

# Generation of robust CD8<sup>+</sup> T-cell responses against subdominant epitopes in conserved regions of HIV-1 by repertoire mining with mimotopes

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HLA-A\*0201-restricted virus-specific CD8<sup>+</sup> CTL do not appear to control HIV effectively *in vivo*. To enhance the immunogenicity of a highly conserved subdominant epitope, TV9 (TLNAWVKVV, p24 Gag<sub>19–27</sub>), mimotopes were designed by screening a large combinatorial nonapeptide library with TV9-specific CTL primed *in vitro* from healthy donors. A mimic peptide with a low binding affinity to HLA-A\*0201, TV9p6 (KINAWIKVV), was studied further. Parallel cultures of *in vitro*-primed CTL showed that TV9p6 consistently activated cross-reactive and equally functional CTL as measured by cytotoxicity, cytokine production and suppression of HIV replication *in vitro*. Comparison of TCRB gene usage between CTL primed from the same donors with TV9 or TV9p6 revealed a degree of clonal overlap in some cases and an example of a conserved TCRB sequence encoded distinctly at the nucleotide level between individuals (a “public” TCR); however, in the main, distinct clonotypes were recruited by each peptide antigen. These findings indicate that mimotopes can mobilize functional cross-reactive clonotypes that are less readily recruited from the naïve T-cell pool by the corresponding WT epitope. Mimotope-induced repertoire diversification could potentially override subdominance under certain circumstances and enhance vaccine-induced responses to conserved but poorly immunogenic determinants within the HIV proteome.

**Key words:** Agonist peptide · CD8<sup>+</sup> CTL · HIV vaccine · *In vitro* immunization

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## Introduction

Virus-specific CD8<sup>+</sup> CTL play a central role in the control of HIV infection [1, 2]. However, the ability of such CTL to impose immune pressure on HIV varies according to specificity, with some responses seemingly exerting no influence on the virus [3]. Strong linkages have been reported between a few HLA-B class I alleles and control of infection [4]. The impact of these molecules on disease outcome is mediated largely by potent CTL activities that target highly conserved determinants [5], the majority of which reside within the p24 Gag protein [2]. Aside from their character, these CTL responses may be effective because the selected escape mutations exact a significant cost to viral fitness, thereby favoring the host even after immune evasion [2]. Thus, the issue of whether similar epitope targets restricted by common HLA alleles remain within the HIV proteome to allow for meaningful population coverage is fundamental to the prospects for a broadly applicable T-cell-based vaccine.

Virus-specific CTL responses restricted by the most prevalent allele, HLA-A\*0201, do not appear to suppress HIV effectively *in vivo* [6], consistent with the notion that “inactive” epitopic forms may have been fixed in the circulating viral strains [7, 8]. Paradoxically, however, there is one well-defined epitope in p24 Gag (TV9, TLNAWVKVV, residues 19–27) that shares many features in common with known protective determinants. Thus, TV9 is highly conserved across HIV clades, with only one common variant in which valine at position 9 is “conservatively” replaced by isoleucine [9]. This epitope resides in the first  $\alpha$ -helix at the N-terminus of p24 and overlaps by five residues with the protective HLA-B\*57-restricted ISPTLNAAW (IW9) determinant recognized early by slow progressors [10]. Despite the small number of reports of TV9 reactivity in patients, there are indications that CTL responses to this epitope can control virus [11]. Above all, an elevated and sustained TV9-specific response was noted in one person who remained uninfected despite parenteral exposure to a highly replicating HIV strain [12]. The functional sensitivity of the *in vivo* TV9 reactivity, a factor considered important for the inhibition of HIV *in vivo* [13], was also relatively high [11]. Thus, the potential of TV9 as a vaccine target deserves further exploration, particularly since there may be few alternatives within the HIV proteome for the most prevalent HLA class I allele.

Previously, we studied the pre-infection TCR repertoire for TV9 by priming naive CD8<sup>+</sup> T cells from healthy seronegative donors [11]. Stable, homogeneous and immunologically reactive TV9-specific CTL (TV9-CTL) cultures were generated from most donors, thereby suggesting that the cognate TCR repertoire is not limiting in HLA-A\*0201 carriers. TV9-CTL were polyfunctional and suppressed HIV replication *in vitro*. Thus, while *in vitro* immunization suggests that TV9 is potentially highly immunogenic, data from patients indicate that it would be necessary to devise immunization strategies that selectively elicit clonotypes with high functional sensitivities to overcome the typical subdominance of this epitope in the context of viral infection. Here, we identified mimic peptides (“mimotopes”) by probing a large positional scanning synthetic combinatorial nonapeptide library (PS-SCL)

with homogeneous, well-characterized *in vitro*-primed TV9-CTL [14]. We selected a peptide, TV9p6 (KINAWIKVV), with the lowest binding affinity to HLA-A\*0201 for detailed analysis, reasoning that an unstable peptide-HLA-A\*0201 complex and the attendant low epitope density would preferentially select clonotypes with high functional sensitivity. Parallel CD8<sup>+</sup> T-cell cultures specific for TV9 and TV9p6 were established to compare specificity and cross-recognition based on functional assays that included cytotoxicity, cytokine production and suppression of HIV replication *in vitro*. Concomitant repertoire analyses were conducted using a quantitative molecular approach to evaluate antigen-driven clonotype selection in relation to these functional attributes.

## Results

### Prediction of TV9 mimotopes by screening a positional scanning synthetic nonapeptide combinatorial library

A polyclonal TV9-CTL culture (TV9-1, index culture) was screened for cytolytic activity with 180 nonapeptide library mixtures as described previously [14]. Each well contained 10<sup>4</sup> CTL and 2 × 10<sup>3</sup> <sup>51</sup>Cr-labeled TAP-deficient, HLA-A\*0201-expressing T2 cells with 100 µg/mL of a peptide mixture. Significant and reproducible differences in lysis were observed between mixtures. Figure 1A is representative of three scans performed. Remarkably, the percent lysis with some mixtures, each containing 1.7 × 10<sup>10</sup> peptides at femtomolar concentrations, was equivalent to that observed with the TV9 peptide (30–50% versus 60% at 1 µg/mL). However, T-cell activation may be achieved *via* degenerate stimulation by many peptides within each mixture [15].

Six mixtures defined with residues corresponding to the TV9 sequence (hollow bars) were among the most stimulatory mixtures for sublibraries within that position. They were L in position 2 (P2), N in P3, W in P5, V in P6 and V in P8 and P9. Results with the P2 and P9 anchor positions confirmed the power and accuracy of this assay. L and M are known preferred residues in P2 of HLA-A\*0201; the scans showed that these mixtures were equally stimulatory. Likewise, mixtures that contained the preferred anchor residues in P9 (I, L and V) were almost equally active. N was the only stimulatory mixture in P3, suggesting that it may be a primary TCR contact site. P1 and P6 appeared degenerate, with at least five mixtures that were more or equally stimulatory than matching TV9 residues. Curiously, the mixtures corresponding to the native A and K in P4 and P7 were less stimulatory than those for W and P, respectively, suggesting that the natural residues are not optimal for recognition by TV9-1 CTL.

Based on the library scans, candidate variant peptides were designed for subsequent testing. To limit their number, substitutions were not made in positions thought to be important for TCR recognition (P3, P5 and P7 [16]) or in P9. Substitutions made in P1, P2, P4, P6 and P8 were mostly conservative or semi-conservative, except for the non-conservative replacement of A by W in P4 (Fig. 1B). A panel of 48 peptides with two to five replacements in the TV9 sequence was synthesized.

## Recognition of candidate peptides by TV9-CTL cultures

The candidate peptides were tested for recognition by TV9-CTL from five donors. Cytotoxicity was measured at four concentrations (1, 10, 100 and 1000 ng/mL) to estimate relative antigenicity. A peptide was considered cross-recognized if it elicited  $\geq 50\%$  of the lysis observed at the two highest concentrations of TV9.

Thirteen of the 48 peptides tested (27%) were cross-recognized by TV9-1 CTL and by at least one other culture (group I, Table 1). Four peptides (TV9p6, TV9p29, TV9p30 and TV9p18) were recognized by all cultures. The ability of non-index TV9-CTL to recognize 6–28 peptides encoding two to five substitutions suggests that recognition of the TV9/HLA-A\*0201 complex is quite degenerate. Curiously, TV9-2 and TV9-4 CTL were sensitized by 6 and 18 sequences not recognized by index CTL (group II, Table 1), thereby suggesting differences in the degree of repertoire overlap between donors.

## Immunogenicity of four broadly cross-recognized peptides

The ability of the first four peptides listed in Table 1 to prime TV9-cross-reactive CTL was determined (Fig. 2). TV9p30 was not immunogenic in three different donors (data not shown). TV9p6

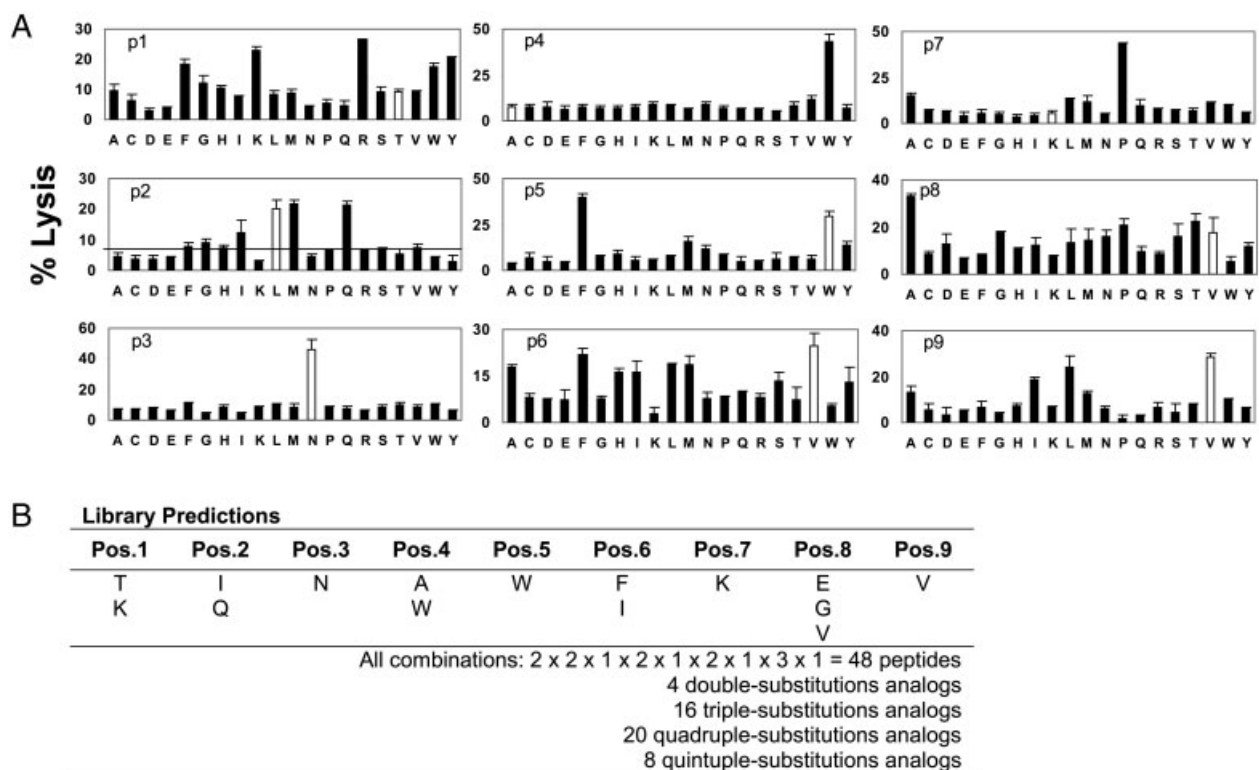
immunized CTL (TV9p6-2, TV9p6-5, TV9p6-6 and TV9p6-7) that cross-recognized TV9 consistently (Fig. 2A). TV9p5 was also immunogenic and elicited TV9-cross-reactive CTL from two donors (Fig. 2B). TV9p29 was immunogenic but induced cross-reactive CTL from only one of two cultures (p29-8, Fig. 2C). That double to quadruple substituted mimotopes stimulated cross-reactive CTL is consistent with our contention that the available naive T-cell pool for TV9 may be broad [11].

## Binding affinities of TV9 and its analogs to HLA-A2 class I antigens

Table 2 shows the binding affinities of TV9 and its mimotopes to members of the HLA-A2 supertype. Since HIV-specific CTL epitopes restricted by protective HLA-B alleles [17] show lower average peptide binding affinities than those of non-protective HLA-A-restricted epitopes [18], we selected TV9p6 with the lowest affinity to determine whether it is possible to consolidate the TV9 response around higher avidity clonotypes.

## CD8<sup>+</sup> cultures to TV9p6 and to TV9

Parallel CD8<sup>+</sup> cultures to TV9p6 or TV9 were generated from four donors and induction of peptide-specific T cells was monitored with



**Figure 1.** Scanning a nonapeptide PS-SCL to identify TV9 agonist peptides. (A) Index TV9-1 cytotoxicity elicited by the 180 mixtures of a nonapeptide PS-SCL. Each graph, designated p1–p9, represents a set of 20 mixtures having the defined amino acid listed on the x-axis at a given position. The y-axis denotes the percentage lysis of T2 cells in the presence of each mixture. The hollow bars represent the native TV9 amino acid sequence. Each mixture was assayed in triplicate and scanning was repeated three times. (B) Design of 48 candidate peptides. The rationale for the selection of a particular active mixture in each position is discussed in the text.

**Table 1.** Recognition of predicted candidate peptides, presented by T2 target cells, by the index and four allogeneic HLA-A\*0201+ TV9-CTL cultures as determined by cytotoxicity<sup>a)</sup>

Native	TV9	T	L	N	A	W	V	K	V	V	O	Residues substituted	% Lysis at peptide concentrations (ng/mL)																								
													TV9-1 (index)					TV9-2					TV9-3					TV9-4					TV9-5				
													1	10	100	1000	1	10	100	1000	1	10	100	1000	1	10	100	1000	1	10	100	1000	1	10	100	1000	1
Group I	p30	-	I	-	-	-	I	-	-	-	2	36	80	75	2	37	56	60	0	5	58	53	0	58	53	0	36	67	76								
	p6	K	I	-	-	-	I	-	-	-	3	31	77	78	10	34	51	51	0	0	30	45	2	8	52	76											
	p29	-	I	-	-	-	I	-	G	-	15	37	47	64	29	63	77	74	9	40	48	59	6	57	68	55	5	48	56	86							
	p5	K	I	-	-	-	I	-	G	-	0	20	44	51	64	84	84	85	14	30	30	34	1	0	25	53	8	4	13	58							
	p42	-	Q	-	-	-	I	-	-	-	2	7	27	41	9	63	75	74	0	24	46	48	0	9	59	83	2	2	29	28							
	p18	K	Q	-	-	-	I	-	G	-	0	11	29	44	29	66	75	73	7	28	41	46	0	43	81	0	5	35	49								
	p41	-	Q	-	-	-	I	-	G	-	13	32	42	52	47	79	84	77	8	31	40	53	0	37	65	80	0	3	28	28							
	p17	K	Q	-	-	-	I	-	G	-	4	33	44	61	66	83	86	82	11	24	29	28	0	15	69	87	0	2	3	14							
	p40	-	Q	-	-	-	I	-	E	-	0	17	39	46	33	73	78	70	17	26	32	34	0	0	0	1	0	5	5	14							
	p35	-	I	-	-	-	I	-	G	-	1	3	24	47	0	1	6	35	0	0	0	0	0	0	0	0	0	2	1	6	13						
	p47	-	Q	-	-	-	I	-	G	-	4	17	30	47	2	6	30	54	0	0	0	0	0	0	79	79	81	77	0	2	36	41					
	p23	K	Q	-	-	-	I	-	G	-	0	2	24	43	1	0	4	11	0	0	0	0	0	0	76	92	90	93	0	2	5	18					
Group II	p28	-	I	-	-	-	I	-	E	-	0	1	14	46	19	64	77	72	31	37	34	37	0	0	0	0	0	14	25	nd							
	p36	-	I	-	-	-	I	-	E	-	3	0	8	15	3	8	15	43	0	0	0	0	nd	34	66	78	73	0	4	8	29						
	p34	-	I	-	-	-	I	-	E	-	0	0	0	0	2	3	3	12	0	0	0	4	4	1	13	56	67	0	0	nd							
	p4	K	I	-	-	-	I	-	E	-	43	72	79	74	43	72	79	74	25	32	32	34	0	0	0	0	0	8	0	9	36						
	p10	K	I	-	-	-	I	-	E	-	1	9	15	51	1	9	15	51	0	0	2	11	33	83	87	89	0	3	16	31							
	p11	K	I	-	-	-	I	-	E	-	0	0	0	0	0	1	2	1	0	0	0	0	0	0	0	11	37	1	0	10	nd						
	p39	-	Q	-	-	-	I	-	G	-	0	0	0	0	0	4	13	16	0	0	0	0	0	57	84	84	82	3	2	14	nd						
	p37	-	Q	-	-	-	F	-	E	-	2	2	30	52	2	2	30	52	0	1	1	20	0	0	0	0	1	19	0	7	23	52					
	p38	-	Q	-	-	-	F	-	G	-	0	6	26	38	0	0	12	32	0	0	0	1	17	0	0	0	0	2	0	1	7						
	p15	K	Q	-	-	-	F	-	E	-	0	0	17	40	15	66	69	69	0	0	0	7	35	0	0	3	38	68	0	6	9	47					
	p13	K	Q	-	-	-	F	-	E	-	0	0	0	29	0	12	48	57	1	19	28	29	0	0	0	0	0	6	6	9	14						
	p14	K	Q	-	-	-	F	-	G	-	0	0	8	36	19	8	38	72	0	0	0	17	0	0	0	0	0	0	6	6	9	38					
	p45	-	Q	-	-	-	F	-	E	-	0	0	0	0	0	0	9	54	0	14	22	27	31	77	84	79	0	0	0	1							
	p43	-	Q	-	-	-	F	-	E	-	1	0	0	0	0	0	0	3	0	0	0	7	12	65	74	82	0	4	0	2							
	p21	K	Q	-	-	-	F	-	G	-	0	0	0	32	0	1	0	4	0	0	0	3	46	71	76	83	0	0	4	0							
	p19	K	Q	-	-	-	F	-	E	-	0	0	0	0	3	0	10	48	0	20	29	39	43	89	89	95	1	4	9	16							
	p20	K	Q	-	-	-	F	-	E	-	0	0	0	0	0	0	0	0	0	0	0	0	0	47	87	83	0	3	11	23							
	p46	-	Q	-	-	-	I	-	E	-	2	7	20	51	2	7	20	51	0	0	0	10	32	68	84	83	0	3	20	5							
	p24	K	Q	-	-	-	I	-	E	-	3	0	1	15	31	31	31	nd	2	27	34	40	78	86	87	0	0	5	6								
	p22	K	Q	-	-	-	I	-	E	-	0	0	0	0	0	0	0	3	0	0	0	4	17	34	66	92	90	0	2	4							
	p25	-	I	-	-	-	F	-	E	-	0	0	0	0	1	4	33	61	0	0	0	3	21	0	0	0	5	1	5	37	nd						
	p26	-	I	-	-	-	F	-	G	-	0	0	0	0	6	12	27	0	0	0	0	15	0	0	0	0	8	36	0	1	7	nd					
	p3	K	I	-	-	-	F	-	E	-	12	63	68	58	12	63	68	58	0	0	0	15	0	0	0	0	0	0	1	0	31	68					
	p1	K	I	-	-	-	F	-	E	-	1	0	4	37	1	0	4	37	0	0	0	10	0	0	0	0	0	0	0	2	10						
	p2	K	I	-	-	-	F	-	G	-	0	6	52	64	0	6	52	64	0	0	0	0	0	0	0	0	0	1	0	10	19						
	p33	-	I	-	-	-	F	-	E	-	1	2	2	13	1	2	2	13	0	4	29	36	9	47	73	77	0	0	13	6							
	p32	-	I	-	-	-	F	-	G	-	0	0	0	0	0	0	1	8	0	0	0	1	19	59	84	78	0	4	5	20							
	p9	K	I	-	-	-	F	-	E	-	0	0	0	0	0	1	0	3	0	6	29	38	0	23	79	86	8	9	13	nd							
	p7	K	I	-	-	-	F	-	E	-	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	14	7	4	3	40						
	p8	K	I	-	-	-	F	-	G	-	0	0	0	0	0	0	0	6	0	0	0	0	0	0	18	81	87	0	5	9	43						

No. of the 48 artificial sequences recognized

<sup>a)</sup> Positive recognition for TV9 and reactive artificial sequences is boldfaced and shaded. nd, not determined.

tetramers (Fig. 3). For donors 2, 6 and 7, the results were similar (Fig. 3A–C). TV9p6-specific T cells were detected as early as day 20. By day 35, the TV9p6-cultures were essentially homogeneous by tetramer staining. Moreover, the staining patterns of TV9p6-T cells with both TV9p6 and TV9 tetramers were essentially identical, indicating that TV9p6-T cells recognized TV9. Parallel TV9-cultures showed ~90% TV9-T cells by day 40.

Differential tetramer staining was observed in the TV9p6-8 culture (Fig. 3D). The majority (74%) of the cells stained intensely and exclusively with the TV9p6-tetramer on day 41; only weak and poorly distinguished staining was observed with the TV9-tetramer. A similar pattern of low intensity staining was detected in the parallel TV9-8 culture, thereby indicating that the naïve repertoire for TV9 from this individual might consist primarily of low avidity clonotypes. Thus, TV9p6 primed both TV9-specific clonotypes and a distinct non-cross-reactive population in this donor.

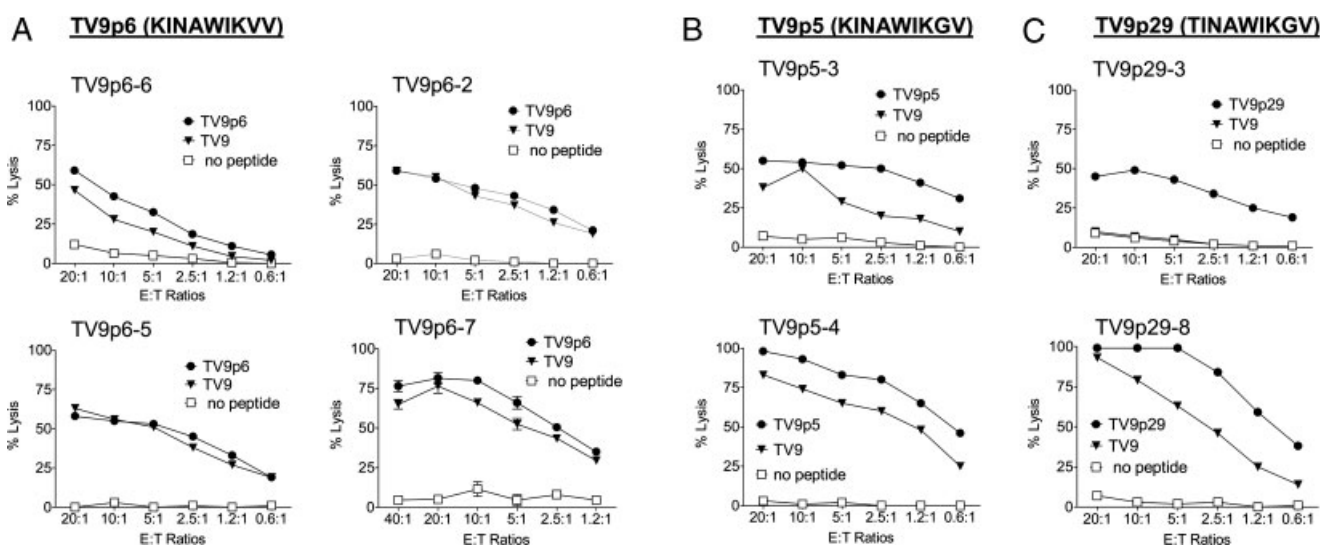
### CD8 coreceptor dependence of cytotoxicity mediated by TV9- and TV9p6-CTL

Homogeneous TV9- and TV9p6-CTL cultures were assessed for their dependence on CD8 compensation using peptide-pulsed C1R target cells expressing WT HLA-A\*0201 (C1R<sup>WT</sup>) or point-mutated HLA-A\*0201 that cannot bind CD8 (C1R<sup>CD8null</sup>) [19] in chromium release cytotoxicity assays. Lysis of TV9-pulsed C1R<sup>CD8null</sup> cells by TV9-CTL was substantially reduced compared to lysis of TV9-pulsed C1R<sup>WT</sup> cells (Fig. 4A–D), thereby indicating that abrogation of CD8 binding impaired but did not obviate downstream functional activation. Figure 4E–H compare lysis mediated by

parallel TV9p6-cultures over an effectual range of TV9p6 or TV9 peptide concentrations. TV9p6-T-cell cultures were equally cytotoxic to C1R<sup>WT</sup> cells pulsed with either peptide (filled symbols). Cytotoxicity was reduced without CD8 participation (open symbols), although this loss was markedly less pronounced for the cognate peptide in most cases. The use of paired target cells revealed that TV9p6-T cells exhibited greater intrinsic avidity than TV9-T cells for their cognate pMHC complexes ( $p = 0.037$ ; comparison of percent change in cytotoxicity at  $10^{-7}$  M with C1R-A2<sup>WT</sup> versus C1R-A2<sup>CD8null</sup> cells for TV9-CTL and TV9p6-CTL using an unpaired two-tailed Student's *t*-test) [20]. However, there was no significant difference between the CD8 dependencies of TV9-CTL and TV9p6-CTL with respect to lysis of target cells pulsed with the natural peptide. Importantly, these data show that CD8 binding to pMHC can fully compensate for any “sensitivity” disadvantages associated with TV9 recognition in terms of the ability of TV9p6-T cells to kill *in vitro*.

### Functional profile of TV9- and TV9p6-CTL

As polyfunctionality of HIV-specific CD8<sup>+</sup> T cells appears to correlate with viral control *in vivo* [21, 22], the ability of cultured T cells to degranulate and secrete IL-2, TNF- $\alpha$  and IFN- $\gamma$  was assessed after stimulation with peptide-pulsed C1R-A2<sup>WT</sup> cells (Table 3). Five of the six cultures were polyclonal, with tetramer<sup>+</sup> cells staining with more than one TCR V $\beta$  mAb; only TV9p6-1 was potentially monoclonal. The percentage of tetramer<sup>+</sup> TV9- or TV9p6-T cells corresponded to that expressing surface CD107a/b upon antigen encounter. IFN- $\gamma$  was produced by most CD107a/b-expressing cells, a proportion of which also produced IL-2 and



**Figure 2.** Evaluation of the ability of three agonist peptides (TV9p6, TV9p5 and TV9p29) listed in Table 1 to induce antigen-specific, TV9-cross-reactive CTL by *in vitro* immunization of naïve CD8<sup>+</sup> T cells from two to four healthy seronegative donors. Cultures are labeled as TV9pX–Y, where TV9pX identifies the inducing peptide and the numeral Y denotes a distinct donor. (A) TV9p6-6, TV9p6-2, TV9p6-5 and TV9p6-7; (B) TV9p5-3 and TV9p5-4; and (C) TV9p29-3 and TV9p29-8. CD8<sup>+</sup> T cells were assayed for specificity and cross-recognition of TV9 using chromium release assays at effector:target (E:T) ratios ranging from 20:1 to 0.6:1. T2 cells without peptide were included as negative controls in each assay. Data are shown as mean  $\pm$  SEM of triplicate assays and are representative of two independent experiments; in most cases, the error bars are smaller than the plot symbols.

**Table 2.** Binding affinities of TV9 and its mimic peptides to HLA-A2 supertype molecules<sup>a)</sup>

Peptide	Sequence										# Substitutions	IC <sub>50</sub> (nM)				
												A*0201	A*0202	A*0203	A*0206	A*6802
TV9	T	L	N	A	W	V	K	V	V	Native	701	179	108	1965	23 937	
TV9p5	<u>K</u>	<u>I</u>	N	A	W	<u>I</u>	K	<u>G</u>	V	4	536	433	10	224	36 760	
TV9p6	<u>K</u>	<u>I</u>	N	A	W	<u>I</u>	K	V	V	3	5120	2950	56	13,449	–	
TV9p29	T	<u>I</u>	N	A	W	<u>I</u>	K	<u>G</u>	V	3	1062	706	15	2248	269	
TV9p30	T	<u>I</u>	N	A	W	<u>I</u>	K	V	V	2	4228	1835	112	12 581	6218	
SL9	S	L	Y	N	T	V	A	T	L	Native	367	79	19	–	–	

<sup>a)</sup> Dash indicates IC<sub>50</sub> > 10 000 nM.

TNF- $\alpha$ . Thus, both TV9p6- and TV9-CTL exhibited a degree of polyfunctionality.

We next quantified the amount of IFN- $\gamma$  secreted by parallel TV9- and TV9p6-CTL after antigenic stimulation over a range of concentrations (Fig. 5). Interestingly, both TV9- and TV9p6-CTL cultures showed greater sensitivity to stimulation by TV9 compared to TV9p6 as shown by the lower EC<sub>50</sub> values for TV9. Although this may reflect in part the different binding affinities of TV9 and TV9p6 peptides to HLA-A\*0201 (Table 2), these data suggest that CTL elicited by the artificial TV9p6 sequence would release IFN- $\gamma$  as readily as those sensitized by the natural epitope when engaging HIV-infected cells.

### TV9p6-CTL suppress HIV replication *in vitro*

The ability of TV9- and TV9p6-CTL to suppress replication of NL4-3.1 and JR-CSF *in vitro* was assessed (Fig. 6). Both viruses encode for TV9. Increasing suppression of NL4-3.1 replication in T1 cells on day 3 was observed with increasing E:T ratios of TV9-8 and TV9p6-8 CTL (Fig. 6A). Moreover, no significant difference in suppression was observed over the range of E:T ratios. Similar antiviral efficacy between four sets of parallel TV9- and TV9p6-CTL cultures was observed at two time points (Fig. 6B). Lastly, all CTL cultures inhibited the replication of JR-CSF in non-CD8<sup>+</sup> PBMC (Fig. 6C). Thus, mimotope-primed CTL inhibited HIV replication *in vitro* at least as efficiently as those activated by the WT peptide.

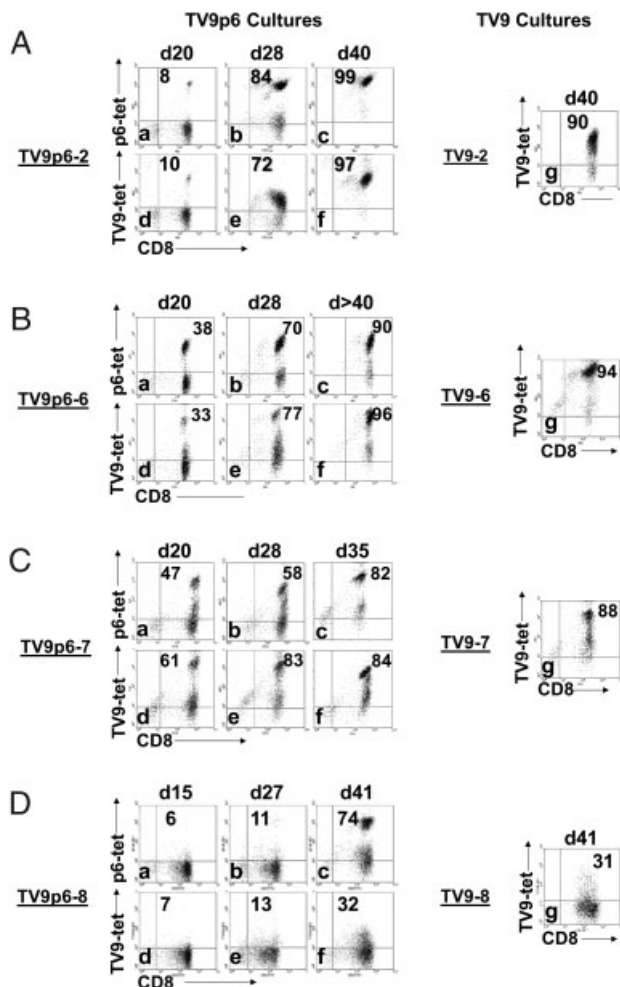
### TCRBV gene usage by parallel cultures of TV9- and TV9p6-CTL

To characterize further the nature of TV9- and TV9p6-CTL, we examined clonotype usage within TV9 tetramer-binding CD8<sup>+</sup> T-cell populations in parallel cultures from several donors using a template-switch anchored RT-PCR to amplify all expressed *TCRB* gene products without bias as described previously [23] (Table 4). All TV9-CD8<sup>+</sup> T-cell populations were oligoclonal, comprising six or fewer TCR clonotypes; distinct hierarchical frequencies were also apparent. Furthermore, despite starting

naïve T-cell pools derived from the same source, parallel TV9- and TV9p6-CTL cultures were largely distinct in terms of TV9-specific clonal composition. However, a degree of intra-individual clonal overlap was observed between TV9-6 and TV9p6-6 cultures and between TV9-7 and TV9p6-7 cultures (light shading, italicized sequences). Importantly, this demonstrates that at least some identical clonotypes were present within the initial naïve T-cell pools and provides evidence that differential priming was not solely a result of stochastic “sampling” bias. Of note, a public clonotype, differentially encoded at the nucleotide level, was shared between two individuals (TV9p6-6 and TV9-9; darker shading, bold sequences).

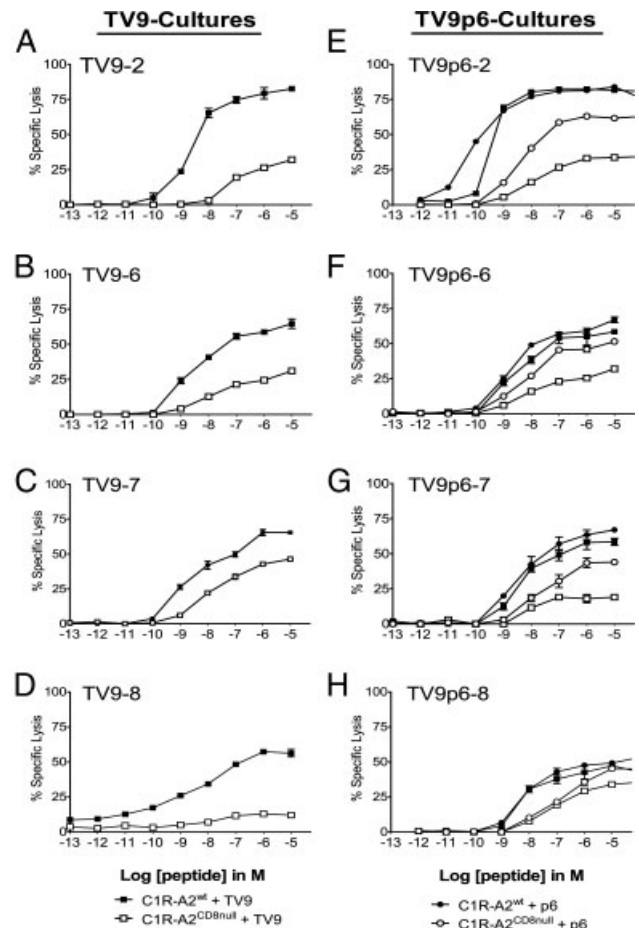
### Discussion

The value of using conserved regions of the HIV proteome for the purposes of vaccination has been recognized for some time [24–26]. However, although these domains are populated with CD8<sup>+</sup> T-cell epitopes, most have not been correlated with protection in carriers of the majority of HLA class I alleles. We propose that the use of conserved regions within HIV as vaccines for non-protective class I alleles will depend on whether it is feasible to improve the intrinsic immunogenicity of these natural sequences. This may be achieved by various means, such as modulating the usual patterns of immunodominance [27], improving processing and presentation [28, 29], or stimulating with mimotopes [14]. Indeed, there is suggestive evidence that protection can be achieved by vaccine-induced responses to a subdominant HLA-A\*02-restricted epitope [30]. TV9 is an example of a conserved subdominant epitope with characteristics of a useful vaccine target. Our data with CTL primed *in vitro* from healthy donors revealed that it may be necessary to stimulate high avidity clonotypes selectively within the structurally diverse TV9-T cell precursor pools [11]. In this study, we identified and tested mimotopes of TV9 for the purpose of recruiting more appropriate HIV-specific CD8<sup>+</sup> T cells. This approach is based on three principles: (i) a single TCR can respond to many different peptides [31]; (ii) the pool of precursor T-cell clonotypes specific for a particular epitope can be very diverse [32, 33]; and (iii) the requirements for T-cell priming and the triggering of downstream effector functions are different [34].



**Figure 3.** Specificity and cross-reactivity of TV9p6-CTL monitored over time by tetramer staining. TV9p6-tetramer binding by the CD8<sup>+</sup> cultures TV9p6-2, TV9p6-6, TV9p6-7 and TV9p6-8 at three time points is shown in panels A-a to A-c, B-a to B-c, C-a to C-c and D-a to D-f. Cross-reactivity of the TV9p6-cultures was assessed concomitantly by staining with TV9-tetramer (A-d to A-f, B-d to B-f, C-d to C-f and D-d to D-f). For comparison, TV9-tetramer staining of the CD8<sup>+</sup> parallel cultures TV9-2, TV9-6, TV9-7 and TV9-8 is shown in Panels A-g, B-g, C-g and D-g, respectively. Numbers in the upper right quadrant represent the percentage of CD8<sup>+</sup> tetramer<sup>+</sup> stained cells. An irrelevant tetramer was used to gate for specific tetramer binding. Data are representative of at least three independent experiments.

We screened a PS-SCL with a TV9-CTL culture to enable *de novo* design of mimotopes that not only stimulate TV9-CTL but also immunize cross-reactive CTL *in vitro*. The peptide library that we used consists of complex nonapeptide mixtures with one defined amino acid in one of the sequence positions and mixtures of all proteogenic amino acids excepting cysteine (which can be oxidized to crosslink peptides) in the remaining positions [35]. Up to  $323 \times 10^9$  different peptides are represented in this library format. This strategy has been applied most extensively in autoimmune diseases and with promising clinical observations in cancer [36]. We have previously applied this approach to the



**Figure 4.** CD8 dependency of cytotoxic activity mediated by TV9- and TV9p6-CTL. Parallel cultures to TV9 or TV9p6, established by *in vitro* immunization using peptide-pulsed DC, were identified as TV9-Y or TV9p6-Y, where the numeral Y denotes a distinct donor. (A) TV9-2, (B) TV9-6, (C) TV9-7, (D) TV9-8 and parallel (E) TV9p6-2, (F) TV9p6-6, (G) TV9p6-7 and (H) TV9p6-8 CD8<sup>+</sup> cultures were assayed for functional sensitivity in a chromium release assay using C1R-A2<sup>wt</sup> and C1R-A2<sup>CD8null</sup> targets loaded with a range of concentrations of TV9p6 or TV9 peptide ( $10^{-5}$  to  $10^{-13}$  M) at an E:T ratio of 10:1 (—■—■—, —●—●—: C1R-A2<sup>wt</sup> pulsed with TV9 and TV9p6, respectively; -□-□-, -○-○-: C1R-A2<sup>CD8null</sup> pulsed with TV9 and TV9p6, respectively). Lysis of C1R cells alone was subtracted to give percent specific lysis. Data are shown as mean  $\pm$  SEM of triplicate assays and are representative of two independent experiments; in most cases, the error bars are smaller than the plot symbols.

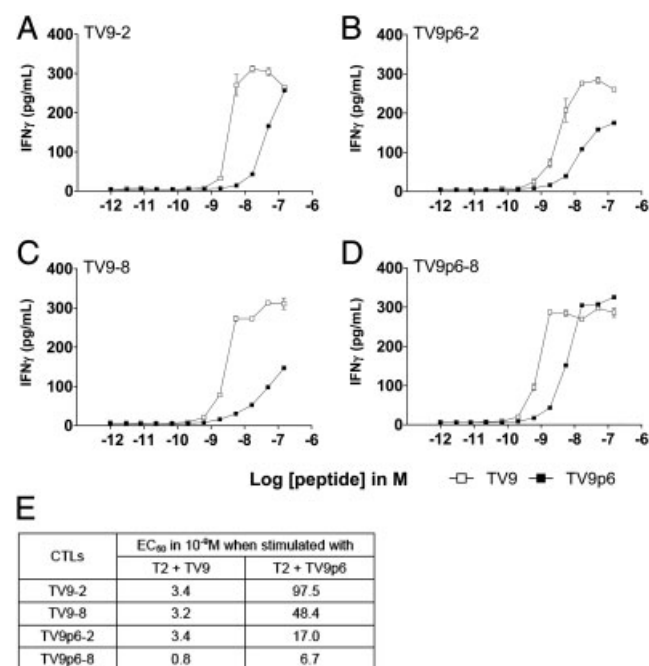
“chronic immunodominant” HLA-A\*0201-restricted SL9 (p17 Gag<sub>77–85</sub>) epitope and identified a mimotope that primed qualitatively superior CTL from the naïve repertoires of healthy donors *in vitro* and triggered more extensive expansion of antigen-experienced CD8<sup>+</sup> T cells from patients [14].

Very large numbers of mimotopes with different sequences and T-cell activation potencies can be identified for each epitope, with each mimotope addressing a different repertoire that intersects around the natural HIV epitope. Here, we specifically explored whether a variant peptide (TV9p6) with significantly lower binding affinity to HLA-A\*0201 than the native epitope, presumably leading

**Table 3.** CD107a/b mobilization and production of cytokines after stimulation with TV9p6 or TV9 loaded onto C1R-A2<sup>wt</sup> cells in three sets of parallel CTL cultures<sup>a)</sup>

CTL cultures (% tetramer <sup>+</sup> cells)	V $\beta$ Staining (% tetramer <sup>+</sup> cells)	Stimulating peptide	% T cells in culture staining for			
			CD107a/b	CD107a/b IL-2	CD107a/b TNF- $\alpha$	CD107a/b IFN- $\gamma$
TV9p6-1 (100)	V $\beta$ 2 (100)	TV9p6	81	33	33	81
		TV9	71	22	23	69
TV9-1 (98)	V $\beta$ 8 (32), V $\beta$ 17 (19), V $\beta$ 21.3 (4), V $\beta$ 22.3 (4)	TV9	81	8	9	72
TV9p6-4 (52)	V $\beta$ 2 (40), V $\beta$ 8 (2), V $\beta$ 14 (1), V $\beta$ 21.3 (2)	TV9p6	68	20	10	48
		TV9	61	15	9	46
TV9-4 (23)	V $\beta$ 8 (2), V $\beta$ 14 (1), V $\beta$ 22 (2), V $\beta$ 9 (14)	TV9	81	1	11	41
		TV9	81	1	11	41
TV9p6-5 (40)	V $\beta$ 8 (3), V $\beta$ 12 (1), V $\beta$ 22 (4)	TV9p6	44	25	21	20
		TV9	25	2	8	9
TV9-5 (57)	V $\beta$ 22 (50)	TV9	61	34	48	61

<sup>a)</sup> No staining was detected using an irrelevant peptide (YV9).

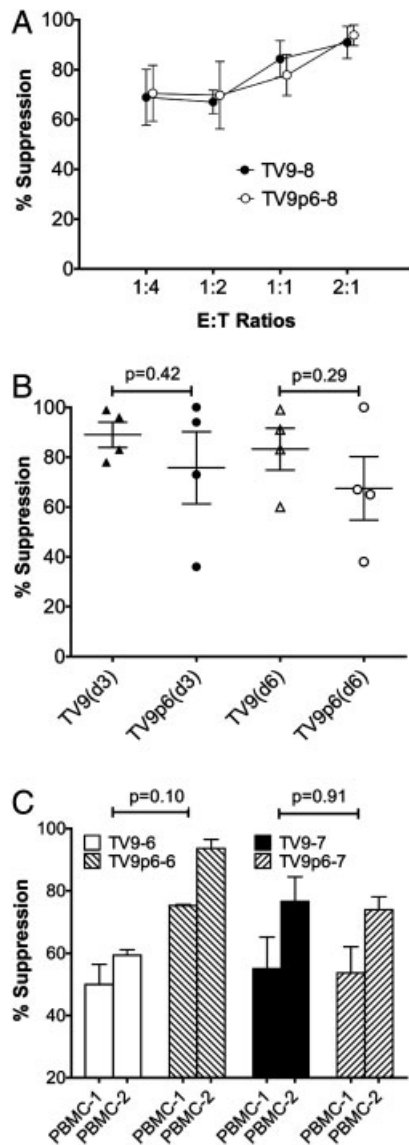


**Figure 5.** Functional sensitivity for two sets of parallel cultures determined by IFN- $\gamma$  secretion. (A) TV9-2, (B) TV9p6-2, (C) TV9-8 and (D) TV9p6-8 cultures were stimulated at an E:T ratio of 1:10 with T2 cells pulsed with either TV9 or TV9p6. IFN- $\gamma$  secretion into the supernatant was quantified by ELISA after 48 h. (E) EC<sub>50</sub> values were calculated for the TV9 and TV9p6 peptides in each case with Graphpad Prism V software. Data are shown as mean  $\pm$  SEM of triplicate assays and are representative of two independent experiments; in most cases, the error bars are smaller than the plot symbols.

to a reduced pMHC density on the surface of APC, would focus the subdominant TV9-CTL response. In fact, a reciprocal relationship between binding affinity and functional sensitivity was recently reported for a cancer epitope and its agonist [37]. CTL cultures specific for TV9 and TV9p6 were almost always cross-reactive as

determined by a shared capacity to lyse target cells loaded over a broad range of peptide concentrations, thereby suggesting considerable overlap between their recognition properties despite differences in three of nine positions between the two peptides. Moreover, CTL primed with both specificities consistently suppressed HIV replication *in vitro*, thereby indicating that they cross-recognized naturally processed and presented TV9. Although altered peptide ligands for other CTL epitopes can trigger differential responses [14], modification of the responding TCR repertoire by TV9p6 did not significantly alter any of the CTL functions or properties assessed, including antigen sensitivity measured by cytotoxicity or avidity measured by the intensity of tetramer staining. Moreover, the fact that highly functional CTL were generated by TV9p6 indicates that either CTL activation does not necessarily depend on peptide binding affinity to class I antigens or, alternatively, that the low binding affinity of TV9p6 to HLA-A\*0201 exceeded a minimal threshold. Our finding is in line with recent reports showing that neither immunodominance nor the efficacy of CTL responses correlates with the binding affinity of the epitopic peptides to class I antigens [18, 38]. Indeed, substantially greater selection pressure on HIV is imposed by HLA-B compared to HLA-A alleles [17] despite the lower average peptide binding affinity of the former [18].

The quality of a virus-specific CD8<sup>+</sup> T-cell response ultimately rests on the availability of “appropriate” clonotypes in the host T-cell repertoire [39]. In HIV infections, most studies have relied on the analysis of patient responses to infections *in vivo*. Although informative, they provide only partial insight into the diversity of the immune repertoire among individuals. Analysis of the pre-infection virus-specific T-cell pools will help complete the picture and may be particularly cogent for the design of prophylactic vaccines. For example, we noted a non-cross-reactive, TV9p6-tetramer binding population in one of the four donors shown in Fig. 3, indicating inter-individual differences in the intersection between the TCR repertoires specific for TV9 and TV9p6. This finding underscores the difficulty in selecting appropriate



**Figure 6.** TV9p6-CTL suppress HIV replication at least as efficiently as TV9-CTL. (A) Percent suppression of NL4-3.1 replication in T1 cells at day 3 post-infection by tetramer<sup>+</sup> T cells at different E:T ratios as indicated. Data are shown as mean  $\pm$  SEM of triplicate assays and are representative of two independent experiments. (B) Suppression of NL4-3.1 virus in T1 cells by TV9- and TV9p6-CTL cultures. The average percent suppression by TV9-2, TV9-6, TV9-7 and TV9-8 T cells at day 3 and 6 are indicated by  $\blacktriangle$  and  $\triangle$ , respectively. Likewise, suppression mediated by TV9p6-2, TV9p6-6, TV9p6-7 and TV9p6-8 T cells at the two time points are denoted by the symbols  $\bullet$  and  $\circ$ , respectively. The E:T ratio for this assay was 2:1. Data are representative of two independent experiments. The concentrations of p24 in infected T1 cell cultures in the absence of CTL were  $0.3 \pm 0.03$  and  $0.8 \pm 0.1$  ng/mL on day 3 and  $93 \pm 14$  and  $684 \pm 48$  ng/mL on day 6. (C) Suppression of HIV JR-CSF replication in two acutely infected, allogeneic HLA-A\*0201-matched CD8-depleted PBMC cultures by two parallel CTL cultures (TV9-6 and TV9p6-6; TV9-7 and TV9p6-7) at day 9 post-infection. The concentrations of p24 in infected PBMC-1 and PBMC-2 cultures in the absence of CTL were 311 and 210 ng/mL, respectively. The *p* values comparing suppression by TV9- and TV9p6-CTL were determined using an unpaired, two-tailed Student's *t*-test with Graphpad Prism V software.

epitope variants that will raise vigorous cross-reactive CD8<sup>+</sup> T cells without inadvertently activating responder cells that are not specific for the virus. These non-HIV responses can be potentially deleterious, perhaps working against the emergence of new responses to viral variants [40, 41]. On the other hand, subsequent HIV infection may selectively activate only the virus-specific memory pool generated by pre-emptive vaccination with mimotopes, thus promoting immunological control of HIV. The use of artificial sequences may be particularly important for “obligate” subdominant epitopes, which are recognized by intrinsically small T-cell precursor populations or suppressed by emerging T cells that target other epitopes. This study examines one strategy to heighten the intrinsic immunogenicity of known conserved subdominant determinants.

Molecular analysis of *TCRB* gene expression showed that CTL cultures specific for TV9 and TV9p6 were oligoclonal and generally dominated by one or two clonotypes (Table 4). Although no TCR motifs were apparent, biased usage of certain sequences was found in two of three parallel cultures (TV9-6/TV9p6-6 and TV9-7/TV9p6-7). This suggests considerable clonal overlap and a high precursor frequency of certain cross-reactive clonotypes. Conservation within the CDR3 loop encoded distinctly at the nucleotide level was also detected between individuals, indicating a common antigen-specific “public TCR”. A recent study showed that usage of public clonotypes within a particular protective Gag-specific CD8<sup>+</sup> T-cell response in acute SIV infection can act as a molecular signature of biological outcome [42]. These findings emphasize the need to develop immunization strategies that selectively recruit efficacious epitope-specific CD8<sup>+</sup> T-cell clonotypes [43].

In summary, despite its low binding affinity to HLA-A\*0201, TV9p6 did not selectively mobilize high avidity clonotypes, although it did effectively activate fully functional HIV-specific CTL. Comparison of *TCRB* gene usage between CD8<sup>+</sup> T-cell populations specific for TV9 and TV9p6 showed that distinct clonotypes were recruited in each case from the same initial naïve T-cell pool. Thus, mimotope-based vaccination represents a potentially viable approach to recruit additional cognate CD8<sup>+</sup> T-cell clonotypes that could overcome the intrinsic limitations of repertoire mobilization and thereby help to override natural patterns of epitope subdominance.

## Materials and methods

### Healthy seronegative donors

Heparinized blood was collected from healthy seronegative HLA-A\*0201<sup>+</sup> volunteers. High resolution HLA genotyping was performed at the Department of Transfusion Medicine, National Institutes of Health. This study was approved by the Human Investigation Committee at Wayne State University School of Medicine and the University of Texas at El Paso. All subjects provided written informed consent.

**Table 4.** TCRBV and TCRJ usage, CDR3 amino acid sequence and percent frequency of TV9-specific CD8<sup>+</sup> T cell clonotypes in parallel CTL cultures

CTLs	HLA class I alleles	Vβ (Arden)	IMGT TRB	CDR3	TRBJ	Frequency
TV9-7	A*0201,A11 B18,B27	13	6-1	CASSEEGAWNTGELF	2-2	90
		9	3-1	CASHTSGGLKDTQY	2-3	10
	TV9p6-7	7	4-3	CASSPRGDWEEKAGELF	2-2	77
		13	6-1	CASSEEGAWNTGELF	2-2	15
		4	29-1	CSVEDPDRGYEQY	2-7	8
TV9-6	A*0201,A29 B40,B44	14	27	CASSRRGDGFQPH	1-3	36
		13	6-6	CASRDFYNEQF	2-1	29
	2	20-1	CSAFSSGTGGVTGELF	2-2	14	
	7	4-3	CASSQDPSPSTDTQY	2-3	7	
	6	7-8	CASSLWGLAPDNEQF	2-1	7	
	14	27	CASSLWGGPMETQY	2-5	7	
TV9p6-6		2	20-1	CSAFSSGTGGVTGELF	2-2	82
		6	7-2	CASSLVPQGEEQY	2-7	6
		2	20-1	CSAFSSGTGGVTGEPF	2-2	6
		2	20-1	CSAFSSGTGGVTGELF	2-2	6
TV9-9	A*0201,A24 B15,B40	6	7-2	CASSLVPQGEEQY	2-7	88
		6	7-2	CASSLVPQGEPEY	2-7	12
TV9-3	A2	3	28-0	CASSYRGQSYEQY	2-7	94
		4	29-1	CSGRGDSYTEAF	1-1	4
		5, 31	5-8	CASSEYRGPSSYEQY	2-7	1
		3	28-0	CASSCRGQSYEQY	2-7	1
TV9-2	A*0201 B15,B40	2	20-1	CSARDAGGDYGYT	1-2	69
		8	12-4	CASSDTGELF	2-2	26
		9	3-1	CASSQVKIGQGNEKLF	1-4	6
TV9p6-2		2	20-1	CSASRDSYEQY	2-7	100

### Generation of *ex vivo* primed peptide-specific CD8<sup>+</sup> T-cell cultures from healthy seronegative donors

The procedures for the generation of peptide-specific CTL by *in vitro* immunization of naïve CD8<sup>+</sup> T cells have been reported [44]. In brief, DC were derived from adherent peripheral blood monocytes after culturing for 7 days in complete medium (RPMI 1640 medium containing 10% autologous serum, 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine) supplemented with GM-CSF (1000 U/mL; Leukine Sargramostim, Bayer HealthCare Pharmaceuticals, Wayne, NJ, USA) and IL-4 (500 U/mL; Peprotech, Rocky Hill, NJ, USA). Positively selected autologous CD8<sup>+</sup> T cells (Dynabeads; Dynal) were primed with irradiated (4000 cGy) DC pulsed for 2 h with 10 µg/mL peptide at a T cell–DC ratio of 5:1 in 48-well cluster plates. T cells were subsequently re-stimulated every 7–10 days with autologous monocytes pulsed with peptides [14, 44]. Ten ng/mL IL-7 (Genzyme) was added on the day of priming and re-stimulation; 20 U/mL IL-2 was added 1 and 4 days later.

### Target cell lines

Cytotoxicity or intracellular cytokine secretion was assessed against peptide-pulsed T2 cells (TAP-deficient T-B lymphoblast hybrids [45]) or C1R cells expressing either full-length WT

HLA-A\*0201 (C1R-A2<sup>wt</sup>) or a point-mutated variant (C1R-A2<sup>CD8null</sup>) that does not bind the CD8 coreceptor [46]. The HIV-1 replication-permissive T1 cell line expressing HLA-A\*0201 was used for viral suppression assays. All cell lines were maintained in RPMI with 10% heat-inactivated fetal calf serum.

### Flow cytometric analysis

Directly conjugated mAb to CD8 (FITC- and PE-RPA-T8), CD107a (FITC-H4A3), CD107b (FITC-H4B4), HLA-A2 (FITC-BB7.2), IFN-γ (PE-B27), IL-2 (PE-MQ1-17H12) and TNF-α (PE-Mab11) were purchased from BD Pharmingen (San Diego, CA, USA). Vβ family-specific mAb were obtained from Beckman Coulter (Miami, FL, USA). Tetramer stains were performed on ice for 30 min; cells were then washed and stained for CD8 at 4°C for an additional 20 min. Staining with an irrelevant tetramer was used as a control. Stained cells were acquired with a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) and data analyzed with WinMDI software. Intracellular cytokine production and degranulation were determined after stimulation for 4 h with peptide-pulsed (10 µg/mL) T2 or C1R cells at 37°C as described previously [47]. Gating was performed on CD8<sup>+</sup> T cells and greater than 10 000 events were collected for each sample. TV9- and TV9p6-tetramers were obtained from the NIH Tetramer Core Facility (Atlanta, GA, USA).

## Nonapeptide library and assay for cytotoxicity with library mixtures

A TV9-CTL culture [11] was used to scan an L-amino acid nonapeptide PS-SCL to identify candidate mimotope sequences [14]. All nonapeptides present in the library contained a free N-terminus and an amidated C-terminus. The PS-SCL comprised 180 mixtures in an OX8 format, where O represents one of the 20 L-amino acids in a defined position and X represents all natural L-amino acids except L-cysteine at each of the remaining positions. Each mixture was composed of  $1.7 \times 10^{10}$  peptides and the total X9 library consisted of some  $323 \times 10^9$  different nonapeptides in approximately equimolar concentration. For stimulation of the index TV9-1, CTL were washed and resuspended at  $1 \times 10^5$  cells/mL in complete medium 7 days after specific re-stimulation. Then, 100  $\mu$ L of this cell suspension was added to triplicate wells of 96-well U-bottom plates containing  $2 \times 10^3$   $^{51}\text{Cr}$ -labeled T2 cells and the various peptide library mixtures (100  $\mu\text{g}/\text{mL}$ ). Cells were cultured for 4 h at 37°C prior to harvesting and calculation of percent lysis determined with respect to control wells. Each mixture was assayed in triplicate and scanning was repeated three times. The library was prepared at Multiple Peptide Systems (San Diego, CA, USA).

## Peptides

TV9 (TLNAWVKVV) and agonist peptides p30 (TINAWIKVV), TV9p6 (KINAWIKVV), p29 (TINAWIKGV) and p5 (KINAWIKGV) peptides were purchased at > 90% purity from Genemed Synthesis (San Francisco, CA, USA).

## MHC-peptide binding assays

Quantitative assays to measure the binding of peptides to HLA A\*0201, A\*0202, A\*0203, A\*0206 and A\*6802 molecules were based on the inhibition of binding of a radiolabeled standard peptide as described previously [48]. Peptides were tested at six different concentrations in three or more independent assays and the concentration of peptide yielding 50% inhibition of the binding of the radiolabeled probe peptide ( $\text{IC}_{50}$ ) was calculated. Under the conditions used, where [radiolabeled probe] < [MHC] and  $\text{IC}_{50} \geq [\text{MHC}]$ , the measured  $\text{IC}_{50}$  values are reasonable approximations of the true  $K_d$  values.

## IFN- $\gamma$ detection by ELISA

IFN- $\gamma$  secretion was determined by ELISA with an OptEIA Set (BD Pharmingen) in supernatants of T cells re-stimulated with peptide-pulsed T2 cells for 48 h. The range of sensitivity for IFN- $\gamma$  was 15–500 pg/mL.  $\text{EC}_{50}$  values (concentrations of peptide required to elicit 50% of the maximal reactivity) were calculated with GraphPad Prism V software.

## In vitro viral suppression assays

The capacity of TV9-CTL to inhibit HIV infection was determined *in vitro*. HIV JR-CSF and NL4-3.1 stocks were generated and titers were determined as previously described [49]. Infected PBMC or T1 cells (infected by NL4-3.1 virus at 500 pg p24 *per*  $10^6$  cells) were cultured at 50 000 cells *per* flat-bottomed well in 96-well plates with CTL at the indicated ratios in complete medium containing IL-2 (25 U/mL). PBMC from HLA-A\*0201<sup>+</sup>, HIV-uninfected donors were depleted of CD8<sup>+</sup> cells (Miltenyi Biotec, Auburn, CA, USA), activated with phytohemagglutinin A in complete medium containing IL-2 (25 U/mL) for 2 days and then infected by overnight incubation with HIV JR-CSF at 80 TCID<sub>50</sub>/mL (GenBank Accession Number AAB05598). Supernatants were tested for p24 antigen concentration by ELISA (HIV-1 p24<sup>CA</sup> antigen capture assay kit, AIDS and Cancer Virus Program, NCI Frederick Cancer Research) every 3 days up to day 9. The range of sensitivity for p24 was 7.5–400 pg/mL.

## Molecular analysis of expressed TCRB gene products by quantitative clonotypic PCR

Tetramer-binding CD8<sup>+</sup> T cells were sorted viably to > 98% purity for RNA-based analysis of *TCRB* gene expression using an unbiased template-switch anchored RT-PCR as described previously [23]. The IMGT nomenclature is used for all molecular analyses conducted in this study.

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**Conflict of interest:** The authors declare no financial or commercial conflict of interest.

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**Abbreviations:** Tetramer: tetrameric HLA-A\*0201-peptide complex · TV9: HIV p24 Gag<sub>19–27</sub> epitope (TLNAWVKVV)

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