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ORIGINAL ARTICLE

Expression and immunological characterization of cardamom mosaic virus coat protein displaying HIV gp41 epitopes

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ABSTRACT

The coat protein of cardamom mosaic virus (CdMV), a member of the genus *Macluravirus*, assembles into virus-like particles when expressed in an *Escherichia coli* expression system. The N and C-termini of the coat protein were engineered with the Kennedy peptide and the 2F5 and 4E10 epitopes of gp41 of HIV. The chimeric proteins reacted with sera from HIV positive persons and also stimulated secretion of cytokines by peripheral blood mononuclear cells from these persons. Thus, a system based on the coat protein of CdMV can be used to display HIV-1 antigens.

Key words coat protein, cytokine, HIV epitopes.

Cardamom mosaic virus is a member of the *Macluravirus* genus of *Potyviridae* (1). It is the causative agent of cardamom mosaic disease. Potyviral CPs are multifunctional proteins (2–4). Several studies on the expression and assembly of potyviral CPs in various expression systems have been published. In the cases of JGMV (5), tobacco etch virus (3), PVY (6–8), (PPV) (9), and pepper vein banding virus (10), expression of CP has led to formation of virus-like particles in *E. coli* and plant systems. The CPs of CdMV also form filamentous virus-like particles on expression in an *E. coli* expression system, as observed by immunogold electron microscopy (11).

The capsids of most plant viruses consist of multiple copies of one or a few types of protein subunits arranged with either icosahedral or helical symmetry. Because of their relative simplicity, stability and ease of production, plant viruses and virus-like particles from plant viral CPs have attracted attention as potential systems for display of foreign antigens. The CP of potyvirus is reportedly a good candidate for a carrier molecule for presenting epitopes. Researchers have studied several potyviruses like zucchini yellow mosaic virus (12), PPV (13), PVY (14), JGMV (15, 16) and papaya ringspot virus (17) for their ability to present epitopes on their surfaces.

The envelope glycoprotein gp41 of HIV anchors the infectious spike to the viral membrane and plays an important role in cell entry. It is composed of ~345 amino acids with a molecular mass of 41 kDa and is highly conserved. The Kennedy peptide, which is located in the cytoplasmic tail of gp41, corresponds to amino acid sequence 735–752 of the precursor envelope glycoprotein and contains three epitopes: 734PDRPEG739 (18), 740IEEE743 and 746ERDRD750 (19), the epitope ERDRD is reportedly neutralizing (20).

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List of Abbreviations: CD, cluster of differentiation; CdMV, cardamom mosaic virus; CP, coat protein; DPBS, Dulbecco's phosphate buffered saline; *E. coli, Escherichia coli*, ELISPOT, enzyme-linked immunosorbent spot; GFP, green fluorescent protein; IFN, interferon; IgG, immunoglobulin gamma; IL, interleukin; IPTG, isopropyl-1-thio-β-D-galactopyranoside; JGMV, Johnson grass mosaic virus; MIP, macrophage inflammatory protein; MPER, membrane proximal external region; PBMC, peripheral blood mononuclear cell; PPV, plum pox virus; PVY, potato virus Y; RANTES, regulated on activation, normal T cell expressed and secreted; rpm, revolutions per minute; tris, tris-(hydroxymethyl)-aminoethane.

The highly conserved MPER comprises the last 24 C-terminal amino acids of the gp41 ectodomain (21) and contains epitopes recognized by three HIV-1 broadly neutralizing monoclonal antibodies, namely 2F5, 4E10 and Z13 (22–25). The 2F5 epitope has been mapped to the motif ELDKWA at the end of the heptad repeat 2 region of gp41 (22, 25). The monoclonal antibody 4E10 recognizes a contiguous epitope at the C terminus of the 2F5 binding region (26). 2F5 and 4E10 neutralize the virus by interfering with its fusion with the target cell membrane, during which the MPER is assumed to be exposed (27).

In the current study, we have displayed various combinations of the Kennedy peptide (E1), 2F5 and 4E10 epitopes at the N and C terminals of the full length CP of CdMV (Fig. 1). We have also analyzed the ability of chimeric proteins to react with sera from HIV infected persons and to induce cytokines in their PBMCs.

MATERIALS AND METHODS

Cloning and expression of full length coat protein of cardamom mosaic virus

Using the primers 5′-GG ATCCATGGATTTAGTAGG-CACG-3′ and 5′-AAGCTTTCATATA GAGTTGCTCGT-GG-3′, the CP was amplified from the construct pTom20 (28), which contains the full length CP and part of the NIb gene. The full length CP was amplified and cloned into T-tailed vector pXcmKn12 and then subcloned into pHT7 vector (28) at *Bam*HI and *Hin*dIII sites for expression in *E. coli*. The protein was found to over-express optimally when the construct was trans-formed into Rosetta strain and induced with 0.8 mM IPTG.

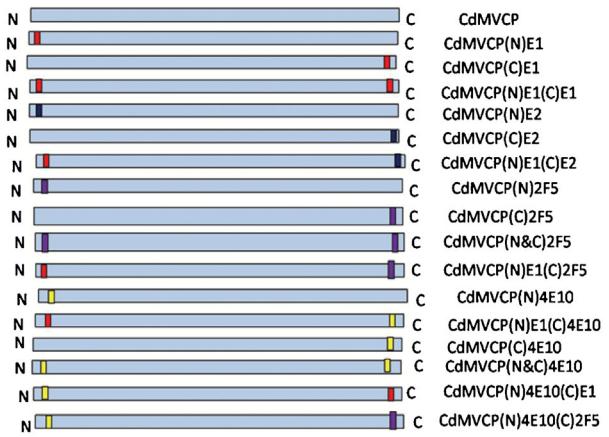

Fig. 1. Schematic representation of the chimeric proteins. The epitopes and their positions on the CdMV CP are indicated as colored bands (red, E1; violet, 2F5; yellow, 4E10).

Refolding and purification of full length coat protein of cardamom mosaic virus

The inclusion bodies formed by the over-expressed protein were purified and solubilized in unfolding buffer (8 M urea, 50 mM Tris–HCl [pH 8.5], 100 mM NaCl). The suspension was centrifuged at 14,000 rpm for 30 min at 4°C to obtain unfolded protein in the supernatant, which was passed through a 0.45 μm filter. The unfolded protein was bound to an Ni-NTA column and refolded in the column by treatment with 10 column volumes of refolding buffer (50 mM Tris, pH 8.5, 0.1 M NaCl, and 10% glycerol). It was then eluted with refolding buffer containing different concentrations of imidazole. The whole process was performed at 4°C.

Oligonucleotides coding for epitopes for engineering on cardamom mosaic virus

The oligonucleotides coding for the epitopes to be engineered were designed as follows. The peptide sequence corresponding to the epitope was back-translated to obtain the DNA sequence, the codons optimized for *E. coli* expression and then synthesized as oligonucleotides.

KE Forward 5′-CCGGGTGGTCTGGATCGTCTGG-TCGTATCGAAGAAGAAGGTGG TGAACAGGATCGT-GATCGTAGC-3′ and KE Reverse 5′-GCTACGATCAC-TATCCTG TTCACCACCTTCTTCTTCGATACGACCC-AGACGATCCAGACCACCCGG-3′, 2F5 Forward 5′-AATGAAAAAGATCTGCTGGCGCTGGATAGCTGGA-AAAATCTGTGG-3′ and 2F5 Reverse 5′-CCACAGATT-TTTCCAGCTATCCAGCGCCAGCAGATCTTTTTCAT-T-3′, 4E10 forward 5′-AGCTGGTTTGATATTGAG-CAATTGGCTGTGGTATATTCG C-3′ and 4E10 Reverse 5′-GCGAATATACCACAGCCAATTGCTAATATCAAAC-CAGCT-3′

Cloning of epitopes into cardamom mosaic virus gene

Polymerase chain reaction-based site-directed mutagenesis was used to create point mutations for the introduction of an *Eco*RV site. For creating an *Eco*RV site at the N and C termini of CdMV CP, the primer pairs 5′-GGATCCATGGATATCGTAGGCACGCCAACA-3′, 5′-TGT TGGCGTGCCTACGATATC CATGGATCC-3′ and 5′-CACTGATATCGACGAATTTG-3′, 5′-CAAATT-CGTCGATATC AGTG-3′, respectively, were used. The oligonucleotides coding for the epitopes were annealed by the following procedure: 1 μg each of epitope

oligonucleotides were heated at 94°C for 10 mins and the tubes left at room temperature till they reached 30°C, they were then precipitated, washed with 70% ethanol and re-suspended in 25 μL ultra-pure water. The CP gene was linearized with *Eco*RV, subjected to alkaline phosphatase treatment and ligated with annealed T4 polynucleotide kinase-treated oligonucleotides. The clones were screened for loss of *Eco*RV site caused by insertion of the epitope and then sequenced to confirm the orientation of the epitopes.

Cloning, expression, and purification of chimeric coat protein engineered with epitopes from HIV gp41

The chimeric CP genes were subcloned into the *E. coli* expression vector pHT7 at *Xba*I (from the vector) and *Hin*dIII (incorporated in the primer) sites. The chimeric proteins were expressed, refolded, and purified following the same procedure as for the wild type full length CdMVCP.

Western blotting of the chimeric proteins with sera from HIV positive persons

About 20 μg of protein samples were subjected to SDS–PAGE and the resolved protein bands transferred to a nitrocellulose membrane. Sera from HIV positive persons (1:20 dilution) were used as the primary antibody and rabbit anti-human IgG conjugated with horse radish peroxidase as the secondary antibody. The reactions were detected by using the substrate 4-chloro 1-napthol.

Collection of blood samples

Ten milliliters of venous blood was collected, with informed consent, from HIV positive persons whose CD4 count was greater than 350 cells/mm^3. At the time of blood collection, these persons were free of opportunistic infections and naïve to anti-retroviral therapy.

Enzyme-linked immunosorbent spot assay

Wells washed with DPBS were coated with 50 μL of 5 μg/mL of primary antibody and incubated at 4°C overnight. On Day 2, the wells were washed with DPBS and blocked with 100 μL of complete RPMI and incubated at 37°C for 2 hrs. A mixture of 100 μL of PBMCs (isolated from the blood samples) suspension (2×10^5 cells per well) and 1 μg of the chimeric proteins was added and incubated at 37°C in a CO_2 incubator overnight. On Day 3, the plates were decanted and washed three times with 100 μL DPBS and then three times with 100 μL PBS + 0.1% Tween 20. Fifty microliters of 2 μg/mL of secondary antibody was added to each well and incubated at room temperature for 3 hrs. 50 μL of streptavidin–horseradish peroxidase (1 μL/mL) was added and incubated for 1 hr. One hundred microliters of substrate (two drops buffer + three drops 3-amino-9-ethyl carbazole) was added and the plates incubated in the dark for 10 mins, then read.

Stimulation of peripheral blood mononuclear cells with chimeric proteins and assay for cytokine induction

The PBMCs (0.5×10^6 cells/500 μL) were cultured in complete RPMI medium. Chimeric protein was added at 10 μg/mL concentration. The cultures were incubated at 37°C for 3 days in a CO_2 incubator and then the cells spun at 10,000 rpm for 15 mins and the supernatant used for cytokine assays. Bio-Plex Pro cytokine and chemokine assays (Bio-Plex Pro assay kit; Biorad, Hercules, CA, USA) were performed to assess the ability of the chimeric proteins to induce these immune system signals. Two-way ANOVA was performed on the data and P values of < 0.05 considered significant.

RESULTS

Cloning, expression and purification of full length coat protein of cardamom mosaic virus

The 924 bp full length CdMVCP was amplified and cloned into an *E. coli* expression vector. In members of *Potyviridae*, the viral proteins are derived by cleavage at specific sites in the polyprotein by viral proteases. Multiple sequence alignment between the members of the *Macluravirus* genus, namely Chinese yam necrotic mosaic virus, narcissus latent virus, Maclura mosaic virus, Alpinia mosaic virus, yam chlorotic necrotic mosaic viruses, large cardamom chirke virus, Ranunculus latent virus and CdMV (Fig. 2a), revealed that QM is the most conserved possible cleavage site between NIb and CP. Therefore the start site of the full length coat protein was considered to be at methionine.

Cloning, expression and purification of chimeric cardamom mosaic virus coat protein displaying epitopes from HIV gp41

Oligonucleotides coding for the Kennedy peptide, 2F5 and 4E10 epitopes from a HIV isolate subtype C (Accession number EF469243) were inserted at the N and C terminal regions of the coat protein. The chimeric proteins obtained by engineering the Kennedy peptide (E1), the 2F5 epitope and the 4E10 epitope in different

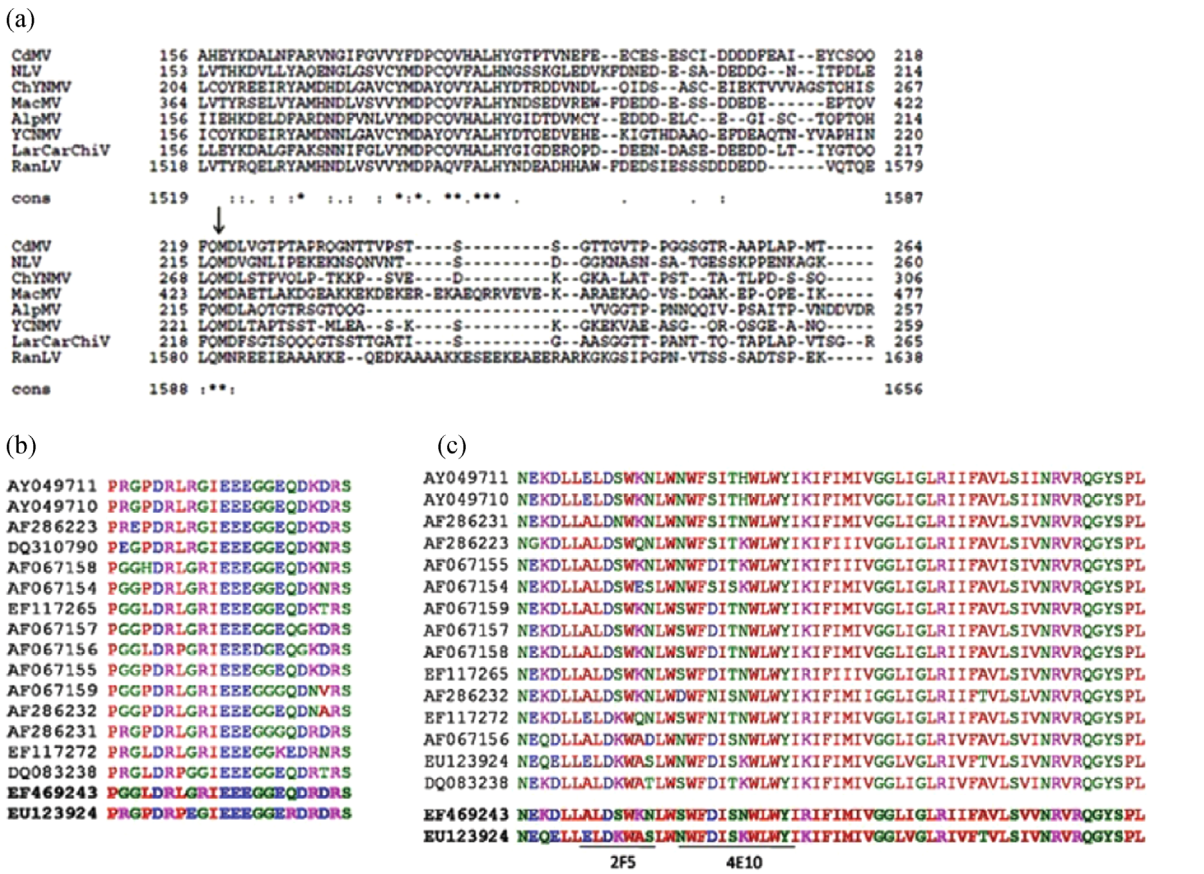

Fig. 2. (a) Alignment of NIb–CP cleavage region of *Macluravirus* members. Arrow indicates the highly conserved possible cleavage site QM. (b) Alignment of Kennedy epitope sequence from Indian isolates with subtype B sequence. (c) Alignment of 2F5 and 4E10 epitope sequences from Indian isolates with subtype B sequence.

combinations at the N and the C termini of CdMVCP (Fig. 1) were designated as follows: (i) CdMVCP(N)E1, (ii) CdMVCP(C)E1, (iii) CdMVCP(N&C)E1, (iv) CdMVCP(N)2F5, (v) CdMVCP(C)2F5, (vi) CdMVCP (N&C)2F5, (vii) CdMVCP(N)E1(C)2F5, (viii) CdMVCP (N)4E10, (ix) CdMVCP(C)4E10, (x) CdMVCP(N&C) 4E10, (xi) CdMVCP(N)E1(C)4E10, (xii) CdMVCP(N) 4E10(C)E1 and (xiii) CdMVCP(N)4E10(C)2F5.

The chimeric proteins derived from the 13 constructs listed above were refolded and purified in the same manner as the wild type coat protein. Figure 3a–l shows the gel pictures of CdMVCP and all but the last two of the above proteins.

The epitope sequences of the Kennedy epitope, 2F5 and 4E10 sequences from all the sequenced Indian isolates of HIV (subtype C) were aligned with the type B sequence (Fig. 2b,c) which is mostly used for other display studies. As the monoclonals for these epitopes were also developed for subtype B sequence, the alignment was designed to identify the corresponding epitope regions in subtype C sequence. Both globally and in India, the commonest subtype of HIV-1 infection is subtype C. Though cross clade neutralization has been demonstrated (29, 30), it is reportedly weak against some epitopes, such as 2F5 (27, 31). Therefore, in order to obtain a subtype C specific immune response the sequence from the Bangalore (India) isolate (EF469243) was used.

Reactivity of chimeric proteins with sera from HIV positive persons

The chimeric proteins had positive reactions with the sera from HIV positive persons (Fig. 4a–f, Table 1), indicating that the epitopes displayed on CdMVCP are in the correct conformation for recognition by the antibodies in sera. Wild-type CdMVCP did not react with the sera, showing that the reaction is specific towards the epitopes. Serum from a volunteer who had been vaccinated with TBC-M4 (32) was also used in this study. Because this vaccine construct has full length gp41 in the modified vaccinia *Ankara* vector, it recognized the chimeric proteins

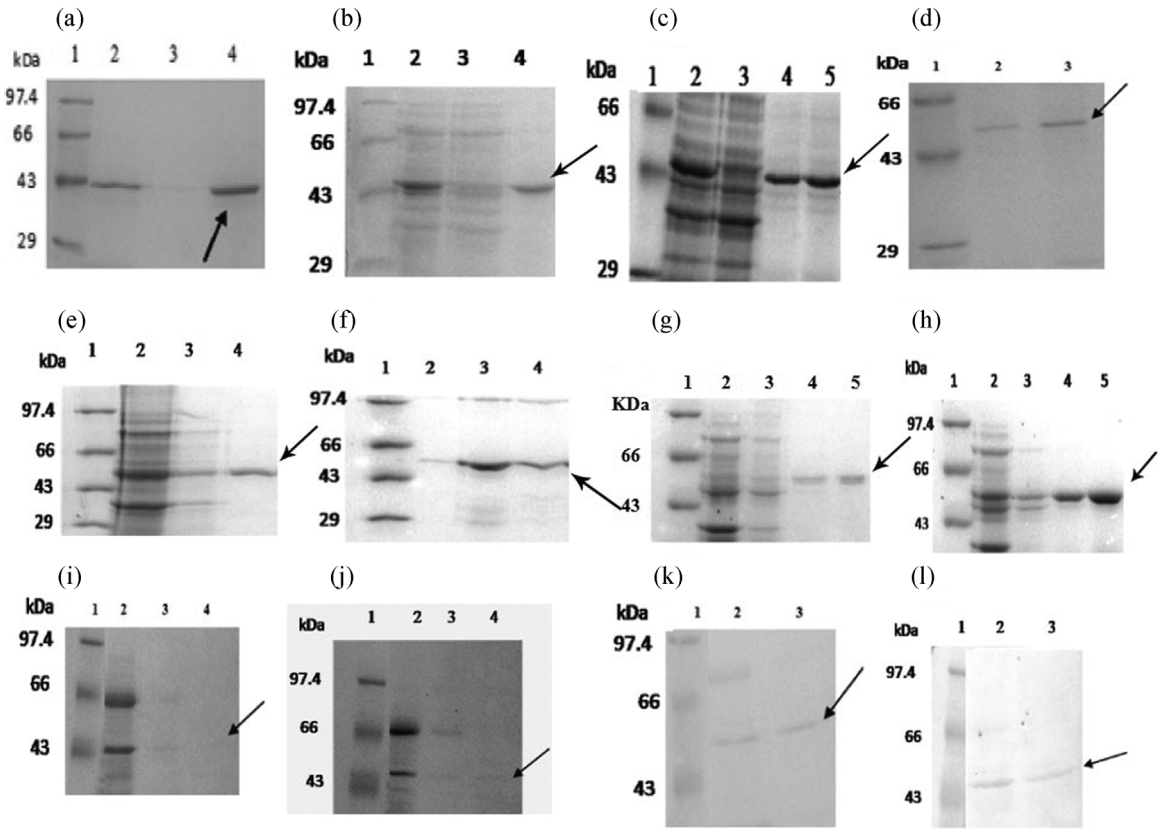

Fig. 3. Purificaton of CdMVCP and the chimeric proteins. (a) Purification of CdMVCP. Lane 1, marker; Lane 2, input; Lane 3, flowthrough; Lane 4, purified CdMVCP. (b) Purification of CdMVCP(N)E1. Lane 1, marker; Lane 2, input; Lane 3, flowthrough; Lane 4, purified CdMVCP(N)E1. (c) Purification of CdMVCP(C)E1. Lane 1, marker; Lane 2, input; Lane 3, flowthrough; Lanes 4 and 5, purified CdMVCP(C)E1. (d) Purification of CdMVCP(N&C)E1. Lane 1, marker; Lanes 2 and 3, purified CdMVCP(N&C)E1. (e) Purification of CdMVCP(N)2F5. Lane 1, marker; Lane 2, flowthrough; Lane 3, wash; Lane 4, purified CdMVCP(N)2F5. (f) Purification of CdMVCP(C)2F5. Lane 1, marker; Lane 2, wash; Lanes 3 and 4, purified CdMVCP(C)2F5. (g) Purification of CdMVCP(N&C)2F5. Lane 1, marker; Lane 2, flowthrough; Lane 3, wash; Lanes 4 and 5, purified CdMVCP(N&C)2F5. (h) Purification of CdMVCP(N)E1(C)2F5. Lane 1, marker, Lane 2, flowthrough; Lane 3, wash; Lanes 4 and 5, purified CdMVCP (N)E1(C)2F5. (i) Purification of CdMVCP(N)4E10. Lane 1, marker; Lane 2, first elute; Lanes 3 and 4, purified CdMVCP(N)4E10. (j) Purification of CdMVCP(C)4E10. Lane 1, marker; Lane 2, first elute; Lanes 3 and 4, purified CdMVCP(N)4E10. (k) Purification of CdMVCP(N&C)4E10. Lane 1, marker, Lanes 2 and 3, purified CdMVCP(N&C)4E10. (l) Purification of CdMVCP(N)4E10(C)2F5. Lane 1, marker; Lanes 2 and 3, purified CdMVCP (N)4E10(C)2F5. The arrows indicate the bands of purified chimeric proteins in the gel.

CdMVCP(N)E1 and CdMVCP(N&C)E1, as expected (Fig. 4g).

The sera from the HIV positive persons that recognized the chimeric proteins possibly contain 2F5-like, 4E10-like and Kennedy peptide specific antibodies. The chimeric proteins with two copies of epitopes (CdMVCP[N&C]E1, CdMVCP[N&C]2F5, CdMVCP[N&C]4E10) reacted with 58% (14/24, 7/12 and 7/12, respectively) of the samples tested. However not all sera recognized the chimeric proteins, because these antibodies are not produced in all such patients. Several studies assessing the presence of such antibodies in the sera of HIV patients have shown that antibodies specific to Kennedy peptide, 2F5 and 4E10 epitopes are not uniformly present in persons infected with HIV(33–38).

It is noteworthy that the chimeric proteins with two copies of epitopes at the N and C termini (CdMVCP [N&C]E1,CdMVCP[N&C]2F5 and CdMVCP[N&C] 4E10) reacted more strongly with sera from HIV infected persons than did the other chimeric proteins. Due to this high reactivity, these three chimeric proteins were subjected to further immunological studies.

Stimulation of cytokines by chimeric proteins

A preliminary screening of the samples for the production of IFN-γ was performed by ELISPOT assay. Table 2 shows the ability of the chimeric proteins to stimulate the production of IFNγ, leading to the formation of spots.

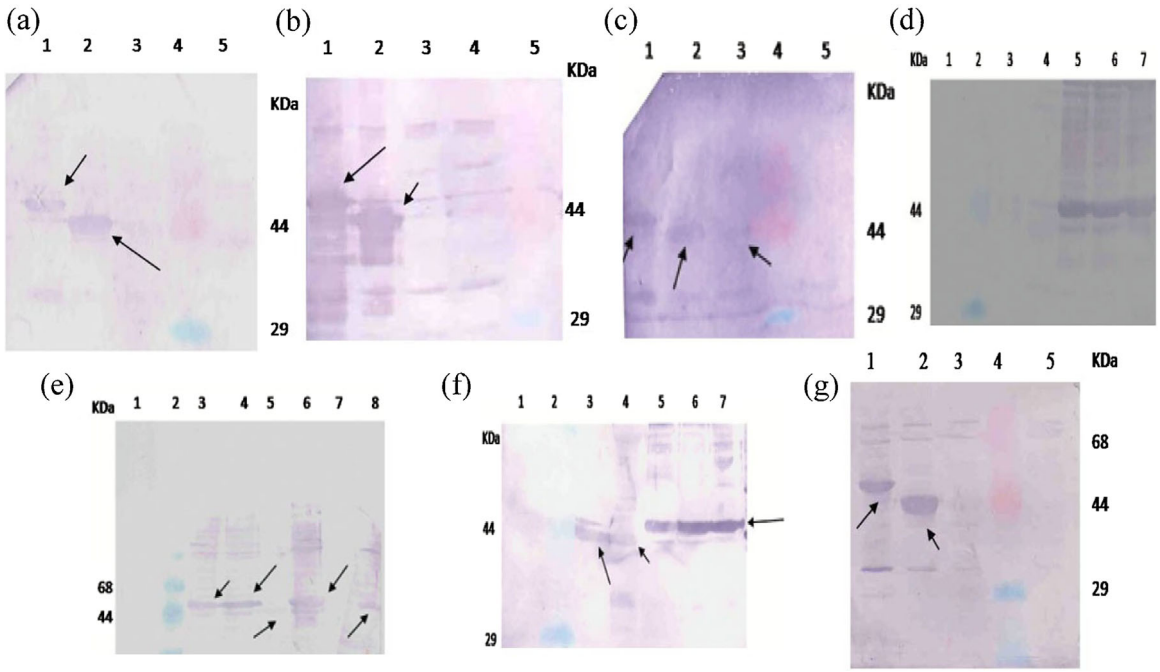

Fig. 4. (a–f) Western blots of chimeric proteins with sera from HIV positive persons. Each blot shown in the figure was treated with a serum sample from a different HIV infected person. Arrows indicate the bands of reactivity between sera and chimeric proteins. The different chimeric proteins loaded in each lane in the blots are as follows. (a) Lane 1, CdMVCP(N&C)E1; Lane 2, CdMVCP(C)E1; Lane 3, CdMVCP(N)E1; Lane 4, colored marker; Lane 5, CdMVCP. (b) Lane 1, CdMVCP(N&C)E1; Lane 2, CdMVCP(C)E1; Lane 3, CdMVCP(N)E1; Lane 4, CdMVCP; Lane 5, colored marker. (c) Lane 1, CdMVCP(N&C)E1; Lane 2, CdMVCP(C)E1; Lane 3, CdMVCP(N)E1; Lane 4, colored marker; Lane 5, CdMVCP. (d) Lane 1, CdMVCP; Lane 2, colored marker; Lane 3, CdMVCP(C)2F5; Lane 4, CdMVCP(N)4E10; Lane 5, CdMVCP(N&C)4E10; Lane 6, CdMVCPCP(N&C) 2F5; Lane 7, CP(N)E1(C)4E10. (e) Lane 1, CdMVCP; Lane 2, colored marker; Lane 3, CdMVCP(N&C)4E10; Lane 4, CdMVCP(N&C)2F5; Lane 5, CdMVCP(N)2F5; Lane 6, CdMVCP(N)E1(C)4E10; Lane 7, CdMVCP(N)4E10(C)2F5; Lane 8, CdMVCP(N&C)E1. (f) Lane 1, CdMVCP; Lane 2, marker; Lane 3, CdMVCP(C)2F5; Lane 4, CdMVCP(N)4E10; Lane 5, CdMVCP(N&C)2F5; Lane 6, CdMVCP(N&C)4E10; Lane 7, CP(N)E1(C)4E10. (g) Western blot with serum from a volunteer vaccinated with TBC-M4. Lane 1, CdMVCP(N&C)E1; Lane 2, CdMVCP(C)E1; Lane 3, CdMVCP(N)E1; Lane 4, colored marker; Lane 5, CdMVCP.

PBMCs stimulated with hemagglutinin served as positive, and unstimulated cells as negative, controls. Samples with more than thrice the number of spot-forming units in negative controls were considered significant. This experiment was followed by a detailed analysis of stimulation of different cytokines.

Because the primary immune response is minimal and therefore more difficult to recognize or quantify than the secondary immune response, PBMCs from HIV infected persons were used in the study. Responses of PBMCs from five normal persons were also studied. The chimeric proteins were found to be stimulating the cytokines and

Table 1. Summary of the results of western blot of the chimeric proteins with sera from HIV positive persons

S. no	Name of the protein	No. of samples tested	No. of samples giving positive reaction
1	CdMVCPN(E1)	21	8
2	CdMVCPC(E1)	14	6
3	CdMVCPN&C(E1)	24	14
4	CdMVCPN(4E10)	12	2
5	CdMVCPN&C(4E10)	12	7
6	CdMVCPN(2F5)	12	2
7	CdMVCPC(2F5)	12	3
8	CdMVCPN&C(2F5)	12	7
9	CdMVCPN(4E10)C(2F5)	12	1
10	CdMVCPN(4E10)C(E1)	12	4
11	CdMVCPN(E1)C(4E10)	12	3

Table 2. Summary of ELISPOT assay results

	No. of spot forming units				
Sample ID	Positive	Negative	CdMVCP(N&C)E1	CdMVCP(N&C)2F5	CdMVCP(N&C)4E10
100	373	2	**48**	**47**	**42**
101	234	1	**21**	**20**	**14**
104	511	112	83	56	130
105	74	31	**120**	**55**	**213**
108	185	11	**70**	8	**24**
109	443	101	40	10	20
112	305	8	**29**	**40**	**80**
113	33	15	**144**	**188**	**33**
115	591	39	14	15	14
116	420	25	9	**60**	**35**
117	486	6	3	5	**38**

Significant results are shown in bold.

chemokines IL-10, IL-6, IL-8, IL-1β, MIP-1α, MIP-1β and RANTES. The significant results of the study are shown as box and whisker plots (Figs. 5, 6). When the chimeric proteins were compared, CdMVCP(N&C)E1 more strongly stimulated IL-6, IL-1β, MIP-1α, MIP-1β and RANTES than did CdMVCP(N&C)2F5 and CdMVCP(N&C)4E10. When the chimeric proteins were compared with respect to IL-6 stimulation in PBMCs, it was evident that CdMVCP(N&C)E1 provided stronger stimulation than the chimeric proteins having the 2F5 and 4E10 epitopes. The responses to CdMVCP (N&C)E1 in the HIV patients' PBMCs had a median value of 90021.38 pg/mL, whereas the median values for CdMVCP(N&C)2F5 and CdMVCP(N&C)4E10 were 107.48 and 1387.66 pg/mL, respectively. Similar responses were observed with the samples from normal subjects. Again, CdMVCP(N&C)E1 was found to more strongly induce IL-1β in patients' PBMCs (median value 144.96 pg/mL) than CdMVCP(N&C)2F5 (31.3 pg/mL) and CdMVCP(N&C)4E10 (31.3 pg/mL). In the case of MIP-1α, the chimeric protein CdMVCP(N&C)E1 stimulated both patient and normal PBMCs (3659.2 pg/mL and 66×10^9 pg/mL, respectively) significantly more strongly than did CdMVCP(N&C)2F5 (56.83 and 65.76 pg/mL, respectively) and CdMVCP(N&C)4E10 (515.59 and 9196.74 pg/mL, respectively). For MIP-1 β, CdMVCP (N&C)E1 also provided stronger stimulation than did the other two chimeric proteins CdMVCP(N&C)2F5 and CdMVCP(N&C)4E10 in both the normal and patient PBMCs. In the normal controls CdMVCP(N&C)E1 stimulated more secretion of RANTES (22903.62 pg/mL) than did CdMVCP(N&C)2F5 (8586.36 pg/mL) and CdMVCP(N&C)4E10 (6474.99 pg/mL). Thus the chimeric proteins, in particular CdMVCP(N&C)E1, stimulate many cytokines and chemokines in PBMCs.

DISCUSSION

In members of the *Potyviridae* family, the viral proteins are derived by cleavage at specific sites in the polyprotein by viral proteases. Q/M is highly conserved among the Maclura viruses and, in addition to Q/S, Q/G, E/S, and E/G, is also one of the preferred cleavage sites of Como, Picorna, and Potyvirus proteases (39). Therefore, we considered methionine as the starting point of the coat protein.

Because there is no three dimensional structural data for potyviral CP, the exposed regions have to be identified based on studies of the coat protein of other potyviruses. Mild proteolysis by trypsin of the particles of six potyviruses (bean yellow mosaic virus, clover yellow vein virus, JGMV, passion fruit woodiness virus, PVY and watermelon mosaic virus II) revealed that the N and C terminal regions of their coat proteins are exposed on the particles (40). Pepscan analysis of PPV coat protein has confirmed that the N and C terminal domains of PPVCP are the immuno-dominant regions of the protein (13). These data suggest that this may be a general feature of all potyviruses. The predicted secondary structure of CdMVCP also shows random coils without any helix or strand at the N and C termini. Therefore, we chose the N and C termini of CdMVCP for display of epitopes from HIV gp41.

There are many reports in which Kennedy peptide has been engineered on a variety of display systems (19, 41, 42), including the CP of a plant virus cowpea mosaic virus, which leads to a strong neutralizing antibody response that provides 99% neutralization of HIV 1 IIIB (43). Antibodies against the Kennedy epitope region can reportedly perform post-attachment neutralization, neutralization of infectious progeny and strong standard neutralization (20, 44–47), making it a suitable candidate for display.

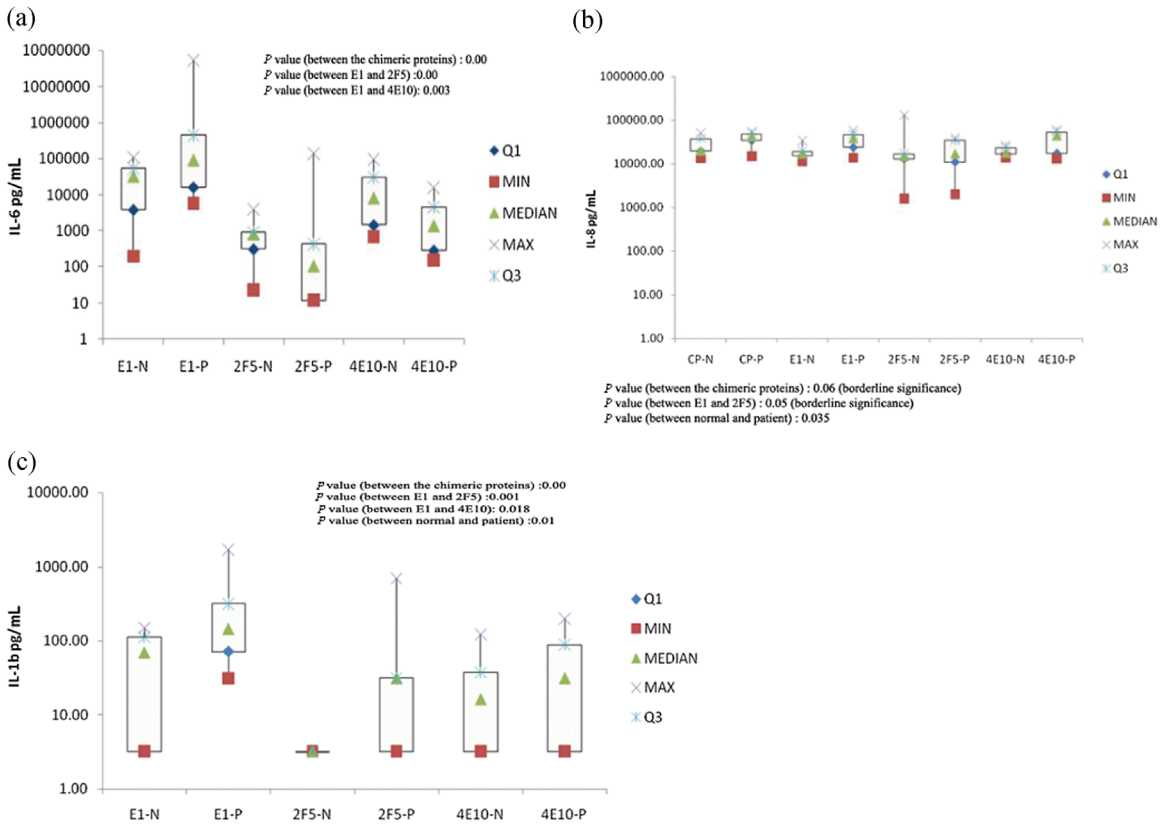

Fig. 5. (a–c): Box and whisker plots showing stimulation of cytokines IL-6, IL-8, and IL-1beta by chimeric proteins. A two-way ANOVA was performed for the data; a *P* value of < 0.05 was considered significant. N, normal; P, patient; Q1, first quartile; Q3, third quartile.

Several chimeric constructs developed with 2F5 epitope "ELDKWA" displayed on various carriers (48–53) give rise to varying degrees of immune response. An enhanced response has been observed in the case of recombinant immunogens in which there is a high density of ELDKWA epitopes (54). Thus, display of the 2F5 epitope on CdMVCP, which forms virus like particles, is expected to induce a good immune response with neutralizing antibodies. The 4E10 epitope, the most broadly cross reactive monoclonal antibody described to date, neutralizes all viruses so far tested, making it a suitable candidate for display on CdMVCP because it provides immune responses against all subtypes of HIV.

The recognition of the chimeric proteins displaying the 2F5, 4E10 and Kennedy peptide shows that the epitopes are displayed by CdMVCP. The 2F5-like, 4E10-like and Kennedy epitope specific antibodies are not consistently produced by all HIV positive persons. Possible explanations include immune diversion by non-neutralizing MPER epitopes (55), down modulation of neutralizing MPER antibodies (56) and immune tolerance mechanisms (2F5- and 4E10-like antibodies are autoreactive) (57).

Many studies of the presence of antibodies specific to Kennedy peptide, 2F5 and 4E10 epitopes have been performed. About 67% of individuals with asymptomatic HIV infection and 37% of those with symptomatic disease have antibodies against the Kennedy epitope and are capable of recognizing the chimera of polio virus with Kennedy epitope (38). Immunization of humans with gp160 elicits antibodies against the immunodominant Kennedy peptide region (35). About 56% of 50 samples of HIV positive sera (33) and 70% of 20 maternal HIV positive sera (37) reportedly have antibodies that can react with peptides containing the 2F5 epitope. Neutralizing antibodies reacting with the 2F5 epitope are reportedly responsible for serum neutralizing activity (36). The presence of 4E10-like antibodies in the broadly cross neutralizing plasma of an HIV positive person and their importance in virus neutralization have been reported (34). These studies show the presence of antibodies to these epitopes in some, but not all, HIV infected persons, explaining the reactivity of the chimeric proteins with sera from HIV infected persons.

We found that the chimeric proteins stimulated the cytokines and chemokines IL-10, IL-6, IL-8, IL-1β, MIP-1α, MIP-1β and RANTES. Genetic

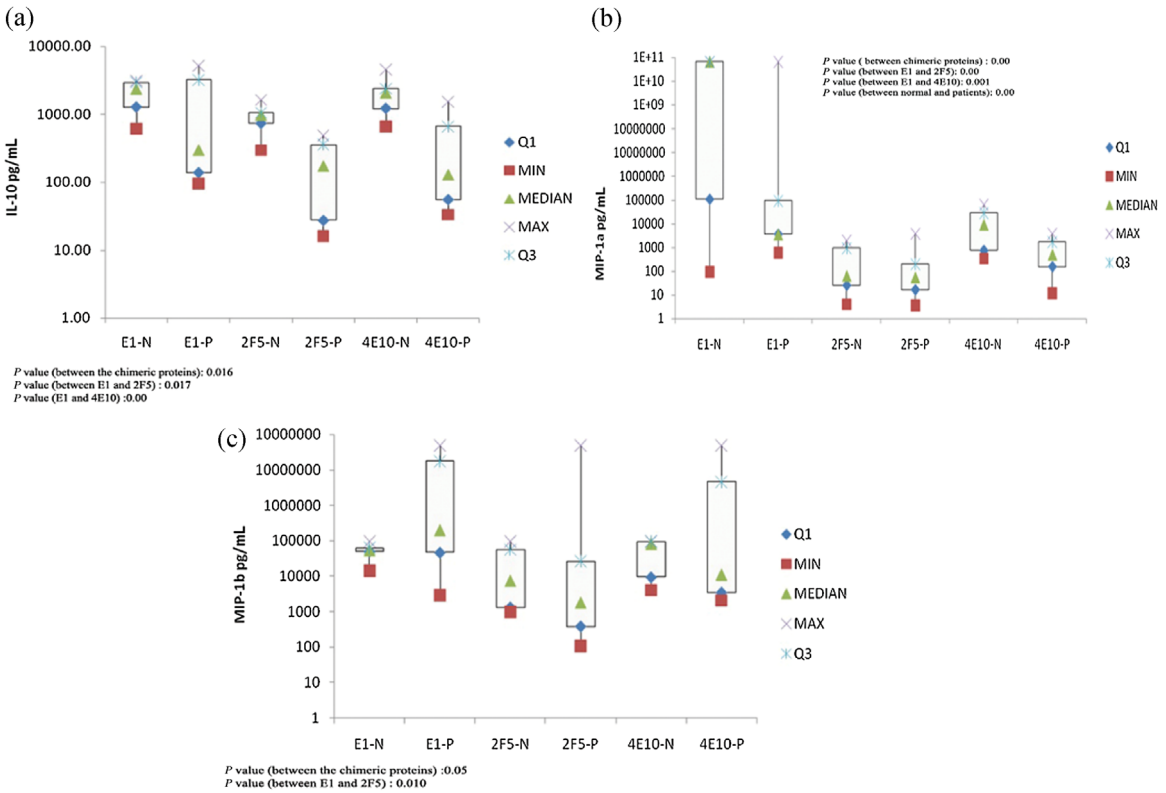

Fig. 6. (a–c): Box and whisker plots showing the stimulation of cytokines IL-10, MIP-1α, and MIP-1β by chimeric proteins. A two-way ANOVA was performed for the data; a *P* value of < 0.05 was considered significant. N, normal; P, patient; Q1, first quartile; Q3, third quartile.

polymorphisms associated with strong IL-10 production reportedly slow disease progression (58) and reduce susceptibility to HIV infection (59, 60). Treatment with recombinant IL-10 has been shown to prevent HIV infection in a humanized mouse model (61). These studies show the importance of IL-10 in HIV prevention. The chimeric proteins' ability to stimulate IFN-γ, which plays an important role in adaptive and innate immune responses to viral infection (62), is evident from the ELISPOT assay. IFN-γ has been shown to mediate antiviral activity against R5-HIV-1 in thymocytes (63). IFN-γ production is down-modulated in HIV patients (64, 65). IL-8, which is increased in the peripheral blood and lymphoid tissue of HIV positive individuals, reportedly decreases replication of primary R5-tropic HIV-1 by transcriptional mechanisms (66). Among the chimeric proteins, CdMVCP(N&C)4E10 stimulates the most IL-8 secretion, closely followed by CdMVCP(N&C)E1. According to previous studies, exposure of human PBMCs to HIV strongly induces IL-6 (67).

Chimeric proteins stimulate the chemokines MIP-1α, MIP-1β and RANTES. These chemokines bind to chemokine receptor type 5 and, by promoting internalization of this critical HIV coreceptor, decrease the ability of HIV to gain entry into otherwise susceptible cells (68). Recombinant human RANTES, MIP-1α and MIP-1β induce dose dependent inhibition of strains of HIV-1, HIV-2 and simian immunodeficiency virus. Autocrine production of MIP-1α and MIP-1β by antigen-specific $CD4^+$ T cells is reportedly protective against HIV-infection *in vivo* (69). Stimulation of these chemokines by chimeric proteins might lead to protection of the cells against HIV.

Thus the chimeric proteins, in particular CdMVCP (N&C)E1, stimulate many cytokines and chemokines that help in preventing HIV infection.

We have shown that the chimeric proteins developed displaying the epitopes from HIV-1 on the coat protein of CdMVCP are immunogenic *in vitro*. The successful display of epitopes from HIV shows the possibilities for development of diagnostics: the CdMVCP system could be used for display of epitopes. With further studies on antibody production patterns by these chimeric proteins, their potential to be used as vaccine candidates could be ascertained.

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DISCLOSURE

There is no conflict of interest for any of the authors of the manuscript caused by financial, commercial or other affiliations.

REFERENCES

1. Jacob T., Usha R. (2001) 3′-Terminal sequence analysis of the RNA genome of the Indian isolate of cardamom mosaic virus: a new member of genus Maclura virus of potyviridae. *Virus Genes* **23**: 81–8.
2. Atreya P.L., Atreya C.D., Pirone T.P. (1991) Amino acid substitutions in the coat protein result in loss of insect transmissibility of a plant virus. *Proc Natl Acad Sci USA* **88**: 7887–91.
3. Dolja V.V., Haldeman-Cahill R., Montgomery A.E., Vandenbosch K.A., Carrington J.C. (1995) Capsid protein determinants involved in cell-to-cell and long distance movement of tobacco etch potyvirus. *Virology* **206**: 1007–16.
4. Mahajan S., Dolja V.V., Carrington J.C. (1996) Roles of the sequence encoding tobacco etch virus capsid protein in genome amplification: requirements for the translation process and a cis-active element. *J Virol* **70**: 4370–9.
5. Jagadish M.N., Ward C.W., Gough K.H., Tulloch P.A., Whittaker L.A., Shukla D.D. (1991) Expression of potyvirus coat protein in Escherichia coli and yeast and its assembly into virus-like particles. *J Gen Virol* **72**: 1543–50.
6. Stram Y., Chetsrony A., Karchi H., Karchi M., Edelbaum O., Vardi E., Livneh O., Sela I. (1993) Expression of the "helper component" protein of potato virus Y (PVY) in *E. coli* : possible involvement of a third protease. *Virus Genes* **7**: 151–6.
7. Jagadish M.N., Huang D., Ward C.W. (1993) Site-directed mutagenesis of a potyvirus coat protein and its assembly in *Escherichia coli*. *J Gen Virol* **74**: 893–6.
8. Jagadish M.N., Hamilton R.C., Fernandez C.S., Schoofs P., Davern K.M., Kalnins H., Ward C.W., Nisbet I.T. (1993) High level production of hybrid potyvirus-like particles carrying repetitive copies of foreign antigens in *Escherichia coli*. *Biotechnology* **11**: 1166–70.
9. Varrelmann M., Palkovics L., Maiss E. (2000) Transgenic or plant expression vector-mediated recombination of plum pox virus. *J Virol* **74**: 7462–9.
10. Joseph J., Savithri H.S. (1999) Determination of 3′-terminal nucleotide sequence of pepper vein banding virus RNA and expression of its coat protein in *Escherichia coli*. *Arch Virol* **144**: 1679–87.
11. Jacob T., Usha R. (2002) Expression of Cardamom mosaic virus coat protein in *Escherichia coli* and its assembly into filamentous aggregates. *Virus Res* **86**: 133–41.
12. Arazi T., Shiboleth Y.M., Gal on A. (2001) A nonviral peptide can replace the entire N terminus of Zucchini yellow mosaic potyvirus coat protein and permits viral systemic infection. *J Virol* **75**: 6329–36.
13. Fernández-Fernández M.R., Martínez-Torrecuadrada J.L., Roncal F., Domínguez E., García J.A. (2002) Identification of immunogenic hot spots within Plum pox potyvirus capsid protein for efficient antigen presentation. *J Virol* **76**: 12646–53.
14. Kalnciema I., Skrastina D., Ose V., Pumpens P., Zeltins A. (2011) Potato virus Y-like particles as a new carrier for the presentation of foreign protein stretches. *Mol Biotechnol* **52**: 129–39.
15. Saini M., Vrati S. (2003) A Japanese encephalitis virus peptide present on Johnson grass mosaic virus-like particles induces virus-neutralizing antibodies and protects mice against lethal challenge. *J Virol* **77**: 3487–94.
16. Choudhury S., Kakkar V., Suman P., Chakrabarti K., Vrati S., Gupta S.K. (2009) Immunogenicity of zona pellucida glycoprotein-3 and spermatozoa YLP12 peptides presented on Johnson grass mosaic virus-like particles. *Vaccine* **27**: 2948–53.
17. Chatchen S., Juricek M., Rueda P., Kertbundit S. (2006) Papaya ringspot virus coat protein gene for antigen presentation in *Escherichia coli*. *J Biochem Mol Biol* **39**: 16–21.
18. Abacioglu Y.H., Fouts T.R., Laman J.D., Classen E., Pincus S.H., Moore J.P., Roby C.A., Kamin-Lewis R., Lewis G.K. (1994) Epitope mapping and topology of baculovirus-expressed HIV-1 gp160 determined with a panel of murine monoclonal antibodies. *AIDS Res Hum Retrovir* **10**: 371–81.
19. Vella C., Ferguson M., Dunn G., Meloen R., Langedijk H., Evans D., Minor P.D. (1993) Characterization and primary structure of a human immunodeficiency virus type 1 (HIV-1) neutralization domain as presented by a poliovirus type 1/HIV-1 chimera. *J Gen Virol* **74**: 2603–07.
20. Cleveland S.M., McLain L., Cheung L., Jones T., Hollier M., Dimmock N.J. (2003) A region of the C-terminal tail of the gp41 envelope glycoprotein of human immunodeficiency virus type 1 contains a neutralizing epitope: evidence for its exposure on the surface of the virion. *J Gen Virol* **84**: 591–602.
21. Zwick M.B., Jensen R., Church S., Wang M., Stiegler G., Kunert R., Katinger H., Burton D.R. (2005) Anti-human immunodeficiency virus type 1 (HIV-1) antibodies 2F5 and 4E10 require surprisingly few crucial residues in the membrane-proximal external region of glycoprotein gp41 to neutralize HIV-1. *J Virol* **79**: 1252–61.
22. Muster T., Steindl F., Purtscher M., Trkola A., Klima A., Himmler G., Ruker F., Katinger H. (1993) A conserved neutralizing epitope on gp41 of human immunodeficiency virus type 1. *J Virol* **67**: 6642–7.
23. Purtscher M., Trkola A., Gruber G., Buchacher A., Predl R., Steindl F., Tauer C., Berger R., Barrett N., Jungbauer A. (1994) A broadly neutralizing human monoclonal antibody against gp41 of human immunodeficiency virus type 1. *AIDS Res Hum Retrovir* **10**: 1651–8.
24. Salzwedel K., West J.T., Hunter E. (1999) A conserved tryptophan-rich motif in the membrane-proximal region of the human immunodeficiency virus type 1 gp41 ectodomain is important for Env-mediated fusion and virus infectivity. *J Virol* **73**: 2469–80.
25. Zwick M.B., Labrijn A.F., Wang M., Spenlehauer C., Saphire E. O., Binley J.M., Moore J.P., Stiegler G., Katinger H., Burton D.R., Parren P.W. (2001) Broadly neutralizing antibodies targeted to the membrane proximal external region of human immunodeficiency virus type 1 glycoprotein gp41. *J Virol* **75**(10): 892–905.
26. Stiegler G., Kunert R., Purtscher M., Wolbank S., Voglauer R., Steindl F., Katinger H. (2001) A potent cross-clade neutralizing human monoclonal antibody against a novel epitope on gp41 of

human immunodeficiency virus type 1. *AIDS Res Hum Retrovir* **17**: 1757–65.
27. Binley J.M., Wrin T., Korber B., Zwick M.B., Wang M., Chappey C., Stiegler G., Kunert R., Zolla-Pazner S., Katinger H., Petropoulos C.J., Burton D.R. (2004) Comprehensive cross-clade neutralization analysis of a panel of anti-human immunodeficiency virus type 1 monoclonal antibodies. *J Virol* **78**: 13232–52.
28. Jacob T. (2002) Investigations on the molecular biology of two phytopathogens: *Cardamom mosaic virus* and *Erwinia amylovora*. PhD thesis. Madurai Kamaraj University.
29. Ferrantelli F., Kitabwalla M., Rasmussen R.A., Cao C., Chou T.C., Katinger H., Stiegler G., Cavacini L.A., Bai Y., Cotropia J., Ugen K.E., Ruprecht R.M. (2004) Potent cross-group neutralization of primary human immunodeficiency virus isolates with monoclonal antibodies implications for acquired immunodeficiency syndrome vaccine. *J Infect Dis* **189**: 71–4.
30. Xu W.D., Smith-Franklin B.A., Li P.L., Wood C., He J., Du Q.J., Bhat G.J., Kankasa C., Katinger H., Cavacini L.A., Posner M.R., Burton D.R., Chou T.C., Ruprecht R.M. (2001) Potent neutralization of primary human immunodeficiency virus clade C isolates with a synergistic combination of human monoclonal antibodies raised against clade B. *J Hum Virol* **4**: 55–61.
31. Bures R., Morris L., Williamson C., Ramjee G., Deers M., Fiscus S.A., Abdool-Karim S., Montefiori D.C. (2002) Regional clustering of shared neutralization determinants on primary isolates of clade C human immunodeficiency virus type 1 from South Africa. *J Virol* **76**: 2233–44.
32. Ramanathan V.D., Kumar M., Mahalingam J., Sathyamoorthy P., Narayanan P.R., Solomon S., Panicali D., Chakrabarty S., Cox J., Sayeed E., Ackland J., Verlinde C., Vooijs D., Loughran K., Barin B., Lombardo A., Gilmour J., Stevens G., Smith M.S., Tarragona-Fiol T., Hayes P., Kochhar S., Excler J.L., Fast P. (2009) A Phase 1 study to evaluate the safety and immunogenicity of a recombinant HIV type 1 subtype C-modified vaccinia Ankara virus vaccine candidate in Indian volunteers. *AIDS Res Hum Retroviruses* **25**: 1107–16.
33. Broliden P.A., Von Gegerfelt A., Clapham P., Rosen J., Fenyo E. M., Wahren B., Broliden K. (1992) Identification of human neutralization-inducing regions of the human immunodeficiency virus type 1 envelope glycoproteins. *Proc Natl Acad Sci USA* **89**: 461–5.
34. Dieltjens T., Heyndrickx L., Willems B., Gray E., Nieuwenhove L. V., Grupping K., Vanham G., Janssens W. (2009) Evolution of antibody landscape and viral envelope escape in an HIV-1 CRF02_AG infected patient with 4E10-like antibodies. *Retrovirology* **6**: 113.
35. Pincus S.H., Messer K.G., Schwartz D.H., Lewis G.K., Graham B. S., Blattner W.A., Fisher G. (1993) Differences in the antibody response to human immunodeficiency virus-1 envelope glycoprotein (gp160) in infected laboratory workers and vaccinees. *J Clin Invest* **91**: 1987–96.
36. Shen X., Parks R.J., Montefiori D.C., Kirchherr J.L., Keele B.F., Decker J.M., Blattner W.A., Gao F., Weinhold K.J., Hicks C.B., Greenberg M.L., Hahn B.H., Shaw G.M., Haynes B.F., Tomaras G.D. (2009) *In vivo* gp41 antibodies targeting the 2F5 mAb epitope mediate HIV-1 neutralization breadth. *J Virol* **83**: 3617–25.
37. Ugen K.E., Goedert J.J., Boyer J., Refaeli Y., Frank I., Williams W. V., Willoughby A., Landesman S., Mendez H., Rubinstein A. (1992) Vertical transmission of human immunodeficiency virus (HIV) infection. Reactivity of maternal sera with glycoprotein 120 and 41 peptides from HIV type 1. *J Clin Investig* **89**: 1923–30.
38. Vella C., Minor P.D., Weller I.V.D., Jenkins O., Evans D., Almond J. (1991) Recognition of poliovirus/HIV chimeras by antisera from individuals with HIV infection. *AIDS* **5**: 425–30.
39. Hellen C.U., Kräusslich H.G., Wimmer E. (1989) Proteolytic processing of polyproteins in the replication of RNA viruses. *Biochemistry* **28**: 9881–90.
40. Shukla D.D., Strike P.M., Tracy S.L., Gough K.H., Ward C.W. (1988) The N and C termini of the coat proteins of potyviruses are surface-located and the N terminus contains major virus-specific epitopes. *J Gen Virol* **69**: 1497–508.
41. Kalyan N.K., Lee S.G., Wilhelm J., Pisano M.R., Hum W.T., Hsiao C.L., Davis A.R., Eichberg J.W., Robert-Guroff M., Hung P. P. (1994) Immunogenicity of recombinant influenza virus haemagglutinin carrying peptides from the envelope protein of human immunodeficiency virus type 1. *Vaccine* **12**: 753–60.
42. Newton S.M.C., Joys T.M., Anderson S.A., Kennedy R.C., Hovi M.E., Stocker B.A.D. (1995) Expression and immunogenicity of an 18-residue epitope of HIV-1 gp41 inserted in the flagellar protein of a Salmonella live vaccine. *Res Microbiol* **146**: 203–16.
43. McLain L., Durrani Z., Wisniewski L.A., Porta C., Lomonossoff G.P., Dimmock N.J. (1996) Stimulation of neutralizing antibodies to human immunodeficiency virus type 1 in three strains of mice immunized with a 22 amino acid peptide of gp41 expressed on the surface of a plant virus. *Vaccine* **14**: 799–810.
44. Cleveland S.M., Buratti E., Jones T.D., North P., Baralle F.E., McLain L., McInerney T.L., Durrani Z., Dimmock N.J. (2000) Immunogenic and antigenic dominance of a non-neutralizing epitope over a highly conserved neutralizing epitope in the gp41 transmembrane envelope glycoprotein of HIV-1: its deletion leads to a strong neutralizing antibody response. *Virology* **266**: 66–78.
45. Cleveland S.M., Jones T.D., Dimmock N.J. (2000) Properties of a neutralizing antibody that recognizes a conformational form of epitope ERDRD in the C-terminal tail of human immunodeficiency virus type 1. *J Gen Virol* **81**: 1251–60.
46. Dimmock N.J. (2005) The complex antigenicity of a small external region of the C-terminal tail of the HIV-1 gp41 envelope protein: a lesson in epitope analysis. *Rev Med Virol* **15**: 365–81.
47. Heap C.J., Reading S.A., Dimmock N.J. (2005) An antibody specific for the C-terminal tail of gp41 of HIV-1 mediates post-attachment neutralization probably through inhibiting virus-cell fusion. *J Gen Virol* **86**: 1501–9.
48. Coeffier E., Clement J.M., Cussac V., Khodaei-Boorane N., Jehanno M., Rojas M., Dridi A., Latour M., El Habib R., Barre-Sinoussi F., Hofnung M., Leclerc C. (2000) Antigenicity and immunogenicity of the HIV-1 gp41 epitope ELDKWA inserted into permissive sites of the MalE protein. *Vaccine* **19**: 684–93.
49. Ho J., Uger R.A., Zwick M.B., Luscher M.A., Barber B.H., MacDonald K.S. (2005) Conformational constraints imposed on a pan-neutralizing HIV-1 antibody epitope result in increased antigenicity but not neutralizing response. *Vaccine* **23**: 1559–73.
50. Kusov Y.Y., Zamjatina N.A., Poleschuk V.F., Michailov M.I., Morace G., Eberle J., Gauss-Muller V. (2006) Immunogenicity of a chimeric hepatitis A virus (HAV) carrying the HIV gp41 epitope 2F5. *Antivir Res* **73**: 101–11.
51. Marusic C., Rizza P., Lattanzi L., Mancini C., Spada M., Belardelli F., Benvenuto E., Capone I. (2001) Chimeric plant virus particles as immunogens for inducing murine and human immune responses against human immunodeficiency virus type 1. *J Virol* **75**: 8434–9.
52. Muster T., Guinea R., Trkola A., Purtscher M., Klima A., Steindl F., Palese P., Katinger H. (1994) Cross-neutralizing activity

against divergent human immunodeficiency virus type 1 isolates induced by the gp41 sequence ELDKWAS. *J Virol* **68**: 4031–4.
53. Zhang H., Huang Y., Fayad R., Spear G.T., Qiao L. (2004) Induction of mucosal and systemic neutralizing antibodies against human immunodeficiency virus type 1 (HIV-1) by oral immunization with bovine papillomavirus-HIV-1 gp41 chimeric virus-like particles. *J Virol* **78**: 8342–8.
54. Wang Z., Liu Z., Cheng X., Chen Y.H. (2005) The recombinant immunogen with high-density epitopes of ELDKWA and ELDEWA induced antibodies recognizing both epitopes on HIV-1 gp41. *Microbiol Immunol* **49**: 703–9.
55. Moore P.L., Crooks E.T., Porter L., Zhu P., Cayanan C.S., Grise H., Corcoran P., Zwick M.B., Franti M., Morris L., Roux K.H., Burton D.R., Binley J.M. (2006) Nature of nonfunctional envelope proteins on the surface of human immunodeficiency virus type 1. *J Virol* **80**: 2515–28.
56. Alam S.M., Scearce R.M., Parks R., Plonk K., Plonk S.G., Sutherland L.L., Gorny M.K., Zolla-Pazner S., Vanleeuwen S., Moody M.A., Xia S.M., Montefiori D.C., Tomaras G.D., Weinhold K.J., Karim S.A., Hicks C.B., Liao H.X., Robinson J., Shaw G.M., Haynes B.F. (2008) Human immunodeficiency virus type 1 gp41 antibodies that mask membrane proximal region epitopes: antibody binding kinetics, induction, and potential for regulation in acute infection. *J Virol* **82**: 115–25.
57. Haynes B.F., Fleming J., St. Clair E.W., Katinger H., Stiegler G., Kunert R., Robinson J., Scearce R.M., Plonk K., Staats H.F., Ortel T.L., Liao H.X., Alam S.M. (2005) Cardiolipin polyspecific autoreactivity in two broadly neutralizing HIV-1 antibodies. *Science* **308**: 1906–8.
58. Erikstrup C., Kallestrup P., Zinyama-Gutsire R.B., Gomo E., Butterworth A.E., Pedersen B.K., Ostrowski S.R., Gerstoft J., Ullum H. (2007) Reduced mortality and CD4 cell loss among carriers of the interleukin-10-1082G allele in a Zimbabwean cohort of HIV-1-infected adults. *AIDS* **21**: 2283–91.
59. Naicker D.D., Wang B., Losina E., Zupkosky J., Bryan S., Reddy S., Jaggernath M., Mokgoro M., Goulder P.J., Kaufmann D.E., Ndung'u T. (2011) Association of IL-10-promoter genetic variants with the rate of CD4 T-cell loss, IL-10 plasma levels, and breadth of cytotoxic T-cell lymphocyte response during chronic HIV-1 infection. *Clin Infect Dis* **54**: 294–302.
60. Shin H.D., Winkler C., Stephens J.C., Bream J., Young H., Goedert J.J., O'Brien T.R., Vlahov D., Buchbinder S., Giorgi J., Rinaldo C., Donfield S., Willoughby A., O'Brien S.J., Smith M.W. (2000) Genetic restriction of HIV-1 pathogenesis to AIDS by promoter alleles of IL10. *Proc Natl Acad Sci USA* **97**: 14,467–72.
61. Kollmann T.R., Pettoello-Mantovani M., Katopodis N.F., Hachamovitch M., Rubinstein A., Kim A., Goldstein H. (1996) Inhibition of acute in vivo human immunodeficiency virus infection by human interleukin 10 treatment of SCID mice implanted with human fetal thymus and liver. *Proc Natl Acad Sci USA* **93**: 3126–31.
62. Malmgaard L. (2004) Induction and regulation of IFNs during viral infections. *J Interferon Cytokine Res* **24**: 439–54.
63. Pedroza-Martins L., Boscardin W.J., Anisman-Posner D.J., Redsar B.N., Bryson Y.J., Yang O.O., Uittenbogaart C.H. (2006) Interferon-[gamma] decreases replication of primary R5 HIV-1 isolates in thymocytes. *AIDS* **20**: 939–42.
64. Imami N., Antonopoulos C., Hardy G., Gazzard B., Gotch F. (1999) Assessment of type 1 and type 2 cytokines in HIV type 1-infected individuals: impact of highly active antiretroviral therapy. *AIDS Res Hum Retro* **15**: 1499–508.
65. Meyaard L., Hovenkamp E., Keet I., Hooibrink B., de Jong I., Otto S., Miedema F. (1996) Single cell analysis of IL-4 and IFN-gamma production by T cells from HIV-infected individuals: decreased IFN-gamma in the presence of preserved IL-4 production. *J Immunol* **157**: 2712–8.
66. Rollenhagen C., Asin S.N. (2010) IL-8 decreases HIV-1 transcription in peripheral blood lymphocytes and ectocervical tissue explants. *J Acquir Immune Defic Syndr* **54**: 463–9.
67. Breen E., Rezai A., Nakajima K., Beall G., Mitsuyasu R., Hirano T., Kishimoto T., Martinez-Maza O. (1990) Infection with HIV is associated with elevated IL-6 levels and production. *J Immunol* **144**: 480–4.
68. Cocchi F., DeVico A., Garzino-Demo A., Arya S., Gallo R., Lusso P. (1995) Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by CD8+ T cells. *Science* **270**: 1811–5.
69. Cassaza J.P., Brenchley J.M., Hill B.J., Ayana R., Ambrozak D., Roderer M., Douek D.C., Betts M.R., Koup R.A. (2009) Autocrine production of β-chemokines protects CMV-specific CD4+ T cells from HIV infection. *PLoS Pathog* **5**(10): e1000646. doi: 10.1371/journal.ppat.1000646