

Characterization of Gag and Nef-specific ELISpot-based CTL responses in HIV-1 infected Indian individuals

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Abstract Cytotoxic T lymphocyte (CTL) responses to Gag have been most frequently linked to control of viremia whereas CTL responses to Nef have direct relationship with viral load. IFN- γ ELISpot assay was used to screen CTL responses at single peptide level directed at HIV-1 subtype C Gag and Nef proteins in 30 antiretroviral therapy naive HIV-1 infected Indian individuals. PBMCs from 73.3% and 90% of the study population showed response to Gag and Nef antigens, respectively. The magnitude of Gag-specific CTL responses was inversely correlated with plasma viral load ($r = -0.45$, $P = 0.001$), whereas magnitude of Nef-specific responses was directly correlated ($r = 0.115$). Thirteen immunodominant regions (6 in Gag, 7 in Nef) were identified in the current study. The identification of Gag and Nef-specific responses across HIV-1 infected Indian population and targeting epitopes from multiple immunodominant regions may provide useful insight into the designing of new immunotherapy and vaccines.

Keywords HIV-1 specific · ELISpot · Gag · Nef · CTL responses · India

Introduction

The development of an efficacious vaccine against HIV-1 is one of the most pressing challenges facing modern medicine [1, 2]. An ideal HIV-1 vaccine candidate should generate both humoral and cellular immune responses against the virus [3]. However, broad range of virus strains found in patient poses a mammoth challenge in generating a vaccine capable of inducing neutralizing antibodies against them all. Therefore, much of the current attention in AIDS vaccine design and research has been focused on vaccines that elicit cytotoxic T lymphocyte (CTL) responses.

Role of virus-specific CD8⁺ T-cell responses in the control of HIV infection has already been documented [4–8]. Potent CTL responses are responsible for dramatic drop of plasma viral load in HIV-1 infection [9, 10] and contain viral replication [11, 12]. The emergence of HIV-1-specific CTLs in acute infection coincides with a plasma viral load decline. Long-term non-progressive infection has been associated with both strong virus-specific CTL and with robust Gag p24-specific CD4⁺ T-cell proliferative responses [6, 13–15]. Vaccine strategies capable of generating HIV specific CD8⁺ T-cell responses have been shown to control virus replication and to prevent the onset of disease in monkeys [16–18]. Additionally, the documentation of CTL escape variants suggests that selective pressures are imposed on the virus through this arm of the immune system [9, 19–21].

Cytotoxic T lymphocyte targets multiple antigens in HIV infection, but CTL responses to Gag have been most frequently linked to a better clinical outcome or control of viremia [4, 13, 22–27] whereas CTL responses to Nef has

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direct relationship with viral load and poor prognosis [4, 13]. HIV-1 *Gag* is not only the most conserved protein of HIV but also its p17 and p24 regions are most frequently targeted and have been shown to have highest magnitude of immune response with a wide array of conserved epitopes for different clades of HIV [28, 29]. HIV-1 *Nef* is one of the earliest genes to be expressed during the viral life cycle [30] and the conserved central region of Nef protein is most intensely targeted by CTL [31].

Delineation of HIV-specific CTL response, an immune correlate of protection, during the course of a natural infection and after vaccination is an elemental step in the process of CTL based vaccine development. Also, the identification of immunodominant epitopes or epitope-rich immunodominant regions that can stimulate a broad range of HIV-specific CTLs offers best way of resistance and is a justifiable approach to vaccine design [32, 33].

The accumulating genetic diversity of HIV-1 in the global AIDS epidemic, driven by a relatively high-evolution rate of the virus, is the foremost obstacle in vaccine design and characterizing CTL responses in HIV-1 infected individuals [12, 34]. The extent to which this genetic diversity impacts T-cell epitope recognition is not completely understood. Subtype C is the most prevalent in the current AIDS epidemic and responsible for the largest proportion of HIV infection in the global scenario. However, only a limited number of the HIV-1-specific CTL immune response studies have targeted non-B subtype in non-Caucasian populations, underscoring the dearth of information relevant to countries like India, which bear the brunt of global HIV pandemic [29, 35, 36].

Hence we aimed to characterize HIV-1 *Gag* and *Nef*-specific CTL responses, the two most frequently targeted [30] HIV proteins. We investigated the magnitude and frequency of CTL responses against overlapping subtype C based peptides corresponding to HIV-1 *Gag* and *Nef* region using interferon (IFN)- γ enzyme linked immunospot (ELISpot) assay to screen and further confirm immunodominant regions in HIV-1 *Gag* and *Nef* proteins. The association between HIV-1-specific CTL responses and markers of disease progression though addressed in previous studies [6, 37–42], the findings are controversial. Therefore, we studied the correlations between CTL responses for HIV-1 proteins *Gag* and *Nef* and for the subregions of these viral proteins, with the CD4⁺ T-cell counts and plasma viral load in infected Indian population.

Materials and methods

Study population

A total of 30 HIV-1 infected individuals of both sexes ranging from 19 to 40 years of age, attending the Antiretroviral

Center and AIDS Clinic at Department of Microbiology of the All India Institute of Medical Sciences (AIIMS) hospital, New Delhi (from February 2005 to January 2007) were included in the study. The diagnosis of HIV-1 infection was established serologically as recommended by the National AIDS Control Organization (NACO, India, 1986–1999) guidelines. All the infected subjects were antiretroviral therapy naive at the time of enrollment into the study. Pregnant women and patients with blood dyscrasias were excluded. The Human Ethics Committee, All India Institute of Medical Sciences, New Delhi, approved the study protocol. Informed consent was obtained from all the study subjects according to the guidelines of the committee.

Isolation of peripheral blood mononuclear cells

Peripheral blood samples from all study subjects were collected by venipuncture into K3-EDTA vacutainers (Becton Dickinson, USA). An aliquot of the sample was processed for immunophenotyping. The remainder was processed for peripheral blood mononuclear cell (PBMC) isolation by density gradient centrifugation on Ficoll-Hypaque (Sigma diagnostic Inc, St. Louis, USA) as per AIDS Clinical Trials Group (ACTG) guidelines. An aliquot of the isolated PBMC was used in ELISpot assay for screening of cellular response and remaining cells were cryopreserved. The frozen PBMCs were thawed as per ACTG guidelines. The PBMC samples with $\geq 90\%$ viability and 70% recovery were used in the further ELISpot assay to confirm the results of the screening ELISpot assay.

CD4 counts and CDC classification

Absolute CD4 and CD8 counts were determined on a FACS Calibur by the Cell Quest software (Becton Dickinson) using a set of criteria for quality control.

HIV-1 subtype C peptides

A set of 121 overlapping peptides corresponding to the consensus HIV-1 subtype C *Gag* (510 amino acids) sequence was obtained from NIH, Bethesda, USA. Similarly a set of 49 overlapping peptides corresponding to the consensus C *Nef* (206 amino acids) sequence was synthesized (Fred Hutch Cancer Research Center, Seattle, USA). The *Nef* peptides were based on the consensus of African and Indian subtype C sequences. Each peptide was 15 ± 1 amino acids (aa) in length, overlapping the next peptide by 11 aa. The lyophilized peptides were dissolved in DMSO at a concentration of 10 mg/ml (stock) and stored in aliquots at -70°C until use. HIV-1 *Gag* and *Nef* peptides pools were designed in a matrix format such that each peptide was represented once in a column pool and once in a row pool. The 121 *Gag*

peptides were pooled in an 11×11 matrix format to form 11 column pools designated C1–C11 and 11 row pools designated R1–R11. Similarly, Nef peptides were pooled in 7×7 matrix format (C1–C7 and R1–R7). Positive responses to both the pools indicated reactivity to that specific peptide, which was then retested using a confirmatory ELISpot assay at single peptide level.

ELISpot assays

Screening for CTL responses was performed by IFN- γ ELISpot using a strategy based on a matrix of peptide pools. Cryopreserved PBMCs were thawed rapidly and cultured overnight at 37°C, 5% CO₂ atmosphere in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% FCS, 10 mM HEPES buffer, 2 mM L-glutamine and 50 U of penicillin–streptomycin. The 96-well polyvinylidene plates (Millipore, Bedford, MA, USA) were coated overnight with capture anti-IFN- γ antibody (Mabtech, Cincinnati, OH, USA) at a concentration of 5 μ g/ml in phosphate-buffered saline (PBS). The plates were washed and blocked as per manufacturer's instructions. PBMC were added at a concentration of 100,000–200,000 per well in 200 μ l of RPMI medium. The Gag and Nef peptide pools were plated in duplicates and the final concentration of each peptide in the pool was 2 μ g/ml. Negative (cells and medium only) and positive control wells (cells and phytohaemagglutinin, cells and CEF peptides pool at concentration of 2 μ g/ml each) were run in duplicate for each patient sample to ensure that the cells were responsive. The plates were incubated for 18–20 h at 37°C and 5% CO₂ and then washed six times with PBS 0.05% Tween 20. The plates were then incubated at room temperature for 2 h with the corresponding biotinylated secondary antibody (Mabtech) followed by washes and a 1-h incubation with a streptavidin–horseradish peroxidase conjugate (BD). After the plates were washed, AEC peroxidase substrates (Vector Lab, CA, USA) were added for color development.

The number of spots per well was determined using an automated ELISpot plate reader (Carl Zeiss Vision GmbH, Germany). The results were expressed as spot-forming cells (SFC) per million PBMC. The negative response was <50 SFC/10⁶ PBMC in all cases. The response was considered positive if >50 SFC/10⁶ PBMC were detected and they were at least 3 standard deviations (SD) above background. Responses to two adjacent overlapping peptides were imputed as one epitopic region, because some CTL epitopes can be located in the overlapping region of two adjacent peptides, resulting in responses to both overlapping peptides. The matrix was analyzed for each patient, and candidate positive peptides were retested individually by a confirmatory ELISpot assay.

Quantification of plasma HIV-RNA

Viral RNA was extracted from 30 collected plasma samples of the patients and quantified by Amplicor HIV Monitor Assay version 1.5 (Roche diagnostics, Meylan, France) with a lower limit of detection of 400 copies/ml, strictly following the manufacturer's instructions.

Statistical analysis

The correlation between HIV-1-specific response and markers of disease progression; plasma virus load and CD4⁺ T-cell count, was assessed by non-parametric Spearman test. Values for *P* of <0.05 were considered significant. All data were analyzed using STATA 8.0 software.

Results

Subjects

A total of 30 HIV-1 infected (20 males and 10 females) antiretroviral naive individuals at different stages of HIV-1 infection were included in the study. The median age of subjects was 29 years (range, 19–40). The subjects had a median CD4⁺ T-cell count of 319 cells/ μ l (range, 11–954 cells/ μ l) and a median plasma HIV-1 virus load of 35,263 copies/ml (range, 400–874,033 copies/ml) (Table 1).

Distribution of HIV-specific CTL responses across subtype C Gag and Nef

A total of 29 of 30 patients responded to either of the HIV-1 antigens. Of the 29 responders, 22 (73%) showed a response to HIV-1 subtype C Gag, and 27 (90%) responded to HIV-1 subtype C Nef protein. Twenty-one (70%) study subjects responded to both Gag and Nef.

A wide range in the magnitude of responses was observed among the responders for HIV-1 subtype C Gag (50–2,718 SFC/10⁶ cells) and Nef proteins (54–3,179 SFC/10⁶ cells) (Fig. 1a). The total magnitude of CTL responses ranged from 255 to 5,474 SFC/10⁶ cells (mean 1,741 SFC/10⁶ cells).

Overall, 29 (96%) subjects recognized at least one peptide from either Gag or Nef regions, and in persons with detectable responses, there was a broad range in the number of responses to individual peptides targeted, from 1 to 12 (mean 4.76 epitopic or immunodominant regions per responding individual). The dominant targets in terms of breadth of responses were p24 Gag and central region of Nef (Fig. 1b). The mean breadth for Gag and Nef antigens were 2.03 and 2.73 epitopic or immunodominant regions

Table 1 Patient cohort characteristics at time of analysis

Patient	Age	Sex	CD4 count (cells/ μ l)	Viral load (RNA copies/ml)	Duration of infection (in years)
1	36	Female	166	874,033	9
2	26	Male	59	684,517	1
3	40	Male	68	487,017	1
4	40	Male	103	131,647	2
5	30	Female	143	31,146	2
6	28	Male	164	92,039	2
7	33	Female	11	6,325	2
8	40	Male	169	121,818	8
9	26	Male	191	621,918	2
10	30	Male	216	432,785	2
11	26	Male	219	22,427	2
12	27	Male	257	367,522	2
13	34	Female	291	13,461	2
14	35	Male	296	514,178	4
15	32	Male	311	423,659	3
16	27	Male	328	14,301	3
17	28	Female	337	9,450	5
18	22	Male	351	118,972	5
19	25	Female	366	111,369	2
20	36	Male	420	400	3
21	40	Male	473	204,328	6
22	22	Male	482	6,210	6
23	19	Female	492	14,465	4
24	25	Male	531	29,509	7
25	28	Male	535	2,971	1
26	29	Male	632	8,372	4
27	22	Female	694	2,161	3
28	40	Male	751	39,380	4
29	30	Male	841	3,817	2
30	28	Female	954	1,207	2

per responding subject, respectively. The magnitude of both Nef ($r = 0.83$, $P < 0.001$) and Gag specific responses correlated directly with the number of epitopic or immunodominant regions targeted ($r = 0.70$, $P < 0.001$).

Distribution of HIV-specific CTL responses within subtype C Gag and Nef

Cytotoxic T lymphocyte responses identified in the screening ELISpot assay using overlapping peptide pools were confirmed at single peptide level using a confirmatory ELISpot to further map the epitopes. Multiple regions of Gag and Nef were targeted by HIV-specific CTLs. Peptides contained within p17 and p24 were frequently targeted, as were central conserved regions of Nef. The highest magnitude of response was detected to

the central conserved region of Nef_{aa 65–147} (mean = 1,519 SFC/ 10^6 cells) and to Gag p24_{aa 152–362} (mean = 528 SFC/ 10^6 cells). Responses to these domains dominated both in terms of magnitude and frequency of recognition, followed by p17, p15 and p2.

Most of the immunodominant regions were located in central conserved regions of Nef_{aa 65–147}. The most frequently targeted region was Nef_{aa 73–95} (QVPLRPMTYK AAFDLSFFLKEKG) with 63% frequency of recognition. Nef_{aa 89–103} (FFLKEKGGLEGLIYS), and Nef_{aa 129–147} (PGPGVRYPLTFGWCFKLV) were recognized by 25.9 and 41% of the study subjects responding to Nef antigens (Table 2). Among Gag subunits most frequently recognized subunits were p24 (63%) followed by p17 (40%) and very few peptides were recognized from p15, p7, p6 and p2 regions (Fig. 1b).

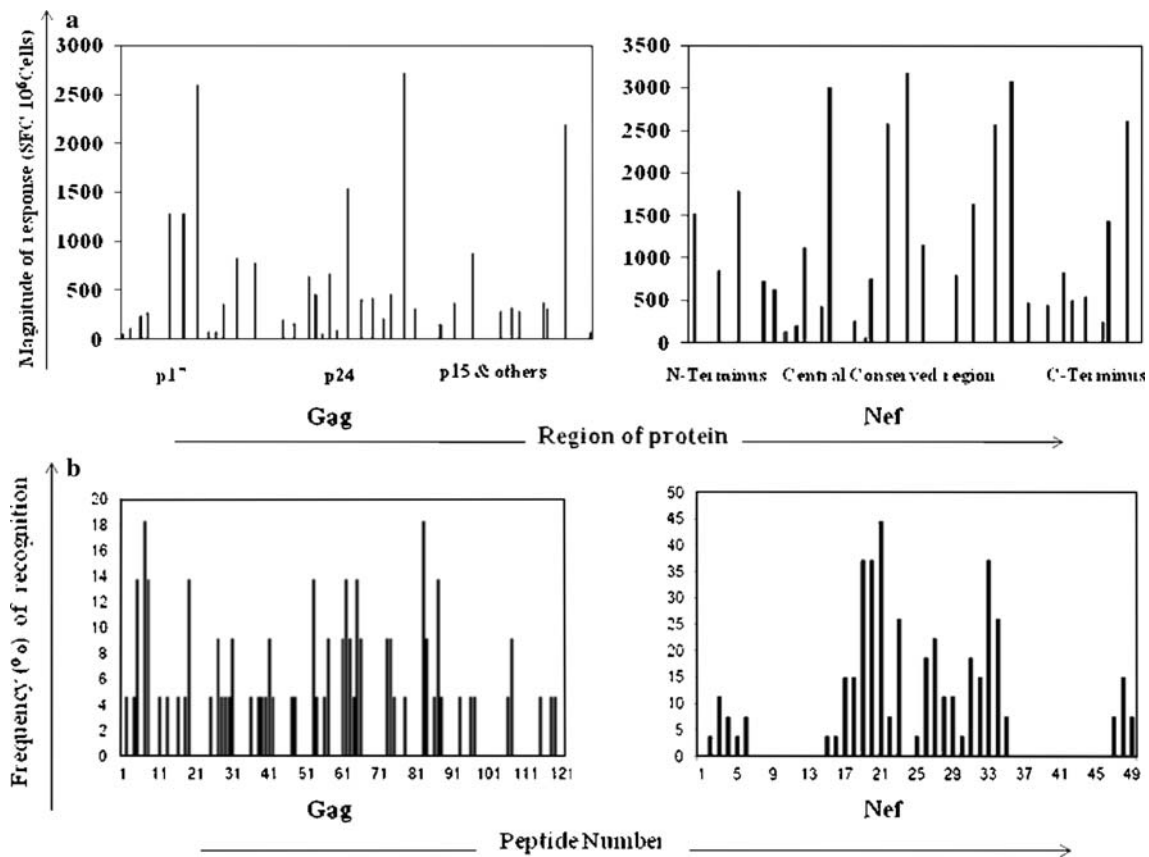


Fig. 1 **a** Graphical representation of magnitude of responses in the different protein regions of Gag and Nef. The x axis of each graph was scaled according to the number of HIV-1 C synthetic peptides used for a particular region of the viral protein, and the length of the graph does not necessarily correspond to the actual size of the viral protein

because of the differences in the lengths of the synthetic peptides. Bars represent magnitude (median SFU/10⁶ cells) of HIV-1 C-specific CTL responses to a particular synthetic peptide for the two proteins. **b** Frequency of recognition of individual Gag and Nef peptides by the study subjects

Correlation between HIV-specific CTL responses, plasma viremia and CD4⁺ T-cell counts

The association of CD4⁺ T-cell count and viral load with the breadth of response (number of HIV antigens recognized by the study subjects as estimated in screening using single peptides) and magnitude of response (SFU per 10⁶ PBMCs) were studied. The CD4⁺ T-cell count did not show any significant association with breadth or magnitude of HIV-specific CTL responses. Additionally, no significant association was found between virus load and the breadth of response. A significant indirect correlation was observed between magnitude of response against Gag antigen and viral load ($r = -0.45$, $P = 0.012$). A weak direct correlation was observed for Nef-specific responses and viral load, but this correlation was not statistically significant ($r = 0.115$) (Fig. 2). A significant negative association was found between CD4⁺ T-cell count and plasma virus load ($r = -0.63$, $P < 0.001$).

Discussion

Of the estimated 33.2 million people living with HIV/AIDS in the world, 95% are inhabitants of developing countries like India, which is home to 2.5 million people already infected with HIV. The uncontrolled HIV infection continues to spread at a ruthless pace, with 28,000 new infections by end 2006 [43].

India, with a predominant subtype C infection (78.4%) [44] is at the epicenter of HIV/AIDS epidemic. Although antiretroviral therapy has augmented lives of HIV infected individuals, these expensive drugs are neither easily available in the developing world, nor can they entirely eradicate the infection. In contrast, a successful AIDS vaccine offers the promise of preventing symptomatic disease, if not a complete cure.

Hypothetically an ideal vaccine should contain multiple, highly immunogenic epitopes (CTL, T-helper, neutralizing) derived from locally circulating viral strains that would protect the host from HIV infection, reduce the rate of HIV

Table 2 Summary of most frequently recognized immunodominant regions/peptides in the study subjects and variation from subtype B

Protein	Amino acid positions	Peptide number	Sequence	Subjects with response (%)	Subtype B epitopes in subtype C peptide sequence
Nef	65–79	17	EVGFVVRPQVPLRPM	11	EVGFVVRPQVPLRPM, FVPTPQVPLR, FVPTPQVPL
Nef	73–95	19, 20, 21	QVPLRPMTYKAADFLSFFLKEKG	63	QVPLRPMTYKGGALDLSHFLKEKG, QVPLRPMTYKAADVLSHFLKEKG, VPLRPMTY, RPMTYKAAL
Nef	89–103	23	FFLKEKGGLEGLIYS	26	HFLKEKGGLEGLIYS, HFLKEKGGLEGLVHS, KEKGGLEGL
Nef	101–115	26	IYSKKRQEIIDLWVY	18	IYSQKRQDILDWVY, VYSQKRQDILDWVY, RRQDILDWVY
Nef	125–139	32	QNYTPGPGVRYPLTF	15	QNYTPGPGIRYPLTF, QNYTPGPGIRFPLTF, TPGPGVRYPL
Nef	129–147	33, 34	PGPGVRYPLTFGWCFKLV	41	PGPGIRYPLTFGWCFKLV, PGPGTRFPLTFGWCFKLV, PGPGTRYPLTFGWCFKLV, RYPLTFGW, YPLTFGW
Nef	189–203	48	LARRHMARELHPEYY	15	LAFHHMAREKHPEYY, LAFHHMAREKHPEFY
p17	17–31	5	EKIRLRPGGKKHYML	14	EKIRLRPGGKKYKL, KIRLRPGGK, KIRLRPGGKK, KIRLRPGGKKYKL, IRLRPGGKK, IRLRPGGKK, RPPGKKKYML
p17	25–39	7	GKKHYMLKHLVWASR	18	GKKKYKLKHIVWASR, GKKKYRLKHIVWASR, GKKKYQLKHIVWASR, GKKKYRLKHLVWASR
p24	248–262	62	AWMTSNPPVPVGDYI	14	GWMTNPPVPIVGEIY, GWMTSNPPVPIVGEIY, PPIPVGDYI
p24	260–274	65	DIYKRWILGLNKIV	18	EIYKRWILGLNKIV, KRWILGLNK, GLNKIVRMY
p24	332–346	83	TILRALGPGASLEEM	18	TILKALGPAATLEEM, TILKALGPGATLEEM
p24	348–362	87	TACQGVGGPSSHARV	14	TACQGVGGPSSHARV, TACQGVGGPSSHARV, GPGHKARVL, GVGGPGHK

Bold amino acid positions mark differences from subtype B and previously identified subtype C epitopes sequence

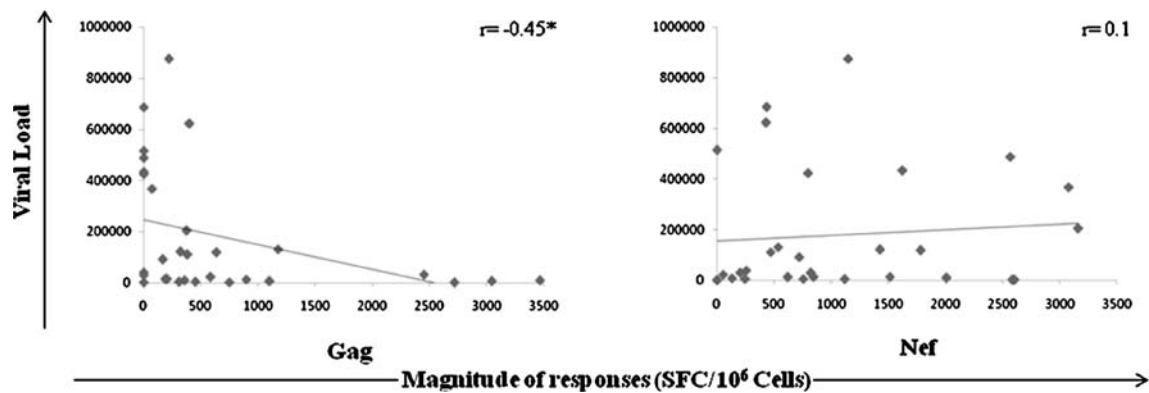


Fig. 2 Bivariate scatter plot for correlation between plasma viral loads and SFU/10⁶ PBMC values showing responses to Gag and Nef peptides

transmission, prevent progression to AIDS, and control HIV infection [35, 45, 46]. Much of the current HIV-1 vaccine research has been aimed at eliciting HIV-specific cell mediated immunity.

We aimed to characterize T-cell immune responses in HIV-1 subtype C infected Indian population and to identify immunodominant regions which can be included into vaccine constructs to restrain HIV-1 subtype C epidemic in India. Thirteen immunodominant regions have been identified in the current study: seven in Nef, two in p17 and four in p24. The results of our study are consistent with earlier study on subtype B from US and Europe and subtype C study from South Africa [47]. Importantly, among the 13 immunodominant regions identified in our study Nef_{aa 65–79} EVGFVPRPQVPLRPM, Nef_{aa 73–95} QVPLRPMTYKAA FDLFFLKEKG, Gag p24_{aa 332–346} TILRALGPGASLEEM have not been reported previously in Indian population and hence, are being documented for the first time in subtype C infected Indian subjects.

Comprehensive studies have characterized CTL epitopes in HIV-1 subtype B infections; few studies have targeted HIV-1 subtype C infected populations. We have earlier attempted to identify immunodominant regions in Gag protein using peptides based on Indian Isolates in HIV-1 subtype C infected individuals [28]. The present study extends to both Nef (using subtype C peptides based on Indian and African isolates) and Gag regions (using peptides based on consensus sequences for subtype C as a kind gift from NIH) [28]. A similar study has been done for HIV-1 subtype C infected western Indian population for screening of immunodominant regions within Gag and Nef [48]. However, our results differ significantly (90% Nef-specific responses observed in our study in comparison to 36% reported in study conducted at Pune). The reasons for these variations in responses may be due to the use of subtype C specific peptide sequence in our study and regional variation of the patients as the current study involves North and North West Indian population.

Magnitude and frequency of CTL responses are important characteristics of cell mediated responses. HIV-1 Gag is one of the important genes to target for the development of a successful candidate vaccine. Gag is not only the most conserved protein of HIV but it also has highest cross clade epitope density. In the present study Gag antigen was targeted by 73% of the study population. The dominant target in terms of magnitude and breadth of responses was observed to be p24 subunit of Gag protein. The reasons for this high frequency and magnitude of response are likely due to amino acid sequence conservation in this protein subunit. The broad and strong response to p24 Gag underscores the dominant role of Gag-specific CTL epitopes in natural course of HIV-1 subtype C infection [28].

The Nef-specific responses are the earliest responses observed in HIV-1 infection. This may explain the robust and frequent responses (90% study subjects responded to Nef antigen) observed in present study. Most of the immunodominant regions were located in central conserved domains of Nef (Nef_{65–147}). The central domain of Nef has been reported as epitope-rich region by other investigators, as well [29, 31, 33, 49, 50]. The robust Nef-specific responses observed in the current study suggests the importance of subtype-specific CTL responses of higher magnitude might be efficacious in vaccine design.

The immunodominant regions identified in the p24 region of Gag protein, and in central region of Nef in the present study corroborates the finding of studies reported on South African subtype C infected individuals [29, 35]. Moreover, most of the identified HIV-1 Gag subtype C peptide sequences matched with previously described epitope sequences for Gag derived from subtype B (obtained from hiv-web.lanl.gov), although some of the subtype B epitope sequences were variant from the subtype C peptide sequences.

Several studies have been able to correlate HIV-1-specific CTL responses in chronically infected patients with disease progression. Some previously reported studies have

shown an inverse correlation between the frequency of HIV-specific T cells and plasma virus load [4, 24, 27, 29, 36, 39, 51], whereas no such correlation was observed in other studies [4, 26, 52, 53]. The discrepancies in correlation between plasma viral load and virus-specific CTL responses might be explained, at least in part, by differences in assay methodologies, the targeting of different epitopes across the viral genome, difference in autologous virus and peptide sequence, different disease stages, and differences between diverse population groups [20, 38, 54].

In our study, we observed no correlation for frequency of Gag and Nef-specific CTL response and CD4⁺ T-cell counts or plasma viral load. However, direct relationship of magnitude of Nef-specific responses and inverse trend of magnitude of Gag responses with viral load are consistent with previous findings. The significant indirect correlation of Gag-specific responses suggests the protective role of these responses in the containment of HIV infection. Direct correlation of viral load with Nef suggests Nef-specific responses are unable to control viremia. This also raise the notion of relative importance of HIV-1 proteins for vaccine design and suggests that HIV gag p24 could serve as an attractive region to include in vaccine candidates to induce CTL responses that would in turn contain viremia.

We conclude that the identification of these responses at single peptide level across HIV-1 subtype C infected Indian population may provide useful insight into the designing of new immunotherapies and vaccines for effective control of HIV-1 infection. The pattern of immunodominance and epitope clustering is comparable with those found in previous studies on subtypes B and C infection. Identification of conserved epitopes across a diverse population is a necessary step in augmenting vaccine immunogenicity, minimizing viral escape and better approach for development of a multiclade vaccine. These results emphasize studies on larger populations and other HIV antigens and suggested the inclusion of identified multiple, highly immunogenic immunodominant regions into vaccine constructs to restrain the HIV-1 C epidemic in India.

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References

- McMichael AJ, Hanke T (2003) HIV vaccines 1983–2003. *Nat Med* 9(7):874–880. doi:10.1038/nm0703-874
- Klausner RD, Fauci AS, Corey L, Nabel GJ, Gayle H, Berkley S et al (2003) Medicine. The need for a global HIV vaccine enterprise. *Science* 300(5628):2036–2039. doi:10.1126/science.1086916
- Pantaleo G, Koup RA (2004) Correlates of immune protection in HIV-1 infection: what we know, what we don't know, what we should know. *Nat Med* 10(8):806–810. doi:10.1038/nm0804-806
- Novitsky V, Gilbert P, Peter T, McLane MF, Gaolekwe S, Rybak N, Thior I, Ndung'u T, Marlink R, Lee TH, Essex M (2003) Association between virus-specific T-cell responses and plasma viral load in human immunodeficiency virus type 1 subtype C infection. *J Virol* 77(2):882–890. doi:10.1128/JVI.77.2.882-890.2003
- Jin X, Bauer DE, Tuttleton SE, Lewin S, Gettie A, Blanchard J et al (1999) Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus-infected macaques. *J Exp Med* 189(6):991–998. doi:10.1084/jem.189.6.991
- Klein MR, van Baalen CA, Holwerda AM, Kerkhof Garde SR, Bende RJ, Keet IP et al (1995) Kinetics of Gag-specific cytotoxic T lymphocyte responses during the clinical course of HIV-1 infection: a longitudinal analysis of rapid progressors and long-term asymptomatics. *J Exp Med* 181(4):1365–1372. doi:10.1084/jem.181.4.1365
- Schmitz JE, Kuroda MJ, Santra S, Sasseville VG, Simon MA, Lifton MA et al (1999) Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. *Science* 283(5403):857–860. doi:10.1126/science.283.5403.857
- Walker CM, Moody DJ, Stites DP, Levy JA (1986) CD8+ lymphocytes can control HIV infection in vitro by suppressing virus replication. *Science* 234(4783):1563–1566. doi:10.1126/science.2431484
- Borrow P, Lewicki H, Hahn BH, Shaw GM, Oldstone MB (1994) Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. *J Virol* 68(9):6103–6110
- Koup RA, Saffrit JT, Cao Y, Andrews CA, McLeod G, Borkowsky W, Farthing C, Ho DD (1994) Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. *J Virol* 68:4650–4655
- Ogg GS, Kostense S, Klein MR, Jurriaans S, Hamann D, McMichael AJ et al (1999) Longitudinal phenotypic analysis of human immunodeficiency virus type 1-specific cytotoxic T lymphocytes: correlation with disease progression. *J Virol* 73(11):9153–9160
- Kostense S, Ogg GS, Manting EH, Gillespie G, Joling J, Vandenberghe K et al (2001) High viral burden in the presence of major HIV-specific CD8(+) T cell expansions: evidence for impaired CTL effector function. *Eur J Immunol* 31(3):677–686. doi:10.1002/1521-4141(200103)31:3<677::AID-IMMU677>3.0.CO;2-M
- Rosenberg ES, Billingsley JM, Caliendo AM, Boswell SL, Sax PE, Kalams SA et al (1997) Vigorous HIV-1-specific CD4+ T cell responses associated with control of viremia. *Science* 278(5342):1447–1450. doi:10.1126/science.278.5342.1447
- Pantaleo G, Menzo S, Vaccarezza M, Graziosi C, Cohen OJ, Demarest JF et al (1995) Studies in subjects with long-term nonprogressive human immunodeficiency virus infection. *N Engl J Med* 332(4):209–216. doi:10.1056/NEJM199501263320402
- Pitcher CJ, Quittner C, Peterson DM, Connors M, Koup RA, Maingo VC et al (1999) HIV-1-specific CD4+ T cells are detectable in most individuals with active HIV-1 infection, but decline with prolonged viral suppression. *Nat Med* 5(5):518–525. doi:10.1038/8400
- Barouch DH, Santra S, Kuroda MJ, Schmitz JE, Plishka R, Buckler-White A et al (2001) Reduction of simian-human immunodeficiency virus 89.6P viremia in rhesus monkeys by recombinant modified vaccinia virus Ankara vaccination. *J Virol* 75(11):5151–5158. doi:10.1128/JVI.75.11.5151-5158.2001

17. Barouch DH, Santra S, Schmitz JE, Kuroda MJ, Fu TM, Wagner W et al (2000) Control of viremia and prevention of clinical AIDS in rhesus monkeys by cytokine-augmented DNA vaccination. *Science* 290(5491):486–492. doi:10.1126/science.290.5491.486
18. Seth A, Ourmanov I, Schmitz JE, Kuroda MJ, Lifton MA, Nickerson CE et al (2000) Immunization with a modified vaccinia virus expressing simian immunodeficiency virus (SIV) Gag-Pol primes for an anamnestic Gag-specific cytotoxic T-lymphocyte response and is associated with reduction of viremia after SIV challenge. *J Virol* 74(6):2502–2509. doi:10.1128/JVI.74.6.2502-2509.2000
19. Allen TM, Horton H, Venham JE, Rudersdorf RA, Vogel T, Pauza CD et al (1999) Virus-specific cytotoxic T-lymphocyte responses select for amino-acid variation in simian immunodeficiency virus Env and Nef. *Nat Med* 18(18):1389–1393
20. Goulder PJ, Tang Y, Brander C, Betts MR, Altfeld M, Annamalai K et al (2000) Functionally inert HIV-specific cytotoxic T lymphocytes do not play a major role in chronically infected adults and children. *J Exp Med* 192(12):1819–1832. doi:10.1084/jem.192.12.1819
21. Price DA, Goulder PJ, Klenerman P, Sewell AK, Easterbrook PJ, Troop M et al (1997) Positive selection of HIV-1 cytotoxic T lymphocyte escape variants during primary infection. *Proc Natl Acad Sci USA* 94(5):1890–1895. doi:10.1073/pnas.94.5.1890
22. Masemola AM, Mashishi TN, Khoury G, Bredell H, Paximadis M, Mathebula T et al (2004) Novel and promiscuous CTL epitopes in conserved regions of Gag targeted by individuals with early subtype C HIV type 1 infection from southern Africa. *J Immunol* 173(7):4607–4617
23. Zuniga R, Lucchetti A, Galvan P, Sanchez S, Sanchez C, Hernandez A et al (2006) Relative dominance of Gag p24-specific cytotoxic T lymphocytes is associated with human immunodeficiency virus control. *J Virol* 80(6):3122–3125. doi:10.1128/JVI.80.6.3122-3125.2006
24. Ogg GS, Jin X, Bonhoeffer S, Dunbar PR, Nowak MA, Monard S et al (1998) Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of viral RNA. *Science* 279(5359):2103–2106. doi:10.1126/science.279.5359.2103
25. Evans DT, O'Connor DH, Jing P, Dzuris JL, Sidney J, da Silva J et al (1999) Virus-specific cytotoxic T-lymphocyte responses select for amino-acid variation in simian immunodeficiency virus Env and Nef. *Nat Med* 5(11):1270–1276. doi:10.1038/15224
26. Buseyne F, Scott-Algara D, Porrot F, Corre B, Bellal N, Burgard M et al (2002) Frequencies of ex vivo-activated human immunodeficiency virus type 1-specific gamma-interferon-producing CD8+ T cells in infected children correlate positively with plasma viral load. *J Virol* 76(24):12414–12422. doi:10.1128/JVI.76.24.12414-12422.2002
27. Buseyne F, Le Chenadec J, Corre B, Porrot F, Burgard M, Rouzioux C et al (2002) Inverse correlation between memory Gag-specific cytotoxic T lymphocytes and viral replication in human immunodeficiency virus-infected children. *J Infect Dis* 186(11):1589–1596. doi:10.1086/345482
28. Kaushik S, Vajpayee M, Wig N, Seth P (2005) Characterization of HIV-1 Gag-specific T cell responses in chronically infected Indian population. *Clin Exp Immunol* 142(2):388–397. doi:10.1111/j.1365-2249.2005.02926.x
29. Masemola A, Mashishi T, Khoury G, Mohube P, Mokgotho P, Vardas E et al (2004) Hierarchical targeting of subtype C human immunodeficiency virus type 1 proteins by CD8+ T cells: correlation with viral load. *J Virol* 78(7):3233–3243. doi:10.1128/JVI.78.7.3233-3243.2004
30. Kaufmann DE, Bailey PM, Sidney J, Wagner B, Norris PJ, Johnston MN et al (2004) Comprehensive analysis of human immunodeficiency virus type 1-specific CD4 responses reveals marked immunodominance of gag and nef and the presence of broadly recognized peptides. *J Virol* 78(9):4463–4477. doi:10.1128/JVI.78.9.4463-4477.2004
31. Mashishi T, Loubser S, Hide W, Hunt G, Morris L, Ramjee G et al (2001) Conserved domains of subtype C nef from South African HIV type 1-infected individuals include cytotoxic T lymphocyte epitope-rich regions. *AIDS Res Hum Retroviruses* 17(17):1681–1687. doi:10.1089/088922201753342103
32. Gallimore A, Hengartner H, Zinkernagel R (1998) Hierarchies of antigen-specific cytotoxic T-cell responses. *Immunol Rev* 164:29–36
33. Novitsky V, Cao H, Rybak N, Gilbert P, McLane MF, Gaolekwe S, Peter T, Thior I, Ndung'u T, Marlink R, Lee TH, Essex M (2002) Magnitude and frequency of cytotoxic T-lymphocyte responses: identification of immunodominant regions of human immunodeficiency virus type 1 subtype C. *J Virol* 76(20):10155–10168. doi:10.1128/JVI.76.20.10155-10168.2002
34. Foley BT (2000) An overview of the molecular phylogeny of lentiviruses: HIV sequence compendium 2000: theoretical biology and biophysics
35. Novitsky V, Rybak N, McLane MF, Gilbert P, Chigwedere P, Klein I, Gaolekwe S, Chang SY, Peter T, Thior I, Ndung'u T, Vannberg F, Foley BT, Marlink R, Lee TH, Essex M (2001) Identification of human immunodeficiency virus type 1 subtype C Gag-, Tat-, Rev-, and Nef-specific elispot-based cytotoxic T-lymphocyte responses for AIDS vaccine design. *J Virol* 75(19):9210–9228. doi:10.1128/JVI.75.19.9210-9228.2001
36. Edwards BH, Bansal A, Sabbaj S, Bakari J, Mulligan MJ, Goepfert PA (2002) Magnitude of functional CD8+ T-cell responses to the gag protein of human immunodeficiency virus type 1 correlates inversely with viral load in plasma. *J Virol* 76(5):2298–2305. doi:10.1128/jvi.76.5.2298-2305.2002
37. Cao Y, Qin L, Zhang L, Safritz J, Ho DD (1995) Virologic and immunologic characterization of long-term survivors of human immunodeficiency virus type 1 infection. *N Engl J Med* 332(4):201–208. doi:10.1056/NEJM199501263320401
38. Greenough TC, Brettler DB, Somasundaran M, Panicali DL, Sullivan JL (1997) Human immunodeficiency virus type 1-specific cytotoxic T lymphocytes (CTL), virus load, and CD4 T-cell loss: evidence supporting a protective role for CTL in vivo. *J Infect Dis* 176:118–125
39. Betts MR, Krowka JF, Kepler TB, Davidian M, Christopherson C, Kwok S et al (1999) Human immunodeficiency virus type 1-specific cytotoxic T lymphocyte activity is inversely correlated with HIV type 1 viral load in HIV type 1-infected long-term survivors. *AIDS Res Hum Retroviruses* 15(13):1219–1228. doi:10.1089/088922299310313
40. Lubaki NM, Shepherd ME, Brookmeyer RS, Hon H, Quinn TC, Kashamuka M et al (1999) HIV-1-specific cytolytic T-lymphocyte activity correlates with lower viral load, higher CD4 count, and CD8 + CD38-DR- phenotype: comparison of statistical methods for measurement. *J Acquir Immune Defic Syndr* 22(1):19–30
41. Dalol M, Dupuis M, Deschemin JC, Sicard D, Salmon D, Del-fraissy JF et al (1999) Broad, intense anti-human immunodeficiency virus (HIV) ex vivo CD8(+) responses in HIV type 1-infected patients: comparison with anti-Epstein-Barr virus responses and changes during antiretroviral therapy. *J Virol* 73(9):7108–7116
42. Rinaldo C, Huang XL, Fan ZF, Ding M, Beltz L, Logar A et al (1995) High levels of anti-human immunodeficiency virus type 1 (HIV-1) memory cytotoxic T-lymphocyte activity and low viral load are associated with lack of disease in HIV-1-infected long-term nonprogressors. *J Virol* 69(9):5838–5842
43. UNAIDS/WHO (2007) AIDS epidemic update: December 2007. UNAIDS, Geneva 2007. UNAIDS/07.27E/JC1322E.ISBN 978 92 9 173621 8

44. Sahni AK, Prasad VV, Seth P (2002) Genomic diversity of human immunodeficiency virus type-1 in India. *Int J STD AIDS* 13(2):115–118. doi:[10.1258/0956462021924749](https://doi.org/10.1258/0956462021924749)
45. Altfeld M, Rosenberg ES (2000) The role of CD4(+) T helper cells in the cytotoxic T lymphocyte response to HIV-1. *Curr Opin Immunol* 12(4):375–380. doi:[10.1016/S0952-7915\(00\)00103-5](https://doi.org/10.1016/S0952-7915(00)00103-5)
46. Hendel H, Caillat-Zucman S, Lebuane H, Carrington M, O'Brien S, Andrieu JM et al (1999) New class I and II HLA alleles strongly associated with opposite patterns of progression to AIDS. *J Immunol* 162(11):6942–6946
47. Alter G, Merchant A, Tsoukas CM, Rouleau D, LeBlanc RP, Cote P et al (2002) Human immunodeficiency virus (HIV)-specific effector CD8 T cell activity in patients with primary HIV infection. *J Infect Dis* 185(6):755–765. doi:[10.1086/339338](https://doi.org/10.1086/339338)
48. Thakar MR, Bhonge LS, Lakhashe SK, Shankarkumar U, Sane SS, Kulkarni SS et al (2005) Cytolytic T lymphocytes (CTLs) from HIV-1 subtype C-infected Indian patients recognize CTL epitopes from a conserved immunodominant region of HIV-1 Gag and Nef. *J Infect Dis* 192(5):749–759. doi:[10.1086/432547](https://doi.org/10.1086/432547)
49. Malhotra U, Li F, Nolin J, Allison M, Zhao H, Mullins JI et al (2007) Enhanced detection of human immunodeficiency virus type 1 (HIV-1) Nef-specific T cells recognizing multiple variants in early HIV-1 infection. *J Virol* 81(10):5225–5237. doi:[10.1128/JVI.02564-06](https://doi.org/10.1128/JVI.02564-06)
50. Culmann B, Gomard E, Kieny MP, Guy B, Dreyfus F, Saimot AG et al (1991) Six epitopes reacting with human cytotoxic CD8+ T cells in the central region of the HIV-1 NEF protein. *J Immunol* 146(5):1560–1565
51. Chouquet C, Autran B, Gomard E, Bouley JM, Calvez V, Katlama C et al (2002) Correlation between breadth of memory HIV-specific cytotoxic T cells, viral load and disease progression in HIV infection. *AIDS* 16(18):2399–2407. doi:[10.1097/00002030-200212060-00004](https://doi.org/10.1097/00002030-200212060-00004)
52. Betts MR, Ambrozak DR, Douek DC, Bonhoeffer S, Brenchley JM, Casazza JP et al (2001) Analysis of total human immunodeficiency virus (HIV)-specific CD4(+) and CD8(+) T-cell responses: relationship to viral load in untreated HIV infection. *J Virol* 75(24):11983–11991. doi:[10.1128/JVI.75.24.11983-11991.2001](https://doi.org/10.1128/JVI.75.24.11983-11991.2001)
53. Addo MM, Yu XG, Rathod A, Cohen D, Eldridge RL, Strick D et al (2003) Comprehensive epitope analysis of human immunodeficiency virus type 1 (HIV-1)-specific T-cell responses directed against the entire expressed HIV-1 genome demonstrate broadly directed responses, but no correlation to viral load. *J Virol* 77(3):2081–2092. doi:[10.1128/JVI.77.3.2081-2092.2003](https://doi.org/10.1128/JVI.77.3.2081-2092.2003)
54. Goepfert PA, Bansal A, Edwards BH, Ritter GD Jr, Tellez I, McPherson SA et al (2000) A significant number of human immunodeficiency virus epitope-specific cytotoxic T lymphocytes detected by tetramer binding do not produce gamma interferon. *J Virol* 74(21):10249–10255. doi:[10.1128/JVI.74.21.10249-10255.2000](https://doi.org/10.1128/JVI.74.21.10249-10255.2000)