The Journal of Immunology

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J Immunol 2004; 173:4607-4617; ;
doi: 10.4049/jimmunol.173.7.4607
http://www.jimmunol.org/content/173/7/4607

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Novel and Promiscuous CTL Epitopes in Conserved Regions of Gag Targeted by Individuals with Early Subtype C HIV Type 1 Infection from Southern Africa[1]

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Characterization of optimal CTL epitopes in Gag can provide crucial information for evaluation of candidate vaccines in populations at the epicenter of the HIV-1 epidemic. We screened 38 individuals with recent subtype C HIV-1 infection using overlapping consensus C Gag peptides and hypothesized that unique HLA-restricting alleles in the southern African population would determine novel epitope identity. Seventy-four percent of individuals recognized at least one Gag peptide pool. Ten epitopic regions were identified across p17, p24, and p2p7p1p6, and greater than two-thirds of targeted regions were directed at: TGTEELRSLYN TVATLY (p17, 35%); GPKEPFRDYVDRFFKTLRAEQATQDV (p24, 19%); and RGGKLDKWEKIRLRPGGKKHYMLKHL (p17, 15%). After alignment of these epitopic regions with consensus M and a consensus subtype C sequence from the cohort, it was evident that the regions targeted were highly conserved. Fine epitope mapping revealed that five of nine identified optimal Gag epitopes were novel: HLVWASREL, LVWASRELERF, LYNTVATLY, PFRDYVDRFF, and TLRAEQATQD, and were restricted by unique HLA-Cw*08, HLA-A*30/B*57, HLA-A*29/B*44, and HLA-Cw*03 alleles, respectively. Notably, three of the mapped epitopes were restricted by more than one HLA allele. Although these epitopes were novel and restricted by unique HLA, they overlapped or were embedded within previously described CTL epitopes from subtype B HIV-1 infection. These data emphasize the promiscuous nature of epitope binding and support our hypothesis that HLA diversity between populations can shape fine epitope identity, but may not represent a constraint for universal recognition of Gag in highly conserved domains. ***The Journal of Immunology,*** **2004, 173: 4607–4617.**

Although there have been numerous advances in successful antiretroviral drug treatments of HIV-1 infection, the eventual public health control of the global HIV-1 epidemic will rely on an effective vaccine. Currently, our knowledge of immune correlates of protection against HIV-1 infection or disease progression remains uncertain, and the mutatable and diverse nature of HIV-1 represents an obstacle to vaccine development. From the host perspective, numerous studies have shown that $CD8^+$ T lymphocytes play an important role in the control of viral replication in HIV-1-infected individuals (1–5), in which there is a temporal association between the appearance of HIV Ag-specific $CD8^+$ T cells and the initial decline in plasma viral RNA in the acute infection stage (6–8). Additionally, the presence of viral escape mutants in response to $CD8^+$ CTL pressure (6, 9) suggests that this arm of the immune response is associated with the control of viral replication. In preclinical macaque studies, various reports have provided evidence that $CD8^+$ T cells are an effective mediator of viral control, in which control of SIV infection is directly correlated with the presence of Ag-specific $CD8^+$ T cells (10–15). Depletion of $CD8^+$ T cells in SIV-infected macaques resulted in a rapid and dramatic rise in plasma viremia and progression to disease and death (16). Vaccine-induced $CD8^+$ T cells protected macaques against the development of AIDS following challenge with

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Received for publication April 8, 2004. Accepted for publication August 2, 2004.

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[1] Funding was through National Institutes of Health Grant N01-AI-45202. A.M.M. is a South African AIDS Vaccine Initiative fellow; A.M.M. and T.M. are both Fogarty International Fellows.

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0022-1767/04/$02.00

Simian-human immunodeficiency virus 89.6P (16–18). More specific data have shown that rhesus macaques immunized with recombinant HIV-1 canarypox vaccine, expressing SIV-Gag-Pol-Env genes, could control viral replication after challenge with SIVmac251 and was associated with Gag-specific $CD8^+$ CTL targeting a dominant epitope, without detectable Pol or Env responses (19).

Clinical data, from natural history studies, have shown that CTL targeting of Gag may or may not be important for viral control. Some studies have shown that no correlations exist between the overall $CD8^+$ T cell response and plasma viral load (20–22), and others show that HIV-1 Gag-specific responses associate with a lower viral set point within the first 12 mo of HIV-1 infection (23). Data also exist that show a correlation between CTL responses targeting Gag with proviral DNA, but not plasma viral RNA (24). Other studies have observed an inverse correlation between viremia and the breadth and magnitude of p24 Gag-specific CTL responses (20, 25–27). These observations alone provide strong evidence that MHC class I presentation of Gag epitopes will be essential for the effectiveness of HIV-1 vaccines and concur with the data found from macaque studies. In agreement with other studies (28, 29), we have recently shown (30) that there is a positive correlation between total HIV-1-specific $CD8^+$ T cell responses and viral load, but a significant correlation between preferential targeting of Gag by $CD8^+$ T cells and control of plasma viremia.

In this study, we extend these observations (30), and the primary objective of this study was to characterize epitope-specific regions in Gag from subtype C HIV-1-infected southern Africans. We have mapped epitopes in Gag and reveal the presence of a number of novel epitopes overlapping with previously described epitopes from subtype B HIV-1 infection. We hypothesize that unique HLA-restricting alleles in the southern African population account for the identity of novel epitopes, and that these occur in highly conserved domains of Gag. These data: 1) highlight the degeneracy of HIV-1 Ag recognition in Gag; 2) highlight the notion that global HLA diversity between populations may not be a limiting factor for common epitope recognition in Gag; and 3) provide baseline information to compare with vaccine-induced Gag-specific $CD8^+$ responses.

Materials and Methods

Patient cohort

Thirty-eight HIV-1-infected individuals were analyzed for responses against Gag, and were derived from those enrolled into HIVNET 028, a natural history study of recent HIV-1 infection from four southern African countries: Malawi, Zimbabwe, Zambia, and South Africa. Table I shows details of the individuals analyzed, including time from seroconversion, CD4 counts, and viremia. The median time from seroconversion was 7 mo, in which time from seroconversion was estimated as the midpoint between the last Ab-negative and the first Ab-positive ELISA, using the ELISPOT assay date as the reference time point. All individuals analyzed were antiretroviral drug naive. The median RNA copies/ml were 12,936 (4.11 $\log_{10}$), and there were no significant associations between country of origin and viremia. Thus, for the purposes of this study, individuals were grouped as one cohort.

Synthetic peptides and design of peptide pools

A total of 66 peptides based on consensus C Gag was used to screen and confirm Gag-specific responses and was synthesized using Fmoc-based solid-phase chemistry (Natural and Medical Sciences Institute, University of Tuebingen, Reutlingen, Germany). All peptides were checked for the correct m.w. using Elektrospray-quadruple time-of-flight-mass spectrometry, and peptide purity ranged from 70 to 80%. Peptides were dissolved in 100% DMSO at an initial concentration of 10 mg/ml and pooled at 40 μg/ml/peptide stock in PBS, in which the final DMSO concentration was always $<0.5\%$. The peptide set consisted of 15–18 mers overlapping by 10 aa and was aliquoted to be arranged in a pool and matrix design, such that each peptide appeared twice in two different pools. In total, there were five major pools containing 14 peptides/pool plus a further 14 matrix pools containing 5 peptides/pool. Pools were arranged so that peptide responses identified in any one of the major pools could be cross-referenced to a response in one of the matrix pools. In so doing, multiple peptide responses could be narrowed down to likely single peptide response. Single peptide responses were then confirmed in subsequent assays. Truncated 9- to 12-mer peptides were used for fine epitope mapping and for HLA restrictions.

Preparation of cells

PBMC were prepared using Ficoll gradient centrifugation and cryopreserved in 90% FCS plus 10% DMSO. At the time of analysis, cryopreserved PBMC were thawed and rested overnight in R10 (RPMI 1640 (Invitrogen Life Technologies, Paisley, U.K.) supplemented with 10% FCS (Invitrogen Life Technologies) and 50 U of gentamicin (Invitrogen Life Technologies) at $2–4 \times 10^6$ PBMC/ml at 37°C in 5% CO_2 before use in the ELISPOT assay (30). After incubation, cells were counted using trypan blue dye exclusion and used in assays. EBV-transformed B cell lines, for use as class I HLA-matched targets, were established for each individual and maintained in RPMI 1640 supplemented with 20% (R20) FCS and 50 U of gentamicin at 37°C/5% CO_2.

IFN-γ ELISPOT assays

ELISPOT assays were performed, as previously described (30). Briefly, cryopreserved PBMC were plated on 96-well polyvinylidene difluoride plates (MAIP S45; Millipore, Johannesburg, South Africa) that were coated with 50 μl of anti-IFN-γ mAb 1-D1k (2 μg/ml; Mabtech, Stockholm, Sweden) overnight at 4°C. Peptides were added directly to the wells at various final concentrations, ranging between 0.002 to 2 μg/ml along with $1–2 \times 10^5$ cells in a volume of 50 μl of R10 and incubated at 37°C in 5% CO_2. After 16–18 h, plates were extensively washed with PBS and wash buffer (PBS, 1% FCS, 0.001% Tween 20), followed by incubation with biotinylated anti-IFN-γ mAb (2 μg/ml; clone 7-B6-1; Mabtech) at room temperature for 3 h. After a further six washes with wash buffer, 2 μg/ml streptavidin HRP (BD Pharmingen, Cupertino, CA) was added to the wells, and the plates were incubated for an additional 1 h at room temperature. Spots were visualized using Novared substrate (Vector Laboratories, Burlingame, CA), according to the manufacturer's instructions. Wells containing PBMC and R10 medium, as well as R10 medium alone, were used as negative controls, in which PBMC and medium controls were run in duplicate. Wells containing PBMC and PHA served as positive controls. The number of spots per well was counted using the Immunospot (Cellular Technology, Cleveland, OH) automated cell counter. Peptide responses were confirmed using individual peptides in the ELISPOT assay. The criteria used to define a positive response are as described previously (30).

Intracellular cytokine staining (ICS)[4]

PBMC ($0.5–1 \times 10^6$) were incubated with 2 μg of peptide and anti-CD28 and anti-CD49d mAbs (1 μg/ml; BD Pharmingen) at 37°C and 5% CO_2 for 1 h. Brefeldin A (10 μg/ml; Sigma-Aldrich, St. Louis, MO) was added, and cells were incubated for a further 5 h at 37°C and 5% CO_2. Cells were transferred to 4°C overnight. PBMC were washed, permeabilized, and stained with mAbs against CD8 (PerCP), CD3 (allophycocyanin), CD69 (PE), and IFN-γ (FITC) for 30 min at room temperature in the dark. Cells were washed, fixed, and acquired on a FACSCalibur using the CellQuest software. Analysis for IFN-γ-positive cells was done using Paint-a-gate software. For HLA restriction, B cells expressing a single matched HLA allele with autologous B cells were pulsed with 2 μg of peptide for 1 h at 37°C and 5% CO_2. B cells were washed four times with RPMI 1640 supplemented with 1% FCS (R1), and the B cells were mixed with Ag-specific cells (effectors) at a ratio of 5:1 T cells to B cells in 1 ml of R10 and incubated with anti-CD28/CD49d mAbs, and the assay continued as described for ICS.

^{51}Cr release assay

PBMC at 1×10^6 were stimulated with 10 μg of peptide and 330 U/ml IL-7 (Roche, Basel, Switzerland), and cells were transferred to a 24-well plate and incubated at 37°C and 5% CO_2. Following 1 wk in culture, 25 U/ml IL-2 was added, and after 14–21 days in culture, PBMC (effectors) were harvested for use in a standard ^{51}chromium release assay. HLA-matched B cells were pulsed with 10 μg of peptide and 100–150 μCi of ^{51}Cr for 1 h at 37°C and 5% CO_2. Following extensive washing with R1,

[4] Abbreviations used in this paper: ICS, intracellular cytokine staining; MHR, major homology region; sfu, spot-forming unit; BLCL, B lymphoblastoid cell line.

Table I. *Details of the 38 patients studied*[a]

	Months from Seroconversion	Viral Load	CD4 Counts	CD8 Counts	Gag Peptide Pools					Cumulative Magnitude	Number of Gag pools Recognized
					P1	P2	P3	P4	P5		
P1	2.3	1,852	444	677	1,007	268	412	235		1,922	4
P2	2.3	14,514	466	831	355					355	1
P3	2.5	27,687	472	629	350	100	795	780	180	2,205	5
P4	2.6	24,793	215	1,146						0	0
P5	3.7	34,176	236	712						0	0
P6	4.1	5,926	692	1,296			800		115	915	2
P7	4.2	ND	572	534						0	0
P8	4.7	34,167	913	548			422	178	133	733	3
P9	5.3	146,916	481	577		105				105	1
P10	5.4	37,406	282	846	696	1,446	446			2,588	3
P11	5.4	2,959	629	708	110	100	280			490	3
P12	5.9	10,141	299	299						0	0
P13	6.1	12,1336	140	495	490	150	225		180	1,045	4
P14	6.7	19,270	ND	ND			160			160	1
P15	6.7	225	373	568	470					470	1
P16	6.8	22,332	ND	ND	225					225	1
P17	6.9	5,340	408	1,052						0	0
P18	7.1	2,478	714	1,068	1,515	1,690	150			3,355	3
P19	7.2	9,402	185	622						0	0
P20	7.7	14,045	358	464	200	480	220			900	3
P21	8.4	70,526	295	1,878	200					200	1
P22	10.1	1,392	515	558						0	0
P23	11.0	9,262	355	549	510	430	630		130	1,700	4
P24	11.2	73,753	242	907		265			115	380	2
P25	13.0	46,699	187	597						0	0
P26	13.4	8,754	284	447						0	0
P27	13.9	1,694	321	408						0	0
P28	14.0	1,241	568	1,021			466		121	587	2
P29	14.4	774	670	2,307	930					930	1
P30	14.8	14,528	266	430	290	930	130	170		1,520	4
P31	15.3	15,557	114	716	200					200	1
P32	15.6	194,236	511	644		160				160	2
P33	15.6	12,936	412	1,433	435	975	365	595		2,370	4
P34	18.0	26,197	357	516	130	377	1,013			1,520	3
P35	22.5	1,262	343	566	691		571	167	333	1,762	4
P36	ND	709	365	486	262	1,292			292	1,846	3
P37	ND	ND	ND	ND	700		240			940	2
P38	ND	302	204	323			230			230	1
Median	7	12,936	356	587	435	430	323	174	132	380 (908)	1 (3)
25% IQR	5	2,478	260	511	200	160	224	169	120	0 (324)	0 (1)
75% IQR	14	34,167	512	936	691	975	492	282	208	992 (1715)	3 (4)

[a] Details of the 38 patients studied, showing time from estimated seroconversion, viral load and CD4/CD8 counts, ELISPOT responses (sfu/10^6 PBMC) to each of the five Gag pools, cumulative magnitude, and number of peptide pools recognized. IQR, Interquartile range.

B lymphoblastoid cell line (BLCL) targets were resuspended in R10 and mixed with effectors in triplicate at a ratio of 50:1 and 25:1 E:T ratio in V-bottom 96-well plate, and the killing assay was incubated for 4–5 h at 37°C and 5% CO_2. Twenty-five microliters of the supernatant were harvested onto LumaPlates-96 (Packard Bioscience, Billerica, MA) and allowed to dry overnight, and the radioactivity was detected using a gamma counter (TopCount.NXT; Packard Bioscience). Spontaneous release of ^{51}Cr was determined by incubating targets with R10, and the maximum release was determined from the total radioactivity released by targets in the presence of 0.01% Triton X-100. Specific lysis (%) was calculated as: $(\text{experimental}_{\text{average}} - \text{spontaneous}_{\text{average}}) \div (\text{maximum}_{\text{average}} - \text{spontaneous}_{\text{average}}) \times 100$.

HLA typing

DNA was extracted from EBV-transformed B cell lines using the Pel-Freez DNA Isolation Kit (Pel-Freez Clinical Systems, LLC, Brown Deer, WI). Low to medium resolution typing of class I HLA A, B, and C loci was performed using the Pel-Freez sequence specific primers UniTray PCR-based method (Pel-Freez Clinical Systems). High resolution typing of HLA class I A and B loci was performed using sequencing-based typing kits (Applied Biosystems, Foster City, CA). Sequencing products were purified and analyzed using the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Analysis of the resulting sequences and subsequent allele assignment were performed using the MatchMaker Allele Identification Software (Applied Biosystems).

Statistical and data analysis

All data were expressed as medians with interquartile ranges and analyzed using nonparametric statistics. Krukal-Wallis nonparametric ANOVA was used to test for significant differences across pools of Gag and Dunn's pairwise analysis to identify differences between pools. Fisher's exact test was used to identify differences in HLA allele frequencies between populations. Statistical analysis was performed using SigmaStat 2.0 (SPSS, Chicago, IL) and Arlequin (Zero G Software, San Francisco, CA).

Results

The frequency of responses to Gag in HIV-1-infected individuals

Table I shows the magnitude and range of IFN-γ ELISPOT responses (spot-forming units (sfu)/10^6 PBMC) of each of the HIV-1-infected individuals to the five pools of overlapping Gag peptides. There were no significant differences between responses found in pool 1 (P1) to pool 4 (P4), although there was a significantly ($p < 0.05$) lower magnitude of recognition to pool 5 (P5, p2p7p1p6 region). Seventy-four percent (28 of 38) of individuals

recognized at least one of the peptide pools, where only one individual recognized all five pools. The median cumulative response across all five pools was 380 sfu/10^6 PBMC for the whole cohort and 908 sfu/10^6 PBMC for only the 28 responders, and the median number of pools recognized was 1 for the cohort and 3 for the 28 responders. There was no significant difference ($p = 0.763$) between viral loads in those that did respond to any of the Gag pools (median of 9,402 RNA copies/ml) and those that did not respond to any pool (median of 14,280 RNA copies/ml). There was no correlation between the number of Gag pools recognized and viral load ($r = 0.05$; $p = 0.74$), although there was a negative nonsignificant trend between sfu/10^6 PBMC and $\log_{10}$ viral load ($r = -0.353$, $p = 0.09$). Using a matrix approach (outlined in *Materials and Methods*), a range of possible peptides was identified and single peptides were then used to confirm epitopic region reactivity.

Fig. 1 shows a peptide density plot of the frequency of recognized epitopic regions after confirming the ELISPOT screens with single Gag peptides in 26 of the 28 responders. When the overlapping amino acid sequences were conjoined, four peptide stretches in p17 were identified: ^{9}RGGKLDKWEKIRLRPG GKKHYMLKHL^{34} was recognized at a frequency of 15.4% (4 of 26); ^{16}KHLVWASRELERFAL^{30} was recognized at a frequency of 7.7% (2 of 26); ^{70}TGTEELRSLYNTVATLY^{86} was recognized at a frequency of 34.6% (9 of 26); and the frequency of recognition for ^{119}KAAADKGKVSQNYPIV^{134} was 11.5% (3 of 26).

Analysis of epitopic regions in p24 revealed the following conjoined peptide sequences (Fig. 1): ^{157}WVKVIEEKAFSPE VIPMF^{174} was recognized at a frequency of 19% (5 of 26); ^{170}PM FTALSEGATPQDLNTMLNTVGGH^{196} at a frequency of 11.5% (3 of 26); ^{259}PVGDIYWKRWIILGLNKIVRMYSPVSI^{285} at a frequency of 7.7% (2 of 26); and ^{289}GPKEPFRDYVDRFFKTL RAEQATQDV^{314} at a frequency of 19.2% (5 of 26). Although there was some evidence of epitope recognition in the p2p7p1p6 region of Gag, this constituted only 7.7% of responses across Gag. There was no significant association ($r = -0.29$, $p = 0.2038$) when the breadth of response, measured as the number of peptides recognized, was correlated with viral loads.

Optimal epitope recognition and HLA restrictions in p17

To determine the optimal epitopes for the peptide stretches identified in Fig. 1, we used truncated peptides ranging from 9 to 12 aa in length. The optimal epitope was defined as the peptide that gave the highest number of sfu/10^6 cells at the lowest peptide concentration in the IFN-γ ELISPOT assay. HLA restrictions were performed using either ^{51}Cr release CTL assays or ICS, and the restricting allele was defined as the single HLA allele that gave comparable or greater responses to autologous target BLCL. Fig. 2 shows that within the parent peptide stretch ^{16}KHLVWAS RELERFAL^{46} (Fig. 1, epitopic region 2), two novel epitopes were identified. The first was ^{17}HLVWASREL^{25} (HL-9), shown to give the highest sfu/10^6 PBMC at the lowest peptide concentration upon peptide titration (Fig. 2*A*). The HLA-restricting allele of the HL-9 epitope was shown to be HLA-Cw*0804 (Fig. 2*B*), in which comparable target cell lysis with autologous BLCL was observed

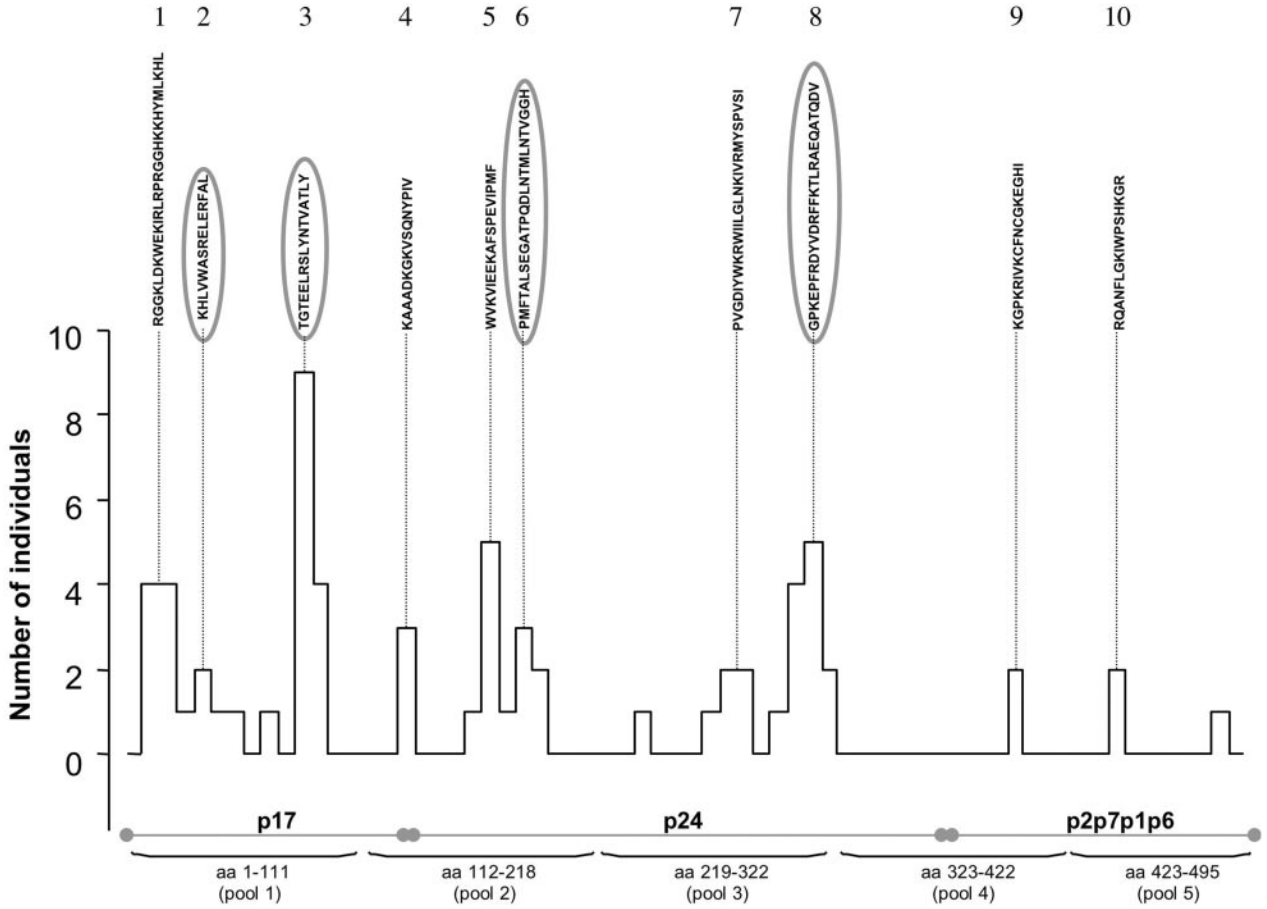

FIGURE 1. Peptide density map showing the number of individuals studied and the location of confirmed peptide responses within Gag. A total of 38 individuals was screened with consensus C Gag peptides, and of the 28 responders, 26 were confirmed with individual peptides. Ten epitopic regions (numbered 1–10) were identified, in which four amino acid stretches (circled) were analyzed in greater detail for fine epitope mapping and HLA restrictions. Five consecutive pools were used: pool 1 (aa 1–111), pool 2 (aa 112–218), pool 3 (aa 219–322), pool 4 (aa 323–422), and pool 5 (aa 11,423–495).

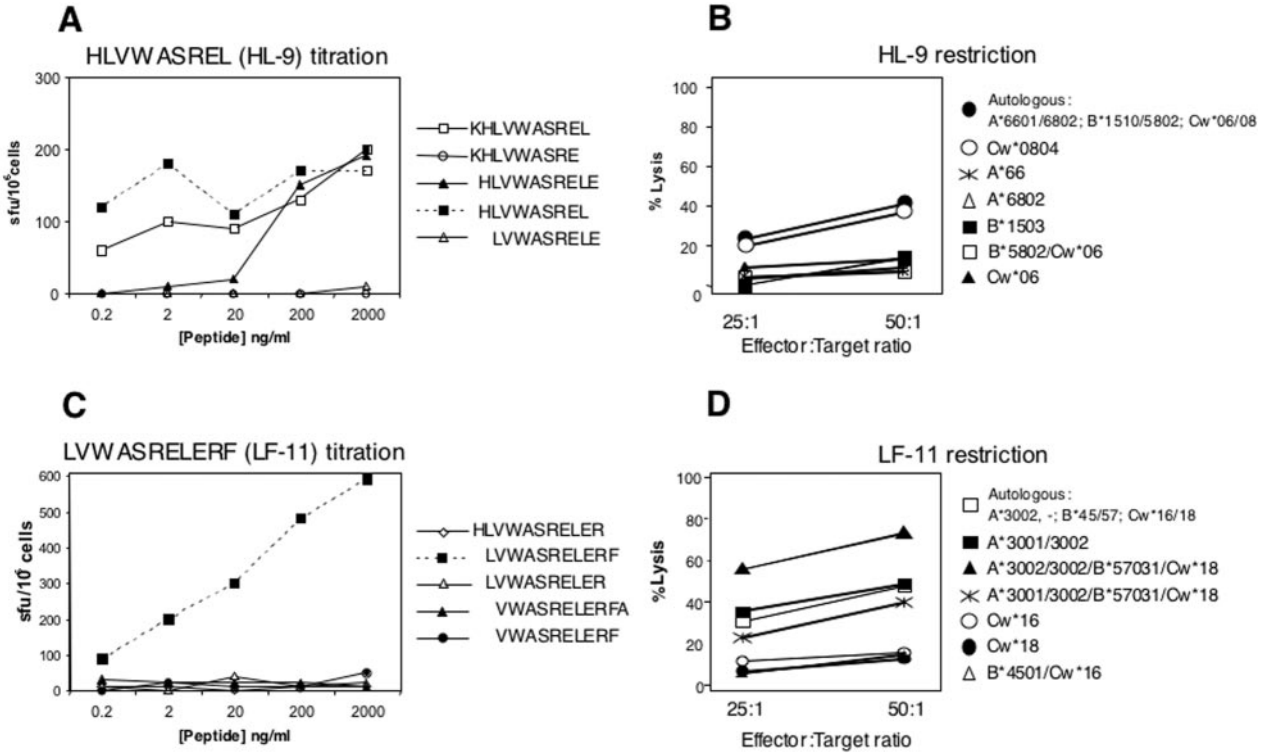

FIGURE 2. Titration of the HL-9 (*A*) and LF-11 (*C*) epitopes using truncated peptides in the IFN-γ ELISPOT assay, and HLA restrictions of HL-9 (*B*) and LF-11 (*D*) using autologous and partially mismatched B cell lines in a CTL killing assay. Two E:T ratios were used at 25:1 and 50:1, in which titratable percentage of lysis was a criteria for accepting the restriction data. The restricting allele was defined as the single HLA allele that gave comparable responses to autologous target BLCL. *B*, Shows that Cw*0804 was closest to autologous, and A*3001/3002 and/or B*57031 were both closest to autologous percentage of lysis in *D*. The full HLA class I types of the mismatched targets were: *B*, (○), A*0205/2902, B*1401/4403, Cw*07/**08**; (*), A*23/**66**, B*39/42, Cw*12/17; (△), A*2301/**6802**, B*0702/4403, Cw*03/07; (■), A*2301/7401, B***1503**/4504, Cw*02/07; (□), A*02/30, B*4501/**5802**, Cw*06/16; (▲), A*2301/3004, B*4403/5301, Cw*02/**06**; *D*, (■), A*3002/-, B*0801/57031, Cw*07/**18**; (▲), A*3001/**3002**, B*0801/4201, Cw*07/-; (*), A*3001/**3002**, B***57031**/5802, Cw*02/**18**; (○), A*02011/7401, B*1503/5101, Cw*02/**16**; (●), A*0101/2902, B*4201/8101, Cw*17/**18**; (△), A*02/03, B*1510/**4501**, Cw*03/**16** (matching alleles are shown in bold).

with partially matched target cells at the –Cw*0804 locus (Fig. 2*B*). The second epitope within the parent stretch was ^{18}LVWAS RELERF[28] (LF-11), which was clearly recognized as the only truncated peptide in the titration experiment (Fig. 2*C*). Restriction experiments using the optimal LF-11 epitope revealed two possible HLA restrictions. Fig. 2*D* shows titratable CTL killing when BLCL partially matched at HLA-A*3001/3002 or HLA-A*3001/3002/B*5703 were used. These experiments show that LF-11 is restricted by either HLA-A*3001/3002 or HLA-A*57031. The higher percentage of lysis using BLCL expressing both HLA-A*3002 and HLA-B*570301 (Fig. 2*D*) infers that the epitope may be presented by both HLA restriction alleles in culture. The lower CTL lysis using almost identical HLA-matched targets, except for HLA-A*3001 difference, suggests that the epitope may be restricted optimally through HLA-A*3002 and not HLA-A*3001. The ambiguous nature of these restrictions highlights the promiscuous nature of LF-11.

The second peptide stretch we focused on in p17 was ^{70}TGTEELRSLYNTVATLYCVHAGIEV^{94}, which is known to contain two previously described CTL epitopes: ^{77}SLYNT VATL^{86} (SL-9) and ^{76}RSLYNTVATLY^{86} (RY-11), restricted by HLA-A*0201 and HLA-A*30, respectively (28, 29). In our study, we also identified the RY-11 epitope (Fig. 3*A*), and, in addition, a novel CTL epitope embedded within this stretch: ^{78}LYNT VATLY^{86} (LY-9) (Fig. 3*B*). HLA restriction experiments showed that LY-9 was restricted by HLA-A*2902 (Fig. 3*C*) and HLA-B*44 (Fig. 3*E*) when partially HLA-mismatched BLCL were used in the CTL assay. Fig. 3*D* shows repeat experiments in which an additional four different HLA-A*29-expressing BLCL were used: three expressing HLA-A*2902 and one expressing HLA-A*290201. It was evident that the latter BLCL could not restrict the epitope and highlighted the fine specificity of LY-9 restriction through HLA-A*2902.

Optimal epitope recognition and HLA restrictions in p24

Next, we fine mapped two epitopic regions in p24 of subtype C Gag. The first region, ^{172}PMFTALSEGATPQDLNTMLNTVGGH^{196}, is known to contain a previously described TL-9 epitope recognized by both subtype B and C HIV-1-infected individuals (39–41), and has been shown to be restricted by HLA-B*07 and HLA-B*42 or HLA-B*81. We mapped fine responses within the longer peptide to the known TPQDLNTML (TL-9) epitope (Fig. 4*A*) and confirmed with the ^{51}Cr release assay that it was restricted by both HLA-B*42 and HLA-B*81 in an individual expressing both alleles (Fig. 4*B*). Confirmation of these HLA restrictions using ICS, a more ex vivo assay, also revealed that the TL-9 epitope was restricted by both HLA-B*42 and HLA-B*81 in the same individual (Fig. 4*C*) and confirmed these were CD8$^+$ T cell mediated.

The second region within p24 that we fine mapped was the peptide stretch, ^{289}GPKEPFRDYVDRFFKTLRAEQATQDV^{314}, which revealed the presence of four different overlapping epitopes within the 26-aa stretch. This region is known to contain previously identified epitopes, and we confirmed the recognition of the HLA-B*14-restricted DRFFKTLRA (DA-9) epitope (41) (results not shown). In addition, we showed the previously described HLA-B*15-restricted YVDRFFKTL (YL-9) epitope (Fig. 5, *A* and

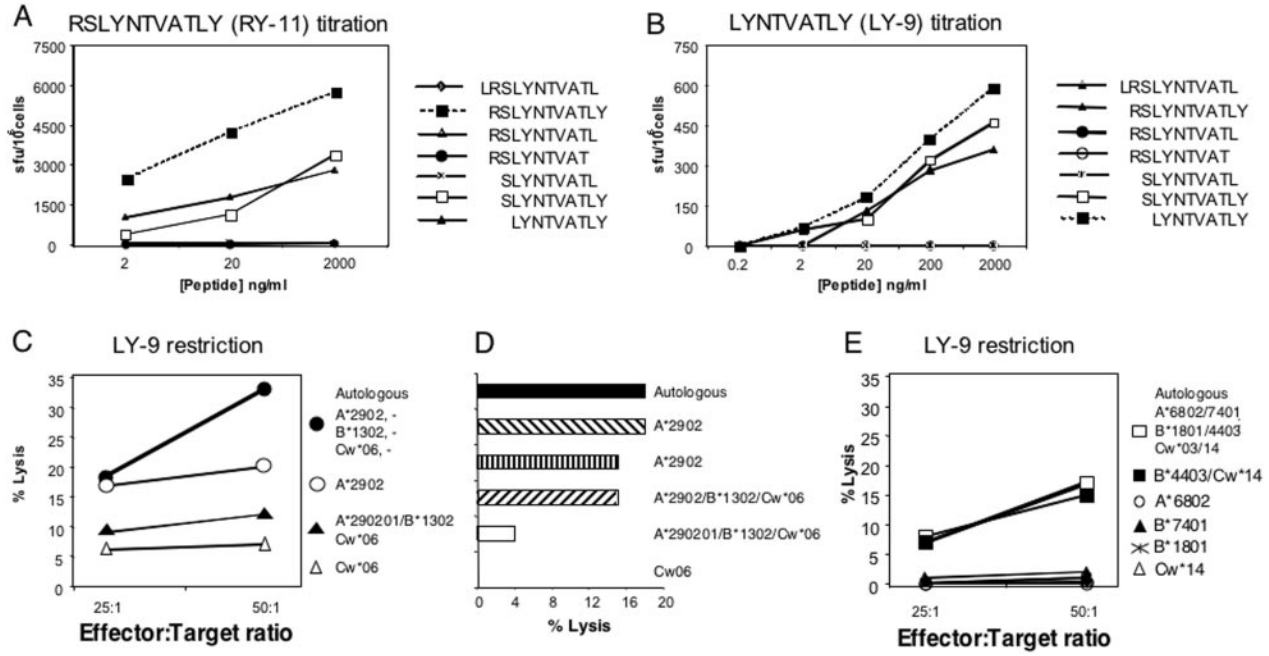

FIGURE 3. Titration of the RY-11 (*A*) and LY-9 (*B*) epitopes using truncated peptides. LY-9 HLA restriction was identified in two individuals, in which *C* and *D* are replicate experiments in one of the individuals showing the A*2902-restricting allele. *E*, Shows that HLA-B*4403 is also the restricting allele for LY-9 in the second individual. Two E:T ratios were used at 25:1 and 50:1, in which titratable percentage of lysis was a criteria for accepting the restriction data. The HLA class I types of the partially mismatched targets were: *C*, (○), A*2902/8001, B*15/44, Cw*04/07; (▲), A*290201/A6802, B*1302/4403, Cw*04/06; (△), A*3601/-, B*5301/5802, Cw*04/06; *D*, (■), A*2902/8001, B*4901/5801, Cw*07/-; (▨), A*2902/4301, B*15/44, Cw*04/07; (▥), A*2301/2902, B*08/13, Cw*06/07; (▨), A*290201/6802, B*1302/440301, Cw*04/06; (□), A*3601/-, B*5301/5802, Cw*02/06; *E*, (■), A*0202/2301, B*1516/4403, Cw*04/14; (○), A*6801/6802, B*0702/1510, Cw*04/07; (▲), A*2901/7401, B*1503/5801, Cw*02/06; (∗), A*3002/-, B*1801/5703, Cw*07/18; (△), A*0206/2402, B*1502/1506, Cw*08/14 (matching HLA class I alleles are shown in bold).

B), as well as two novel optimal epitopes: PFRDYVDRFF (PF-10; Fig. 5*C*) and HLA-Cw*0304-restricted TLRAEQATQD (TD-10) epitope (Fig. 5, *D* and *E*). The restricting allele for PF-10 was not determined due to shortage of material.

CTL epitopes lie in conserved domains of Gag

When each of the epitopic regions in p17, p24, and p2p7p1p6 (Fig. 1) were aligned with the consensus Gag sequence from the HIV-NET 028 cohort, consensus C from southern Africa and consensus M (Fig. 6), it was evident that each of the targeted regions was highly conserved. Our rationale for these alignments was to highlight the level of conservancy across these regions. Although there were a few amino acid substitutions identified, mostly in the consensus M sequence, the mapped epitope identities (Fig. 6) revealed the highly conserved nature of the epitopes. Both the RY-11 and LY-9 epitopes in p17 were identified using the RSLYNTVATLY peptide sequence, although the corresponding consensus cohort and autologous sequence was identified to be RSLFNTVATLY. The phenylalanine (F) substitution for tyrosine (Y) is known to abrogate T cell recognition in the context of HLA-A*0201 restriction (37), but appears not to influence recognition for HLA-A*2902/B*44 and HLA-A*30 (Fig. 6). The ^{289}GPKEP FRDYVDRFKTLRAEQATQDV^{314} epitopic region, at the -C terminus of the major homology region in p24, is completely conserved and possesses four highly targeted overlapping epitopes.

Multiple and unique HLA restrictions of CTL epitopes

Table II shows a summary of the nine optimal epitopes identified in four of the peptide stretches described in Fig. 1. Three epitopes (LVWASRELERF, LYNTVATLY, and TPQDLNTML) were restricted by more than one HLA allele, showing the promiscuity of epitope binding. Five of the epitopes have not been previously reported, and two epitopes (LVWASRELERF and LYNTVATLY) were restricted by frequently expressed HLA-A*30 (HLA-A*3002) (allele frequency = 0.226) and HLA-A*2902 (allele frequency = 0.119) alleles in the cohort. Similarly, three epitopes (RSLYNTVATLY, TPQDLNTML, and YVDRFFKTL) identified were not novel, although the restricting HLA allele was significantly more common in the cohort examined; namely HLA-B*4201, HLA-B*8101, and HLA-B*15 (Table II). Conversely, two further epitopes (LYNTVATLY and TLRAEQATQD) were restricted by HLA-B*4403 and HLA-Cw*0304 that are significantly more common in Caucasian populations (Table II). When we noted the HLA backgrounds of individuals recognizing each peptide, we could make accurate associations for single HLA alleles with the identified restricting allele for eight of the epitopes. For example, all individuals ($n = 3$) recognizing the LF-11 epitope expressed HLA-A*30, and all individuals ($n = 13$) recognizing either the RY-11 or LY-9 epitope expressed HLA-A*30, HLA-A*29, or HLA-B*44 (Table II). All individuals ($n = 5$) recognizing the TL-9 epitope expressed either or both HLA-B*4201 or HLA-B*8101. For the cluster of epitopes within the peptide stretch, GPKEPFRDYVDRFFKTLRAEQATQDV, all individuals ($n = 10$) expressed either a variant of HLA-B*15 or HLA-B*1401 or Cw*03. These data show that epitopes recognized frequently in the cohort were associated with more than one HLA allele, and partly substantiate the promiscuous nature of epitope restriction within this cohort.

When the frequency of the HLA alleles that restricted these epitopes was compared with Caucasians, it was evident that the HLA restrictions in our study were unique due to the significantly higher allele frequencies in our cohort (Table II). For example, there was a cumulative HLA-A*, B*, and Cw* allele frequency of 0.919 in single alleles that were found to restrict the identified

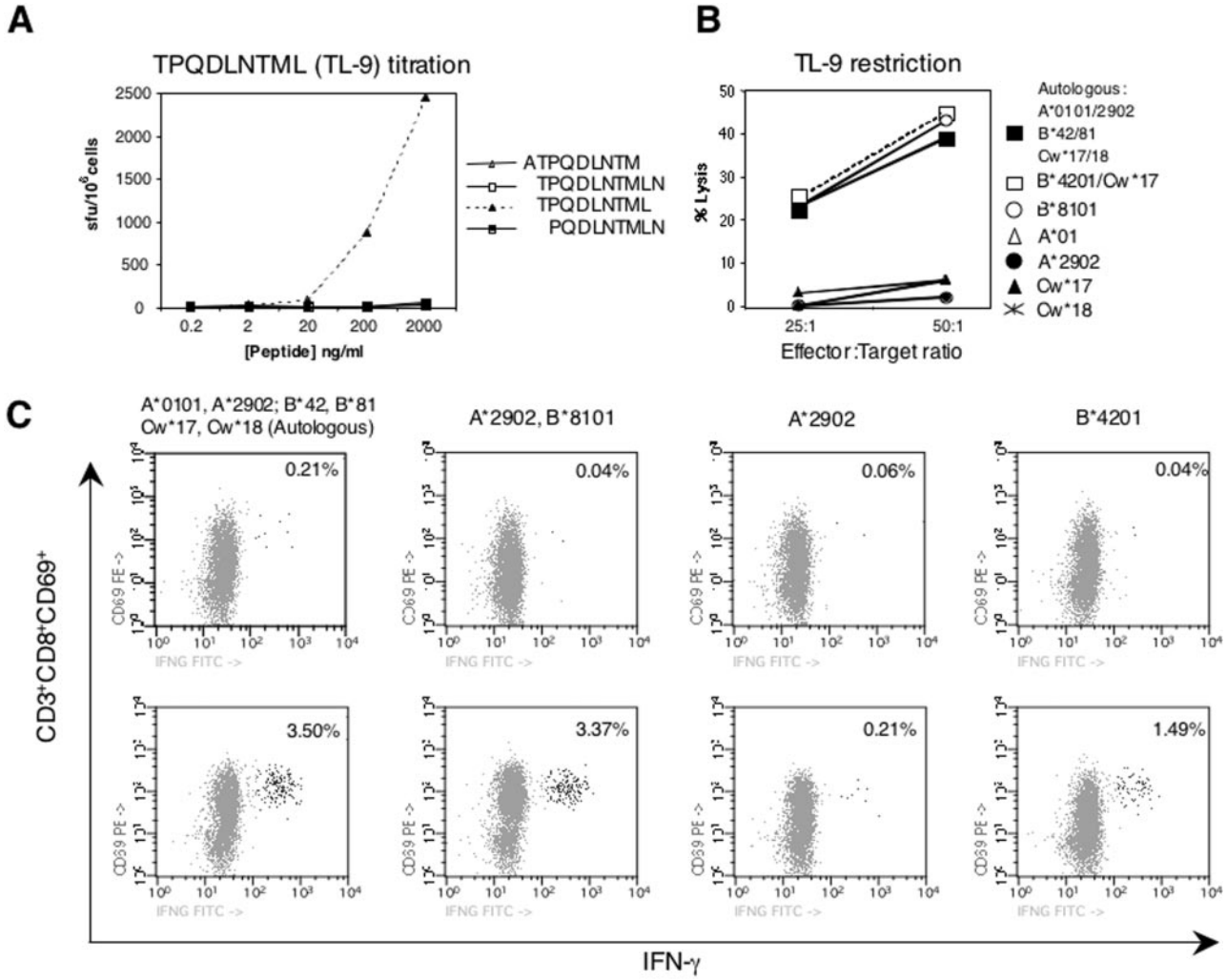

FIGURE 4. Fine epitope mapping of the TL-9 epitope (*A*) and HLA restrictions using the ^{51}Cr release assay (*B*) and ICS (*C*). HLA restriction from bulk cultured cells shows that HLA-B*42 and HLA-B*81 are the restricting alleles for TL-9 in the same individual. This was confirmed using a direct ex vivo ICS assay, showing the response to be $CD3^+CD8^+$ T cell mediated. IFN-γ-positive cells were gated from the $CD3^+/CD8^+/CD69^+$ T cell population, and 50,000 gated $CD3^+$ events were counted. The HLA class I types of the partially mismatched targets in *B*, (□), A*2301/2601, B*0801/**8101**, Cw*04/07; (○), A*30/34, B*4201/4501, Cw*06/**17**; (△), A*01/11, B*07/52, Cw*07/12; (●), A*2902/8001, B*4901/5801, Cw*07/-; (▲), A*3201/68011, B*4101/5802, Cw*06/**17**; (∗), A*3002/-, B*45/57, Cw*16/**18** (matching HLA alleles are shown in bold).

optimal CTL epitopes (Table II) compared with 0.572 for the same HLA alleles found in Caucasians (34). By comparison, there was a cumulative HLA allele frequency of 1.022 in a healthy Zulu population (35, 36), inferring that there was minimal skewing of HLA due to HIV-1 infection in our cohort.

Discussion

The role of HIV-1-specific CTL in the control of viral replication has been studied extensively in individuals with acute infection, long-term nonprogressive disease, highly exposed but persistently seronegative, and in SIV animal models (1–5, 7, 8, 10–18, 31–33, 37, 38). The majority of studies have focused on identification and description of subtype B epitopes recognized mostly by Caucasians, and considerably fewer subtype C epitopes have been identified from HIV-1-infected people in Africa. The identification of epitopes in geographic regions and populations that are affected by the epidemic is crucial for the development of a relevant vaccine. In this study, we report on the characterization of epitope-rich regions in Gag targeted by individuals from Zambia, Malawi, Zimbabwe, and South Africa, where all individuals were infected with subtype C HIV-1 (C. Gray, C. Williamson, H. Bredel, A. Puren, X. Xia, R. Filter, L. Zijenah, H. Cao, L. Morris, E. Vardas, et al., manuscript in preparation). We used Gag peptides that were derived from a consensus subtype C sequence and found that 74% of subjects recognized one or more regions in Gag. We identified that highly conserved regions in Gag were recognized, and that the defined optimal epitopes were restricted by more than one HLA allele. We also show that these optimal CTL epitopes were more likely recognized by populations in southern Africa due to unique HLA types found in the region. However, the epitopes identified overlap and are embedded within regions known to contain several previously described epitopes. Taken together, these data highlight the promiscuous nature of epitope binding and that the frequently targeted regions of Gag that would need to be included in a vaccine candidate would be recognized by multiple HLA.

Two separate studies have shown that while p17 is highly targeted in subtype B HIV-1-infected individuals, p24 was mostly targeted by subtype C-infected individuals in Africa (39, 40). In contrast, we identified epitope-rich regions in both p17 and p24 that are targeted by $CD8^+$ T cells, and the peptide stretch, TGTEELRSLYNTVATLYCVHAGIEV, in p17 was recognized at the highest frequency (35%) and contained multiple epitopes.

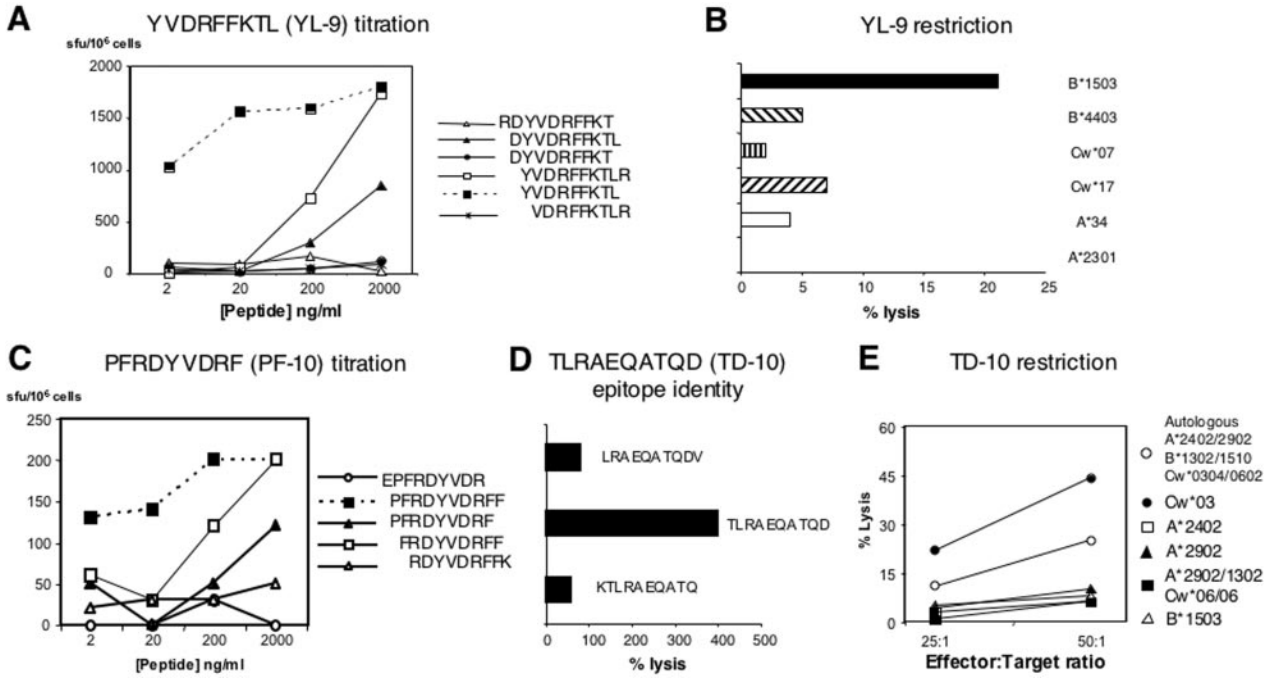

FIGURE 5. Identification of epitopes and HLA restrictions in the C-terminal region of the MHR of p24 Gag. Titration of the YL-9 epitope (*A*) and its restriction by HLA-B*1503 at an E:T ratio of 50:1 (*B*); titration of the PF-9 epitope (*C*) and TD-10 epitope identity (*D*). HLA restriction of the TD-10 epitope by HLA-Cw*03 at E:T ratios of 50:1 and 25:1 (*E*) is shown. The HLA class I types of the partially mismatched targets for the TD-10 epitope were: (●), A*2301/6802, B*0702/4403, Cw***03**/07; (□), A***2402**/6801, B*0702/4101, Cw*07/17; (▲), A*0101/**2902**, B*42/81, Cw*17/18; (■), A***2902**/-, B***1302**/-, Cw***06**/-; (△), A*2301/7401, B***1503**/4504, Cw*02/16 (matching HLA alleles are shown in bold).

Characterization of targeted regions in p17 revealed the presence of both previously described epitopes, identified from subtype B and C HIV-1 infection, and three novel epitopes. The HLA-A*02-restricted SL-9 epitope has been widely characterized in subtype B-infected individuals, in which earlier studies showed an inverse correlation between the magnitude of SL-9-A02 tetramer-positive cells with plasma viral load (41, 42). Our data show that individuals in this cohort recognized the RY-11 epitope restricted by HLA-A*30 (43) and LYNTVATLY (LY-9), which was found to be restricted by both HLA-A*2902 and HLA-B*4403. Peptide-binding specificities for HLA-A*2902 and B*4403 are the same at positions 2 (E) and 9 (Y) (44), and these observations provide evidence that epitopes in p17 recognized by subtype C HIV-1-infected individuals overlap with epitopes defined from subtype B HIV-1 infection and are promiscuous in nature. The fine nature of LY-9 restriction was further highlighted by the inability of HLA-A*290201 to present the epitope. A previous study demonstrated that a phenylalanine (F) substitution for tyrosine (Y) at position 3 within SL-9 resulted in loss of recognition of an HLA-A*0201 peptide/MHC tetramer folded around SLYNTVATL, suggesting that TCR recognition is sensitive to a single amino acid change at this position (45). It was interesting to note that 52% of the major viral population infecting the individuals investigated had an F to Y substitution at position 3, even though the consensus peptides used in this study contained the SLYNTVATL sequence. It is likely that the LY-9 and RY-11 epitope restrictions and TCR recognition do not have the same constraints as for SL-9 and HLA-A*0201. We also identified two further novel epitopes, HLVWAS REL (HL-9) and LVWASRELERF (LF-11), in p17 between aa 9–46, restricted by Cw*0804 for HL-9 and HLA A*30 for LF-11. A similar epitope in this region, WASRELERF, restricted by HLA-B*35, has been described for subtype B-infected individuals (41).

Regions recognized in p24 contain previously described epitopes that also matched previously described restricting HLA alleles. For example, the TL-9 epitope has been shown to be restricted by B*07 in Caucasians and by B*42 or B*81 in Africans (39–41). Our study has confirmed TL-9 epitope recognition and has further shown that it can be HLA restricted by HLA-B*81 (39–41). Additionally, we demonstrate that the TL-9 epitope can be restricted by both HLA- B*81 and HLA-B*42 alleles in the same individual with a B*42/B*81 haplotype. The highly targeted region in p24 (aa 289–314) located on the C-terminal side of the major homology region (MHR) has been shown to contain multiple epitopes from subtype B HIV-1 infection. The MHR is a 20-aa highly conserved stretch among all replication-competent retroviruses, with the exclusion of spumaretroviruses, and is virtually identical between different HIV-1 subtypes, HIV-2, and SIV (41, 46). The functional importance of this region has been highlighted when site-specific mutations abolished viral replication and significantly reduced the particle-forming ability of the mutant *gag* gene products. The DA-9 epitope, an immunodominant epitope described in a long-term nonprogressing individual (47), is located at the C terminus of the MHR, and we report that it was recognized by subtype C-infected individuals and is restricted by HLA-B*14. Mutations in this epitope that abrogated CTL recognition strongly impaired viral replication, whereas replication-competent viral variants were recognized by CTL (47). Previous studies have shown that the YVDRFFK*T*L epitope variant is restricted by A*2601 and B*70 in Caucasians (41, 44), and the YVDRFFKRL variant is restricted by B*1510 in Africans (32), and both variants are equally recognized by A*0207 in Thais (45). We show that the YVDRFFK*T*L variant is restricted by B*1503 in this cohort (40, 45, 48, 49). It was interesting to note that some of the individuals with an HLA-B*1510 background and who recognized the peptide stretch aa 289–314 recognized the TD-10 epitope, which we show

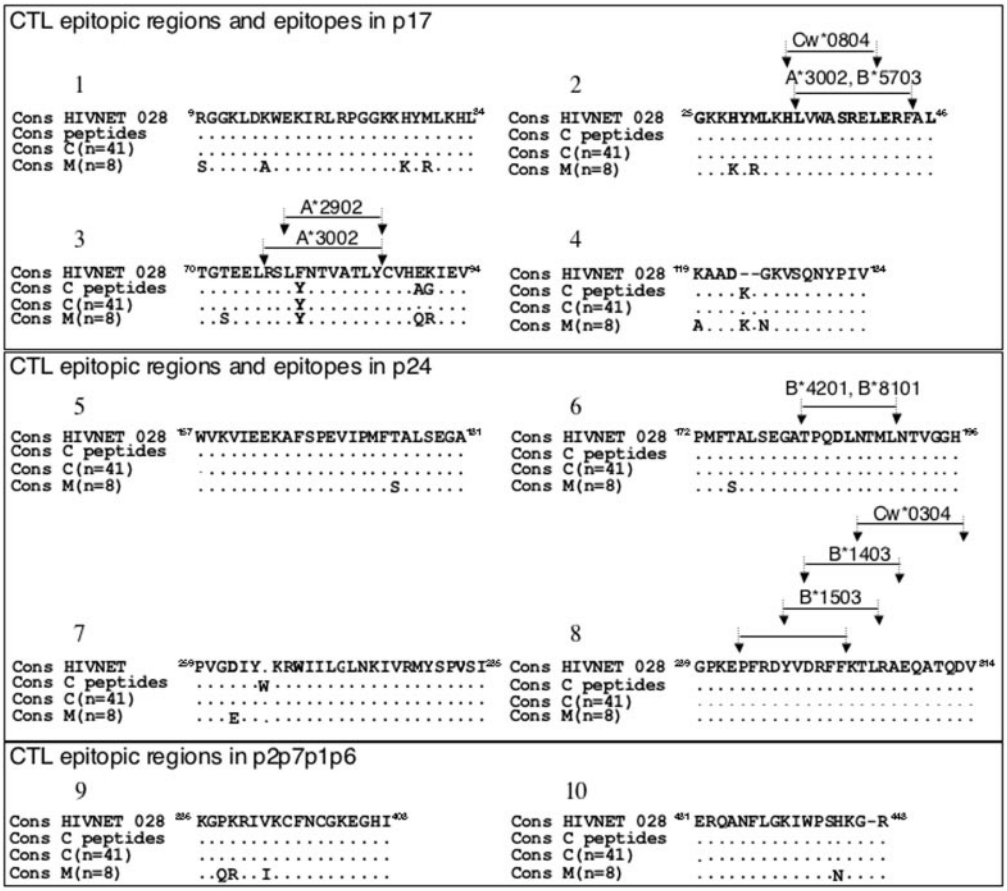

FIGURE 6. Alignment of 10 epitopic regions identified in Gag showing a high level of sequence conservation. A consensus HIVNET 028 Gag sequence was constructed from infecting viral populations from the 38 individuals studied, and, using BioEdit software (Los Alamos database; http://hiv-web.lanl.gov/), was aligned with consensus C Gag peptide reagents and consensus C and M sequences. Mapped epitopes and their restricting HLA alleles are indicated with arrows.

to be restricted by -Cw*0304, suggesting preferential epitope processing. The known peptide-binding specificity for HLA-Cw*0304 indicates a requirement for alanine at position 2 and either L or M at position 9, and judging from the sequence of this novel epitope, it is evident that the epitope does not follow this prediction (44). A second novel epitope, PF-10, also located on the

Table II. *Summary of optimal epitopes identified within each peptide stretch and the HLA restricting alleles*[a]

Peptide Sequence	Optimal Epitope	Restricting HLA Allele	Frequency of HLA Associations (%)	HLA Allele Frequency			p^b
				Cohort[c]	Zulu[d]	Caucasians[e]	
[25]GKKHYMLKHLVWASRELERFAL[46]	**HLVWASREL**	Cw*08	33	0.06	0.066	0.038	
	LVWASRELERF	A*30	100	**0.226**	0.195	0.019	<0.05
		B*57	33	0.042	0.051	0.043	
[70]TGTEELRSLYNTVATLYCVHAGIEV[94]	RSLYNTVATLY	A*30	58	**0.226**	0.195	0.019	<0.05
	LYNTVATLY	A*29	25	**0.119**	0.125	0.045	<0.05
		B*44	25	0.091	0.107	**0.149**	<0.05
[172]PMFTALSEGATPQDLNTMLNTVGGH[196]	TPQDLNTML	B*42	100	**0.085**	0.122	0.004	<0.05
		B*81	60	**0.018**	0.036	0	<0.05
	PFRDYVDRFF	ND					
[289]GPKEPFRDYVDRFFKTLRAEQATQDV[314]	YVDRFFKTL	B*15	40	**0.188**	0.153	0.079	<0.05
	DRFFKTLRA	B*14	10	0.036	0.066	0.038	
	TLRAEQATQD	Cw*03	50	0.054	0.101	**0.157**	<0.05
			Cumulative frequency	0.919	1.022	0.572	

[a] Novel epitopes are shown in bold. The frequency of individuals in the cohort who recognized the single peptide stretch and who shared the HLA-restricting allele is also shown along with the HLA frequencies of restricting alleles within the cohort compared with those found in Zulu and Caucasian populations. Cumulative allele frequency in each population that have or would be expected to recognize the CTL epitopes are also shown.
[b] Using Fisher's exact test.
[c] Full cohort of 84 individuals.
[d] Of 98 individuals (27, 28).
[e] Of 265 individuals (29).

C-terminal end of the MHR region, was identified, but due to shortage of cells, we were unable to determine the restricting allele.

These observations suggest that there might be preferences in epitope processing and presentation, and would argue against the use of a polyepitope vaccine to prime the immune system, despite taking into account a comprehensive HLA coverage of the target population group. We have provided evidence that it is possible to measure CTL responses to Gag using consensus peptides in subtype C-infected individuals, and that the majority of these responses target conserved regions in Gag. However, fine epitope mapping of these regions has revealed the presence of a number of novel and promiscuous epitopes that overlap or are embedded within previously described epitopes from subtype B-infected Caucasians. We propose that HLA allele divergence between populations will dictate which epitopes are recognized in infected individuals, and the presence of a specific allele does not always translate into presentation of a predicted epitope. For example, in this study, an individual expressing HLA-B*1510 recognized the peptide stretch that contained the B*1503-restricted YL-9 epitope. However, this individual restricted the TD-10 epitope through HLA-Cw*0304, and not the B allele. Thus, although both B*1510 and B*1503 belong to the same supertype family, we did not observe the expected restriction. Our observations suggest that a vaccine based on known poly-epitopes may not elicit expected epitope-specific responses, or possibly no responses, from different populations with unique HLA backgrounds. It is therefore important to allow expression and processing of epitopes using full-length Gag. In summary, despite the highly conserved nature of this region, we have shown the presence of novel epitopes and novel restricting HLA alleles, confirming the immunogenicity of this region of p24. These data highlight the degenerate nature of epitope binding in which different class I HLA molecules across -A, -B, and -Cw alleles obviously share some level of common sequence motifs. Structural similarities in MHC-binding clefts have given rise to HLA supertype groupings (50, 51), although our data show that degenerate epitope binding does not adhere to known supertypes. These data extend and confirm recent data, inferring that similar epitope specificities are observed between different populations expressing divergent HLA class I alleles (52).

To conclude, we have characterized responses in Gag and provide evidence that subtype C HIV-1-infected southern Africans target conserved regions in Gag. Epitope mapping has identified the presence of novel epitopes that are embedded and overlap with previously described epitopes. We have identified novel restricting alleles, due to the unique HLA class I distribution in southern Africa, and provided evidence for promiscuous CTL epitope recognition. Collectively, these data support our hypothesis that HLA diversity between populations can shape fine epitope specificity, but may not represent a constraint for eliciting universal responses, regardless of HIV-1 subtype or host genetic diversity.

Acknowledgments

We thank the expert technical help of Moses Mashiloane and Ewalde Cutler for CD4 and viral load estimations, Stephina Nyoka for assistance with flow cytometry analysis, and Phineas Mohube for expert technical assistance in the CTL laboratory. We also thank the clinical staff at each of the clinical sites for their invaluable help in collecting and shipping samples. Finally, we thank the HIV-1-infected participants of this study.

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