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Enhanced recognition of HIV-1 Cryptic Epitopes Restricted by HLA-Class I alleles Associated with a Favorable Clinical Outcome

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Abstract

Background—Cryptic Epitopes (CE) are peptides derived from the translation of one or more of the five alternative reading frames (ARFs; 2 sense and 3 antisense) of genes. Here, we compared response rates to HIV-1 specific CE predicted to be restricted by HLA-I alleles associated with protection against disease progression to those without any such association.

Methods—Peptides (9–11mer) were designed based on HLA-I binding algorithms for B*27, B*57 or B*5801 (protective alleles) and HLA-B*5301 or B*5501 (non-protective allele) in all five ARFs of the nine HIV-1 encoded proteins. Peptides with >50% probability of being an epitope (n=231) were tested for T cell responses in an IFN-γ ELISpot assay. PBMC samples from HIV-1 seronegative donors (n=42) and HIV-1 seropositive patients with chronic clade B infections (n=129) were used.

Results—Overall, 16%, 2%, and 2% of CHI patients had CE responses by IFN-γ ELISpot in the protective, non-protective, and seronegative groups, respectively (p=0.009, Fischer's exact test). Twenty novel CE specific responses were mapped (median magnitude of 95 SFC/10^6 PBMC) and the majority were both anti-sense derived (90%) as well as represented ARFs of accessory proteins (55%). CE-specific CD8 T cells were multifunctional and proliferated when assessed by intracellular cytokine staining.

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AUTHOR CONTRIBUTIONS:
Conceived and designed the experiments: Anju Bansal and Paul Goepfert
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Wrote the paper: Anju Bansal and Paul Goepfert

Conclusions—CE responses were preferentially restricted by the protective HLA-I alleles in HIV-1 infection suggesting that they may contribute to viral control in this group of patients.

Keywords

Cryptic epitopes; alternate reading frames; chronic HIV-1 infection; protective alleles; HLA-B*53; HLA-B*57

INTRODUCTION

Development of a vaccine for HIV-1 that confers protection against HIV-1 infection or delays disease progression remains a formidable challenge[1–3]. Perhaps the biggest hurdle to an efficacious vaccine development is the enormous sequence diversity of HIV-1 and the propensity of the virus to escape cytotoxic CD8 T lymphocytes (CTL) and/or antibody recognition[4–7,8]. For a CTL based vaccine, novel approaches to vaccine design are currently being investigated in an attempt to circumvent this obstacle[9–14]. A dominant theme common to these studies is the use of vaccines designed to enhance the breadth (targeting more epitopes) of T-cell responses. The rationale for this line of research largely stems from the immunogenicity results obtained in non-human primates that have suggested that for a CTL based HIV-1 vaccine to be effective, it will need to induce an enhanced breadth of CD8 T-cell responses [15,16].

Several studies in human and non-human primates have shown that sense and antisense transcription and translation are fairly common likely owing to aberrations in gene expression that are driven by one or more of the multiple molecular mechanisms [17–32]. Involvement of multiple reading frames is biologically plausible as among the 6 potential reading frames encoded in a double stranded DNA, one is designated as the ORF (open reading frame; frame usually associated with a functional protein) and five others represent ARFs (alternate reading frames) [33–38]. Most HIV-1 epitopes, comprehensively characterized and studied to date in context of infection and vaccination, are those derived from known HIV-1 proteins encoded by the primary ORFs of the HIV-1 viral genome (traditional epitopes or TE). Cryptic epitopes (CE) represent a class of non-conventional epitopes, which have been shown in prior studies to be immunologically relevant in context of viral infections and cancers [30,32,39–63]. Furthermore, studies in HIV-1 and SIV have shown that CE specific CTLs are under immune pressure to escape immunosurveillance [39,40,52,64,65]. Despite the known immunogenicity of CE and its potential to increase the T cell breadth, most HIV-1 vaccines in clinical development do not induce pertinent responses directed against these epitopes [41].

A plethora of data in HIV-1 infection has now established a definite association of certain HLA class I alleles and disease progression[66–74]. Broadly, two groups of alleles can be delineated based on their disparate disease outcomes: protective allele (HLAs-B*27, B*57 and B*58) and non-protective allelic group (HLAs-B*53 and B*55) [66–74]. Although studies on protective alleles and recognition of traditional epitopes are abundant, very few studies have addressed this association for cryptic epitopes. The first study that evaluated HLA-I restricted CE was done by Cardinaud et al [42] where CTL responses to CE were observed in

HLA-B*07 expressing HIV-1 infected individuals. Using a selected set of CE (9–11 mers) based on HLA-I associated HIV-1 polymorphisms; we and others have previously shown that CE are frequently targeted during HIV-1 infection[39,40]. A recent paper illustrated enhanced ARF encoded immune responses in HIV infected patients receiving antiretroviral therapy (ART) [44]. Similar to our prior work [39] most of the peptides used in this study were derived from reverse frames. The T-cell responses in this study were examined in a small subset of HIV-1 infected individuals expressing HLA A*02, B*07 and B*58 alleles [44]. However, no study to date has assessed the frequency, functionality and biological significance of CE in context of specific HLA class I alleles associated with differential disease outcome.

In the current study, we compared predicted CE specific responses presented by "protective" (HLAs-B*58, B*57 and B*27) and the "non-protective" alleles (B*53 and B*55) in disease progression. We demonstrate that CD8 T-cell targeting by protective- CE is not uncommon and may therefore contribute to not only an increased breadth of responses but also durable viral control in individuals who are endowed with protective HLA-I alleles.

MATERIALS AND METHODS

Cryptic epitope prediction

Using consensus clade B sequences and the Epipred program (http://boson.research.microsoft.com/bio/epipred.aspx), we predicted HLA-I restricted (9–11mer peptides) that bind HLA-B*27, B*57, B*5801 **(protective)** or B*5301, B*5501 **(non-protective)** alleles for all the five alternative reading frames of the nine HIV-1 encoded proteins (ARF-CE, Table 1). A total of 231 predicted epitopes with posterior probability (pp) >0.5 were synthesized and tested for immunogenicity. Posterior probability of a predicted peptide is a statistical measure of an amino-acid sequence being a true epitope, as estimated from the interaction of peptide and HLA sequence characteristics [75]. The epipred model was trained on all optimally defined ("A-list) HLA-epitope pairs listed in the Los Alamos database, in addition to all HLA-epitope pairs listed in the IEDB database (www.iedb.org) as eliciting a functional response. Negative training data were derived from randomly sampled peptides from the human proteome and from HLA-peptide pairs shown to exhibit low binding affinity and deposited in IEDB.

Study cohort

PBMC samples from clade B HIV-1 chronically (n=129, Table 2) infected individuals were used for immune assays. These individuals were enrolled from the 1917 HIV-1 clinic at the University of Birmingham at Alabama. In addition, PBMC from healthy seronegative donors (n=42) from the **A**labama **V**accine **R**esearch **C**linic (AVRC) were used as controls. IRB approval was obtained and all participants consented for this study. Details on the demographics and clinical features of these individuals are shown in detail in Table 2.

HIV-1 and non-HIV-1 antigens

The predicted protective and non-protective allele specific CE were synthesized in a 96 well peptide array format from New England peptides (Gardner, MA). Individual stock peptides

were reconstituted at 10–40mM in DMSO. The immunogenicity was evaluated for each specific HLA specific pool (Table 1). For B*58, both pools and subpools were tested initially. As positive controls, pools representing immunodominant epitopes, previously shown to be restricted by the protective alleles in the Los Alamos database, were used (ORF-TE, Table 1). In addition, overlapping peptides (OLP, 15 mers overlapping by 11) for Gag (# 8117) and Pol (#6208) from NIH AIDS reagent program were also used (ORF-OLP, Table 1). Due to protein length, the latter was split in 2 pools (Pol-N OLP and Pol-C OLP). For the non-HIV-1 antigens, we used CEF (9-mers, #9808, 2ug/ml) and CMV pp65 peptide pool (15 mers overlapping by 11, #11549, 5 μg/ml), both from NIH AIDS Reagent program. These peptides were tested in both HIV-1 seronegative and seropositive individuals.

IFN-γ ELISpot assay

An *ex-vivo* IFN-γ ELISpot assay was performed as described previously [39,66,76]. In brief, cryopreserved PBMC were thawed and allowed to rest overnight at 37° C. Nitrocellulose 96-well plates were also coated overnight at 4° C with anti-IFN-γ monoclonal antibody. The following day, PBMCs (100,000 cells/well) in duplicate were incubated with appropriate peptide pool or single peptide at 2uM or 10uM respectively for 20–22 hours at 37° C in 5% CO2. The media or unstimulated controls were plated in quadruplicate. After washing, biotinylated anti-IFN-γ was added to the plates for 2 hours at room temperature. Following another round of washing, SA-conjugated alkaline phosphatase was added for 1 hr. and then NBT/BCIP added for color development. Individual cytokine-producing cells were counted by the ImmunoSpot CTL ELISpot reader. The criteria for a positive response was >50 SFC/10^6 PBMC and 2 times the unstimulated control. In addition, PHA was used as a positive control (duplicate) and samples with a PHA response of less than 500 SFC/10^6 were excluded from analysis.

Polychromatic flow cytometry (PFC)

Cryopreserved PBMC were stained in a PFC assay as described previously [39]. In brief, PBMC were washed in RPMI containing 10% human AB sera (R-10 media) and co-stimulatory monoclonal antibodies (anti-CD28 and anti-CD49d; Becton Dickinson, San Jose, CA) at 1 ug/ml each and 50U/ml of Benzonase (Novagen, Madison, WI) were added to each tube containing 1×10^6 PBMC in 500μl R-10 media. For co-culture, CD107a-FITC was added. Cells were pulsed with the appropriate peptide, monensin and brefeldin for 12 hours at 37°C. Staphylococcus enterotoxin B (SEB) [1 ug/ml] was used as a positive control. The cells were stained for the dead cell dye marker and surface labeled for 20 min before Cytofix/cytoperm reagent was added. The fluorescent labeled antibodies used were: anti-CD3 (Alexa Fluor 780) and anti-CD8 (V500). After 20 min, the cells were labeled with intracellular antibodies i.e. anti-IL2 (APC), anti-TNFα (PECy7), anti-IFN-γ (Alexa Fluor 700) and anti-perforin (PE) for 20 min at room temperature. CD14 and CD19 labeled with PercpCy5.5 were used as a dump channel. The cells were fixed in 2% paraformaldehyde and analyzed on an LSR II flow cytometer. At least 100,000 CD3+ events were acquired and the data was analyzed using FlowJo Version 8.6.2 software. Lymphocytes were analyzed based on forward and side scatter profiles, and the gates were set based on the media control (irrelevant peptides) and applied to all samples from the same individual. Cytokines produced were measured from CD3+CD8+ cells. A response was considered positive if the

value was greater than >2X the media control for that individual with a magnitude of ≥0.05%. All fluorochrome-conjugated antibodies were obtained from Becton Dickinson, San Jose, CA, USA.

In Vitro expansion of antigen specific CD8 T-cells

Peptide specific CD8 T-cells lines were expanded for 14 days *in-vitro* using monocytes as APC as described before [77]. In brief, after 2 rounds of weekly antigenic stimulation, the CD8 T-cells were re-stimulated with the cognate antigen in the presence of co-stimulatory antibodies and intracellular transport inhibitors for 6 hours and analyzed as mentioned above. For HLA-I restriction experiments, HLA-class I matched and mismatched BLCL pulsed for 1 hr with the cognate peptide (10uM). The BLCL were washed three times before adding to the in-vitro expanded CD8 T-cells. The effector (CD8 T-cells) and targets (BLCL) were co-cultured at 5:1 (E:T) ratio for 6 hours in the presence of co-stimulatory antibodies and intracellular transport inhibitors as described above under ICS and the production of IFN-γ from CD8 T-cells was quantified.

Statistics

Comparisons of continuous variables within each group were done using the non-parametric Wilcoxon rank sum test. Analyses of variables between each group were performed using the non-parametric Mann-Whitney U test. Differences in the responder frequencies were compared using Fishers exact test.

RESULTS

Predicted peptides for HLA-I alleles with opposing disease outcomes

The total number of the CE tested along with a select set of HLA-I restricted traditional epitopes (TE) and overlapping peptides (OLPs) representing the entirety of Gag and Pol are detailed in Table 1. The evaluated peptides included representatives from all nine known HIV proteins and were enriched in the antisense direction (63–90%, Table 1) for all HLA alleles but B*27 (when correcting for the number of alternative reading frames in the sense vs. antisense directions).

Frequency of CE responses in individuals with favorable HLA subtypes

We evaluated CE specific immunogenicity in PBMC obtained from 42 HIV seronegative donors and 129 chronically HIV-1 infected individuals with B*27, B*57 or B*5801 (protective, n=79) or HLA-B*5301 or B*55 (non-protective, n=50) alleles. We first evaluated T-cell responses in 42 HIV-1 seronegative donors who were predominately Caucasian (30/42, 71%), females (25/42, 60%) with a median age of 42 years. Among the seronegative donors, HLA-I information was available for 39 of which thirteen had alleles of interest i.e. B*27, B*57, B*58, B*53 and/or B*55. HIV-1 seronegative donors elicited a high frequency (25/42, 60%) responses to the non-HIV-1 antigens represented by CMV-pp65 and/or CEF peptide pools; data not shown. However, a CE specific T-cell response was seen in only one seronegative donor (2%). The single seronegative donor that responded had a positive CE specific response to an HLA-B*58 pol subpool, and the net magnitude of

the response was 85 SFC/10^6 PBMC. This sub-pool response could not be mapped to a single peptide.

The demographics and clinical features of the chronically HIV-1 infected individuals whose samples were used in this study are shown in Table 2. Our cohort was enriched with individuals with HLA-B*57 and B*53 among the protective and non-protective groups, respectively. Although both allelic groups were composed of African Americans, the non-protective group was largely African American, female and receiving ART; this composition is reflective of the high HIV-1 disease burden observed for this racial group in US. The median CD4 and plasma viral load were similar between the 2 allelic groups (Table 2). The relatively preserved CD4 T cells and low viral load in both groups is likely due to the fact that most patients in our HIV clinic are being treated with antiretroviral therapy (ART) unless their infection is fairly well controlled. Initially peptides in pools were tested (Table 1) and positive responses were enumerated using a criteria of >50 SFC/10^6 PBMC and 2 times the unstimulated control. These responses were further mapped to the sub-pool or to the single peptide level.

Overall 16% of the patients with a protective allele responded to at least one CE (Figure 1A). For each protective allele, the overall response rate was 16%, 14% and 23% for B*27, B*57 and B*5801, respectively (Figure 1A). At the peptide level, 13%, 18% and 10% responses were detected for all B*27, B*57 and B*58 restricted peptides tested respectively (Figure 1B). Furthermore, nearly all of these mapped peptides were antisense derived (90%,) and were predominately located in the ARFs of HIV-1 accessory genes (>55%, Nef, Vpr, Vif and Vpu); data not shown. Regarding the magnitude of response in the protective allele group, the median magnitude of the response was higher at the single peptide level compared to the sub-pool and pools of peptide (data not shown, $p<0.05$).

For the non-protective allele group, we evaluated responses in a total of 50 CHI individuals with HLA-B*53 (N=46) and HLA-B*55 (N=4). The total number of peptide tested was 72, with 57 and 9 peptides being unique to B*53 and B*55, respectively, and 6 common to both alleles. In this group, we observed only one response to a B*53 peptide (2%) and the pool which contained it. Taken together, the overall response rate for the protective allele and non-protective allele group was 16% and 2%, respectively ($p= 0.009$, Figure 1A) despite similar median posterior probability (0.60) of predicted epitopes. On the other hand, no differences were observed comparing either the plasma viral loads or the absolute CD4 T cell counts when comparing the individuals with CE responses to those without such responses (1,243 and 1,430 RNA copies/ml and 458 and 571 cells/mm3, respectively). Furthermore CE responses were similar among patients on and off ART (data not shown).

Novel CD8 cryptic epitope specific responses targeted during chronic HIV-1 infection

In our study, we identified a total of twenty novel 9–10 mer peptides restricted by one or more of the protective alleles (Table 3) in seven HIV-1 infected individuals. These responses were restricted nearly equally by each of the 3 protective alleles studied (range 20–45 %).

Polychromatic flow cytometry was used to further characterize the CE specific CD8 T-cell responses. Using an ex-vivo ICS assay, our data showed that these CD8 T-cells elicit multifunctional cytokine and effector responses (Figure 2). In addition to IFN-γ, CE specific cells produce cytokines and effectors molecules including IL-2, TNF-α, CD107 and granzyme-B and perforin. Following our ex-vivo analyses (Figure 2), we expanded the CE specific CD8 T-cells in culture using autologous peptide pulsed monocytes as antigen presenting cells (APC). These cells readily proliferate and retain their multifunctionality (Supplementary figure 1). Using a similar approach for CE-CD8 T-cell expansion, we were also able to confirm the HLA-I restriction of CE to be similar to the computational prediction (Supplementary figure 2).

DISCUSSION

Our understanding of cryptic epitopes (CE) has significantly improved since the first antisense protein was described in 1988 “as a novel protein on the genomic DNA plus strand” [25]. Antisense transcription for HIV-1 was later shown [35,78,79] and He et al elegantly demonstrated its common occurrence in the human genome [23]. Although traditionally derived epitopes (TE) have long been considered to be the sole source of HLA-I presented immunogens, the functional relevance of the ARF encoded cryptic epitopes (CE) has only recently begun to emerge. Therefore, this would imply that the total breadth of epitope presentation has heretofore been largely under estimated.

Consistent with our previous findings that HLA-I CE are commonly targeted in HIV-1 infection [39], we observed a significant rate of CE targeting, especially from anti-sense derived peptides. Our data shows a bias towards the preferential targeting of CE restricted by protective compared to non-protective alleles. This observation could have been skewed by two factors. Firstly, it is possible that the binding prediction algorithms for protective allele are better than non-protective allele since the former group is much better studied. However, this is unlikely to be the main reason since the peptides in both group were selected based on a posterior probability of >0.5 and the number of peptides tested in each group were comparable. Furthermore, the prediction models used in the current manuscript have worked well previously for both allelic groups (i.e. PA and NPA) [39,40,80]. Secondly, based on the observation that at a population level protective alleles are associated with more traditional epitopes that have evidence of escape [81], it can be speculated that the protective allele group has the ability to target more CE as well. Conversely, patients with HLA types associated with rapid disease progression recognize a small fraction of the TE restricted by the non-protective alleles [72]. It is therefore not surprising that the CE restricted by these alleles are also not frequently targeted.

Although previous work [39,40] has shown that the breadth of CE specific CTL do correlate with markers of disease progression, we were unable to see a significant correlation between CE targeting and plasma viral load. One possible explanation is that the median viral load in our entire cohort was very low making correlations difficult. Nevertheless, multiple prior studies have shown that overall, individuals with protective alleles have lower viral load compared to individuals who lack these HLA-I types [8283848586]. Moreover, this effect can be

seen early following viral infection suggesting that CE responses could perhaps be partly responsible for the enhanced viral control afforded to these patients.

There are fairly convincing data that the protective alleles' favorable effect on disease progression is in a large part attributed to HIV-1 Gag targeting [87–899082], although targeting [4,91,92] of other proteins has been shown to be important during acute infection. HIV-1 and SIV specific CE CD8 T cell responses have also been directed towards ARFs of all of the viral proteins that have been analyzed [39,40,44,46,54,93,94]. Consistent with prior work, the current study demonstrated CE responses to ARFs of all of the genes analyzed, including the accessory genes [44,46]. Interestingly, targeting CE in the ARFs of accessory genes such as Tat, Nef, and Vif was also frequently seen in macaques including those exhibiting elite control of SIV [95–98]. Therefore, CE targeting ARFs can be an important source of immmunogens for enhancing the overall repertoire of virus specific T cell responses.

The preferential targeting of antisense peptides is consistent with our prior work that showed that there is potential to encode more antisense peptides [39] relative to sense direction. A recent study also found a higher number of predicted epitopes were derived from antisense ARFs [44]. In the current study, we found that nearly half of CE specific responses were targeted towards peptides translated from the ARFs of accessory genes (vif, vpr, vpu and nef) followed by pol antisense ARFs. The latter is in line with our previous data [39] showing that the potential number of antisense pol-CE far outweighs the number of pol TE. In parallel, the multi-functionality of CE specific T cell responses is comparable with those seen for TE making CE attractive as viable immunogens that can likely act in concert with TE to broaden the overall CD8 T-cell response.

Taken together, data from this study suggests that there are multifaceted players involved in exacting viral control in individuals endowed with protective alleles. Some of the attributes working in tandem or in concert to control a viral infection include the ability to mount fitness cost imposing CD8 T-cell responses, elicit multifunctional responses [70], superior ability of responses to cross-recognize variants [99,100], and a broader TCR usage. Perhaps a greater breadth of responses via CE may yet be another dimension to this equation. In summary, understanding the full extent of immune targeting of protein products derived from ARFs not only provides insight into the full extent of virus specific CTL responses to HIV-1 but could yield information which can be effectively utilized to increase the breadth of future CTL based HIV-1 vaccines.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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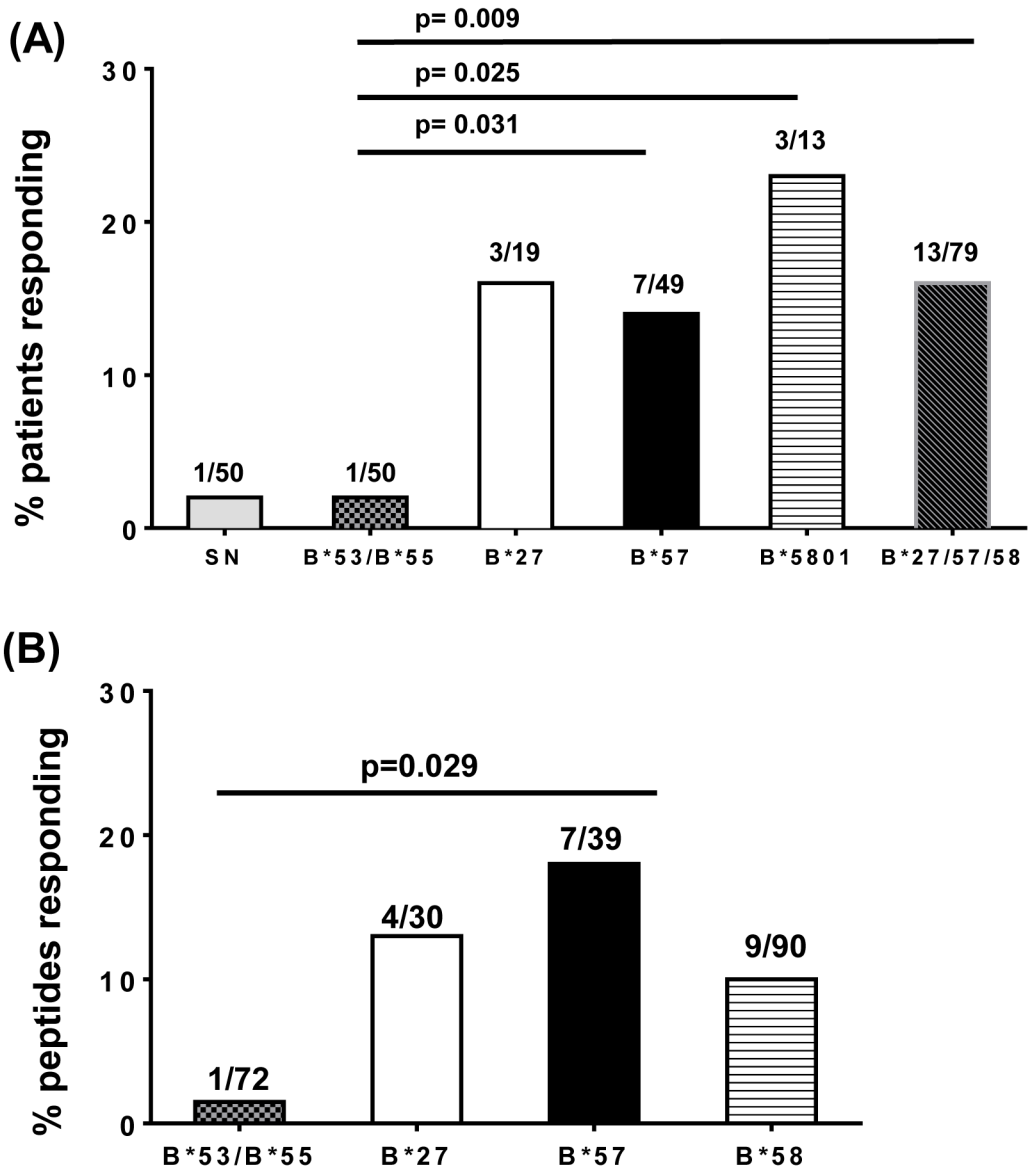

Figure 1. Patient and peptide responder frequency data for CE specific T-cell responses as assessed in an IFN-γ ELISpot assay in a cohort of chronically HIV infected individuals

(A) Percentage patient responder frequency for each group of individuals carrying non-protective (B*53/55) and protective (B*27/57/58) HLA-class I alleles is shown. SN= HIV seronegative donors; (B) Response frequency (%) elicited to each peptide pool. The fraction on top of each bar indicates the number of positive responses over total tested for that group.

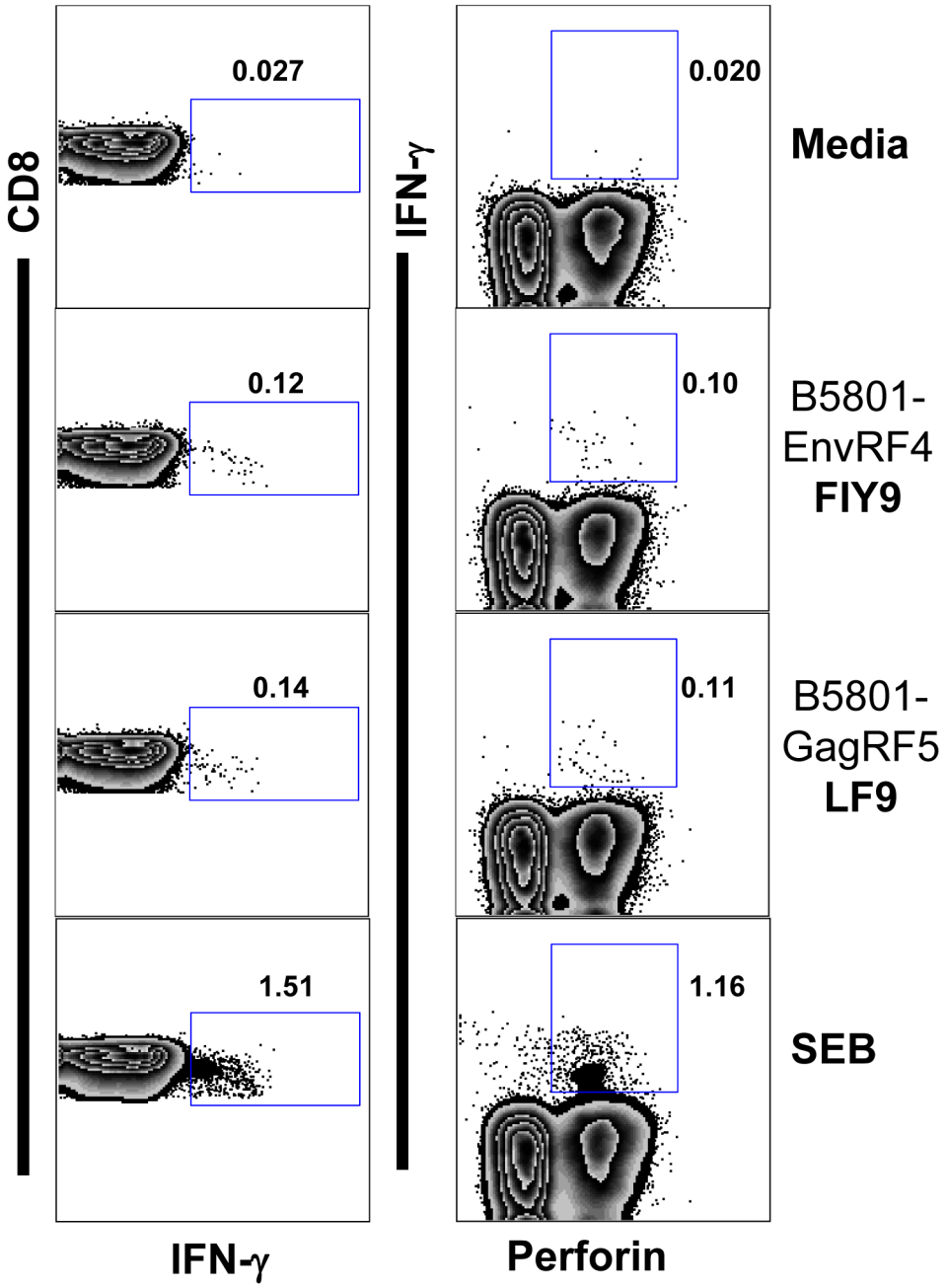

Figure 2. Functionality of CE specific CD8 T-cell responses restricted by protective alleles as measured in an *ex-vivo* ICS assay

PBMC from a chronically HIV infected donor (HLA-A: 0201/0205, HLA-B:0702/5703, HLA-C:0701/0702) were stimulated with 10uM peptide in presence of co-stimulatory antibodies and intracellular transport inhibitors for 12 hours at 37°C. Representative data showing IFN-γ and perforin production from CE specific CD8 T-cells in response to B*5801 restricted Env RF4 (FIY9= FYFSSPSIY) and B*5801 restricted Gag RF5 (LF9= LPLWEGQIF) peptides. Response to SEB is shown as a positive control. Using Fischer's exact test, p-value for FIY9 response relative to media was 1.1×10^{-6} for IFN-γ, and 4.0 ×

10^{-6} for perforin. For LF9 response, the p-values for media compared to IFN-γ and perforin were 2.7×10^{-5} and 4.4×10^{-5} respectively. This patient was on ART and the plasma viral load and absolute CD4 counts were 49 and 611 respectively.

Table 1

Peptide pools of predicted CE, previously described TE or OLPs tested in immunoassays.

Source[a]	Pool name[b]	Type[c]	HLA-I[d]		Pool Size	Location[e]	
			Restriction	Associated Prognosis		Sense	Antisense
ARFs	B*27CE	CE	B*27	Protective	30	11 (37%)	19 (63%)
	B*57CE	CE	B*57	Protective	39	4 (10%)	35 (90%)
	B*58CE	CE	B*58	Protective	90	10 (11%)	80 (89%)
	B*5301/B*5501	CE	B*5301 or B*5501	Non-protective	72	12 (17%)	60 (83%)
Gene	B*27 TE	TE	B*27	Protective	17	17 (100%)	
	B*57/58 TE	TE	B*57 or B*58	Protective	32	32 (100%)	
	Gag	OLP	Multiple	Both	123	123 (100%)	
	Pol-N	OLP	Multiple	Both	125	125 (100%)	
	Pol-C	OLP	Multiple	Both	124	124 (100%)	

[a] ARF=alternative reading frame

[b] CE= cryptic epitope, TE= traditional epitope, and Pol-N and Pol-C contain peptides representing the N or C terminal regions of the protein, respectively

[c] OLP= overlapping peptides

[d] alleles associated with disease progression in multiple prior studies

[e] sense or forward and antisense or reverse alternate reading frames; number (%) of sense and antisense is based on pool size

Table 2

Demographic and clinical charateristics of the chronically HIV-1 infected cohort.

Protective alleles (PA)	Number	%	Non-protective alleles (NPA)	Number	%
Total	79	100	**Total**	50	100
B*57	47	59	B*53	46	92
B*27	17	22	B*55	4	8
B*58	13	16			
B*27/B*57	2	3			
Gender			**Gender**		
Female	32	41	Female	31	62
Male	47	59	Male	19	38
Race			**Race**		
Caucasian	38	45	Caucasian	4	8
African American	41	55	African American	45	90
Other	0	0	Other	1	2
On ART			**On ART**		
Yes	34	43	Yes	35	70
No	45	57	No	15	30
Median*			**Median***		
CD4	543		CD4	490	
Plasma viral load	1186		Plasma viral load	1309	

*Absolute CD4 T-cell count ($X10^6$/l); viral load (RNA copies/ml)

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Table 3

List of novel immunogenic cryptic epitopes (CE)

	Cryptic epitope			
Patient ID	**Sequence**	**Name**	**HLA-I[a]**	**ARF-frame[b]**
CMI-1	HLLAQLSFF	HF9	B57	Nef-RF6
	YMNCYQDNF	YNF9	B57	Pol-RF4
	YCMDFQAQF	YQF9	B57	Pol-RF6
	NYCYYCCYY	NY9	B57	Vpu-RF4
CMI-2	FYFSSPSIY	FIY9	B5801	Env-RF4
CMI-3	WPLVFWGLF	WF9	B57	Vif-RF5
	YYGPHNYCYY	YY10	B57	Vpu-RF4
	FYEYYGPHNY	FNY10	B5801	Vpu-RF4
CMI-4	RQWQQFHQYY	RY10	B27	Pol-RF3
	TRLYTFRRK	TK9	B27	Pol-RF3
	HRFYYSLTL	HL9	B27	Pol-RF4
CMI-5	IYIWCFTKL	IL9	B27	Vif-RF5
	FPKPEALFW	FW9	B57	Gag-RF4
CMI-6	FPHFQQPFF	FF9	B5801	Gag-RF5
	LPLWEGQIF	LF9	B5801	Gag-RF5
	GLFYLLWLNW	GW10	B5801	Nef-RF4
CMI-7	FPCSNPHPVY	FVY10	B5801	Vif-RF4
	HPLAFLEIY	HY9	B5801	Vif-RF5
	DPNASLFLLY	DY10	B5801	Vif-RF6
	YPRKMSNSF	YF9	B5801	Vpr-RF4

[a] EpiPred predicted HLA-I restriction

[b] alternate reading frame of an HIV-1 gene and frame number (RF3=sense and RF4–6=antisense)