Short Communication: HIV-1 Nef Protein Carries Multiple Epitopes Suitable for Induction of Cellular Immunity for an HIV Vaccine in Africa

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Abstract

Using the early protein HIV Nef, new HLA class I binding epitopes of importance for immune responses to HIV were predicted for common African alleles. In total we identified 45 epitopes previously not described for the HLA alleles A*30:01, A*30:02, B*58:01, and C*07:01 and compared them to reported epitopes, primarily from HLA-A*02:01, from the Los Alamos database and our own vaccine studies. Related to its small size, the Nef gene/protein appears to be able to contribute effectively to confer both stronger and broader cellular immunogenicity to an HIV-1 vaccine. We also propose feasible mutations of such an additional vaccine antigen to preserve its immunogenicity, modified not to confer HLA or CD4⁺ down-regulating activities. This article includes data on a valuable HIV immunogenic component for a vaccine in Africa.

IN HIV VACCINOLOGY it is important to induce a broad cellular cytotoxicity that, hypothetically, would induce reactivity to infected cells carrying different viral strains.¹ In HIV infection, cytotoxic T lymphocytes (CTLs) recognize viral peptides (epitopes) presented via human leukocyte antigen (HLA) class I molecules expressed on nucleated cells.^{2,3} The HLA molecules are highly polymorphic, with thousands of different alleles recognized in the human population.⁴ For HIV proteins and glycoproteins, immunogenic epitopes have predominantly been identified for HLA alleles commonly expressed in white populations,⁵ but the need for HIV vaccines is particularly urgent in many African countries. Average frequencies of HLA class I alleles for different populations in African countries were adapted from numbers in the Allele frequency net database, which indicated frequencies of A*02:01 9%, A*30:01 6%, A*30:02 7%, B*58:01 5%, C*07:01 17%, and other HLA-A, B, or C alleles 5% or lower.⁶

The small HIV-1 protein Nef is very immunogenic.^{7–9} A study showed that HIV-infected individuals in the United States overall recognized a median of 14 HIV-derived epitopes, of which the most frequently recognized protein was Nef. These evaluations, based on preclinical assays and supertype associations, showed that Nef contains a high density of epitopes.^{10,11} The Nef gene/protein was early shown to

induce antigen-specific CTL and CD4⁺ cells after immunization of infected humans.¹²

The Nef protein, which is produced early in the virus life cycle, has, however, evolved toxic mechanisms to disturb the induction of innate and adaptive immunity. It down-regulates the expression of host cell HLA (classes I A, B and II) as well as the CD4 molecule.^{13–16} It also interferes with the T cell receptor signal transduction machinery and has untoward effects on the cytokine activation cascade,¹⁷ which reduces adaptive immunity. Mutations in a prospective immunogen should therefore counteract such cellular disturbances.

We examined two HIV-1 Nef proteins, one of consensus subtype B, isolate HXB2, which we have used in a vaccine trial,¹² and one primary subtype C isolate from Tanzania (accession no. AY734561.1) for potential presentation of immunogenic epitopes by common African HLA class I alleles, the direct aim being a theoretical understanding of the potential for induction of cellular responses, if Nef were to be included in existing vaccines. Nef is present in a few HIV vaccine candidates, but often as the last protein in a fusion protein, or only as a DNA, and has therefore not been fully explored.^{18–20}

The HLA-I binding predictions for the HLA-C*07:01 were made using the Immune Epitope Database (IEDB) analysis resource NetMHCpan tool.^{21,22} This tool performs quantitative

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affinity predictions of peptide-major histocompatibility complex (MHC) interactions using artificial neural networks (ANN). Several MHC molecules were concurrently used to train a single network ensemble, incorporating both the MHC peptide and contact residues. By exploiting known sequence relationships to binding affinity, estimations of binding predictions for MHC molecules not included in the training can be procured.²³ HLA-I binding predictions for all other alleles were performed using the IEDB analysis resource Consensus tool²⁴ that combines predictions from ANN,^{25,26} SMM,²⁷ and comblib.²⁸ Predictions from the described individual predictors are converted into percentile scores, the definition of which is the percentage of random peptides sampled from naturally occurring proteins yielding a higher score than the given peptide. The percentile scores of the individual predictors are compared, and a median of these is presented as the final binding affinity prediction, here referred to as percentile rank.²⁴ A low percentile rank thus indicates high affinity. These tools were used together with the Los Alamos HIV/AIDS database.⁵

Peptides in antigen-presenting cells (APC) undergo intracellular cleavage by proteases (for the immunoproteasome trypsin and chymotrypsin), transportation by the transporter associated with antigen processing (TAP), and, finally, binding to the MHC molecules at the cell surface of APC. These processing steps are taken into account by the IEDB

Proteasomal cleavage/TAP transport/MHC class I combined predictor,^{22,23} which includes variables for cleavage,²⁹ TAP transport,³⁰ as well as binding to the respective HLA allele.²³ The HLA-I total processing prediction relies on these parameters.³¹

To evaluate the biological relevance of our predictions, a comparison was made between the known epitopes and their prediction variables for A*02:01, using both the IEDB HLA-I binding prediction expressed as percentile rank and the HLA-I total processing prediction tools (Table 1).

Eighteen epitopes had been previously described for HLA-A*02:01, one for A*30:02, and nine for B*58:01 (Table 1). Of these 27 previously described epitopes for HIV-1 viruses of any subtype 13 had good predicted binding affinities, i.e., percentile rankings with a cut-off set at 10. High MHC total processing predictive scores above -1.5 were thus noted for 13/27 known CTL peptides (Table 1). Using these 50% cut-off values, 11 of the 27 known alleles were identified by both programs, and 15/27 by either program. The similarity of epitopes identified by both procedures has been noted before, and is most likely related to the fact that the databases we used are trained on natural ligands.

A complete analysis of predicted epitopes was then performed for the five chosen alleles using the Nef protein of selected strains of HIV-1 subtypes B and C (Supplementary Tables S1 and S2; Supplementary Data are available online at

TABLE 1. MHC TOTAL PROCESSING AND MHC-I BINDING PREDICTIONS OF KNOWN EPITOPES FOR NEF FROM THE LOS ALAMOS HIV IMMUNOLOGY DATABASE⁵ WITH SEQUENCES AND SUBTYPES ACCORDING TO THE ORIGINAL AUTHOR, ARRANGED ACCORDING TO AA AND ALLELES

Allele	HXB2 location	Sequence	Subtype	MHC total processing score	Percentile rank
HLA-A*02:01	42	ALTSSNTAA	—	-2.4	5.4
HLA-A*02:01	42	ALTSSNTAH	C	-3.7	33.3
HLA-A*02:01	66	VGFPVRPQV	B	-2.9	13.7
HLA-A*02:01	73	QVPLRPMTYK	A, B, C, CRF01_AE, CRF02_AG, D	-3.6	59.0
HLA-A*02:01	79	MTYKAAFDL	C	-1.4	5.0
HLA-A*02:01	79	MTYKAALDL	—	-1.8	9.7
HLA-A*02:01	83	AALDLSHFL	B	-0.5	2.9
HLA-A*02:01	90	FLKEKGGL	A, B, C, CRF02_AG, D	-1.3	22.7
HLA-A*02:01	109	ILDLWVYHT	B, C	-1.2	1.8
HLA-A*02:01	136	PLTFGWCYKL	B, CRF01_AE	-1.7	14.0
HLA-A*02:01	136	PLTFGWCFKL	B	-1.7	12.6
HLA-A*02:01	136	PLTFGWCYKLV	B	-2.6	19.1
HLA-A*02:01	137	LTFGWCFKL	A, B, C	0.2	1.1
HLA-A*02:01	180	VLEWRFD SRL	B, CRF01_AE	-1.6	13.4
HLA-A*02:01	180	VLMWQFDSRL	B	0.1	1.5
HLA-A*02:01	181	LEWRFDITL	B	-1.8	18.5
HLA-A*02:01	190	AFHHVAREL	B, CRF01_AE	-2.4	31.1
HLA-A*02:01	190	ALKHRAYEL	A	-1.1	4.8
HLA-B*58:01	82	KAAVDLSHF	B, C	0.9	0.3
HLA-B*58:01	82	KAAFDLSFF	C	1.1	0.6
HLA-B*58:01	82	KGALDLSHF	—	-0.7	2.6
HLA-B*58:01	116	HTQGYFPDW	B, C, CRF02_AG	0.2	0.7
HLA-B*58:01	120	YFPDWQNY	B, C	-1.7	24.0
HLA-B*58:01	120	YFPDWQNYT	A1, B, C	-4.0	12.1
HLA-B*58:01	127	YTPGPGVRY	C	-1.2	16.1
HLA-B*58:01	130	GPGIRYPLTFGWCF	—	-2.1	50.0
HLA-A*30:02	120	YFPDWQNY	B, C	-0.5	1.9

A high MHC total processing score indicates a high efficiency; the score combines predictors of TAP transport, proteasomal cleavage, and MHC-I binding. Percentile rank refers to MHC-I binding affinity prediction where a low number indicates a high MHC-I binding affinity.

Bold numbers indicate the epitopes with best MHC total processing score/percentile rank.

www.liebertpub.com/aid). The more complete analysis included proteosomal cleavage, TAP transport, and affinity prediction (Fig. 1A and B and Supplementary Tables S1 and S2). Thus, we found that several of the best predicted A*02:01 molecules were the same as or very similar to the known ones, both for A*02:01 and the few B*58:01 reported epitopes (in italics in Supplementary Tables S1 and S2).

It is notable that one of the previously described epitopes for B*58:01 (starting at aa 130, Table 1) is 14 aa long, and our MHC total processing prediction recognized two peptides within this 14 aa sequence, at aa 133 and at aa 137 (Supplementary Table S1). The only previously described epitope for HLA-A*30:02 is an 8-mer with a starting position at aa 120 (Table 1). Our prediction found a closely located 9-mer

FIG. 1. The top 30 Nef epitopes each of HIV-1 subtypes B (A) and C (B) according to proteasomal cleavage, TAP transport, and MHC binding combined predictions. Peptides marked as black represent the highest scoring epitopes, with scores over 1. Gray peptides represent medium scoring epitopes in the range of -1 and 1, and low scoring epitopes under -1 are colored in white. The cutoffs were selected to clearly distinguish the top scoring peptides from the medium and lower scoring ones.

at starting position aa 119 had a percentile ranking of 1.25, indicating a high affinity (Supplementary Table S1).

When the prediction models were performed using the HXB2 HIV-1 subtype B, several of the previously known peptides were identified. The two methods seemed similarly accurate at identifying presumptive epitopes. When the prediction models were performed with the subtype C sequence, again several previously identified regions were recognized, but also several novel epitopes (altogether 17 novel predicted epitopes among those with high affinity) (Fig. 1B).

In Fig. 1A, the top 30 Nef epitopes of HIV-1 subtype B were aligned according to MHC-I total processing prediction scores by all alleles. Out of these 30, B*58:01 had the highest scoring epitope frequency with nine predicted epitopes followed by A*30:02 and A*30:01, which had seven and six, respectively. A*02:01 and C*07:01 had three to four epitopes among the top 30 predicted epitopes (Fig. 1). Six of these high ranking epitopes were already described (three for A*02:01 and three for B*58:01, Table 1), and 24 are novel predicted peptides described here.

In Fig. 1B, the top 30 predicted Nef epitopes of HIV-1 subtype C were aligned according to MHC-I total processing prediction scores by the chosen alleles. Out of these 30, A*30:02 had the highest scoring epitope frequency with 11 predicted epitopes followed by B*58:01, which had eight, A*02:01 with five, and finally A*30:01 and C*07:01, which had three epitopes each (Fig. 1B). Nine of these high ranking epitopes were already described (taking into account an aa similarity of over 80%, Table 1), and 21 are novel predicted peptides described here. Therefore, in all we predicted 45 novel epitopes.

In these comparisons, the HLA-A*30:02 and B*58:01 alleles had the most reactive predicted allele reactivities. For alleles HLA-A*30:01 and HLA-C*07:01, no epitopes have yet been described. The new epitopes we predicted for these alleles may thus be true CTL epitopes, which in turn may be immunogens for CTL induction in HIV-infected patients or in a preventive vaccination.

To retain Nef immunogenicity but avoid toxicity, the protein may be mutated. Figure 2 illustrates the percentile

FIG. 2. (A) Previously published epitopes for HIV-1 Nef (black) and the 10 best predicted epitopes (hollow) by allele denoted on HXB2 aa positions. Regions with the highest frequency of epitopes are marked by lines for known (black) and predicted novel (hollow) epitopes. Arrows indicate feasible mutations: for myristylation at aa G2, association with polyproline at aa VGFP65-69, and/or dimerization at aa R105, R106, and D123. (B) The Nef protein variation across all subtypes, including variants with additional C-terminal sequences.⁵

ranks of Nef epitopes in relation to position in the protein. Figure 2B illustrates the Nef protein variation across all subtypes,⁵ when unusual Nef proteins up to aa 280 are also taken into account. The most immunogenic regions derived from predicted epitopes are consistent with the overlapping regions found to be immunogenic in previous biological studies, where they were generally found in association with HLA-A*02:01 (Fig. 2A), which is the allele most commonly studied.

Mutations should target functional regions and avoid immunogenic regions. For mutations one may consider the N-terminal myristylation site aa 2, aa R105, R106, and D123 necessary for dimerization of Nef, and the recently identified conserved aa 66-68VGF region, which associates with the proline-rich region of Nef.³² Aa C142 breaks cyclization, but may be unsuitable for mutation, since the cysteine is included in high-affinity epitopes. Sites for such mutations are indicated in Fig. 2A. The sites were selected to be located where the Nef protein associates with cellular proteins. A correct mutation of the Nef protein should further enhance its immunogenicity.

All HIV strains and most genes are subjected to prominent sequence variations.⁵ Variations in sequence indicate that escape may take place once the individual is infected. We studied this in Nef following six immunizations with the Nef gene in HIV-infected adults.³³ We found no increase in nucleotide (nt) substitutions within predicted cytotoxic epitopes compared with epitopes outside these regions. The lack of increased nt variations during repeated immunization was taken to mean that in this case the selective pressure did not cause obvious escape. We have also noted in our predictions here that similar regions/epitopes are found to be predicted for the various alleles, and that Nef has a high density of potential epitopes. If the whole protein/genes of Nef, i.e., of subtype B or C, were to be used in a vaccine, there seems to be a very great chance of CTLs being induced also to other subtypes.

Drawbacks associated with Nef immunization include the dysregulation of certain cellular molecules. Down-regulation of cellular HLA-A and HLA-B as well as of CD4⁺ molecules contributes to a poor immune response against HIV in primary infection and may also participate in progression of the chronic infection. Recent data suggest that Nef-mediated down-regulation of HLA-A is more effective than that of HLA-B,³⁴ which may help explain why HLA-B alleles tend to be more protective against HIV-1 disease progression.³⁵ The STEP HIV vaccine trial incorporated gag/pol/nef DNA in an Ad5 vector. Primary analysis showed that HLA-B alleles indeed were associated with strong CTL interferon- γ responses, as studied by 15-aa-long peptides.²⁰ HLA-C is not downregulated by Nef.¹⁴ Anti-HIV envelope-specific and Gag-specific responses have been described, yet few HLA-C restricted responses to Nef responses have been reported. This is of particular interest when targeting Nef-specific and HLA-C-restricted CTL responses, since HLA-C has been shown to be crucial in HIV control.³⁶ The HLA-C association of Nef CTL epitopes should motivate further attempts to assay HLA-C-related epitopes from Nef in elite controllers.

In a previous study, the best *ex vivo* binding of Nef peptides to cells with A*02:01 expressing cells was found by epitopes starting with aa 83, 106, 109, and 137.¹⁰ After immunization with a complete sequence of Nef DNA, the best

Nef-specific CTL responses were instead directed to epitopes starting at aa 42, 79, 83, 107–109, and 172. This is interesting, since both the binding assay and the immunization procedure revealed epitopes found in the natural infection.

Experimentations to verify the epitopes we describe are desirable. An elegant experimental way to confirm affinity and CD8⁺ CTL activation would be to construct peptides and HLA class I molecules *in vitro*, and use them in binding studies as well as by tetramer production, as has been done for several *Mycobacterium tuberculosis* antigens.³⁷ Synthesis of the African mono-alleles can be performed to reveal affinities of the predicted Nef epitopes. To identify epitopes that induce CD8⁺ CTLs *in vivo*, cells from HIV-1-infected or Nef-vaccinated individuals should be selected, which contain each allele in mono or duplicate conformation, i.e., the individuals being typed to contain A*30:02, A*30:02, or A*02:01, A*30:02, etc. It is not yet clear how the presentation of certain peptides/epitopes is influenced by such heterogeneity, although we hypothesize that the A*30:02 genotype will present the epitopes discovered in this article more effectively than a single A*30:2 allele.

Such a study takes several years to perform, and involves cloning and production of selected African MHC molecules, as well as synthesis of the relevant peptide library, binding assay, and subsequent construction of MHC class I tetramers in order to confirm the existence of antigen-specific T cells directed to these epitopes. This is beyond the scope of this article, but is the topic of a follow-up study that we are currently planning.

Taking into consideration the variability of the Nef viral sequence, single aa substitutions should be inserted in prospective epitopes. We previously demonstrated that cross-reactivity to mutated epitopes occurs after vaccination with peptides, although certain aa changes may also decrease affinity. Affinity was not related to the occurrence of CD107a cytolytic granulae on the surface of CD8⁺ cells, considered to be related to cytotoxicity. The occurrence of CD107a was instead related to allele phenotype rather than any of the total score variables.³⁷ It is therefore likely that the most accurate determination of Nef induction of CD8⁺ CTL and its contribution to protection against HIV infection will have to be performed by including Nef in an HIV vaccine where the African alleles occur frequently. Since Nef was shown here to contain many prospective epitopes for such alleles, a mutated Nef might contribute effectively to such a vaccine.

In this study we predicted 24+21 previously unreported epitopes with high MHC-I total processing prediction for HLA-A*30:01, A*30:02, B*58:01, HLA C*:07:01, and also HLA*A02:01, which are common alleles in Africa. Not yet described epitopes for allele A*02:01 had lower predictive values. Our study of the HIV-1 Nef aa sequence has brought to light several candidate epitopes that should be further studied, and indicates, despite the variation of subtypes found,³⁸ that incorporation of the Nef protein or gene in an HIV vaccine may be immunologically beneficial, related to its size. This appears to be true in particular for African populations, but also for whites.

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Author Disclosure Statement

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