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CTL escape and increased viremia irrespective of HIV-specific CD4$^+$ T-helper responses in two HIV-infected individuals

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Abstract

We investigated whether development of mutations leads to loss of CD8 T-cell recognition in HIV-1 infection and is possibly linked to alterations in HIV-1-specific CD4$^+$ T-cell responses in 2 HIV-infected individuals. In patient, H434 full genome sequencing of HIV-1 biological clones at early and late time points during disease progression showed development of fixed mutations in 16 predicted HIV-specific CTL epitopes. Loss of T-cell recognition and reactivity against wild-type and mutant epitopes was observed primarily for the HLA-B27-restricted KK10 epitope and HLA-A2-restricted SL9 epitope. Similarly, in patient H671, decreasing numbers of HLA-A3-restricted CD8$^+$ T cells specific for the wild-type RK9 epitope was observed after CTL escape. Only in patient H434 loss of CTL responses was paralleled by a decrease in HIV-specific IL-2$^+$ CD4$^+$ T-helper responses. This suggests that loss of T-cell reactivity may not be directly linked to HIV-specific CD4$^+$ T-cell responses but that increased viremia after CTL escape may influence CD4$^+$ T-helper responses.

Keywords: HIV; Escape; CTL; CD4; CD8; Mutation

Introduction

Cellular immune responses play a pivotal role in the control of HIV-1 infection. Resolution of acute viremia was shown in a number of studies to correlate with the development of strong CD8$^+$ T-cell responses in the natural history of HIV-1 infection (Koup et al., 1994; Borrow et al., 1994; Wilson et al., 2000b). During chronic HIV-1 infection, HIV-specific CD8$^+$ T-cell numbers correlated inversely with viral load (Greenough et al., 1997; Ogg et al., 1998), and high numbers of HIV-specific CD8$^+$ T cells have been associated with delayed HIV disease progression (Ogg et al., 1999). Furthermore, depletion of CD8$^+$ T cells in simian immunodeficiency virus (SIV)-infected macaques has been reported to result in increased viremia (Jin et al., 1999; Schmitz et al., 1999) which was subsequently reversed by the restoration of CD8$^+$ T cells. Escape from CD8$^+$ T-cell recognition through mutation of cytotoxic T-lymphocyte (CTL) epitopes, and the subsequent failure of HIV-specific CD8$^+$ T cells to eliminate HIV-1-infected cells, has proven to be a major means whereby HIV-1 evades HIV-1-specific cellular immune responses, leading to disease progression and ultimately AIDS (Letvin and Walker, 2003). Evidence that escape may lead to loss of immune control by CTL as was originally described by Goulder et al. (1997) and has since been confirmed by others (Price et al., 1997; Borrow et al.,

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1997) showing that escape in a single epitope during acute HIV-1 infection precedes progression to AIDS. In addition, CTL escape mutations have been reported in the SIV macaque model, both in the acute (Allen et al., 2000; O'Connor et al., 2002) and in the chronic phase of natural SIV infection (Friedrich et al., 2004) and have also been described in vaccinated rhesus macaques (Barouch et al., 2003). Mutations of residues within epitopes can affect loading of the peptide into the MHC class I molecule or inhibit proper T-cell activation. Alternatively, mutations outside CTL epitopes can influence antigen processing and thereby alter HIV-specific $CD8^+$ T-cell function (Draenert et al., 2004; Allen et al., 2004). It has been shown previously that, although numbers of HIV-1-specific tetramer$^+$ $CD8^+$ T cells persist at high frequencies during progression to AIDS, numbers of IFNγ-producing $CD8^+$ T cells decrease (Kostense et al., 2001, 2002). Thus, loss of HIV-specific $CD8^+$ T-cell function rather than physical depletion of these cells may explain the failing control of viremia during progression to AIDS. Loss of HIV-specific $CD8^+$ T-cell function may be a consequence of impaired maturation as loss of proliferative capacity and expression of perforin of HIV-specific $CD8^+$ T cells have been associated with progressive disease (Migueles et al., 2002). Furthermore, the majority of HIV-specific $CD8^+$ T cells express typical memory markers like CD27 and are therefore not fully differentiated (Appay et al., 2000; Champagne et al., 2001; van Baarle et al., 2002; Jansen et al., 2004). Alternatively, loss of function may be due to CTL escape, causing mutations within $CD8^+$ T-cell epitopes that influence TCR binding or functionality of the corresponding $CD8^+$ T cell.

Another explanation for impaired functionality of HIV-specific $CD8^+$ T cells may be the loss of $CD4^+$ T-cell help. Several studies now reported T-helper dependence of specific $CD8^+$ T-cell functions (Matloubian et al., 1994; Zajac et al., 1998; Kalams et al., 1999). Interestingly, more HIV-specific IL-2-producing $CD4^+$ T cells and $CD4^+$ T-helper cells producing both IL-2 and IFNγ were reported in long-term non-progressors (LTNP) compared to progressors (Wilson et al., 2000a). Furthermore, the presence of these cells was found to be inversely related to viremia (Boaz et al., 2002; Harari et al., 2004), compatible with proliferative $CD4^+$ T-cell responses (Rosenberg et al., 1997; Pontesilli et al., 1999; Palmer et al., 2004). In this study, full genome sequencing of HIV-1 was performed to determine whether genotypic alterations affected T-cell recognition in a qualitative and quantitative manner. Furthermore, Gag-specific $CD4^+$ T-cell responses were measured at several time points in the course of HIV infection in order to investigate whether the increase in viremia after CTL escape may be linked to alterations in HIV-specific $CD4^+$ T-helper responses.

Results

Identification of CTL escape in HIV-1-infected patients

We selected two patients (H434, H671) who demonstrated a sudden increase in HIV-1 viral load after several years of viral control without the occurrence of X4 variants (Fig. 1). In patient H671, a switch in viral phenotype to an SI variant occurred after the increase in viral load at 62 months post-seroconversion (mo. psc.), however, no subsequent increase in viral load was associated with the switch. From these two patients, we choose to select time points corresponding to low, increasing and high viral loads to monitor the association of CTL escape mutations with alterations in HIV-1-specific T cellular responses. We previously reported for patient H671 that mutations in CTL epitopes were linked to alterations in CTL responses and the increase in viral load. In this study, we extended these analyses to further investigate the relation between CTL escape and the subsequent increase in viral load and HIV-specific CD4 T-helper responses. In patient H434, full genome sequencing of HIV-1 biological clones isolated at 12 mo. psc. (clones 8-A3 + 8-F4) and 127 mo. psc. (43-A1, C1 and E12) was performed. Sixty-one CTL epitopes were analyzed of which 16 epitopes showed fixation of mutations between 12 and 127 mo. psc. (Table 1). Seven epitopes were selected for functional T-cell analysis (Table 1), based on observed fixation of mutation at residues critical for MHC

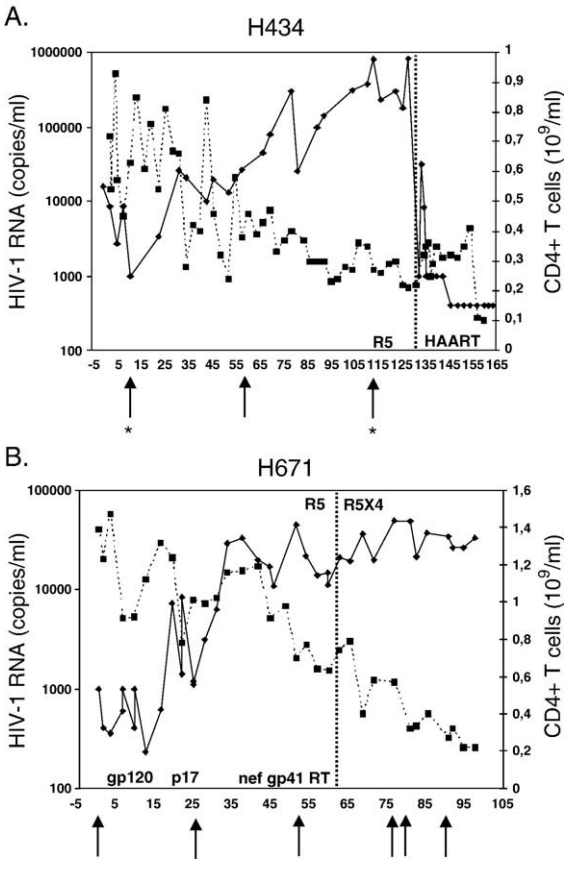

Fig. 1. Clinical parameters for patients H434 and H671. Longitudinal analyses of serum HIV RNA load (solid line) and $CD4^+$ T-cell numbers (dotted line) are shown for patient H434 (A) and H671 (B). Follow up is given in months post-seroconversion on the *x* axis. Asterisks indicate the time points from which biological clones from patient H434 (A) were subjected to whole genome analysis (Table 1). Arrows on the *x* axis indicate time points at which T-cell responses were measured (Figs. 3 and 4).

Table 1
CTL epitopes with fixed mutations during disease progression in patient H434

HIV-1 region[a]	Epitope[b]	HLA	Class I epitopes[c]	HLA-anchor residue affected[d]
p2/p24(1-7+230-231, VV9)	V L A E A M S Q V – – – – – – – – A	A2	X	X
p17 (77-85, SL9)	S L Y N T V A T L – – – – – I – – –	A2	X	X
p24 (19-27, TV9)	T L N A W V K V V – – – – – – – – I	A2	X	
RT (282-290, Pol-316)	Y T A F T I P S I – – – – – – – – V	A2		X
RT (209-220)	L L S W G L T T P D K K – – K – – – – – – – – –	A2		
RT (309-317, IV9)	I L K V P V H G V – – – A – – – – –	A2	X	
Nef (190-198, AL9)	A F H H V A R E L – – R – – – – – –	A2		
Vpr (59-67, AL9)	A I I R I L Q Q L – L – – – – – – –	A2	X	X
P6 (36-45)	Y P L T S L R S L F – – – – – – K – – –	B7		
p17 (22-31)	R P G G K K R Y H L – – – – – – – – R –	B7		
RT (156-164, SM9)	S P A I F Q S S M – – – – – – C – –	B7		
Nef (77-85, RL9)	R P M T Y K G A L – – – – – – A – V	B7	X	
Nef (128-137, TP9)	T P G P G T R F P – S – – – I – Y –	B7	X	
Vif (48-57, HI10)	H P R V S S E V H I N – – – – – – – – –	B7	X	X
Vif (34-42, FL9)	F P R I W L H G L – – – T – – – N F	B7	X	X
p24 (263-272, KK10)	K K W I V M G L N K – R – – I L – – – –	B7, B27	X	X

[a] Position of the epitope relative to protein start in HXB2 and epitope name as listed in the HIV-1 Molecular Immunology Database.
[b] Autologous sequence of the $CD8^+$ T-cell epitopes determined in patient H434 at 12 mo. psc. (upper sequence) and 127 mo. psc. (lower sequence). Sequences are based on 2 respectively 3 biological clones.
[c] Most optimally defined CTL epitopes in the Molecular Immunology Database.
[d] Mutations that disrupt primary or secondary HLA anchor residues. These 7 epitopes were selected for analysis by IFNγ Elispot.

and T-cell receptor binding. Five of these seven epitopes have been previously described to be the most optimally defined HIV-1 CTL epitopes (HIV-1 Immunology Compendium, http://www.hiv.lanl.gov/content/immunology/pdf/2003/immuno2003.pdf).

Functional $CD8^+$ T cells against wild-type and mutant epitopes in H434

To investigate whether the observed fixed mutations in well-known and optimally defined $CD8^+$ T-cell epitopes influenced

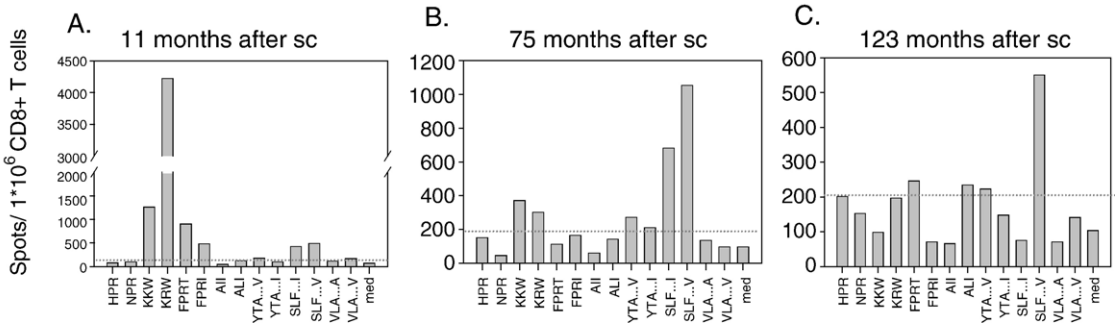

Fig. 2. Elispot analysis in individual 434 using 7 wild-type epitopes with various HLA restrictions and their mutants. To determine whether mutations in CTL epitopes could affect T-cell function, IFNγ Elispot analysis was performed using seven epitopes and their mutated variant at 11 months (A), 75 months (B) and 123 months post-seroconversion (C). Responses are shown as the number of spots/1 * 10^6 $CD8^+$ T cells. Responses higher than 2 times medium are considered positive (indicated by a dotted line).

$CD8^+$ T-cell functionality, IFNγ Elispot assays were performed with 7 wild-type epitopes and their observed variants (Table 1) at several time points in the course of HIV-1 infection. Twelve months after seroconversion, responses were identified against 3 wild-type (wt) peptides (KK10, SL9, FI9) and their respective mutants (mKK10, mSL9 and mFI9) (Figs. 2A and 3A). At 75 months after seroconversion, responses against KK10 and mKK10 had decreased over time but were still detectable. The SL9-specific $CD8^+$ T-cell responses slightly increased over time, while mSL9 responses did not change. Responses against the epitopes FI9 and mFI9 became undetectable. Interestingly, at 123 mo. psc., no KK10 or mKK10-specific production of IFNγ was observed, and SL9-specific $IFN\gamma^+$ $CD8^+$ T cells decreased to undetectable levels. Responses against mSL9 decreased but were still detectable. To further investigate the occurrence of escape mutations in the KK10 and SL9 epitope, we analyzed serum-derived viral sequences for both epitopes at three time points during disease progression (Table 2). At 12 mo. psc, a heterogeneous population of 5 different genotypes was detected for the p24 epitope KK10, including the M268L mutation. This mutation has been shown to be a precursor compensational mutation for the later R264K mutation (Kelleher et al., 2001). At 78 mo. psc., the R264K mutation became fixed. The appearance of mutations coincided with the loss of T-cell reactivity in the IFNγ Elispot to both the WT and MT peptide (Fig. 2 and Table 2), indicative of loss of $CD8^+$ T-cell reactivity due to mutation of the KK10 epitope. For the HLA-A2-restricted p17 epitope SL9, fixation of the mutation on the secondary anchor (V82I) was identified between 78 and 127 mo. psc, which paralleled the loss of T-cell reactivity to the WT peptide in the IFNγ Elispot (Fig. 2 and Table 2).

Numbers of $CD8^+$ T cells specific for wild-type and mutant $CD8^+$ T cell KK10 and SL9 epitopes in patient H434

To determine numbers of HLA-B27 and-A2 restricted $CD8^+$ T cells specific for KK10 and SL9, PBMCs from three time points in the course of HIV infection were analyzed using HLA-B27 tetramers loaded with peptide KK10 and its mutant, as well as HLA-A2 tetramers loaded with peptide SL9 and its mutant peptide (Fig. 3C). HLA-B27-restricted KK10-specific $CD8^+$ T cells decreased during HIV infection from 1.97% at 11 months to undetectable levels at 123 months after seroconversion. Furthermore, HLA-B27 mKK10 specific $CD8^+$ T cells decreased from 2.1% at 11 months to 0.35% at 123 months following seroconversion. HLA-A2-restricted SL9-specific $CD8^+$ T cells initially increased from 0.46% at 11 months after seroconversion to 1.0% at 75 months after seroconversion but eventually decreased again to 0.32% at 123 months after seroconversion. Staining with the mSL9 tetramer did not result in a tetramer-positive population. Thus, both numbers as measured by tetramers and function as identified by IFNγ production of HLA-B27-restricted $CD8^+$ T cells decreased during HIV infection. Numbers and function of HLA-A2-restricted SL9 specific $CD8^+$ T cells showed only a transient increase.

HIV-specific $CD8^+$ T cells specific for two Gag-p17 epitopes in patient H671

The observed escape from $CD8^+$ T-cell responses in patient H434 was compared to a second HIV-1-infected individual who was documented to harbor viruses that had escaped $CD8^+$ T-cell recognition. We previously documented near-complete fixation of a mutation in the C-terminal residue of the HLA-A3-restricted epitope in p17 (RK9: RLRPGGKK**K** → **R**), which coincided with a decrease in T-cell responses as measured by IFNγ (Fig. 3B). However, at the last time point analyzed (65 mo. psc), we detected an increase in $CD8^+$ T-cell responses for both the wild-type and mutant peptide and hypothesized that this observation could be due to possible cross-reactivity of $CD8^+$ T cells recognizing a non-mutating epitope 2 amino acids upstream: KIRLPGGK (KK9; HLA-A3) (Geels et al., 2003). Here, we extend the previous findings by using tetramers loaded with the epitopes of interest to determine numbers of HIV-specific $CD8^+$ T cells specific for the two epitopes in Gag-p17.

The RK9-specific $CD8^+$ T cells initially decreased from 1.0 to 0.8% during the first 25 months at follow-up and then strongly increased to 1.6% at 54 mo. psc. This corresponded with the previously described increase in numbers of IFNγ-producing cells. Finally, the number of RK9 positive $CD8^+$ T

Table 2
Evolution of epitopes KK10 and SL9 in patient H434

Epitope	Mo.psc[a]	Viral genotype		Method
KRWIILGLNK (KK10)	12	KRWI I LGLNK	(29% 4/14)	Cloned sequence
		– – – – – I – – – –	(29% 4/14)	
		– – – – – M – – HK	(21% 3/14)	
		– – – – – M – – – –	(14% 2/14)	
		– – – – – I – – – E	(7% 1/14)	
	78	KRWI I LGLNK		Cloned sequence
		– K – – VM – – – –	(100% 14/14)	
	127	KRWI I LGLNK		Cloned sequence
		– K – – VM – – – –	(100% 14/14)	
SLYNTVATL (SL9)	12	S L Y N T V A T L	(100% 11/11)	Cloned sequence
	78	– – – – – – – – –		Direct sequence
	127	– – – – – I – – –		Direct sequence

[a] Months post-seroconversion.

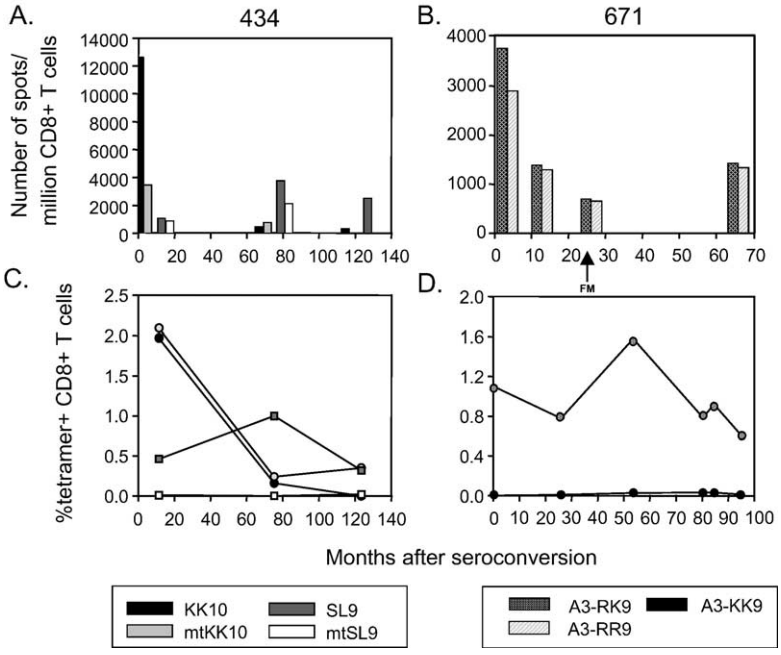

Fig. 3. Analysis of numbers and function of HIV-specific $CD8^+$ T cells in individuals who escape CTL surveillance. In patient H434, $CD8^+$ T-cell responses against the HLA-B27-restricted KK10 and the HLA-A2-restricted SL9 epitope were measured by IFNγ Elispot analysis (A). Tetramer analysis of the number of $CD8^+$ T cells specific for the KK10, mtKK10, SL9 and mtSL9 epitope in patient H434 (C). Analysis of RK9- and RR9-specific $CD8^+$ T-cell responses by IFNγ Elispot in patient H671 (B). Tetramer analysis of $CD8^+$ T cells was performed in patient H671 using tetramers loaded with either RK9 or the non-mutating epitope 2 amino acids upstream KK9 (D).

cells declined to 0.6%. No KK9-positive CD8 T cells were detected in H671 throughout disease progression. These observations exclude the previously hypothesized cross-reactivity and indicate that the IFNγ is produced by the RK9-specific $CD8^+$ T-cell population.

Analysis of mutations in HIV-specific $CD4^+$ T-helper epitopes

To determine whether $CD4^+$ T-helper epitopes were affected by mutations, we analyzed whether predicted HIV-specific $CD4^+$ T-helper cell epitopes were altered through sequence variation. We found that in patient H434 the mutation in the KK10 epitope overlapped with a T-helper epitope TNNPPIPBGEIYKRW (DQ13), whereas no HLA-class-II-restricted $CD4^+$ T-helper epitopes were found to be affected by fixation of mutation in patient H671 (data not shown). Next, we included peptides which are known to be broadly recognized by $CD4^+$ T-helper cells in clade-B-infected patients with different genetic backgrounds (Rosenberg et al., 1997; Younes et al., 2003; Kaufmann et al., 2004) (Table 3) In patient H434, the fixation of nine residues (Gag, Nef and RT) and in patient H671 fixation of two mutated residues (Gag, Nef) was observed between early and late time points in the course of HIV-1 infection. The two mutations in patient H671 overlapped with mutations in HIV-specific CTL epitopes.

Table 3
Mutations in commonly recognized $CD4^+$ T-cell epitopes

Patient	Region[a]	Commonly recognized peptide[b]	Reference
H434	p24 (119–133)	TNNPPIPBGEIYK**R/K**W	Kaufmann et al., 2004
	p24 (23–40)	WKV**V/I**EEKAF**S/N**PEVIPMF	Kaufmann et al., 2004
	p24 (31–48)	AF**S/N**PEVIPMF**S/T**ALSEGAT	Rosenberg et al., 1997
	p24 (81–102)	DRVHPVHAGPI**A/P**VPGQMREPRGS	Kaufmann et al., 2004
	p24 (141–158)	IVRMYSP**T/V**SILDIRQGPK	Kaufmann et al., 2004
	p24 (164–181)	YVDRFYKTLRAEQA**S/T**QEV	Kaufmann et al., 2004
	p17 (77–94)	SLYNT**V/I**ATLYCVHQRIEV	Kaufmann et al., 2004
	Nef (184–199)	KFDS**R/H**LAF**H/R**HMARELH	Younes et al., 2003
	Int (250–267)	VIQDNSDIK**V/A**VPRRKAKI	Kaufmann et al., 2004
H671	p17 (17–34)	EKIRLRPGGKK**K/R**YKLHKI	Kaufmann et al., 2004
	Nef (11–128)	LWVYHTGQGYFPDW**Q/D**NYT	Kaufmann et al., 2004

[a] Amino acid position of the epitope relative to protein start in HXB2 and epitope name as listed in the HIV-1 Molecular Immunology Database.
[b] Autologous sequences of $CD4^+$ T helper epitopes in patients H434 and H671. In bold are amino acid residues which became fixed during disease progression (H434: 12 and 127 mo. psc; H671: 2 and 47 mo. psc).

CTL escape, viremia and CD4$^+$ T-helper responses

Gag-specific CD4$^+$ T cells producing IFNγ, IL-2 or IL-2 and IFNγ were analyzed (Jansen et al., 2005) at the same time points as used for the CD8$^+$ T-cell measurements in both patients to investigate whether the observed loss of CD8$^+$ T-cell responses was accompanied by loss of CD4$^+$ T-helper responses in relation to the observed increase in viral load. Absolute numbers of HIV-specific cytokine-producing CD4$^+$ T cells are shown since this is a more reliable read-out than percentages, as we have previously described (Jansen et al., 2005). For patient H434, Gag-specific IL-2$^+$ CD4$^+$ T cells decreased from 1870 cells/ml 11 mo. psc. to 148 cells/ml 75 mo. psc. and became undetectable 127 mo. psc. (Fig. 3A). Similar results were observed for IL-2$^+$ and IFNγ$^+$ cells (1360 cells/ml 11 mo. psc., 111 cells/ml 75 mo. psc. and undetectable levels at 123 mo. psc. Fig. 3C). As shown in Fig. 3A, Gag-specific IFNγ$^+$ CD4$^+$ T cells decreased from 1955 cells/ml to 74 cells/ml but increased during the last 48 months of follow-up to 240 cells/ml. In patient H671, levels of HIV-specific IFNγ$^+$ and IL-2$^+$ CD4$^+$ T cells fluctuated during the first 80 months of follow-up and eventually decreased to 429 and 165 cells/ml respectively (Figs. 3D, E). As shown in Fig. 3F, numbers of IL-2$^+$ and IFNγ$^+$ CD4$^+$ T cells initially decreased from 417 to 96 cells/μl at 85 months after seroconversion and then increased again to 400 cells/ml at 95 months following seroconversion.

Taken together, loss of HIV-specific CD8$^+$ T-cell responses (both wild-type and mutant) is not per se accompanied by a decrease in HIV-specific T-helper responses. Interestingly, the slope of viral RNA load increase determined by linear regression in patient H434 (3690 cp/ml ± 762) was approximately 10-fold higher than in patient H671 (377.5 cp/ml ± 42.7), suggesting that high viremia may be responsible for the observed decrease in HIV-specific IL-2$^+$ and IL-2 and IFNγ$^+$ CD4$^+$ T cells (Fig. 4).

Discussion

Analysis of the mechanisms associated with failure of T-cell function during HIV-1 infection is of importance when a vaccine is to be realized with the aim of eliciting effective, protective and sustainable T-cell responses. In this report, we describe two HIV-1-infected individuals who escape recognition of functional HIV-specific CD8$^+$ T cells during disease progression and relate these responses to CD4$^+$ T-cell function. From full genome sequence analysis of HIV-1 biological clones generated from patient H434, we identified at least 7 known epitopes acquiring mutations on critical residues during disease progression. However, upon CD8 recognition analysis, we identified that CD8$^+$ T-cell responses against only two epitopes (KK10 and SL9) were readily detected, and mutations of critical residues led to an abrogation of existing T-cell responses.

Previous reports have demonstrated that the HLA-B27-restricted epitope KK10 is associated with effective control of viremia (Goulder et al., 1997; Kelleher et al., 2001) and that a distinct sequence of mutations (L268M, R268K) in this epitope leads to loss of CTL recognition correlating with disease progression and preceding viremia breakthrough (Kelleher et al., 2001; Feeney et al., 2004). Interestingly, in patient H434, we observed the same order in appearance of mutations, again supporting the concept of structural constraint on epitope evolution. The mutant KK10 epitope has previously been shown to be less adequately presented by HLA-B27 compared to the wild-type KK10 due to inadequate processing. Furthermore, recognition of the variant peptide was shown to be reduced, but not fully abrogated, possibly due to rapid dissociation for the B27-molecule (Goulder et al., 1997). Despite this, in our assays using a high concentration of peptide, the mutant KK10 was able to stimulate T cells resulting in IFNγ production. Furthermore, although the peptide will more rapidly dissociate from the B27 molecule, the overall orientation of the peptide in the binding groove was shown not to be altered by the R to K mutation (Goulder et al., 1997). Therefore, we were able to synthesize HLA-B27 tetramers containing the mutant peptide. Even more, we were able to specifically stain T cells recognizing the mutant KK10 in the context of HLA-B27 to the same extent as T cells recognizing the wt KK10.

It is possible that the effects of acute T-cell responses have been missed due to the late start of our analysis at 12 mo. psc. At this time point, minimal T-cell reactivity was detected against the FL9 wild-type and mutant peptides, and this may well represent remnants of T-cell responses elicited earlier during infection. T-cell responses to Vpr and Vif are known to contribute significantly to the total CD8$^+$ T-cell responses in HIV-infected individuals (Altfeld et al., 2001), and the observed mutation in Vpr-AL9 (Table 1) has previously been shown to confer fitness loss upon the virus (Altfeld et al., 2001). The appearance of the well-documented HLA-A2-restricted SL9 T-cell response fits the previous finding as CTL directed against this epitope only appear during chronic infection (Goulder et al., 2001). We previously hypothesized that the increase in CD8$^+$ T-cell responses against the HLA-A3-restricted epitope RK9 and its mutant RR9 could be due to possible cross-reactivity of CD8$^+$ T cells recognizing a non-mutating HLA-A3-restricted epitope 2 amino acids upstream: KK9 (Geels et al., 2003). Analyzing the number of RK9-specific CD8$^+$ T cells as well as the number of KK9-specific CD8$^+$ T cells demonstrated that continued T-cell reactivity despite the occurrence of escape mutations is not likely to be a consequence of cross-reactivity.

In patient H434, the occurrence of escape mutation was paralleled by loss of CTL control, a subsequent increase in viral load and the eventual depletion of HIV-specific CD4$^+$ T cells producing IL-2 or IL-2 and IFNγ. Interestingly, this profile was not observed in patient H671, in whom escape was associated with an on average ten-fold less increase in viral load. These differences in viral load and its effect on CD4$^+$ T-helper cell function fit the model proposed by Younes et al. (2003), which indicates that the presence of IL-2-producing CD4$^+$ T cells with self-renewal capacity is restricted to aviremic individuals. In individuals with a high viral load, HIV-specific CD4$^+$ T cells are preferentially induced to further differentiate into a population of IFNγ-only-producing CD4$^+$ T cells, which lack proliferative potential upon re-stimulation by antigen. Thus, the observed loss of HIV-specific IL-2$^+$ and IL-2$^+$ and IFNγ$^+$ CD4$^+$ T cells

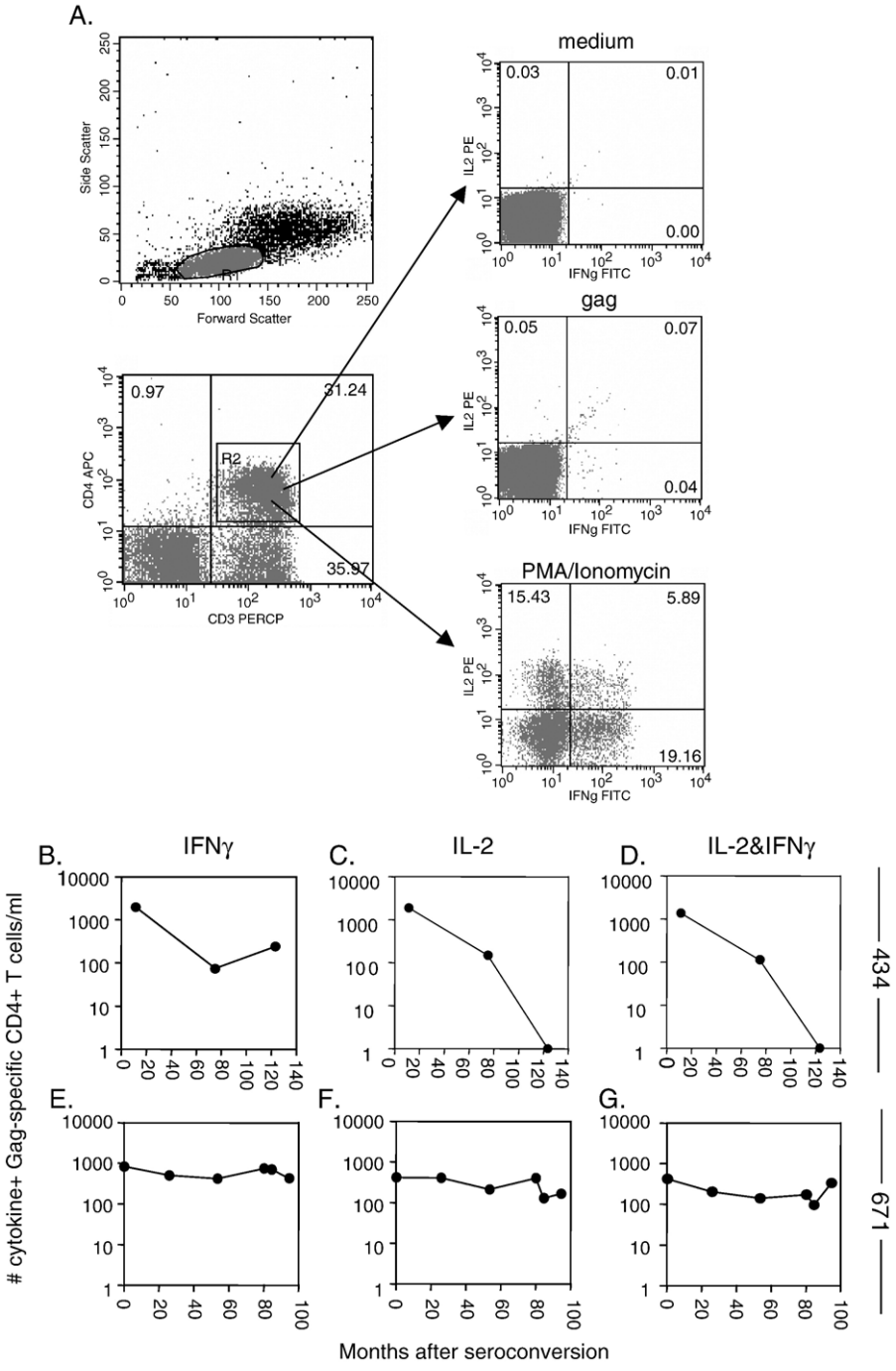

Fig. 4. HIV-specific T-helper responses in two individuals who escape CTL surveillance. Representative FACsplots demonstrating the production of IFNγ and IL-2 of $CD3^+$ $CD4^+$ T cells in the absence of stimulus (medium), after stimulation with a Gag-peptide pool (Gag) and a polyclonal stimulus (PMA/Ionomycin) in patient H671 at 0.2 months post-seroconversion. Percentages of $CD3^+$ $CD4^+$ T cells (left panel) and cytokine-producing $CD3^+$ $CD4^+$ T cells (right panel) are indicated. Longitudinal analyses of the absolute number of $IFN\gamma^+$ (B), $IL\text{-}2^+$ (C) and IL-2, and $IFN\gamma^+$ $CD4^+$ T cells (D) are shown for patient H434 and patient H671 (E–G).

may reflect the differences in viral load observed between the two patients. Furthermore, these data suggest that CTL escape is not necessarily linked to HIV-specific $CD4^+$ T-cell responses but imply that $CD4^+$ T-helper responses are influenced by the level of viremia, as has been reported before (McNeil et al., 2001; Iyasere et al., 2003; Palmer et al., 2004). However, studies in larger cohorts are warranted to further elucidate the role of $CD4^+$ T-cell responses in relation to viral load in subjects who escape CTL recognition.

Concurrent with the viral sequence evolution affecting CTL epitopes, mutations may arise in $CD4^+$ T-cell epitopes that influence loss of T-cell function during HIV-1 infection. Indeed, $CD4^+$ T-cell epitope escape has been described in the mice persistently infected with lymphocytic choriomeningitis virus (LCMV) (Ciurea et al., 2001). Although promiscuity has previously been reported for CD4 epitopes and their restricting haplotypes (Kaufmann et al., 2004), we found mutations in class-II-restricted peptides (p17, p24 and Nef), which are

known to be broadly recognized in clade-B-infected patients with different genetic backgrounds. Of note, mutations in $CD4^+$ T-cell epitopes overlapped with mutations in $CD8^+$ T-cell epitopes and are very likely primarily induced by class-I-restricted CTL responses. Mutations at these residues may prove to be a more effective means of viral escape since not only CTL responses, but also a more diverse array of responses are lost.

Taken together, we presented here two patients escaping HIV-1 CTL recognition. Although both escape CTL recognition, an apparent difference in control of viremia is observed between the two patients, which coincides with differences in HIV-1-specific $CD4^+$ T-cell responses. This suggests that loss of T-cell recognition after CTL escape is not directly linked to HIV-specific $CD4^+$ T-cell responses but that the increased viremia after CTL escape may influence $CD4^+$ T-helper responses as suggested previously (Jansen et al., 2005).

Materials and methods

Patient characteristics

Two patients (H434 and H671) were selected from the Amsterdam Cohort Studies (ACS) on HIV-1 infection and AIDS based on low viral loads and high $CD4^+$ T-cell numbers at study entry and a stable viral load during the first years of follow-up. After some years of follow-up, a sudden increase in viral load was observed which was paralleled by decreasing $CD4^+$ T-cell numbers. Patient H671 demonstrated a switch in virus phenotype to an SI variant at month 62, a time point after the rise in viral loads had occurred (data not shown), while patient H434 demonstrated NSI variants throughout. Both patients were heterozygous for the CCR5Δ32 allele and wild-type for CCR2 and SDF-1 alleles.

Patient H434 who entered the ACS remained therapy naive for 132 months. By linear regression analysis, we determined that the RNA viral load increased with a slope of 3690 copies/ml/month (standard deviation (SD) 762) The slope of $CD4^+$ T-cell decline was found to be 3.7 cells/ml/month (SD 0.5). The patient's HLA type was A2, 28, B7, 27, Cw02, DR09, 13, DQ03 and 06. Characteristics of the second patient (H671) have been previously described (Geels et al., 2003). The HLA type of this patient is HLA A3, 36, B15, 51, Cw3, 6 DR4, 8, DQ7 and for this individual sequential fixation of mutations in documented CTL epitopes or reactive peptides has been reported to coincide with alteration of T-cell responses and increasing viral loads during a 5-year follow up period (Geels et al., 2003). The average increase in viral load was 377.5 copies/ml/month (SD 42.7), whereas $CD4^+$ T-cell decline was 9.8 cells/ml/month (SD 1.1). Informed written consent was obtained from both patients, and this study was approved by the Medical Ethical Committee of the Academic Medical Center.

Biological cloning, full-length and serum HIV-1 sequencing

Viral DNA was extracted ('blood and body fluid DNA' isolation kit, QIAGEN, Valencia, CA, USA) from one million PBMCs infected with biological clones generated through limiting dilution (Koot et al., 1996). Sequencing of viral DNA was performed as previously published (Harris et al., 2002) with minor modifications.

To investigate the occurrence of escape mutations in the HLA-B27-restricted KK10 and the HLA-A2-restricted SL9 epitope, we analyzed serum-derived viral sequences for both epitopes at three time points during disease progression. Serum-derived viral nucleic acids were isolated using a silica-based method (Boom et al., 1991). Reverse transcription and polymerase chain reactions (PCRs) were performed using previously published methods with slight modifications (Cornelissen et al., 1997). SL9 sequences were reversed transcribed using primers Gag-5 (5′ CCC CCACTGTATT-TAACATGGTG 3′) and KK10 with TFSM (5′ TTATG-GAATTCATGAAGCTCTCTTCTGGTGG 3′). First PCR reaction for SL9 was performed using Louw1gag (5′ TTG ACT AGC GGA GGC TAG AA 3′) + Gag 5 and TFSM and SK145 (5′ AGT GGG GGG ACA TCA AGC AGC CAT GCA AAT 3′) for KK10. Secondary p17 PCRs were done with gag-2 (5′ CAT AAG CTT GGG AAA AAA TTC GGT TAA GGC C 3′) + gag-3 (5′ TCG GCT CCC TCC ATG TCT CC 3′) and for p24 epitope with p24/p7 (5′ GGG GAA GTG ACA TAG CAG GAA CTA 3′) and bgcom (5′ TCC CTA AAA AAT TAG CCT 3′).

All positive PCR products (full genome + serum) were bi-directionally sequenced with the BigDye Terminator Cycle Sequencing kit (ABI, Foster City CA, USA) and analyzed using an ABI 377 automated sequencer (ABI). Alignment of the sequences was straightforward and was performed manually using BioEdit 7.0 (T. Hall, Ibis Therapeutics, CA) and Textpad (Helios Software Solutions).

IFNγ Elispot assay

IFNγ-producing antigen-specific $CD8^+$ T cells were enumerated by IFNγ-specific Elispot assays as previously described (Lalvani et al., 1997; van Baarle et al., 2001) using 96-well nylon-backed plates (Nunc, Roskilde, Denmark) and IFNγ-specific monoclonal antibodies (MABTECH, Stockholm, Sweden). PBMCs were added at 10^5 cells per well in triplicate in the absence or presence of 10 μg/ml peptide and incubated overnight at 37 °C. PHA stimulation served as a positive control to test the capacity of PBMCs to produce IFNγ. IFNγ-producing cells were detected as dark spots and counted using an automated spot reader (AELVIS, Software version 3.2, Hanover, Germany). HIV-1-specific IFNγ responses were reported as number of spot-forming units (SFU) per 10^6 $CD8^+$ T cells. Only samples with at least twice the number of spots observed with media alone were considered positive.

Peptides that were used representing wild-type and mutant epitopes used in this study are described in Table 1 and were synthesized by the Dutch Cancer Institute, Amsterdam, The Netherlands. Peptides were dissolved in dimethyl sulfoxide (DMSO) at 100 mg/ml and stored at −20 °C.

Flow cytometry and tetramer staining

MHC class I tetramers complexed with virus-specific peptides were produced as previously described (Altman et al., 1996; Kostense et al., 2002). Monomers were analyzed biochemically for proper folding. Tetramers were tested for T-cell recognition in HLA-matched HIV-positive individuals showing T-cell reactivity against the specific peptide (staining between 0.1 and 0.4%, data not shown). PBMCs were thawed, and 1.5×10^6 cells were stained in PBS supplemented with 0.5% (v/v) bovine serum albumin (PBA) with MHC class I tetramers and fluorochrome-conjugated monoclonal antibodies. Next, cells were fixed in Cellfix (Beckton Dickinson, San José, California, United States), and at least 250,000 events were acquired using a FACSCalibur flow cytometer (BD). Lymphocytes were gated by forward and sideward scatter. Data were analyzed using the software program CELL Quest (BD).

Detection of HIV-specific $CD4^+$ T cells

Gag-specific $CD4^+$ T cells were quantified as previously described (Jansen et al., 2005). In short, cryopreserved PBMCs were thawed using RPMI-1640 medium supplemented with penicillin, streptomycin and 20% inactivated FCS and subsequently washed in 10% FCS medium. Cells were aliquoted at 2×10^6 cells per ml in round-bottom tubes (polystyrene, Falcon, BD). Cells were stimulated with a Gag-specific peptide pool (15 mers with 11 overlap, HXB2, NIH AIDS Research and Reagent program, Bethesda, Maryland, United States) in the presence of co-stimulation (2 μg/ml αCD28 (Sanquin Reagents) and 2 μg/ml αCD49d (Pharmingen, San José, California, United States). The concentration of the individual peptides within the pool was 2 μg/ml. As a positive control, PMA/Ionomycin was used. After 1 h, Brefeldin A (BD) was added, and cells were incubated for another 5 h at 37 °C, 5% CO_2. Next, cells were fixed and permeabilized (permeabilization kit, BD) and stained with αCD3-PerCP, αCD4-APC, αIL-2-PE and αIFNγ-FITC (BD) for 20 min at 4 °C. At least 300,000 events were acquired by flow cytometry as described before. Frequencies of IFNγ and IL-2-producing cells were reported after subtraction of the frequencies in medium controls. Cytokine production was above the background levels observed in HIV-negative individuals (median 0.02%, range 0–0.03, data not shown).

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