Induction of a primary human cytotoxic T-lymphocyte response against a novel conserved epitope in a functional sequence of HIV-1 reverse transcriptase

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Objective: *To identify novel major histocompatibility complex (MHC) class I-restricted cytotoxic T-lymphocyte (CTL) epitopes conserved in HIV-1.*

Methods: Potential conserved CTL epitopes were selected using a predictive computer algorithm based on a human leukocyte antigen (HLA)-A*0201 peptide-binding motif and tested for actual binding to the human processing defective cell line 174.CEM T2 (T2). Hence, the amino-acid sequences of 14 full-length sequenced HIV-1 strains were analysed. An *in vitro* primary peptide-specific human CTL response was induced with responding lymphocytes of an HIV-1-seronegative donor. Responding T cells were cloned by limiting dilution and tested for their ability to recognize naturally processed antigen in a ^{51}Cr-release assay using recombinant vaccinia–HIV protein-infected B-lymphoblastoid cells (B-LCL) as target cells.

Results: The analysis of peptides bearing the HLA-A*0201 motif for conservation resulted in one peptide of Env, three of Gag and 12 of Pol. Only $Gag_{340-348}$, Pol_{83-92}, $Pol_{267-277}$ and $Pol_{960-968}$ showed binding properties to T2 comparable with those of known CTL epitopes Gag_{76-84} SLYNTVATL and $Pol_{468-476}$ ILKEPVHGV. A successful primary MHC class I-restricted CTL response was induced against $Pol_{468-476}$ and $Pol_{267-277}$ VLDVGDAYFSV, a peptide in a functional sequence of reverse transcriptase (RT). The resulting CD8+ CTL clones were peptide-specific and able to specifically lyse recombinant vaccinia–HIV-1 RT-infected HLA-A*0201-matched B-LCL.

Conclusion: The method used to screen proteins sequences for potential CTL epitopes, test selected peptides for binding to MHC class I and induction of an *in vitro* primary response against optimal binding peptides resulted in the identification of at least one novel conserved CTL epitope. The novel epitope is located in an area crucial for RT activity. This study demonstrates the feasibility of identifying highly conserved HIV-1-derived peptides capable of eliciting novel anti-HIV-1 CTL responses.

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Introduction

Cytotoxic T-cell responses against virus-infected cells are of key importance in the clearance and control of most virus infections [1]. Cytotoxic T lymphocytes (CTL) recognize viral peptides presented by major histocompatibility complex (MHC) class I molecules at the surface of infected cells [2–6]. CTL responses against many viruses involve recognition of a single or few immunodominant viral epitopes [7–9]. However, studies of CTL specific for HIV-1 have shown that multiple epitopes of all proteins of HIV-1 may serve as targets and that these epi-

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topes can be presented by the same restricting human leukocyte antigen (HLA) class I molecule [10–13]. To date there has been no systematic study to determine to which of these peptides an HIV-seropositive individual responds. The initial vigour of the CTL response against HIV-1 in infected individuals is shown by CTL activity of freshly isolated peripheral blood mononuclear cells (PBMC) without restimulation [13]. Despite these initial CTL responses, HIV-1 infection nevertheless leads to virus persistence and eventually to deterioration of humoral and cellular immune responses [14–16], resulting in AIDS [17].

One role of CTL in eradicating HIV-infected human cells was shown in studies in which *in vitro* HIV-1 replication was controlled by MHC class I-restricted CTL [18,19]. Studies of SIV-infected macaques showed that animals with broad CTL activity were able to control disease in contrast to SIV-infected macaques with low CTL activity [20,21]. In HIV-1-infected asymptomatic individuals effective CTL responses were found against relatively or highly conserved sequences of HIV-1 Gag [10,22,23], reverse transcriptase (RT) [24] and Nef [25].

HIV-1 exhibits sequence variation due to error-prone RT, recombination and the absence of an error-correction system [26]. Consequent to this lack of control, the number of virus variants increases with disease progression [27]. In longitudinal studies a decline or loss of CTL responses to a particular epitope was found associated with the emergence of epitope-loss virus variants [28,29]. Also, in a comparison of CTL reactivity to an epitope of one particular HIV-1 virus with natural variants of this epitope in other HIV-1 viruses, a decrease or absence of cytotoxicity against peptide-incubated targets was observed [30]. These results combined with those of CTL studies in long term asymptomatic individuals suggest that CTL capable of controlling HIV-1 infection are directed against epitopes that are not readily prone to mutation. Strong and sustained CTL responses against these highly conserved sequences might enable the infected host to effectively control the spread of virus or to establish at least a low grade of infection so that disease progression is delayed.

Conservation of protein sequences may reflect functional importance in the life cycle of the virus. Since HLA-A*0201 is found at high frequency in all human races [31], we selected HLA-A*0201-binding peptides that were completely conserved among different HIV-1 virus strains. *In vitro* induction of peptide-specific class I-restricted primary CTL responses has been achieved in the mouse [32,33] as well as in human [34] systems. We used the processing-defective cell line T2 [35] to detect binding of the selected peptides [36], and a modified strategy as described by Houbiers *et al.* [34] to induce primary CTL responses.

This study demonstrates that novel CTL epitopes in conserved HIV sequences can be identified by the strategy of *in vitro* primary immune response induction by HLA-A*0201 motif-bearing peptides with proven binding ability to HLA-A*0201.

Materials and methods

Cell lines and recombinant vaccinia viruses

The processing defective T2 cell line, which expresses low levels of surface HLA-A*0201, was a gift from Dr P. Cresswell (Yale University, New Haven, Connecticut, USA). Recombinant vaccinia constructs were kindly provided by Dr B. Moss (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, USA). The recombinant vaccinia virus vCF-21 contains the HIV-1_{HXB2} *pol* RT gene (vvRT) [37]. The negative control recombinant vaccinia virus vSC-8 contains the *Escherichia coli* β-*gal* gene (vvLacZ) [38].

Cytotoxicity of CTL against target cells was tested in a standard 4-h ^{51}Cr-release assay. HLA-A*0201-positive B-LCL served as target cells. B-LCL were subtyped with an HLA-A*0201 alloreactive CTL clone [39]. Briefly, B-LCL were either labelled for 1 h at 37°C with 100 μCi ^{51}Cr and preincubated for 10 min with peptide, or target cells were infected (multiplicity of infection, 5) with appropriate recombinant vaccinia constructs 16 h before labelling. A difference of ≥ 10% with background lysis was considered to be specific lysis of target cells. The percentage-specific ^{51}Cr release was calculated by the formula:

$$\%\ \text{specific}\ ^{51}\text{Cr release} = \frac{(\text{release experimental well} - \text{background release})}{(\text{maximum release} - \text{background release})} \times 100\%$$

Target cell lysis by CTL was blocked by incubation of CTL with anti-CD8 monoclonal antibody (MAb) FK18 [40]. FK18 was used as a 1 : 300 dilution of ascitic fluid. Effector cells were mixed with FK18 and preincubated for 1 h at room temperature. MHC class I restriction was determined by 30 min preincubation of T2 cells with anti-HLA class I MAb W6/32.

Peptide selection and T2 peptide-binding assay

All amino-acid sequences of 14 different full-length sequenced HIV-1 virus strains: LAI, MN, NL43, OYI, SF2, RF, MAL, D31, CAM1, HAN, ELI, NDK, JR-CSF and JR-FL [41] were screened for possible HLA-A*0201-restricted CTL epitopes using a scoring system [36]. Virus strains not full-length sequenced or the highly divergent Cameroonian isolates were not included. All predicted high-scoring peptides were compared and those sequences with anchor residues on both anchor positions and completely conserved among all 14 strains were synthesized as described previously [34].

The T2 HLA-A*0201 peptide-binding assay was performed according to Nijman *et al.* [36]. Fluorescence

of viable cells was measured on a FACscan flow cytometer (Becton Dickinson, Franklin Lakes, New Jersey, USA). The fluorescence index (FI) was calculated by the formula: FI =(mean fluorescence sample − mean fluorescence background) ÷ mean fluorescence background.

Peptides with a FI ≥ 0.5 were considered to bind to HLA-A*0201. Subsequent serial dilutions of the concentration of peptide were used to determine the concentration needed to upregulate HLA-A*0201 to half maximum fluorescence on the T2 cell line.

Primary CTL induction

The *in vitro* CTL response induction method recently published [34] was modified as follows. T2 cells were loaded with 100 μg/ml peptide overnight at 37°C and subsequently treated with 50 μg/ml Mitomycin-C (Kyowa Co. Ltd, Tokyo, Japan). After 2 h the cells were washed twice and used as antigen-presenting cells by cocultivation with HLA-A*0201-positive PBMC of a healthy HIV-1-seronegative donor at a T2-to-PBMC ratio of 1 : 4. Cells were cultured for 10 days in 2 ml standard medium [RPMI-1640 Dutch modification (Gibco, Paisley, Scotland, UK) containing L-glutamine, antibiotics, 15% pooled human serum] and 40 μg/ml peptide in 24-well Costar plates (Costar, Cambridge, Massachusetts, USA) at a density of 2 million cells/well. Responder cells were harvested and depleted of CD4+ T cells using CD4+ magnetic beads (Dynal A.S, Oslo, Norway). Two million CD4-depleted responder cells were restimulated with a feeder-mix consisting of 1×10^6 irradiated (3000 rad) autologous PBMC and 2×10^5 irradiated (10 000 rad) autologous B-LCL. Feeder cells were sensitized with 50 μg/ml peptide in serum-free Iscove's modified Dulbecco's medium for 2 h at 37°C, washed and added to the responders in standard medium supplemented with 60 IU/ml human recombinant interleukin-2 (rIL-2; Eurocetus, Amsterdam, The Netherlands). At day 17, responder cells were harvested on Ficoll-lymphoprep (Nycomed Pharma, Oslo, Norway) and cloned by limiting dilution. Ten or less responder cells were cocultured with 1×10^5 irradiated (3000 rad) PBMC and 5000 irradiated (10 000 rad), peptide sensitized washed B-LCL (from at least two different HLA-A*0201-positive donors) in 100 μl standard medium containing 60 IU rIL-2/ml and 1% leucoagglutinin (Phytohaemagglutinin-leucocytes; Sigma, St Louis, Missouri, USA). Growing clones were expanded but stimulated with peptide-sensitized washed B-LCL at a 2-week interval only.

Results

Selection of conserved HIV peptides with binding ability for HLA-A*0201

Analysis of binding to HLA-A*0201 of the amino-acid sequences of Env, Gag, Pol, Rev, Vpu, Vif, Vpr, Tat and Nef of 14 full-length HIV-1 strains resulted in one conserved peptide of Env, three of Gag, and 12 of Pol bearing the HLA-A*0201 motif. The binding capacity of these peptides was tested in the T2-binding assay. Five out of 16 peptides had a FI ≥ 0.5 at a concentration of 100 μg/ml. Subsequent binding tests with serial dilutions of the peptide concentration revealed high affinity binding of one Gag peptide ($Gag_{345-353}$), and three Pol peptides (Pol_{83-92}, $Pol_{267-277}$, $Pol_{960-968}$; Table 1). As a control, the serial dilutions of two known CTL epitopes $Pol_{468-476}$ ILKEPVHGV [42] (highly conserved) and Gag_{77-85} SLYNTVATL (not conserved) [43] resulted in a half maximum of 5 and 20 μg/ml, respectively (Table 1). In accordance with earlier studies [36], 60% of the motif-bearing peptides did not bind.

Table 1. Binding of conserved HIV-1 sequences of Gag, Pol and Env to human leukocyte antigen (HLA)-A*0201.

Protein (position)*	Binding score	Peptides	FI	½ max (μg/ml)
Gag (76–84)†	576	SLYNTVATL	1.3	20.0
Gag (227–236)	576	QMREPRGSDI	0.2	
Gag (235–243)	288	DIAGTTSTL	0.2	
Gag (340–348)	144	EMMTACQGV	1.0	10.0
Pol (78–88)	288	QLKEALLDTGA	0.4	
Pol (83–92)	288	LLDTGADDTV	1.3	17.5
Pol (105–114)	576	KMIGGIGGFI	0.3	
Pol (160–169)	288	PISPIETVPV	0.5	62.5
Pol (163–171)	576	PIETVPVKL	0.3	
Pol (267–277)	288	VLDVGDAYFSV	2.7	15.0
Pol (307–317)	288	VLPQGWKGSPA	0.2	
Pol (460–468)	576	ELAENREIL	0.2	
Pol (468–476)†	2304	ILKEPVHGV	2.9	5.0
Pol (580–588)	288	PLVKLWYQL	0.1	
Pol (909–919)	288	GIGGYSAGERI	0.2	
Pol (931–939)	1152	ELQKQITKI	0.1	
Pol (960–968)	576	LLWKGEGAV	0.7	32.5
Env (114–124)	288	SLKPCVKTLPL	0.4	

*Derived from HIV-1 strain JR-CSF. †The peptides $Pol_{468-476}$ [42] and Gag_{77-85} [43] are known HLA-A*0201-restricted cytotoxic T-lymphocyte epitopes, therefore, the half of maximum (½ max) fluorescence values obtained with these peptides served as reference for good binding. Computer scoring of predicted HLA-A*0201-binding peptides was performed according to Nijman *et al.* [36]. The importance of the anchor positions was stressed by assigning 12 points to allowed residues at these positions [36]. The fluorescence index (FI) is shown at 100 μg/ml of peptide: peptides with a FI ≥ 0.5 were considered to be binding peptides. The affinity of binding peptides was tested by determining the peptide concentration needed to upregulate HLA-A*0201 expression on the T2 cell line to ½ max fluorescence: peptides with ½ max > two times the ½ max of peptide Gag_{76-84} were considered to be weakly binding peptides.

Induction of primary CTL responses

We induced primary CTL responses against the peptides $Pol_{267-277}$ and $Pol_{468-476}$ (Table 1) from PBMC of a HIV-1-seronegative healthy donor. The known HLA-A*0201-restricted peptide $Pol_{468-476}$ was selected as a positive control for CTL induction. Limiting dilution resulted in clones 468-13, 468-16 and 468-22 against peptide $Pol_{468-476}$ and clone 267-18 against peptide $Pol_{267-277}$ showing specific lysis of T2 cells sensitized with peptide (Fig. 1).

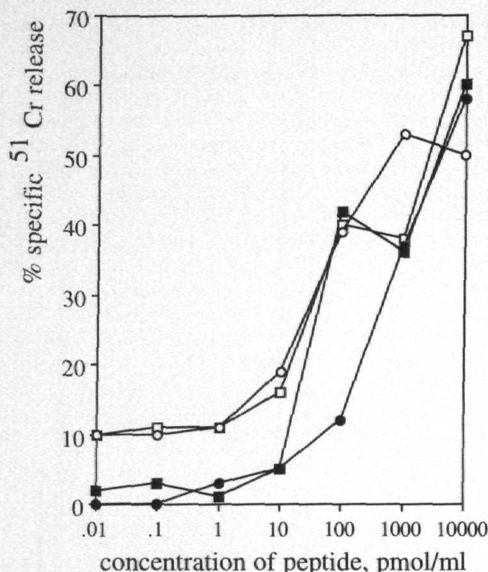

Fig. 1. Lytic activity of clones 468-13 (■), 468-16 (□) and 468-22 (○) obtained against peptide $Pol_{468-476}$ and clone 267-18 (●) against peptide $Pol_{267-277}$ was tested against peptide-loaded T2 cells. T2 cells were pre-incubated for 10 min with increasing concentrations (pmol/ml) of specific peptide and then used as targets. The percentage lysis of T2 cells is represented as the percentage-specific ^{51}Cr-release values and shows for each clone the mean of triplicate measurements at an effector-to-target ratio of 5 : 1 for $Pol_{468-476}$ and 10 : 1 for $Pol_{267-277}$ obtained in two independent experiments.

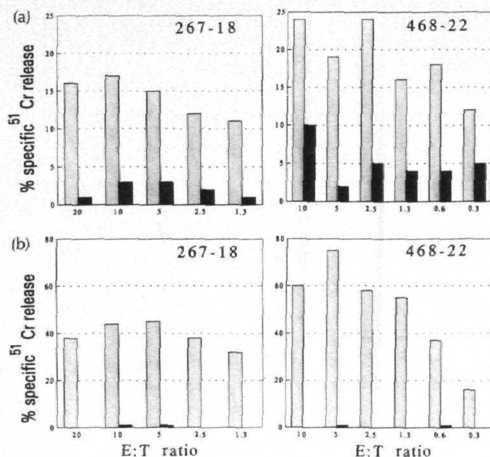

Fig. 2. Recognition of target cells sensitized by exogenously added peptide and of target cells expressing the natural processed epitope by clone 267-18 (left) and clone 468-22 (right). (a) B-lymphoblastoid cells (B-LCL) were infected with recombinant vaccinia–HIV-1 reverse transcriptase (vvRT; □) gene. Aspecific lysis of the B-LCL due to vaccinia infection was monitored by infecting B-LCL with recombinant vaccinia–β-*gal* (vvLacZ; ■) gene. (b) Aspecific lysis of the B-LCL by the cytotoxic T-lymphocyte clones was also tested. The specific lysis of 5 μg/ml peptide-pulsed B-LCL (□) versus background lysis of non-peptide-pulsed B-LCL (■) is shown. The clones were tested at different effector-to-target (E : T) ratios and the percentage-specific lysis of different targets is represented as percentage-specific ^{51}Cr-release values and show for each clone the mean of triplicate values obtained in two independent experiments.

FACscan analysis showed that the clones were CD8-positive. Blocking studies with anti-CD8 or anti-HLA class I antibodies revealed that CTL activity was mediated by CD8+ T cells and was HLA class-I-restricted (data not shown).

The clones were tested at different effector-to-target cell (E : T) ratios to estimate the cytolytic capacity. At an E : T ratio of 0.015 for clone 267-18 and 0.15 for clone 468-22, T2 cells sensitized with 5 nm/ml of peptide are specifically lysed (data not shown).

The fine specificity of clone 267-18 was studied by serine and arginine replacements in the synthetic $Pol_{267-277}$ peptide. Binding of the peptide was strongly influenced by the residues at position 2 and 11. Replacement by a small amino acid such as serine showed that residues 3, 4, 5, 8 and 9 in particular are important for recognition by the CTL. Arginine replacement had more drastic effects in that only a replacement at position 6 allowed recognition of peptide-sensitized target cells (data not shown). Thus, residues at positions 3, 4, 5, 8, 9 and 10 interfere with recognition by the T-cell receptor.

Recombinant vaccinia virus-infected target cells

To investigate whether the clones would not only recognize synthetic peptide but also the naturally processed peptide, the most sensitive CTL clones were tested against B-LCL infected with the recombinant vaccinia viruses vvRT or vvLacZ. Clones 468-22 and 267-18 lysed vvRT but not vvLacZ-infected B-LCL (Fig. 2a). In the same experiments both clones specifically lysed peptide-sensitized HLA-A*0201-matched B-LCL (Fig. 2b).

Discussion

The therapeutic potential of CTL against tumours and viruses has recently been reviewed [1,12,13,44]. The imminent escape of HIV-1 from deteriorating humoral and cellular immune responses requests prompt and specific intervention. Evoking CTL to highly conserved HIV-1-derived epitopes may decrease the risk of emerging escape mutants of HIV-1, which may contribute to the control of HIV-1 infection.

Following our strategy to screen protein sequences for potential CTL epitopes, test selected peptides for MHC class I binding, and induce CTL against the optimally binding peptides resulted in CTL recognizing naturally processed HIV-1-derived peptides, including one novel RT epitope in a protein sequence crucial for RT activity.

The existence of CD4+ CTL in HIV patients [45,46] is acknowledged but CD4+ T cells are more susceptible to HIV infection and compared with CD8+ CTL, display lysis of HIV-infected cells only at higher E : T ratios [45]. *In vitro* it is possible to infect CD8+ T cells [47,48]. The *in vivo* relevance of this phenomenon is debatable because HIV-1 DNA was not detectable by polymerase chain reaction (PCR) in the CD8+ subset of PBMC from AIDS patients [49]. Therefore we have focused primarily on induction of HIV-specific CD8+ CTL.

One Gag and three Pol peptides showed similarly high-affinity binding properties as previously published HLA-A*0201-restricted CTL epitopes [42,43]. Analysis of the Gag sequences of 70 HIV-1 isolates [50] showed the high conservation of the binding $Gag_{345-353}$ peptide in 63 out of 70 isolates. It is noteworthy that CTL from a long-term asymptomatic HIV-1-seropositive individual reacted to target cells pulsed with a 20 amino-acid peptide containing this $Gag_{345-353}$ peptide [15].

Induction of primary CTL responses to peptides *in vitro* has already been shown in mice [51] and humans [34,52]. Our *in vitro* induction protocol, using PBMC from a healthy HIV-1-seronegative blood donor, allowed generation of CTL to a novel highly conserved HLA-A*0201-restricted epitope ($Pol_{267-277}$: VLDVGDAYFSV) derived from HIV-1 *pol* RT. We also elicited a primary CTL response to the already known HLA-A*0201-restricted epitope, published by Walker *et al.* ($Pol_{468-476}$) [42]. The resulting CD8+ CTL clones were able to recognize target cells pulsed with as little as 10 pmol peptide/ml. Observations in mice of CTL against influenza nucleoprotein showed recognition of peptide at concentrations of 0.1–100 pmol peptide/ml [2,3].

Clones 468-22 and 267-18 both directed to RT epitopes were able to specifically kill recombinant vaccinia HIV-1 RT-infected targets in a class I-restricted fashion. Although ^{51}Cr release is lower it is frequently observed that the lower peptide concentration displayed at the cell surface following endogenous processing is often associated with lower ^{51}Cr-release values than following incubation with much higher doses of exogenous peptide. In a recent study from our laboratory [53], low *in vitro* tumour lysis values following incubation with tumour-specific CTL was nevertheless associated with CTL-mediated protection against tumour outgrowth *in vivo* [53]. Thus, the lower CTL response measured *in vitro* against the endogenously processed peptide provided adequate protection *in vivo* [53]. An overall difference in the maximum percentage of lysis can be noticed between clones 267-18 and 468-22. This could be due to a difference in lytic capability of the clones although both clones lysed their targets at similar E : T ratios. The lysis of vvRT-infected cells is similar when compared with the background lysis of vvLacZ-infected cells. On the other hand, variation in peptide affinity can exist between different CTL recognizing the same epitope [2,3]. It will be of interest to study the possible diversity of the T-cell repertoire against epitope $Pol_{267-277}$.

Sequences in which no variation is allowed due to functional or structural constraints on the viral protein may offer CTL an opportunity to attack HIV-1 at its Achilles' heel. The RT-derived peptide $Pol_{267-277}$ may therefore be a particularly interesting epitope. Site-specific mutagenesis of the region overlapping peptide $Pol_{267-277}$ revealed that alterations in the sequence by substitution of single amino acids at position 269 (asp to glu), 272 (asp to gly) or 273 (ala to ser) can profoundly affect RT activity [54]. All of these amino acids are located in peptide $Pol_{267-277}$. The amino-acid replacement study showed that among six of the amino-acid side chains the aspartic acid at position 269 was also important for recognition by clone 267-18. The amino acids at positions 3, 4, 5, 8, 9 and 10 seem essential for T-cell receptor recognition, which largely confirms previous reports on HLA-A*0201-restricted CTL [8,34,55]. Viral escape from T-cell recognition by mutation of the T-cell receptor contact residues [28,56] could be disastrous for the virus in the case of the $Pol_{267-277}$ epitope.

Recently Harrer *et al.* [24] reported a study of anti-HIV CTL responses in long-term asymptomatic HIV-seropositive individuals. A response was found against a CTL epitope that spans the RT region of the highly conserved YMDD amino-acid motif also known to be important for RT function [54,57]. Targeting the immune response to this epitope, the epitope identified in this study and others found in regions that are functionally important for the life cycle of HIV-1 may influence the length of the asymptomatic phase.

Our data show the feasibility of primary *in vitro* targeting to a synthetic viral peptide of human CTL capable of recognizing the naturally processed peptide. Recently, induction of primary CTL in a completely autologous system was reported [52]. These approaches may allow *in vitro* culture of CTL against a viral or autologous peptide of interest for adoptive transfer into patients with viral disease or cancer. Primary CTL induction with lymphocytes obtained from HIV-infected subjects has to be established. We are currently investigating this possibility with PBMC obtained from long-term asymptomatic patients.

A study by Sijts *et al.* [58] in mice showed the relevance of cryptic epitopes: upon infection with the Moloney sarcoma virus–murine leukaemia virus complex C57BL/6 mice ($H2^b$) respond with a class I D^b-restricted CTL response but peptide vaccination with a K^b-restricted epitope resulted in CTL recognizing this epitope both as synthetic peptide and as endogenously processed antigen in the context of K^b. It is therefore feasible to direct the cellular immune response to subdominant epitopes that are not selected as major epitopes for CTL recognition. It can be argued that immunization with peptides might result in relatively low responses *in vitro* and that peptide immunization might therefore result in weak responses *in vivo*. However, *in*

vivo peptide immunization could result in higher levels of specific CTL because the proper cytokine networks and lymphoid architecture are available. In animal models it has been shown that peptide immunization can effectively protect against lymphocytic choriomeningitis virus [9], Sendai virus [7] and human papilloma virus-induced tumours [53]. A peptide-based vaccine also elicited protective CTL in rhesus monkeys against SIV [59].

The exquisite sensitivity of CTL to sequence variation in the epitope itself demonstrates the need for stable epitopes. Thus peptide vaccines should be designed to evoke immune responses against several of these functionally important epitopes in order to minimize the emergence of escape variants of the virus.

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