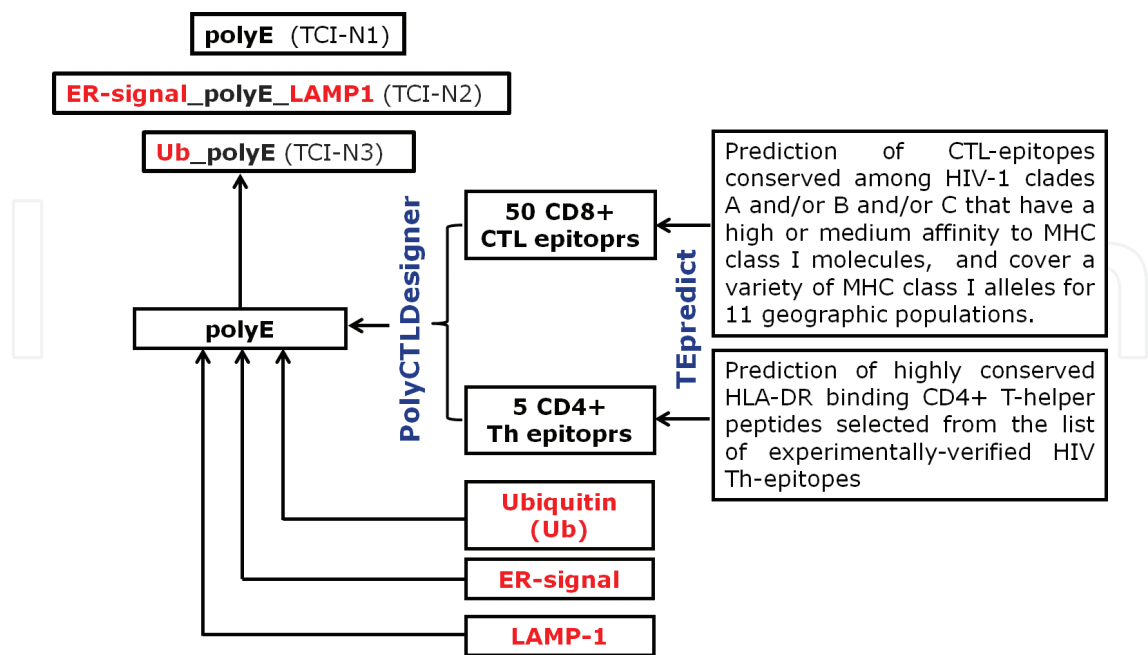


Figure 6. Design of T-cell polyepitope immunogens. polyE – common for all antigens sequence polyE designed using cytotoxic and helper T-cell epitopes of HIV-1; ER-signal – N-terminal signal peptide (in our case MRYMILGLLALAAVCSAA – the signal sequence of the adenovirus protein E3/gp19K); LAMP1 – C-terminal tyrosine-based motif of LAMP-1 glycoprotein (RKRS HAGYQTI); Ub – N-terminal ubiquitin with substitution of the C-terminal Gly to Val to prevent liberation of Ub cleavage by Ub hydrolases.

N-terminal signal peptide and C-terminal tyrosine motif of LAMP-1 protein. N-terminal signal peptides are believed to provide delivery of immunogen in ER, while LAMP-1 protein motif directs immunogen from the secretory pathway to the lysosome and presents epitopes released after the cleavage to CD4⁺ T-lymphocytes through MHC class II pathway. The sequence polyE of TCI-N3 comprises N-terminal ubiquitin for its delivery into proteasome and presentation of epitopes released after the cleavage to CD8⁺ T-lymphocytes through MHC class I pathway.

Immunogenicity of the obtained DNA-vaccine constructs was studied in BALB/c mice according to capacity of CD4⁺ and CD8⁺ T-cells to produce IL-2 and IFN γ in ELISpot. The obtained results revealed that DNA-vaccine constructs encoding TCI-N2 and TCI-N3 immunogens induce responses of HIV-specific CD4⁺ and CD8⁺ T-lymphocytes that are significantly higher than that of the negative control the group of animals immunized with vector plasmid pcDNA3.1 as well as of group of mice that received a construct encoding core immunogen TCI-N1 with no additional signal sequences. At the same time DNA-vaccine construct encoding TCI-N3 immunogen comprising N-terminal ubiquitin induces the highest statistically significant level ($P \leq 0.05$) of CD4⁺ and CD8⁺ T-lymphocytes as compared with two other immunogens.

Thus, the obtained results point to a regular correlation between the structure of polyepitope construct and its antigenic and immunogenic properties:

- it is possible to significantly increase the immunogenic potential of the target polyepitope vaccine via optimization of the immunogen structure using the spacer sequences comprising motifs for binding to TAP and the sites of proteasomal and lysosomal cleavage flanking CTL- and Th-epitopes in the compound of the polyepitope construct;
- ubiquitin-dependent targeting of polyepitope at proteasome is the most efficient strategy to induce specific T-cell immune response as compared to LAMP-dependent targeting at lysosome.

Our findings support the concept of vaccine rational design based on existing knowledge on mechanism of presentation of T-cell antigens through MHC class I and II pathway.

5. Conclusions

We did not set ourselves the task of covering all challenges facing designers of HIV-1 vaccine. The paper presents our experience on designing artificial polyepitope HIV-1 immunogens constructed using a broad spectrum of conservative T- and B-cell epitopes. This approach is believed to be promising for the design of new generation HIV-vaccines. In theory, it makes it possible to overcome HIV-1 antigenic variability, focuses immune responses on protective determinants, and allows to exclude from vaccine composition undesired determinants capable of inducing autoantibodies or antibodies increasing virus infectivity. The results demonstrate that completely artificial molecules designed with the use of bioinformatic and combinatorial biology methods are able to induce production of broad-spectrum neutralizing antibodies and responses of cytotoxic (CD8⁺ CTL) and helper (CD4⁺ Th) T-lymphocytes in laboratory animals and human.

It is our belief that the proposed approach can play an important and positive role in the development of HIV-1 vaccine.

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Conflict of interest

None.

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