

# An unanticipated lack of consensus cytotoxic T lymphocyte epitopes in HIV-1 databases: the contribution of prediction programs

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**Background:** Most consensus HIV-1-specific cytotoxic T lymphocytes epitopes presented via intensively studied HLA molecules are thought to be known

**Objective:** To identify possible novel HIV-1 epitopes for HLA-B27 and HLA-B57; two HLA types which are abundantly studied because of their correlation with slow HIV disease progression.

**Methods:** HIV-1 consensus subtype B sequences were analysed using peptide prediction programs based on major histocompatibility complex binding, proteasomal cleavage and TAP (transporter associated with antigen processing) transport. Recognition of the novel identified epitopes by cytotoxic T lymphocytes was tested using interferon- $\gamma$  ELISpot assay.

**Results:** In total, 22 novel epitopes predicted to be presented by either HLA-B27 or HLA-B57 were selected. Of these, 86% elicited significant immune responses in HIV-1-infected individuals.

**Conclusions:** These data show that numerous HIV-1 epitopes remain to be identified, and that prediction programs are powerful tools for this purpose.

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AIDS 2008, 22:33–37

**Keywords:** CTL epitopes, HIV-1, HLA-B27, HLA-B57, peptide prediction programs

## Introduction

There is increasing evidence that cytotoxic T lymphocytes (CTL) play an important role in the control of HIV infection [1–4]. Studies addressing the role of HIV-1-specific CTL responses are, however, complicated by the large population diversity of human leukocyte antigen (HLA) molecules. Nevertheless, intense research has identified a large variety of HIV-1 peptides presented by different HLA molecules that are known to elicit immune responses in HIV-1-infected individuals. Most of these peptides were identified after stimulation of cells with overlapping peptide pools, followed by the identification of the optimal epitope and the HLA-restriction element. It is commonly believed that most consensus HIV-1-

specific CTL epitopes that are presented via intensively studied HLA molecules are now known. To examine whether HIV-1 databases are indeed nearly complete, the current study sought to identify novel HIV-1 epitopes presented by HLA-B27 or HLA-B57, two HLA alleles that are frequently studied because of their well-known correlation with slow HIV-1 disease progression [5,6].

## Methods

### Peptide predictions

Peptide prediction programs based on major histocompatibility complex (MHC) binding [7,8], proteasomal cleavage [9] and TAP (transporter associated with antigen

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Received: 24 July 2007; revised: 13 August 2007; accepted: 17 August 2007.

processing) transport [10] ([www.cbs.dtu.dk/services](http://www.cbs.dtu.dk/services)) were applied to selected novel epitopes predicted to be presented via either HLA-B27 or HLA-B57. Peptides were only selected if they fulfilled the following criteria: (i) novelty, that is, not reported in the Los Alamos HIV database [11]; (ii) a predicted proteasomal cleavage score  $>0.5$  [9]; (iii) a predicted TAP transport score  $>1$  [10]; and (iv) a normalized [12] HLA-binding affinity  $>1.5$ , which corresponds to good binders within the top 1% for each HLA allele.

### Measurement of epitope recognition by cytotoxic T lymphocytes

Cryopreserved peripheral blood mononuclear cells (PBMC) were thawed and incubated in triplicate at a final concentration of  $10^5$  cells/well in the presence or absence of 20 µg/ml peptide for 20–24 h at 37°C. The capacity of PBMC to produce interferon (IFN)-γ was measured by an ELISpot assay. Phytohemagglutinin (Murex, Dartford, UK) stimulation served as a positive control to test the capacity of PBMC to produce interferon (IFN)-γ, and medium alone served as a negative control. Samples with  $>100$  spot-forming units (SFU) per  $10^6$  PBMC after subtraction of the negative control value were considered positive.

### Statistical analysis

Data were analysed using SPSS 12.01 software (SPSS, Chicago, Illinois, USA). Differences between predicted and known epitopes were analysed using Mann–Whitney or  $\chi^2$  tests.  $P \leq 0.05$  was considered statistically significant.

## Results

### Strong cytotoxic T lymphocyte responses towards peptides predicted to be presented through HLA-B27 or HLA-B57

PBMC were collected from HIV-infected individuals from the Amsterdam Cohort Studies on HIV-1 infection and AIDS. In total, 22 novel HIV-1 epitopes (Table 1) were selected for which recognition by CTL was tested in PBMC from a mean of nine (range, 8–9) individuals carrying the *HLA-B27* allele or six (range, 5–8) individuals carrying the *HLA-B57* allele, as previously described [13,14]. Peptide prediction scores of the novel epitopes were comparable to prediction scores obtained for peptides already known to bind to HLA-B27 or HLA-B57 listed in the Los Alamos HIV database (Mann–Whitney,  $P=0.12$ ).

All 11 peptides predicted to be presented through HLA-B27 induced IFN-γ production in at least one HLA-B27-positive individual [a positive IFN-γ response in a median of 22% of tested individuals (range, 11–89)]. This was also the case for 8 out of the 11 epitopes predicted to be presented through HLA-B57 (a positive IFN-γ response

in a median 20% of tested individuals (range, 0–63)]. In contrast, these peptides did not induce IFN-γ production in five HIV-negative donors expressing HLA-B27 or in five donors expressing HLA-B57, indicating that the observed responses are indeed HIV specific (data not shown). Overall, the magnitude of CTL responses towards the predicted peptides was similar to the responses towards known HLA-B27- or HLA-B57-restricted peptides from the Los Alamos HIV database (Table 1 gives peptide characteristics) analysed in the same HIV-1-infected individuals (Fig. 1). Only two HLA-B27-restricted peptides (TR9, EF9) and one HLA-B57-restricted peptide (DW9) induced significantly lower responses, while one HLA-B27-restricted peptide (KY9) even induced a significantly higher response (Mann–Whitney,  $P<0.01$ ). The latter peptide was recognized by as many as eight out of nine individuals tested ( $\chi^2$  test comparison with the known HLA-B27-restricted peptides:  $P=0.03$ ). None of the predicted peptides induced a positive IFN-γ response in HIV-1-infected individuals who did not express HLA-B27 or HLA-B57 (data not shown).

Although the magnitude of the CTL response against the newly identified peptides was similar to the responses against known HIV-1 peptides from the Los Alamos HIV database, the number of individuals responding to the different predicted peptides was somewhat lower. Of the 11 HLA-B27-restricted peptides, five induced responses in a fraction of individuals comparable to (or in the case of KY9 even higher than) known peptides from the Los Alamos HIV database. Also five of the eight HLA-B57 peptides were recognized by a similar fraction of individuals compared with known HLA-B57 restricted peptides.

## Discussion

After more than 20 years of intensive research, it is believed that most consensus HIV-specific CTL epitopes are known. Unexpectedly, we found that there are still numerous epitopes to be discovered. Using peptide prediction programs, we identified 22 novel epitopes presented via either HLA-B27 or HLA-B57 which could induce strong CTL responses. A possible explanation for the lower fraction of individuals responding to certain predicted epitopes is that only two-digit HLA typing of the individuals participating in this study was available. Since the peptide predictions were performed using the most abundant four-digit HLA type of each HLA serotype, lack of response to certain predicted HLA epitopes may result from absence of the actual four-digit HLA type. The lower number of individuals responding to certain predicted peptides could also reflect higher sequence variability within these epitopes, as all three HLA-B57-restricted peptides and four of the six

**Table 1. Characteristics of the HLA-B27- and HLA-B57-restricted cytotoxic T lymphocyte epitopes studied.**

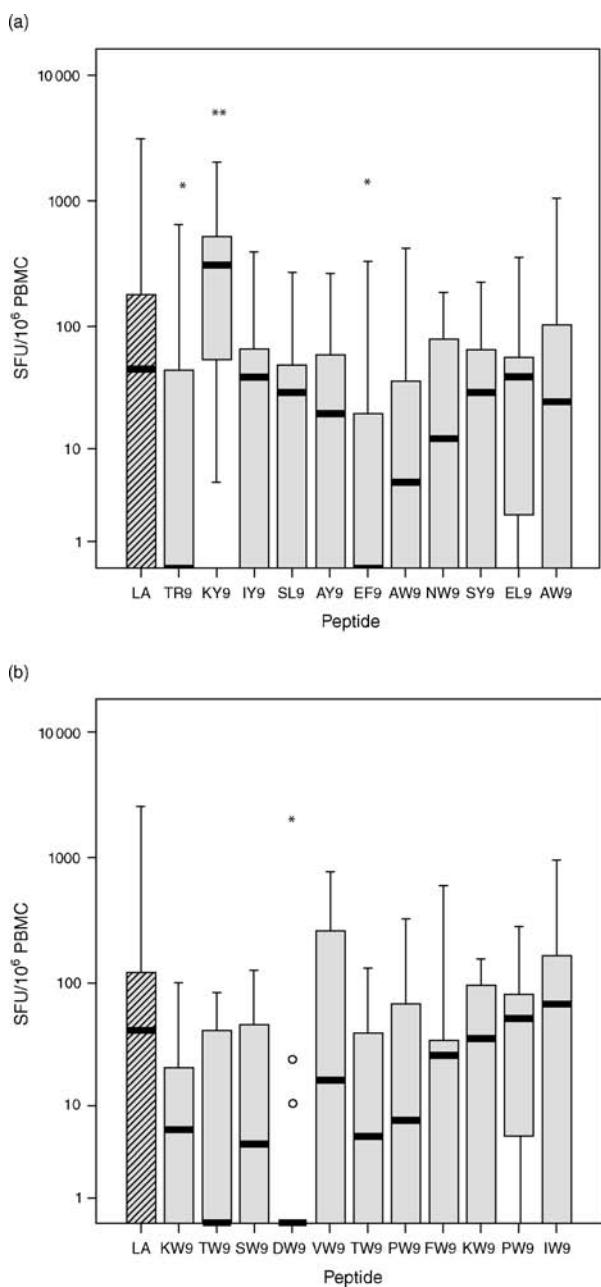
Source	Epitope	Sequence	Gene	Recognition (%)
HLA-B27-restricted epitopes				
Predicted	TR9	TRANSPTRR	pol	22
	KY9	KRKGGIGGY	pol	89
	IY9	IRDKVQKEY	env	22
	SL9	SRAKWNNTL	env	22
	AY9	ARVLAVERY	env	33
	EF9	EREVLVWKF	nef	11
	AW9	ARRNRRRRW	rev	22
	NW9	NRWQVMIVW	vif	25
	SY9	SRKAKGWFY	vif	22
	EL9	ERFAVNPGL	gag	22
	AW9	ARLVITTYW	vif	44
Los Alamos	RL8	RYLKDDQQL	env	44
	GK9	GRRGWEALK	env	88
	RI10	RRQDILDLWI	nef	44
	RW8	RYPLTFGW	nef	44
	KK10	KIRLRPGGKK	gag	11
	KK10	KRWIILGLNK	gag	100
	AW9	AVRHFPRIW	vpr	55
HLA-B57-restricted epitopes				
Predicted	KW9 <sup>a</sup>	KNYQHLWRW	env	0
	TW9 <sup>a</sup>	TTQLFNSTW	env	0
	SW9	STWNVNGTW	env	17
	DW9 <sup>a</sup>	DPEREVLVW	nef	0
	VW9	VTKLTEDRW	vif	60
	TW9	TINEEAAEW	gag	20
	PW9	PTRRELQVW	pol	25
	FW9	FSFPQITLW	pol	25
	KW9	KLPIQKETW	pol	38
	PW9	PVDPRLEPW	tat	13
	IW9	IATESIVIW	pol	63
Los Alamos	GK9	GRRGWEALK	env	22
	AW9	AVRHFPRIW	vpr	25
	GY14 <sup>a</sup>	GPGVRYPLTFGWCY	nef	0
	GP10	GQMVKHQISP	gag	29
	NF20	NAWKVVEEKAFSPEVIPMF	gag	25
	IF9	ISKKAKGWF	vif	25
	YT9	YFPDWQNYT	nef	38
	IW9	ISPRTLNAW	gag	44
	QW9	QASQEVKNW	gag	67
	KY11	KAVERRIKFLY	rev	63
	HQ10	HTQGYFPDWQ	nef	67
	IW9	IVLPKEKDSW	rt	67
	KF11	KAFSPEVIPMF	gag	67
	TW10	TSTLQEIQIGW	gag	78

<sup>a</sup>Epitopes that failed to elicit interferon- $\gamma$  responses of > 100 spot-forming units/10<sup>6</sup> peripheral blood mononuclear cells after subtraction of the negative control value in HIV-1-infected individuals.

HLA-B27-restricted peptides recognized in fewer individuals had a significantly higher entropy score compared with the other HLA-B57- or HLA-B27-restricted peptides ( $\chi^2$  test,  $P < 0.05$ ). A similar negative correlation between the entropy score of a peptide and the number of individuals responding to the peptide has been described previously [15]. In the present study, the selection of potential novel CTL epitopes was based on peptide prediction programs for MHC binding, TAP transport and proteasomal cleavage. Recently, Assarsson *et al.* [16] have shown that binding to MHC molecules is the most restricting step influencing immunodominance, although other factors including T cell receptor repertoire and immunoregulatory mechanisms also play a significant role. Interindividual differences in the last two factors may

also have contributed to the observation that some individuals did while others did not respond to our novel identified epitopes.

Since we aimed to detect novel epitopes that could be recognized by a large number of HIV-1-infected individuals, all peptide predictions were performed using HIV-1 consensus subtype B sequences. The fact that we found a significant physiological CTL response in HIV-1-infected individuals against as much as 86% of the novel predicted epitopes suggests that many consensus HIV epitopes are still missing from the HIV-1 epitope databases despite the intensity of research based on overlapping HIV peptide pools. In line with this, Betts *et al.* [17] found a significantly broader CTL response after



**Fig. 1. Magnitude of cytotoxic T lymphocyte responses towards peptides predicted to be presented through HLA-B27 or HLA-B57.** The responses towards peptides predicted to be presented through HLA-B27 (a) or HLA-B57 (b) were compared with responses towards 7 or 14 peptides described to be presented via these HLA proteins, respectively, in the Los Alamos database (LA). Interferon- $\gamma$  responses were measured as number of spot-forming units (SFU)/10<sup>6</sup> peripheral blood mononuclear cells (PBMC) after subtraction of the medium value. The different predicted peptides are shown on the x axis. Significant differences compared with the peptides from the Los Alamos database were calculated using the Mann–Whitney test; one asterisk indicates that the magnitude of response after stimulation with the peptide was significantly lower compared with that of the database peptides and two asterisks indicates that the response was significantly higher.

stimulation with overlapping peptide pools covering the whole HIV genome than with a pool of optimized peptides. In addition, Liu *et al.* [18] have recently studied an HIV-1-infected individual for 3 years after acute infection and have shown that 14 of the 25 recognized epitopes had not been reported previously in HIV-1 databases. As shown by Altfeld *et al.* [15], the use of peptides based on autologous HIV sequences instead of consensus sequences significantly enhances the detection of CTL responses, especially against the more variable regions of HIV. Our study clearly shows that prediction programs provide powerful tools to help the identification of such autologous HIV epitopes.

In the current study, we detected CTL responses by measuring the production of only one cytokine, IFN- $\gamma$ , which is the last cytokine to be lost during chronic antigen stimulation [19] and which is widely used as a means of identifying specific CTL. It was recently shown that the presence of polyfunctional T cells, which are capable of producing multiple cytokines and chemokines and are able to proliferate, is associated with virus control [20]. Further research focusing on the polyfunctionality of the CTL responses induced by these newly identified epitopes, but also by previously identified HIV epitopes, could reveal information about the relative importance of these CTL responses in virus control.

## Acknowledgements

**Sponsorship:** This work was financially supported by a grant from the Landsteiner Foundation for Blood Transfusion Research (grant 0317). The Amsterdam Cohort Studies on HIV-1 Infection and AIDS (a collaboration between the Amsterdam Health Service, the Academic Medical Center of the University of Amsterdam, Sanquin Blood Supply Foundation and the University Medical Center Utrecht) are part of the Netherlands HIV-1 Monitoring Foundation and financially supported by the Netherlands National Institute for Public Health and the Environment.

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