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For Protection from HIV-1 Infection, More Might Not Be Better: a Systematic Analysis of HIV Gag Epitopes of Two Alleles Associated with Different Outcomes of HIV-1 Infection

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A subset of women in the Pumwani Sex Worker Cohort, established in 1985 in Nairobi, Kenya, remains uninfected despite repeated high-risk exposure (HIV-exposed, seronegative [HESN]) through active sex work. This HESN phenotype is associated with several alleles of human leukocyte antigens (HLAs) and specific CD8$^+$ and CD4$^+$ T cell responses to HIV-1. The associations of HLA alleles with differential HIV-1 infection are most likely due to their different abilities to present antigen and the different immune responses they induce. The characteristics of epitopes of HLA alleles associated with different outcomes of HIV-1 infection might therefore point to a vital clue for developing an effective vaccine. In this study, we systematically analyzed HIV-1 clade A and D Gag CD8$^+$ T cell epitopes of two HLA class I alleles associated with different outcomes of HIV-1 infection. Binding affinity and off-rates of the identified epitopes were determined. Gamma interferon (IFN-γ) enzyme-linked immunospot (ELISpot) assays with patient peripheral blood mononuclear cells (PBMCs) validated the epitopes. Epitope-specific CD8$^+$ T cells were further phenotyped for memory markers with tetramer staining. Our study showed that the protective allele A*01:01 recognizes only three Gag epitopes. By contrast, B*07:02, the allele associated with susceptibility, binds 30 epitope variants. These two alleles differ most importantly in the spectrum of Gag epitopes they can present and not in affinity, off-rates, the location of the epitopes, or epitope-specific Tem/Tcm frequencies. The binding of more epitopes and strong IFN-gamma ELISpot responses are associated with susceptibility to HIV-1 infection, while more focused antigen recognition of multiple subtypes is protective. Rational vaccine design should take these observations into account.

Current candidate vaccines for HIV-1 have failed to provide protection and in some cases may have actually enhanced the likelihood of infection (3, 6–11, 44–46, 48) or produced only modest effects (15, 16, 51, 69, 70). This failure has been attributed to the difficulties of confronting a virus that targets cells which are a key component of the immune system and to the challenges posed by a pathogen that mutates rapidly and occurs with great genetic diversity. More critically, vaccines developed to date have been based on a conventional approach to control viral infection, which does not reflect a sufficient understanding of the correlates of protection against HIV-1. To develop a successful vaccine, we will need to understand which immunological parameters correlate with protection against HIV-1 and why.

Several cohort studies have documented that there is considerable heterogeneity in susceptibility to HIV-1 infection (21, 68). Despite repeated exposures, some individuals do not appear to become infected by HIV-1, and these individuals can be classified as HIV exposed, seronegative (HESN). Understanding why these individuals can escape HIV-1 infection and how their immune system works will help reveal parameters of protective immunity and aid in the development of effective vaccines and control strategies. A subset of women in the Pumwani Sex Worker Cohort (33, 65), established in 1985 in Nairobi, Kenya, remains HIV-1 seronegative and PCR negative despite repeated high-risk exposure through active sex work (21, 52). This resistance to HIV-1 infection is associated with several alleles of human leukocyte antigens (HLAs) and specific CD8$^+$ and CD4$^+$ T cell responses to HIV-1 (4, 5, 27, 28, 34, 43). HLAs constitute a group of host proteins that are central in regulating the immune response through the binding and presenting to T cells of peptides known as epitopes, which are derived from self and foreign proteins. The genes coding for HLAs are extremely polymorphic, resulting in a diversity of HLA alleles with variable recognition ability and affinity to the self and to the pathogenic proteins in the population. The contribution of different HLA alleles to virus control varies because of differences in antigenic recognition. The associations of specific HLA alleles with different outcomes of HIV-1 infection are most likely due to the differences in the antigenic peptides or epitopes of HIV being presented and the resulting immune responses following antigen recognition. Moreover, T cell responses to HIV-1 Gag correlate with viremia control and better outcomes of disease progression (25, 30, 37, 49, 54, 58, 62, 74). Finding out which differences in the Gag epitopes between HLA alleles are associated with different outcomes of HIV-1 infection might therefore offer a vital clue for developing an effective HIV-1 vaccine.

In this study, we systematically analyzed the HIV-1 clade A and D Gag epitope profiles of two HLA class I alleles, A*01:01 and B*07:02, which are independently associated with different outcomes of HIV-1 infection in order to elucidate the correlates of protective immunity to HIV-1. HLA-A*01 is significantly en-

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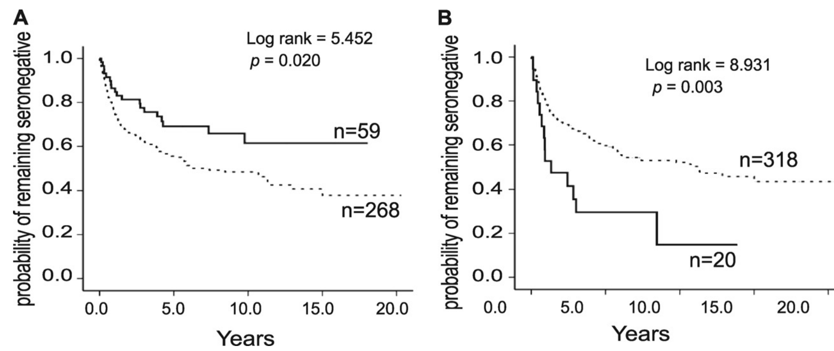


FIG 1 Kaplan-Meier analysis of seroconversion in the Pumwani Sex Worker Cohort. (A) Women with A*01:01 were significantly less likely to seroconvert than individuals without A*01. Solid line, with A*01; dashed line, without A*01. (B) Women with B*07:02 were significantly more likely to seroconvert than individuals without B*07:02. Solid line, with B*07:02; dashed line, without B*07:02.

riched in the HIV-1-resistant women ($P = 0.016$; odds ratio: 1.7; 95% confidence interval [95% CI]: 1.1 to 2.7). HIV-1-negative women with A*01 seroconvert significantly more slowly than women without this allele (Fig. 1A). By contrast, HLA-B*07:02 is associated with susceptibility to HIV-1 infection ($P = 0.035$; odds ratio: 0.38; 95% CI: 0.14 to 2.1) and rapid seroconversion (Fig. 1B) in the Pumwani cohort, as well as high viral loads and rapid disease progression in other populations (26, 67, 68). The peptide binding capacity of these two alleles was compared by screening an HIV-1 Gag overlapping peptide library with the iTopia epitope discovery system (Beckman Coulter). The identified epitopes were further characterized by affinity and off-rates and confirmed by gamma interferon (IFN-γ) ELISpot assays with patient peripheral blood mononuclear cells (PBMCs). Epitope-specific CD8$^+$ T cells were further phenotyped for memory markers with tetramer staining. The broad epitope recognition and induced immune response were seen in the allele associated with susceptibility, while the more focused epitope recognition of multiple subtypes was observed in the allele associated with protection from HIV-1 infection. Thus, more epitopes and broad immune responses might not always be better for preventing HIV-1 infection. These observations should be considered when undertaking to design rational vaccines for HIV-1.

MATERIALS AND METHODS

Ethics statement. All subjects gave informed consent to participate, and the study has been approved by the Institutional Review Boards at the Universities of Manitoba and Nairobi. Written informed consent has been obtained from all study participants.

Study cohort. Subjects involved in this study were women enrolled in the Pumwani Sex Worker Cohort established in 1985 in Nairobi, Kenya (33, 65). The Pumwani Sex Worker Cohort is an open, prospective cohort to study factors influencing sexually transmitted diseases (33, 65). All enrollees have been followed biannually after enrollment, and a subgroup of women remain serologically and PCR negative for HIV-1 despite repeated exposure through high-risk sex work (21).

HLA class I typing. DNA was isolated using QIAmp DNA mini kit and QIAgen EZ1 blood robot (QIAgen. Inc., Mississauga, ON, Canada). HLA-A, -B, and -C genes were amplified by PCR with gene specific primers (41, 42). The purified PCR products were sequenced with BigDye cycle sequencing kits (Applied Biosystems) using sequence specific primers and analyzed with an ABI3100 Prism genetic analyzer. Allele-specific primers were used to resolve ambiguous allele combinations. HLA-A, -B, and -C were typed using CodonExpress, a computer software program developed based on a taxonomy-based sequencing analysis (41, 42). Since exons 2 and 3 encode the peptide binding region of HLA class I molecules, analysis was restricted to these exons and all alleles were typed to 4-digit resolution. Homozygotes were classified based on the 4-digit typing for the peptide binding regions of the HLA genes. Between 1985 and 2001, 338 women who were seronegative at the cohort enrollment were included for the longitudinal seroconversion analysis. Of them, 327 were typed for HLA-A, 338 were typed for HLA-B, and 329 were typed for HLA-C.

Peptide synthesis and epitope screen using iTopia epitope discovery system. Overlapping peptides spanning *Gag* of HIV-1 subtype A and D (predominant HIV subtypes in Kenyan) consensus clades were synthesized (JPT Peptides Technologies, Inc.). The peptide library is consisted of 632 peptides (9-mer overlapping by 8 amino acids) incorporating sequence variations of the subtype A and D consensus. The A*01:01 and B*07:02 kits of the iTopia epitope discovery system were purchased from Beckman Coulter, Inc. (San Diego, CA). The iTopia system consists of 3 assays to identify and characterize epitopes: peptide binding assay, off-rate assay, and affinity assay (72). The peptide binding assay evaluated the bindings of all 632 peptides to A*01:01 and B*07:02. Results are expressed as the percentage of the positive (POS) controls provided in the kits. Peptides showing binding of at least 30% of the positive control peptide were identified as binders and analyzed for binding affinity at the concentration range from 10^{-4} to 10^{-9} M. The binding affinity is expressed as 50% effective dose (ED_{50}), which represents the concentration at which the peptide is bound to the major histocompatibility complex (MHC) at 50% maximum binding

TABLE 1 Multivariate analysis of HLA class I alleles associated with seroconversion

	Cox regression result			
			95% CI[c] for Exp(B)	
Allele	P value[a]	Exp(B)[b]	Lower	Upper
Protective				
A*01	0.031	1.731	1.052	2.849
C*06:02	0.015	1.679	1.105	2.551
C*07:01	0.042	1.520	1.015	2.277
Susceptible				
A*23:01	0.010	0.551	0.351	0.865
B*07:02	0.011	0.483	0.276	0.846
B*42:01	0.015	0.593	0.389	0.905

[a] Significances are indicated.
[b] Exp(B), Exponent of B, hazard ratio.
[c] 95% CI, confidence interval.

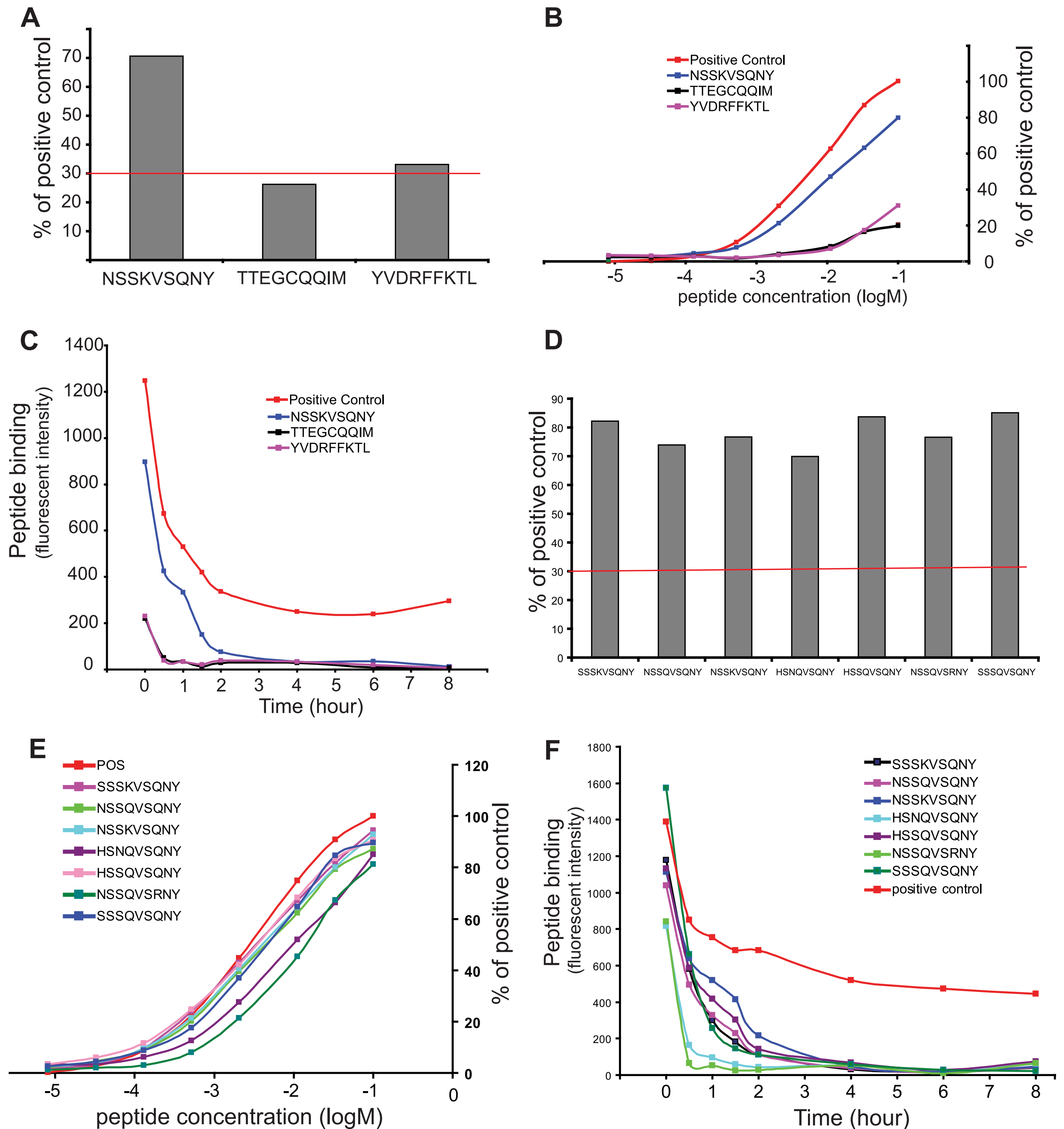


FIG 2 The binding, affinity, and off-rates of A*01:01 HIV-1 Gag epitopes. (A) The level of binding of Gag epitopes to A*01:01. (B) The binding affinity of A*01:01 Gag epitopes. (C) The off-rates of A*01:01 Gag epitopes. (D) The binding of variant peptides of A*01:01 epitope NSSKVSQNY. (E) The binding affinity of epitope variants to A*01:01. (F) The off-rates of A*01:01 epitope variants.

efficiency and is the midpoint of the sigmoidal dose-response curve. The identified peptides were analyzed for off-rate (complex stability) where $t_{1/2}$ represents the time point when 50% of the peptide remains bound to the MHC. The off-rate was calculated as the time to the 50% of the highest binding (fluorescent intensity). Peptide binding, affinity, and off-rate values were calculated using the iTopia software and Graphpad Prism 4. ED_{50} was determined using a sigmoidal dose-response curve, and $t_{1/2}$ was determined using a one-phase exponential decay curve (63, 72).

Validation of epitopes using ELISpot assays. Peptides (9 amino acid) identified by the iTopia epitope discovery system were synthesized (Sigma Genosys, Oakville, ON) for confirmation with IFN-γ ELISpot assays using patient PBMCs. To confirm that peptides identified by the iTopia epitope discovery system are true epitopes capable

TABLE 2 Summary of A*0101 and B*0702 iTopia and ELISpot data

Allele	Peptide sequence	aa[b] no. in Gag	% Peptide binding	Affinity (ED_{50})	Off-rate $t_{1/2}$ (h)	Mean ELISpot response[c]	Reported in HIV database[a]
A*01:01	NSSKVSQNY	124–132	70.6	1.21×10^{-5}	0.94	79	Y
	TTEGCQQIM	53–61	26.2	1.70×10^{-5}	0.33	77	N
	YVDRFFKTL	296–304	33.1	4.91×10^{-5}	0.30	2,148	Y
	SSSKVSQNY	124–132	82.2	5.13×10^{-6}	0.49	115	N
	NSSQVSQNY	124–132	73.9	5.02×10^{-6}	0.48	96	Y
	HSNQVSQNY	124–132	69.9	1.43×10^{-5}	0.31	115	Y
	HSSQVSQNY	124–132	83.7	4.30×10^{-6}	0.79	74	N
	NSSQVSRNY	124–132	76.6	1.21×10^{-5}	0.27	127	N
	SSSQVSQNY	124–132	85.1	5.18×10^{-6}	0.43	130	N
B*07:02	APPAEIFGM	456–464	57.6	1.75×10^{-5}	0.40	100	N
	APRKKGCWK	406–414	145.3	8.96 E-07	0.78	148	Y
	FPQSRPEPT	447–455	55.6	1.50×10^{-5}	0.70	55	N
	GPGATLEEM	338–346	35.1	1.78×10^{-5}	0.81	80	N
	GPGHKARVL	355–363	93.1	4.80×10^{-6}	0.42	84	Y
	GPIPPGQMR	221–229	45.9	4.84×10^{-4}	0.78	215	N
	GPSHKARVL	355–363	91.6	3.14×10^{-5}	0.44	95	Y
	IVGGHQAAM	190–198	46.4	4.49×10^{-5}	0.47	n/a	N
	IVQNAQGQM	134–142	38.8	4.13×10^{-4}	0.72	n/a	N
	RALGPGATL	335–343	52.2	1.49×10^{-5}	0.45	115	Y
	RLRPGGKKK	20–28	32.4	7.83×10^{-5}	0.71	135	Y
	RPGNFPQSR	443–451	37.3	5.37×10^{-5}	0.73	80	N
	SPRTLNAWV	148–156	113.3	1.61×10^{-6}	3.78	245	Y
	TPQDLNMML	180–188	36.9	8.12×10^{-5}	0.83	905	Y
	TPQEQIGWM	242–250	44.8	2.60×10^{-5}	0.67	185	N
	TPQEQLGWM	242–250	43.7	1.04×10^{-4}	0.70	<50	N
	VRMYSPVSI	274–282	41.6	1.69×10^{-4}	0.83	435	Y
	WPSSKGRPG	437–445	81.0	2.71×10^{-5}	0.40	115	N
	YPLVSLKSL	483–491	75.5	2.27×10^{-5}	0.69	703	N
	YVDRFFKTL	296–304	60.0	4.37×10^{-5}	0.50	1280	Y
	KARVLAEAM	359–367	66.5	8.08×10^{-6}	0.44	78	N
	QVQHTNIMM	369–377	46.1	2.55×10^{-5}	0.27	87	N
	QAQQPNVMM	369–377	46.2	1.92×10^{-5}	0.35	96	N
	QVNGNTAIM	369–377	46.3	2.28×10^{-5}	1.23	99	N
	QANANTAIM	369–377	46.0	1.36×10^{-5}	1.35	109	N
	ATNANAAIM	370–378	37.4	2.08×10^{-5}	0.25	95	N
	NIMMQRGNF	375–383	73.4	9.72×10^{-6}	0.25	100	N
	NIMMQRSNF	375–383	73.0	9.31×10^{-6}	0.25	121	N
	NVMMQRSNF	375–383	70.2	8.96×10^{-6}	0.27	117	N
	AIMMQRGNF	375–383	91.6	3.45×10^{-6}	0.38	113	N

[a] Y, yes; N, no.
[b] aa, amino acid.
[c] n/a, not applicable.

of stimulating T cell responses, functional IFN-γ ELISpot assays were carried out as previously described (61). The peptide stocks were dissolved in dimethyl sulfoxide (DMSO), and the stocks were diluted to a final concentration of 10 mg/ml in RPMI medium for ELISpot assays. PBMCs were suspended in RPMI medium, and 10^5 cells were stimulated in duplicate for each peptide. Responses were considered positive if there were at least 50 spot-forming units (SFU)/million PBMCs after background subtraction and the positive control was successful (61).

Tetramer staining and phenotyping. HIV-specific $CD8^+$ T cells were detected with the following allophycocyanin (APC)-conjugated iTAg MHC class I (MHC-I) tetramers: HLA-A*01:01-NSSQVSQNY (HIV Gag 124 to 132), B*07:02-APRKKGCWK (HIV Gag 408 to 416), and B*07:02-SPRTLNAWV (HIV Gag 148 to 156) from Beckman Coulter (14). Fresh PBMCs isolated by Ficoll gradient centrifugation were incubated with antibodies: anti-CD3-Amcyan, anti-CD8-APC-Cy7, anti-CD4-PE-Cy5, anti-CCR7-PE-Cy7, anti-CD28-PE, and anti-CD27 Alexa Fluoro 700 from BD-Bioscience (San Diego, CA) and anti-CD45-RA-ECD (Beckman Coulter) for 30 min at room temperature. Cells were washed in phosphate-buffered saline with 2% fetal calf serum (FCS). After the wash, cells were fixed with 1% paraformaldehyde and analyzed on a BD LSRII system. Data were compensated and analyzed using Flowjo Software.

Statistical analysis. Kaplan-Meier survival analysis was conducted among >300 women who enrolled in the Pumwani cohort between 1985 and 2001 and who were HIV seronegative at enrollment. Cox regression and Kaplan-Meier analysis were conducted for HLA alleles with genotype frequencies above 5% in this population. A total of 12 HLA-A, 14 HLA-B, and 11 HLA-C alleles with genotype frequencies greater than 5% in this population were included in the Cox regression analysis using a backward conditional method. The FACS data were analyzed with Graph Pad Prism 4.0 (Graph Pad Software, San Diego, CA). The nonparametric Kruskal-Wallis test was used to compare values between the independent groups. All differences were considered significant at $P < 0.05$.

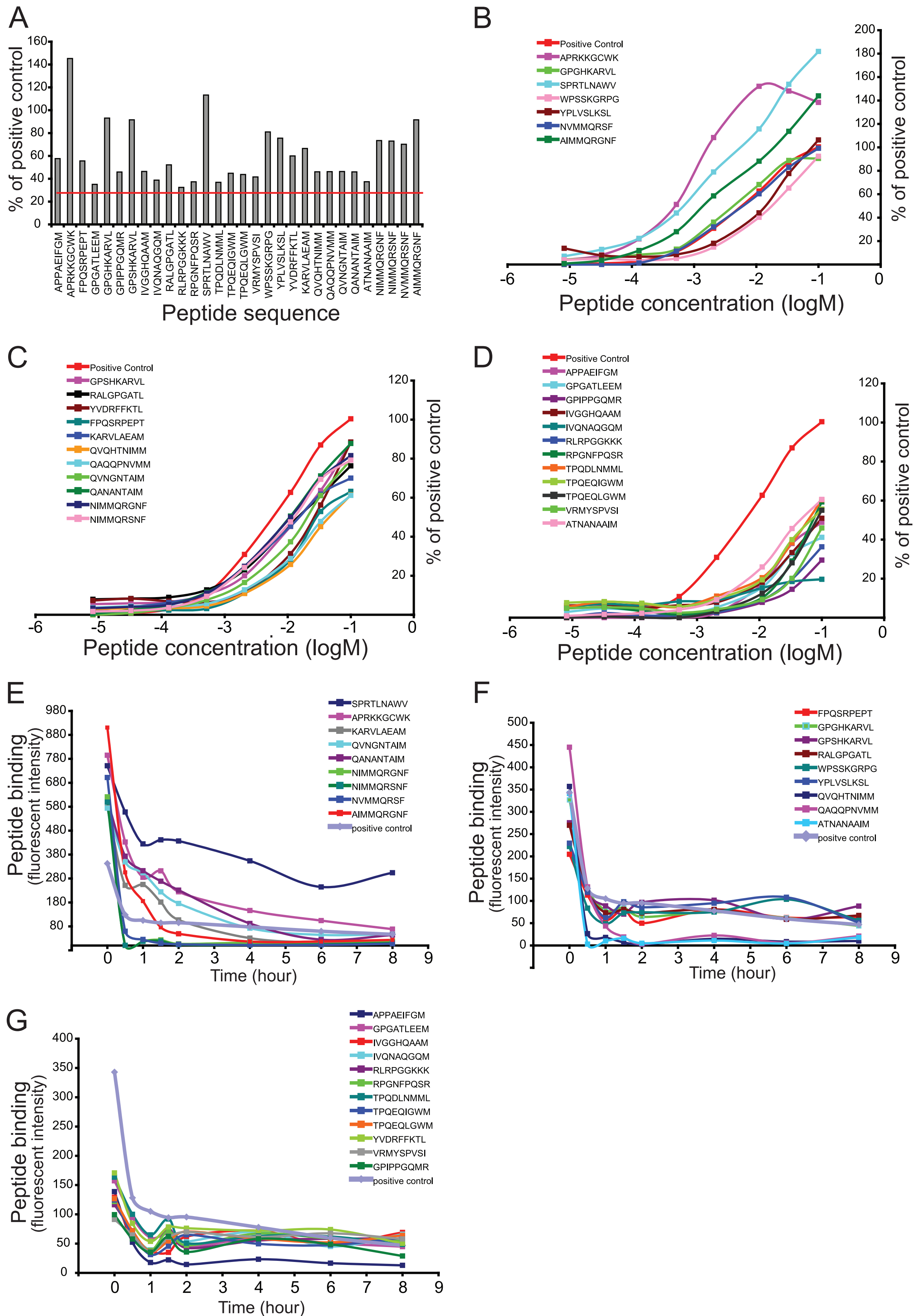


FIG 3 The binding, affinity, and off-rates of B*07:02 HIV-1 Gag epitopes. (A) The level of binding of Gag epitopes to B*07:02. (B to D) The binding affinity of B*07:02 Gag epitopes. (E to G) The off-rates of B*07:02 Gag epitopes.

TABLE 3 Summary of ELISpot responses to Gag peptides identified by iTopia epitope discovery system screening

Peptide		Patient sample		
Allele	Sequence	Tested	Responded	Mean SFU/million
A*01:01	NSSKVSQNY	12	3	79
	TTEGCQQIM	7	3	77
	YVDRFFKTL	7	2	2,148
	SSSKVSQNY	5	2	115
	NSSQVSQNY	5	4	96
	HSNQVSQNY	5	4	115
	HSSQVSQNY	5	4	74
	NSSQVSRNY	5	3	127
	SSSQVSQNY	5	4	130
B*07:02	APPAEIFGM	8	1	100
	APRKKGCWK	8	2	148
	FPQSRPEPT	7	1	55
	GPGATLEEM	5	1	80
	GPGHKARVL	8	2	83
	GPIPPGQMR	5	1	215
	GPSHKARVL	8	3	62
	RALGPGATL	5	3	115
	RLRPGGKKK	5	1	135
	RPGNFPQSR	5	1	80
	SPRTLNAWV	8	2	245
	TPQDLNMML	5	1	905
	TPQEQIGWM	5	1	185
	TPQEQLGWM	4	0	<50
	VRMYSPVSI	4	1	435
	WPSSKGRPG	8	3	115
	YPLVSLKSL	8	2	703
	YVDRFFKTL	8	2	1,280
	GPGHKARVL	8	4	84
	GPSHKARVL	8	6	95
	KARVLAEAM	8	5	78
	QVQHTNIMM	8	6	87
	QAQQPNVMM	8	5	96
	QVNGNTAIM	8	6	99
	QANANTAIM	8	6	109
	ATNANAAIM	8	5	95
	NIMMQRGNF	8	3	100
	NIMMQRSNF	8	6	121
	NVMMQRSNF	8	6	117
	AIMMQRGNF	8	4	113

TABLE 4 Detailed ELISpot data for tested HLA A*01:01 patients[b]

Peptide	Patient no., HIV status[a]												Responses/tested
	893, RES (HESN)	1589, RES (HESN)	2137, POS	1932, POS	2003, POS	2204, POS	1811, POS	**2463**, NSN	**2646**, POS	**2297**, POS	**2261**, POS	**2178**, POS	
TTEGCQQIM	15	15	**85**	**65**	10	**80**	40	N/T	N/T	N/T	N/T	N/T	3/7
NSSKVSQNY	**55**	**80**	15	**65**	25	15	30	**60**	35	**95**	**75**	**120**	7/12
YVDRFFKTL	0	10	30	**50**	**4245**	5	10	N/T	N/T	N/T	N/T	N/T	2/8
SSSKVSQNY	N/T	N/T	N/T	N/T	N/T	N/T	N/T	0	45	30	**90**	**140**	2/5
NSSQVSQNY	N/T	N/T	N/T	N/T	N/T	N/T	N/T	45	**50**	**80**	**130**	**125**	4/5
HSNQVSQNY	N/T	N/T	N/T	N/T	N/T	N/T	N/T	**55**	15	**50**	**115**	**240**	4/5
HSSQVSQNY	N/T	N/T	N/T	N/T	N/T	N/T	N/T	**50**	10	**70**	**60**	**115**	4/5
NSSQVSRNY	N/T	N/T	N/T	N/T	N/T	N/T	N/T	10	**125**	45	**155**	**100**	3/5
SSSQVSQNY	N/T	N/T	N/T	N/T	N/T	N/T	N/T	**75**	20	**65**	**95**	**285**	4/5

[a] RES (HESN), HIV-1 resistant; POS, HIV-1 positive; NSN, newly enrolled seronegative; N/T, not tested.
[b] Bold patient numbers indicate that fresh PBMCs were used. Lightface patient numbers indicate that PBMCs were previously frozen and stored in liquid nitrogen.

RESULTS

A*01:01 and B*07:02 are independently associated with different outcomes of HIV-1 infection. To identify HLA class I alleles associated with resistance or susceptibility to HIV-1 infection, we analyzed the effect of HLA class I alleles on the seroconversion rates of more than 300 women who were HIV uninfected at the cohort enrollment by Kaplan-Meier survival analysis. Kaplan-Meier survival analysis showed that women with A*01 seroconverted significantly slower than those without A*01 (Fig. 1A), whereas, women who have B*07:02 seroconvert significantly faster than those without B*07:02 (Fig. 1B). A total of 12 HLA-A, 14 HLA-B, and 11 HLA-C alleles with genotype frequencies greater than 5% in this population were included in the Cox regression analysis using a backward conditional method. Results showed that A*01, C*06:02, and C*07:01 are independently associated with slower seroconversion, while A*23:01, B*07:02, and

TABLE 5 Detailed ELISpot data for tested HLA B*07:02 patients[b]

Peptide	Patient no., HIV status[a]																Responses/tested
	2044, NSN	2230, NSN	2181, POS	2127, NSN	1961, NSN	2035, NSN	1394, RES (HESN)	2257, POS	**2427**, POS	**2320**, NSN	**2400**, POS	**2545**, POS	**2619**, POS	**2423**, NSN	**2425**, NSN	**2644**, NSN	
APPAEIFGM	0	0	**100**	0	5	0	10	20	N/T	N/T	N/T	N/T	N/T	N/T	N/T	N/T	1/8
APRKKGCWK	35	**240**	0	0	0	20	**55**	0	N/T	N/T	N/T	N/T	N/T	N/T	N/T	N/T	2/8
FPQSRPEPT	0	N/T	25	**55**	35	0	0	0	N/T	N/T	N/T	N/T	N/T	N/T	N/T	N/T	1/7
GPGATLEEM	0	N/T	N/T	**80**	25	35	N/T	45	N/T	N/T	N/T	N/T	N/T	N/T	N/T	N/T	1/5
GPGHKARVL	**90**	0	0	5	0	0	**75**	30	N/T	N/T	N/T	N/T	N/T	N/T	N/T	N/T	2/8
GPIPPGQMR	0	N/T	N/T	15	10	0	N/T	**215**	N/T	N/T	N/T	N/T	N/T	N/T	N/T	N/T	1/5
GPSHKARVL	**55**	0	5	**65**	20	**65**	0	10	N/T	N/T	N/T	N/T	N/T	N/T	N/T	N/T	3/8
RALGPGATL	0	N/T	N/T	**175**	**80**	0	N/T	**90**	N/T	N/T	N/T	N/T	N/T	N/T	N/T	N/T	3/5
RLRPGGKKK	0	N/T	N/T	**135**	10	35	N/T	20	N/T	N/T	N/T	N/T	N/T	N/T	N/T	N/T	1/5
RPGNFPQSR	0	N/T	N/T	0	0	0	N/T	**80**	N/T	N/T	N/T	N/T	N/T	N/T	N/T	N/T	1/5
SPRTLNAWV	**115**	**375**	0	0	35	0	25	20	N/T	N/T	N/T	N/T	N/T	N/T	N/T	N/T	2/8
TPQDLNMML	0	N/T	N/T	15	30	0	N/T	**905**	N/T	N/T	N/T	N/T	N/T	N/T	N/T	N/T	1/5
TPQEQIGWM	0	N/T	N/T	0	20	10	N/T	**185**	N/T	N/T	N/T	N/T	N/T	N/T	N/T	N/T	1/5
TPQEQLGWM	0	N/T	N/T	0	35	0	N/T	N/T	N/T	N/T	N/T	N/T	N/T	N/T	N/T	N/T	0/4
VRMYSPVSI	0	N/T	N/T	**435**	0	0	N/T	N/T	N/T	N/T	N/T	N/T	N/T	N/T	N/T	N/T	1/4
WPSSKGRPG	**70**	**220**	0	0	0	**55**	25	5	N/T	N/T	N/T	N/T	N/T	N/T	N/T	N/T	3/8
YPLVSLKSL	0	0	**1320**	**85**	30	0	10	40	N/T	N/T	N/T	N/T	N/T	N/T	N/T	N/T	2/8
YVDRFFKTL	0	0	**2500**	**60**	40	0	10	15	N/T	N/T	N/T	N/T	N/T	N/T	N/T	N/T	2/8
GPGHKARVL	N/T	N/T	N/T	N/T	N/T	N/T	N/T	N/T	**55**	**75**	**70**	45	5	5	15	**135**	**4/8**
GPSHKARVL	N/T	N/T	N/T	N/T	N/T	N/T	N/T	N/T	**70**	**67**	**70**	**55**	15	**70**	10	**235**	**6/8**
KARVLAEAM	N/T	N/T	N/T	N/T	N/T	N/T	N/T	N/T	45	**50**	**80**	**75**	0	**115**	10	**70**	**5/8**
QVQHTNIMM	N/T	N/T	N/T	N/T	N/T	N/T	N/T	N/T	45	**55**	**95**	**75**	5	**80**	**50**	**165**	**6/8**
QAQQPNVMM	N/T	N/T	N/T	N/T	N/T	N/T	N/T	N/T	**65**	30	**70**	**70**	0	10	**50**	**225**	**5/8**
QVNGNTAIM	N/T	N/T	N/T	N/T	N/T	N/T	N/T	N/T	**65**	**50**	**80**	**90**	5	**120**	5	**190**	**6/8**
QANANTAIM	N/T	N/T	N/T	N/T	N/T	N/T	N/T	N/T	**75**	25	**65**	**80**	5	**115**	**55**	**265**	**6/8**
ATNANAAIM	N/T	N/T	N/T	N/T	N/T	N/T	N/T	N/T	**60**	**60**	**85**	**75**	15	25	5	**195**	**5/8**
NIMMQRGNF	N/T	N/T	N/T	N/T	N/T	N/T	N/T	N/T	35	25	**80**	**50**	10	0	10	**170**	**3/8**
NIMMQRSNF	N/T	N/T	N/T	N/T	N/T	N/T	N/T	N/T	**65**	**70**	**125**	**50**	5	**105**	15	**310**	**6/8**
NVMMQRSNF	N/T	N/T	N/T	N/T	N/T	N/T	N/T	N/T	**55**	**90**	**85**	**100**	5	**55**	15	**315**	**6/8**
AIMMQRGNF	N/T	N/T	N/T	N/T	N/T	N/T	N/T	N/T	**90**	35	35	**65**	0	**80**	30	**215**	**4/8**

[a] RES (HESN), HIV-1 resistant; POS, HIV-1 positive; NSN, newly enrolled seronegative; N/T, not tested.
[b] Bold patient numbers indicate that fresh PBMCs were used. Lightface patient numbers indicate that PBMCs were previously frozen and stored in liquid nitrogen.

B*42:01 are independently associated with rapid seroconversion (Table 1). The enrollment years and ages of women with and without these alleles are very similar.

The spectrum and characteristics of HLA-A*01:01 HIV-1 Gag epitopes. Epitope screening with the iTopia epitope discovery system showed that only 2 out of 632 peptides met the binding criteria (30% of the positive control peptide) for A*01:01 (Fig. 2A). The strongest binder was peptide NSSKVSQNY at 70.6% of the positive control, followed by two weaker peptides, YVDRFFKTL at 33.1% of the positive control and TTEGCQQIM at 26.2% of the positive control peptide. The binding affinity of peptide NSSKVSQNY displayed a typical sigmoidal binding curve over the concentrations tested, and the peptide has the highest affinity for A*01:01 with an ED_{50} at 1.21×10^{-5} M among the three positive peptides tested (Fig. 2B). The other two peptides have a relatively flat curve with ED_{50} values of 1.70×10^{-5} M for TTEGCQQIM and 4.91×10^{-5} M for YVDRFFKTL. The off-rate assay shows that the peptide NSSKVSQNY dissociated from the complex at a rate ($t_{1/2}$) of 0.94 h. The $t_{1/2}$ is 0.33 h for peptide TTEGCQQIM and 0.30 h for peptide YVDRFFKTL, both at a very low binding capacity (Fig. 2C). The peptide binding, affinity, and off-rate values are summarized in Table 2. We further tested 14 variants of peptide NSSKVSQNY representing major variants of HIV subtypes A, B, C, and D. A*01:01 binds 6 major variant peptides of HIV subtypes A, B, and D very well with comparable affinities and off-rates (Fig. 2D to F; Table 2).

The spectrum and characteristics of HIV Gag epitopes of HLA-B*07:02. A total of 30 peptides bound to B*07:02 with greater than 30% of the positive control peptide (Fig. 3A; Table 2) as determined by the iTopia epitope discovery system screen. The peptides were divided into three groups based on their relative binding to the positive control peptide and analyzed their binding affinity to B*07:02 (Fig. 3B to D; Table 2). Group 1 (Fig. 3B) contained 7 peptides, three of which had higher affinity for B*07:02 than the positive control peptide. The binding of APRKKGCWK to B*07:02 was 145.3% of the positive control with an ED_{50} of 8.96×10^{-7} M, the binding of SPRTLNAWV to B*07:02 was 113.3% of the positive control with an ED_{50} of 1.61×10^{-6} M, and the binding of AIMMQRGNF to B*07:02 was 91.6% of the positive control with an ED_{50} of 3.45×10^{-6} M. Group 2 (Fig. 3C) contained 11 peptides with binding between 60% and 90% of the positive control peptide. These peptides were GPSHKARVL, RALGPGATL, YVDRFFKTL, FPQSRPEPT, KARVLAEAM, QVQHTNIMM, QAQQPNVMM, QVNGNTAIM, QANANTAIM, NIMMQRGNF, and NIMMQRSNF. Group 3 (Fig. 3D) contained 12 peptides with more than 30% but less than 60% binding of the positive control peptide. The peptides were APPAEIFGM, GPGATLEEM, GPIPPGQMR, IVGGHQAAM, IVQNAQGQM, RLRPGGKKK, RPGNFPQSR, TPQDLNMML, TPQEQIGWM, TPQEQLGWM, VRMYSPVSI, and ATNANAAIM. The detailed binding and ED_{50} values are shown in Table 2.

The off-rate assay was carried out on all 30 peptides to determine their rate of dissociation from B*07:02 (72). B*07:02 binds to several peptides with much higher capacity than the positive control peptide, and the binding persisted for a very long time; these peptides include SPRTLNAWV, APRKKGCWK,

TABLE 6 HLA class I alleles of all patients tested in ELISpot assays

Allele	Patient no.	HLA class I allele[a]					
		HLA-A		HLA-B		HLA-C	
A*01:01	893	**01:01**	24:02	15:17	35:02	04:01	17:01
	1589	**01:01**	30:02	58:02	58:02	06:02	06:06
	2137	**01:01**	23:01	58:02	44:15	03:02	04:07
	1932	**01:01**	01:01	81:01	37:01	06:02	18:01
	2003	**01:01**	68:02	81:01	15:10	03:04	18:01
	2204	**01:01**	68:02	42:01	49:01	07:01	17:01
	1811	**01:01**	23:01	**07:02**	45:01	16:01	07:02
	2463	**01:01**	02:01	41:01	42:01	07:01	17:01
	2646	**01:01**	29:02	57:01	45:01	07:01	06:02
	2297	**01:01**	30:01	57:03	08:01	18:01	07:04
	2261	**01:01**	49:01	27:26	02:02	02:02	07:01
	2178	**01:01**	29:02	81:01	81:01	07:04	08:04
B*07:02	2044	03:08	29:01	**07:02**	08:01	07:02	07:02
	2230	**01:01**	03:01	**07:02**	58:02	06:06	07:02
	2181	30:01	31:01	**07:02**	53:01	07:14	03:04
	2127	**01:01**	03:01	**07:02**	41:01	07:01	07:23
	1961	03:01	23:01	**07:02**	42:01	17:01	04:01
	2035	23:01	30:01	**07:02**	15:10	07:14	03:04
	1394	02:01	68:02	**07:02**	13:02	06:02	04:01
	2257	30:02	23:01	**07:02**	18:01	07:02	15:05
	2427	68:02	29:02	**07:02**	39:10	07:27	12:03
	2320	33:01	02:01	**07:02**	49:01	07:01	07:02
	2400	68:02	02:01	**07:02**	42:01	08:02	17:01
	2545	68:02	30:02	**07:02**	18:01	07:04	07:02
	2619	02:01	68:01	**07:02**	53:01	07:02	04:01
	2423	68:02	68:02	**07:02**	**07:02**	07:02	07:02
	2425	29:02	68:02	**07:02**	39:10	12:03	07:02
	2644	36:01	68:02	**07:02**	53:01	17:01	06:02

[a] Bold data indicate A*01:01 or B*07:02 allele of the patients in ELISpot assays.

TABLE 7 B*07:02 Gag epitopes identified in this study and binding motifs[a]

Binding motif	Amino acid at indicated anchor position								
	P1	P2	P3	P4	P5	P6	P7	P8	P9
Previously defined motif	**X**	**PVA**	**X**	**X**	**X**	**X**	**X**	**X**	**FLM(AIVYW)**
Epitopes identified in this study	A	P	P	A	E	I	F	G	M
	A	P	R	K	K	G	C	W	**K**
	F	P	Q	S	R	P	E	P	**T**
	G	P	G	A	T	L	E	E	M
	G	P	G	H	K	A	R	V	L
	G	P	I	P	P	G	Q	M	**R**
	G	P	S	H	K	A	R	V	L
	R	A	L	G	P	G	A	T	L
	R	**L**	R	P	G	G	K	K	**K**
	R	P	G	N	F	P	Q	S	**R**
	S	P	R	T	L	N	A	W	V
	T	P	Q	D	L	N	M	M	L
	T	P	Q	E	Q	I	G	W	M
	T	P	Q	E	Q	L	G	W	M
	V	**R**	M	Y	S	P	V	S	I
	W	P	S	S	K	G	R	P	**G**
	Y	P	L	V	S	L	K	S	L
	Y	V	D	R	F	F	K	T	L
	G	P	G	H	K	A	R	V	L
	G	P	S	H	K	A	R	V	L
	K	A	R	V	L	A	E	A	M
	Q	V	Q	H	T	N	I	M	M
	Q	A	Q	Q	P	N	V	M	M
	Q	V	N	G	N	T	A	I	M
	Q	A	N	A	N	T	A	I	M
	A	**T**	N	A	N	A	A	I	M
	N	**I**	M	M	Q	R	G	N	F
	N	**I**	M	M	Q	R	S	N	F
	N	V	M	M	Q	R	S	N	F
	A	**I**	M	M	Q	R	G	N	F

[a] Bolded amino acids are additional residues identified through iTopia peptide binding assays and confirmed by ELISpot assays. Previously described HLA binding motifs are summarized in reference 49.

QANANTAIM, QVNGNTAIM, AIMMQRGNF, and KARVLAEAM (Fig. 3E). Most of the peptides dissociated from B*07:02 to the level of 50% of saturated binding within an hour, and their binding to B*07:02 is comparable to or lower than that of the positive control peptide (Fig. 3 E, F and G; Table 2).

Validation of A*01:01 and B*07:02 epitopes by IFN-γ ELISpot assay. Using the iTopia epitope discovery system, we have determined HIV-1 Gag peptide binding capacity, affinity, and off-rates of A*01:01 and B*07:02. To confirm these peptides are true epitopes capable of stimulating T cell responses, we validated the epitopes with IFN-γ ELISpot assays with patient PBMCs. PBMCs isolated from 5 to 12 A*01:01$^+$ women were tested by IFN-γ ELISpot assay for each of the 9 peptides (including the 6 variants of NSSKVSQNY) identified as A*01:01 epitopes (Table 3). Two or more A*01:01$^+$ patients had positive ELISpot responses to the nine peptides tested ($>$50 SFU/million) (Table 3). We were able to detect responses for 3 epitopes and 6 variants of the epitope NSSKVSQNY for A*01:01 (Table 3). Four of the epitopes and epitope variants of A*01:01 are novel and have not been reported previously (Table 2). Among the 12 A*01:01$^+$ subjects tested for the identified peptides, two are defined as resistant to HIV-1 infection (HESN) (enrolled in the cohort in 1987 and 1992, respectively), one is a seronegative new enrollee (NSN) and the rest are HIV-1 infected (POS). Positive ELISpot responses to the peptides were detected in 28% to 80% of the subjects tested (Tables 3 and 4). The magnitudes of the ELISpot responses among the tested subjects were comparable, except for one HIV$^+$ patient whose response to peptide YVDRFFKTL is $>$10-fold higher than the average ELISpot responses to all peptides tested (Table 4). The HLA class I allele data for the subjects are listed below (see Table 6).

PBMCs from 4 to 10 patients were tested for each of the 28 peptides (peptide IVGGHQAAM and IVQNAQGQM failed to be synthesized for ELISpot assays) binding to B*07:02 (Table 3). All peptides except TPQEQLGQM had at least one positive ELISpot response ($>$50 SFU/million) from the tested patient samples. We were able to confirm 19 Gag epitopes and 8 epitope variants of B*07:02. Twenty-one epitopes or epitope variants are novel (Table 2). Among the 16 B*07:02 subjects tested for the identified peptide by using the iTopia epitope discovery system, one was defined as resistant to HIV-1 infection (HESN), nine subjects were HIV-1-negative new enrollees (NSN), and six subjects were HIV-1 infected (Table 5). Positive ELISpot responses to the peptides were detected in 12.5% to 75% of the subjects tested (Tables 3 and 5). The magnitudes of the ELISpot responses among the tested sub-

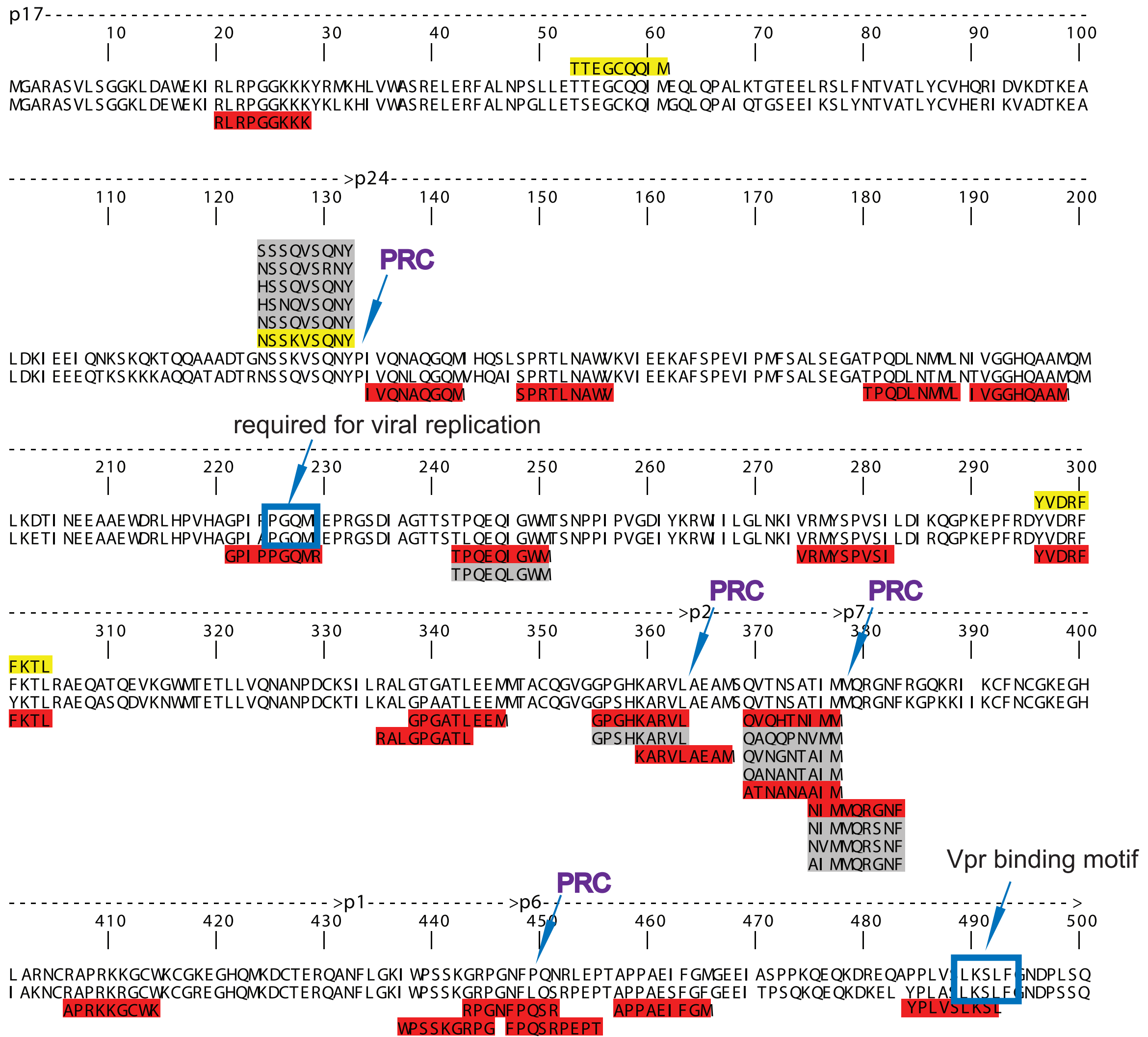


FIG 4 The location of HIV-1 Gag epitopes of A*01:01 and B*07:02. A*01:01 epitopes are highlighted in yellow, and B*07:02 epitopes are highlighted in red. The epitope variants are shaded in gray. PRC, protease cleavage sites. The clade A Gag consensus is at the top of the alignment, and the clade D consensus is at the bottom.

jects were comparable, except for one HIV+ patient whose response to peptide YVDRFFKTL was much higher than the average ELISpot responses to all peptides tested (Table 5). The HLA class I allele data for the subjects are listed in Table 6. Peptide YVDRF FKTL can be recognized by both A*01:01 and B*07:02, and it induced the highest IFN-γ ELISpot responses in some patients. The higher magnitude of ELISpot responses to the peptide may be due to specific T cell receptor usage and need to be investigated further.

The binding motif of nine A*01:01 epitopes and epitope variants fits very well with the reported binding motif in the Immune Epitope database (IDEB; http://www.immuneepitope.org/MHCalleleId/142). However, among many novel B*07:02 Gag epitopes identified in this study (Table 2), 4 new amino acid residues at anchor positions P2 and P9 were identified (Table 7). Polar basic amino acids such as lysine and arginine are the surprising additions to the anchor positions. This suggests that the anchor residues of B*07:02 are more tolerant to different amino acid changes than previously reported (64).

Comparison of A*01:01 and B*07:02 Gag epitopes. We compared the numbers, affinities, off-rates, and ELISpot responses, as well as the locations of the identified A*01:01 and B*07:02 Gag epitopes. As expected, only one epitope of A*01:01 overlaps with the epitopes of B*07:02. However, we were surprised to see the substantial difference in the numbers of Gag epitopes between the two alleles. B*07:02 Gag epitopes (19 epitopes) outnumber those of A*01:01 (3 epitopes) by 6-fold, excluding the epitope variants. B*07:02 epitopes are distributed along the entire Gag, while two of the three A*01:01 epitopes are located in p17 and one in p24 (Fig. 4). The locations of the A*01:01 Gag epitopes do not appear to be special, except for one, NSSKVSQNY, which is located right beside the p17/p24 protease cleavage site (Fig. 4). We further analyzed 15 variants of this peptide from HIV-1 subtypes A, B, C, and D. A*01:01 can bind to major variants of subtype A, B, and D and their recombinants (Fig. 2D to F; Table 1). However, with a few exceptions, A*01:01 does not bind the peptide variants of subtype C. There is no significant difference between the off-rates of the epitopes/epitope variants of A*01:01 and B*07:02 ($P = 0.377$)

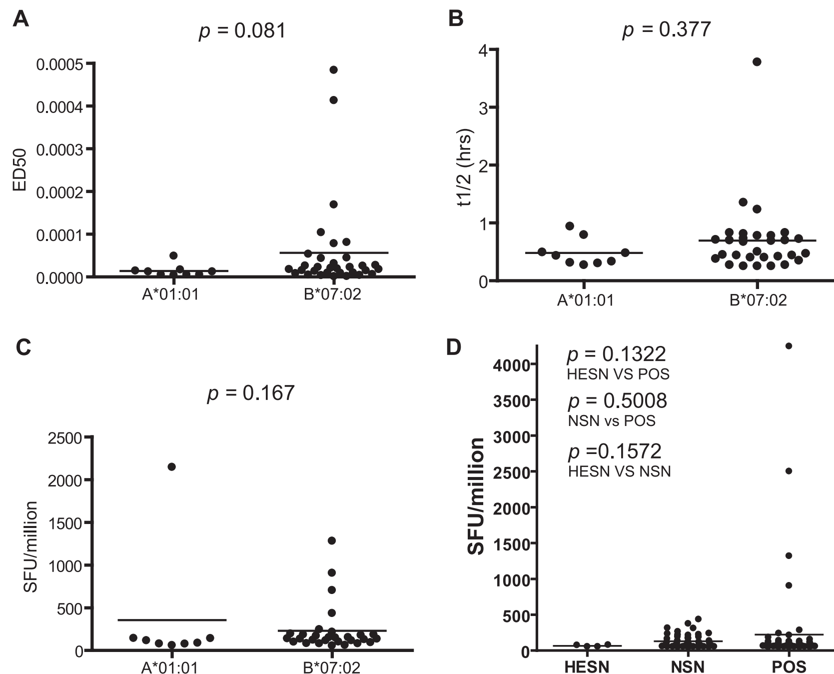


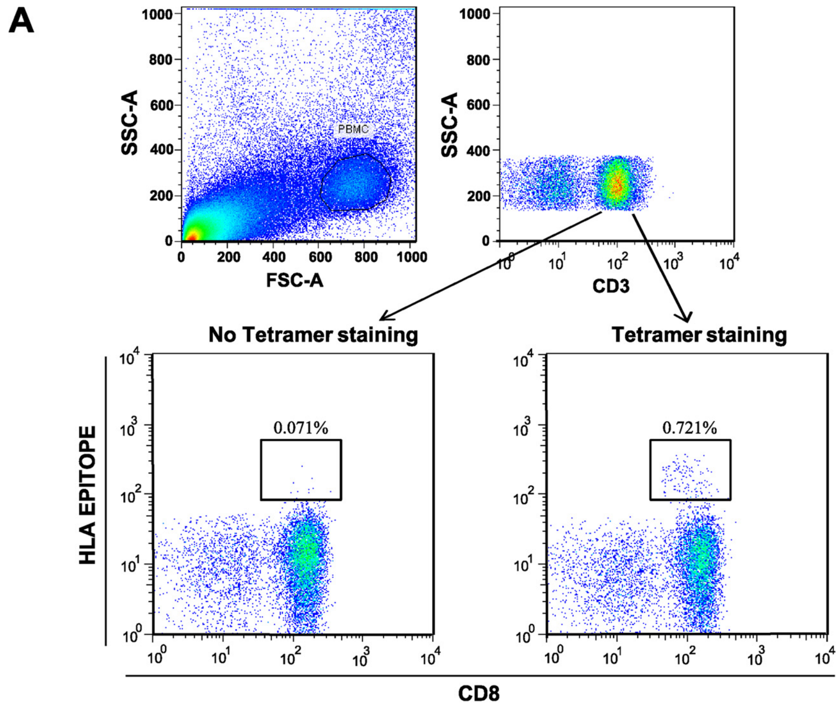
FIG 5 Comparison of the HIV-1 Gag epitopes of A*01:01 and B*07:02. (A) Binding affinities of HIV-1 Gag epitopes and epitope variants of A*01:01 and B*07:02. (B) Off-rates of HIV-1 Gag epitopes and epitope variants of A*01:01 and B*07:02. (C) IFN-γ ELISpot responses of HIV-1 Gag epitopes and epitope variants of A*01:01 and B*07:02. (D) Comparison of ELISpot responses to the A*01:01 and B*07:02 peptides among HESN, NSN, and POS.

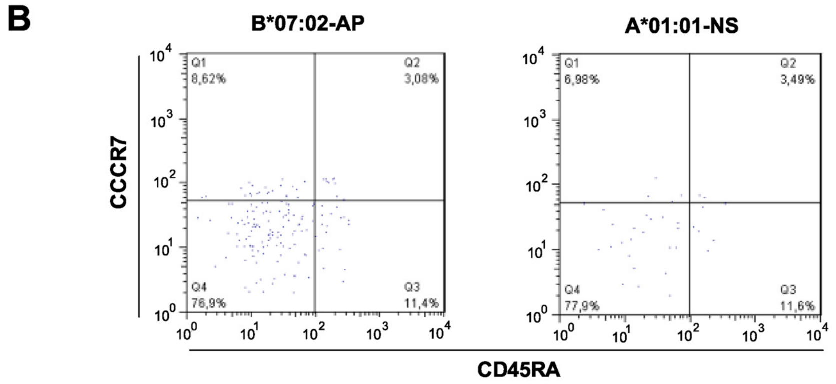
(Fig. 5B), and while B*07:02 binds to its epitopes with higher affinity than A*01:01 does to its epitopes, the difference is not significant ($P = 0.081$) (Fig. 5A). We observed no significant difference in the IFN-γ ELISpot SFU values between the Gag epitopes of A*01:01 and B*07:02 (Fig. 5C). There is no significant difference in magnitude of ELISpot responses to the individual peptides among HESN, NSN, and POS groups (Fig. 5D).

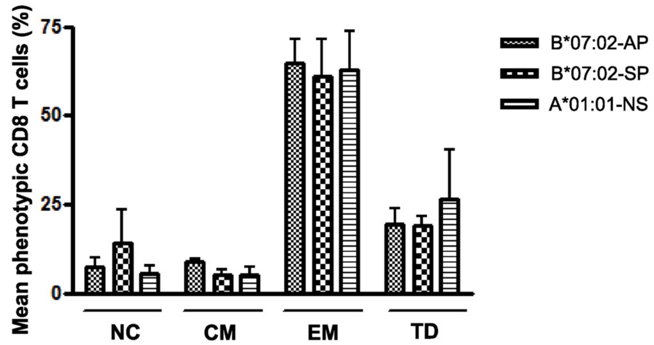
A*01:01 and B*07:02 epitope-specific CD8$^+$ T cells. To determine if differences in potential epitope presentation between A*01:01 and B*07:02 epitopes may affect the functional phenotype of A*01:01- and B*07:02-responsive cells in proof-of-concept studies, we examined the memory phenotype using 3 epitope-specific tetramers and a phenotyping panel to define memory profiles among 6 chronic HIV-1 infected subjects (3 for tetramer A*01:01-NSSQVSQNY, and 3 for both B*07:02-APRKKGCWK and B*07:02-SPRTLNAWV) (Fig. 6A). The proportion of naïve (CD45RA$^+$ CCR7$^+$) and central memory (Tcm$^-$ CD45RA$^-$ CCR7$^+$)-, effector memory (Tem$^-$ CD45RA$^-$ CCR7$^-$)-, and terminal differentiated (Ttd$^-$ CD45RA$^+$ CCR7)-specific CD8$^+$ T cells was determined. We observed no statistically significant differences in the frequencies of memory phenotypes as defined by CD45RA and CCR7 of tetramer-specific HIV-1-specific CD8$^+$ T cells (Fig. 6B). The majority responses classified as effector memory (Tem) and the proportion of terminal differentiated (Td) cells were very similar (Fig. 6B). We further examined the frequencies of different effector memory CD8$^+$ T cell subsets with the co-stimulator markers CD27 and CD28. CCR7$^-$ CD45RA$^-$ CD28$^+$ CD27$^-$ and CCR7$^-$ CD45RA$^-$ CD28$^-$ CD27$^-$ CD8$^+$ T cells were identified within all of the tetramer-positive T cell populations. Higher frequencies of CCR7$^-$ CD45RA$^-$ CD28$^+$ CD27$^-$ CD8$^+$ T cells were identified with NSSKVSQNY-A*01:01 tetramer than with the B*07:02 tetramers (APRKKGCWK-B*07:02 and SPRTL-NAWV-B*07:02), and the difference between NSSKVSQNY-A*01:01 and APRKKGCWK-B*07:02 is significant (Fig. 6C). Conversely, the B*07:02 tetramers identified more CCR7$^-$ CD45RA$^-$ CD28$^-$ CD27$^-$ CD8$^+$ T cells, terminally differentiated effector memory T cells, than the NSSKVSQNY-A*01:01 tetramer, but the difference was not significant (Fig. 6C). This indicates that while the responses were phenotypically similar in Tem/Tcm frequencies, there may be subtle differences in the exhaustion and stimulatory potentials between the tetramer-specific T cell pools in A*01:01 and B*07:02 HIV-infected subjects, and perhaps this could be extrapolated to A*01:01 and B*07:02 responses in HESN subjects.

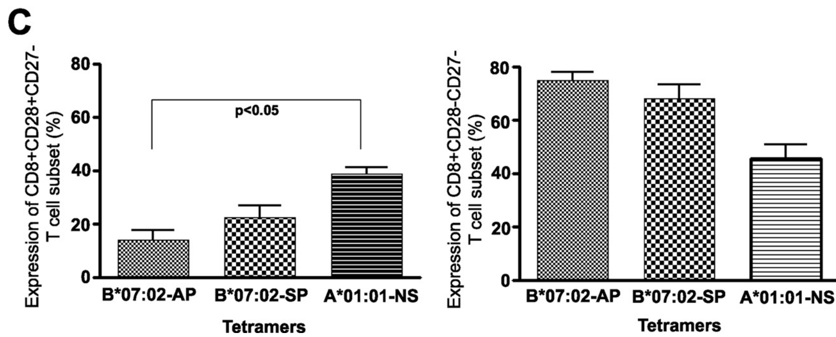
DISCUSSION

Numerous studies have documented the role of CD8$^+$ T cells in controlling HIV-1 (18, 23, 24, 57, 66, 71). Specifically, studies have shown that CD8$^+$ T cell responses to HIV-1 Gag are associated with viremia control (25, 37, 49, 54, 58, 62). Characterization of CD8$^+$ T cell responses correlated with protection from HIV-1 infection needs to be conducted with PBMCs from individuals who are highly exposed but remain uninfected (HESN). Because such individuals represent a rare biological phenotype and identifying such individuals requires long-term careful observations, very few such studies have been reported. Many theoretical and empirical techniques have been used for epitope identification including predictive algorithms, defective cell lines, shotgun methods, and peptide elution techniques (63). These methods can be inaccurate and labor-intensive given the large number of antigens and their variations and the diversity of HLA (22, 63). Furthermore, screening a large number of HIV peptides with patient PBMCs is not practical because the numbers of PBMCs that can be obtained from patients are limited, and pooled peptides can ob-


A
SSC-A
FSC-A
PBMC
CD3
No Tetramer staining
Tetramer staining
HLA EPITOPE
0.071%
0.721%
CD8


B
B*07:02-AP
A*01:01-NS
CCCR7
Q1
8,62%
Q2
3,08%
Q4
76,9%
Q3
11,4%
Q1
6,98%
Q2
3,49%
Q4
77,9%
Q3
11,6%
CD45RA


Mean phenotypic CD8 T cells (%)
B*07:02-AP
B*07:02-SP
A*01:01-NS
NC
CM
EM
TD


C
Expression of CD8+CD28+CD27- T cell subset (%)
p<0.05
B*07:02-AP
B*07:02-SP
A*01:01-NS
Tetramers
Expression of CD8+CD28-CD27- T cell subset (%)
B*07:02-AP
B*07:02-SP
A*01:01-NS
Tetramers

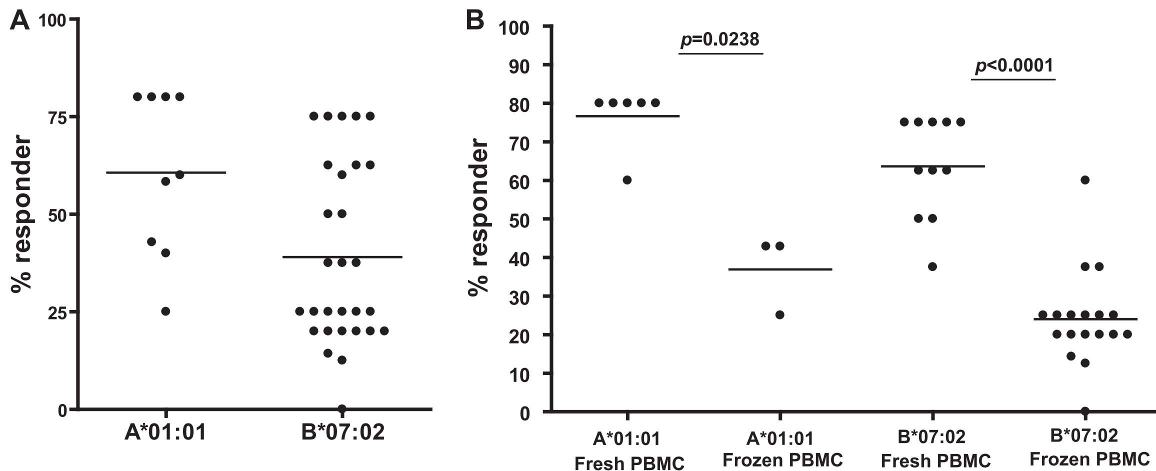


FIG 7 Confirmation of epitopes identified using the iTopia epitope discovery system by IFN-γ ELISpot assays. (A) Percent responders among all subjects with A*01:01 or B*07:02 tested using either fresh PBMCs or PBMCs that were stored in liquid nitrogen. (B) Percent responders among subjects with A*01:01 or B*07:02 tested using fresh PBMCs.

scure weak but real CD8$^+$ T cell responses. In this study, we used an alternative approach to characterize CD8$^+$ T cell epitopes that are more likely to be recognized by the HESN individuals. We first identified HLA class I alleles that are significantly associated with either protection from HIV-1 infection or susceptibility to HIV-1 infection. The epitopes of the identified alleles were then characterized by an *in vitro*, cell-free, peptide binding assay, the iTopia epitope discovery system. The identified epitopes were further confirmed by IFN-γ ELISpot assay with patient PBMCs. Our study showed that the iTopia epitope discovery system is a reliable *in vitro* system to rapidly identify epitopes of specific HLA alleles. Most peptides identified by this approach have been confirmed by IFN-γ ELISpot assays with fresh PBMCs from subjects with specific HLA alleles (Fig. 7) (60). The two alleles, A*01:01 and B*07:02, were chosen because they are independently associated with resistance or susceptibility to HIV-1 infection, and these two alleles are available in the iTopia epitope discovery system. Because studies have shown that CD8$^+$ T cell response to HIV Gag is correlated with protection from disease progression and viremia control, in this study we focused on Gag of two common HIV subtypes in the Kenyan population. By comprehensively analyzing CD8$^+$ T cell Gag epitopes of two HLA class I alleles associated with different outcomes of HIV-1 infection in a well-characterized high-risk sex worker cohort, we aim to characterize the CD8$^+$ T cell responses that protect against HIV-1 infection.

This study has shown that B*07:02, an allele associated with rapid seroconversion, can present a broad spectrum of Gag epitopes and induce strong immune responses, while the narrowly focused epitope presentation by A*01:01 correlates with protection. Because there is no significant difference in the magnitudes of IFN-γ ELISpot responses between A*01:01 and B*07:02 epitopes and between different groups of individuals (HESN, NSN, and POS), the major difference between these two alleles appears to be the spectrum of antigen presentation, although subtle differences may exist. As HLA class I alleles present viral peptides to activate CD8$^+$ T cells, the ability to present more CD8$^+$ T cell epitopes could mean more chances to induce CD8$^+$ T cell responses. Indeed, most of the B*07:02 patients we examined have positive ELISpot responses to more than 3 different Gag epitopes and some have ELISpot responses to 8 different Gag epitopes. Thus, the ability to present more CD8$^+$ T cell epitopes and induce more CD8$^+$ T cell responses is not correlated with protection from HIV-1 infection. A*01:01, by contrast, can present only three Gag epitopes. The narrow spectrum of Gag epitope binding ability of the protective allele A*01:01 can explain previous observations that HIV-specific responses are generally lower in magnitude (4, 56) and narrower in breadth in HIV-1-resistant individuals than in HIV-infected donors (4). Although A*01:01 presents a very narrow spectrum of Gag epitopes, it appears to tolerate the variation of the presented epitope as seen in its binding to the epitope NSSKVSQNY and its variants of major subtypes A, B, and D. Thus, a more focused presentation of major HIV subtype variants appears to confer more-effective protection from infection. The ability of A*01:01 to present multiple major subtype variants of specific epitopes could be advantageous in dealing with multiple circulating subtypes in Kenya (35, 36, 50). To deal with the diver-

FIG 6 Phenotype analysis of A*01:01 and B*07:02 epitope-specific CD8$^+$ T cells. (A) Identification of circulating HIV-specific CD8$^+$ T cells with Gag HLA peptide tetrameric complex. Results represent analyses of samples from representative HIV-1-seropositive subjects. (B) Visualization of virus-specific CD8$^+$ T cells by staining *ex vivo* with MHC-I tetramers and surface expression of memory/effector phenotypic markers. All cells shown are a result of a gate on the specific CD8 subset to a tetramer. (Top) Representative figure showing surface expression of CCR7 and CD45-RA on specific HIV-1 CD8-T cells to A*01:01-NSSKVSQNY and B*07:02-APRKKGCWK tetramers. Numbers in the quadrants indicate percentages of cells. (Bottom) Comparison of percentages of markers expressed by CCR7 and CD45-RA (NC, naive cells; CM, central memory; EM, effector memory; TD: terminal differential) on specific HIV-1 CD8-T cells to A*01:01-NSSKVSQNY, B*07:02-APRKKGCWK, and B*07:02-SPRTLNAWV tetramers. Data are from the participants with HIV-1 tetramer reactivity (n = 3 for A*01:01-NSSKVSQNY and for B*07:02-APRKKGCWK and B*07:02-SPRTLNAWV). (C) Comparison of CD27 and CD28 expression in CD8$^+$ T cells of A*01:01-NSSKVSQNY, B*07:02-APRKKGCWK, and B*07:02-SPRTLNAWV tetramers. Representative figure showing surface expression of CD28$^+$ CD27$^-$ (left) and surface expression of CD28$^-$ CD27$^-$ (right) on specific HIV-1 CD8-T cells to each tetramer. Data are from participants with HIV-1 tetramer reactivity (n = 3 for A*01:01-NSSKVSQNY and for B*07:02-APRKKGCWK and B*07:02-SPRTLNAWV).

sity of HIV-1 virus, a mosaic vaccine approach has been developed (13, 17, 20, 31, 59), and it appears to have enhanced coverage of diverse HIV strains (13, 59). In addition to the reports that Gag specific T cell responses are associated with lower viral loads (25, 30, 37, 49, 54, 58, 62, 74), studies have suggested that the failure of current vaccine approach may be due to targeting immunodominant variable epitopes and vaccine approaches targeting conserved regions of HIV-1 have been proposed and are under investigation (38, 55, 73). The narrowly focused antigen presentation of multiple variants by A*01:01 could be advantageous in dealing with viral diversity, in the meantime avoiding the negative effect of immunodominance of mutable epitopes (55).

Are the specific memory $CD8^+$ T cells to A*01:01-Gag epitope complex phenotypically different from the B*07:02-epitope complex? Our preliminary study showed that although there is no difference in epitope-specific Tem/Tcm frequencies, the effector memory T cells ($CCR7^-$ $CD45RA^-$ $CD28^+$ $CD27^-$) induced by A*01:01-NSSKVSQNY are more numerous than those induced by B*07:02-APRKKGCWK and B*07:02-SPRTLNAWV. Thus, there appears to be a difference in the regulation of the costimulation marker, CD28 of the antigen-specific $CD8^+$ T memory phenotype, between the allele associated with resistance to HIV-1 infection and those alleles associated with susceptibility. Further studies are needed to determine if these effects are independent of disease progression, as all the subjects in this analysis were in the chronic phase of HIV infection. We are conducting further studies to examine tetramer-specific responses in HESN subjects and chronically HIV-infected subjects. CD28 plays a key role in controlling the size and quality of pathogen-specific immune responses (53). CD28-mediated costimulation is needed for effective primary T cell expansion and for reactivating memory $CD8^+$ T cells and secondary responses (46), and it helps generate and maintain virus-specific memory $CD8^+$ T cells. Maintaining CD28 is important for proliferation and survival of $CD8^+$ T cells; loss of these molecules is associated with reduced ability to respond to recurrent infection. The higher level of $CCR7^-$ $CD45RA^-$ $CD28^+$ $CD27^-$ cells in the A*01:01 epitope NSSKVSQNY-specific T cell population could translate into a better ability to proliferate as well as enhanced cell survival. Future studies will address this and examine $CD8^+$ T cell exhaustion and the functionality of these memory $CD8^+$ T cells. Studying these parameters may help us better understand the advantage in chronic HIV-1 infection that this costimulator marker confers on memory CD8 T cells presented by this allele.

Recent studies have shown that in most cases mucosal transmission of HIV-1 is by a single or a few founder viruses (1, 12, 19, 29, 39, 40). Immune mechanisms preventing the establishment of a few founder viruses are likely different from the ones dealing with a full-blown viral infection after the virus has been well established in the host. It is possible that a lower-magnitude, narrowly focused, well-maintained virus-specific $CD8^+$ T cell response to multiple subtypes is sufficient to destroy and eliminate a few founder viruses without inducing inflammatory responses that may activate more $CD4^+$ T cells and provide more targets for HIV-1. More narrowly focused epitope recognition has also been reported for B*57:01, the allele associated with viral control in HIV-1-infected elite controllers (32, 47). There is also a common observation that the alleles with much broader capacity of peptide binding are not associated with viral control (2, 32). This study is the first to show that broader epitope recognition and induced immune response correlate to a detrimental outcome of HIV-1 infection, while the narrowly focused epitope presentation is correlated with protection from HIV-1 infection.

Generating strong immunological memory responses to infectious pathogens is a common vaccine approach. For many reasons, the traditional approaches have failed until recently to yield a successful AIDS vaccine. First, HIV-1 targets $CD4^+$ T cells, and the activated $CD4^+$ T cells are more readily infected. Second, HIV-1 virus exhibits a great diversity and evolves rapidly due to the high error rate of reverse transcriptase. In addressing the profound challenge these factors pose to developing an effective vaccine, we have shown here that the allele associated with protection from HIV-1 infection does not recognize a broad range of Gag epitopes but can present multiple variants of a few specific epitopes. By contrast, the allele associated with susceptibility to HIV-1 infection can recognize a broad spectrum of Gag epitopes and generate strong immune responses. Although we compared only Gag epitopes of two HLA class I alleles, and whether this observation can be extended to other alleles associated with different outcomes of HIV-1 infection remains to be determined, it is clear that recognizing more epitopes and generating stronger T cell responses are not always protective against HIV-1 infection. Since activated $CD4^+$ T cells are more susceptible targets for HIV-1, a broad spectrum of antigen recognition may lead to broad T cell activation and thus provide more targets for HIV-1 and a greater probability of establishing infection. The much narrower epitope spectrum of the allele associated with a better HIV-1 outcome suggests that selectively targeting fewer and important locations of different HIV-1 subtypes might work better by destroying infected cells without overactivation of the immune system.

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