Acute phase cytotoxic T lymphocyte escape is a hallmark of simian immunodeficiency virus infection

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Cytotoxic T-lymphocyte (CTL) responses peak coincident with the decline in acute HIV viremia. Despite two reports of CTL-resistant HIV variants emerging during acute infection, the contribution of acute CTL escape to HIV pathogenesis remains unclear. Difficulties inherent in studying acute HIV infection can be overcome by modeling virus–host interactions in SIV-infected rhesus macaques. We sequenced 21 complete simian immunodeficiency virus (SIV)mac239 genomes at four weeks post-infection to determine the extent of acute CTL escape. Here we show that viruses from 19 of 21 macaques escaped from CTLs during acute infection and that these escape-selecting CTLs were responsive to lower concentrations of peptide than other SIV-specific CTLs. Interestingly, CTLs that require low peptide concentrations for stimulation (high 'functional avidity') are particularly effective at controlling other viral infections. Our results suggest that acute viral escape from CTLs is a hallmark of SIV infection and that CTLs with high functional avidity can rapidly select for escape variants.

CD8$^+$ cytotoxic T-lymphocyte (CTL) responses may have an important role in the containment of human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV). The appearance of CTLs in the acute phase correlates with the initial control of primary HIV and SIV viremia[1–4], and depletion of CD8$^+$ cells during acute and chronic SIV infection results in loss of control of SIV replication[5–7]. However, despite numerous studies delineating the roles of CD8$^+$ T cell responses in controlling HIV and SIV (ref. 8), it is not known which of the many CTL specificities present in infected individuals are important for control of viral replication[9–11]. Certain CTL responses are elicited early after infection and are maintained at relatively high levels[12] whereas other responses present at high levels during the acute phase of infection decline precipitously[13]. A third subset of responses is not present during early infection and arises only during chronic infection[14]. Finally, a fourth set of CTL responses are detected in HIV-exposed, seronegative subjects, but rarely in HIV-infected seropositive subjects[10,11,15,16]. It is unlikely that all of these CTL responses are equally effective, and some may be more important than others in controlling virus replication.

It is now well-established that both HIV- and SIV-specific CTL responses select for viral escape variants during chronic infection[17–24]. However, the extent of CTL escape in acute HIV infection is less clear. Though two groups have documented CTL escape during acute HIV infection[25,26], difficulties in recruiting untreated seronegative patients for studies has made it impossible to generalize these findings. The occurrence of acute phase CTL escape in SIV infection was illustrated by our previous identification of an immunodominant Mamu-A*01-restricted Tat-specific CTL response ($Tat_{28–35}$SL8) that rapidly selects for viral escape variants during acute infection of macaques with molecularly cloned SIVmac239 (ref. 13). This epitope accumulated mutations encoding amino-acid replacements coincident with the resolution of primary viremia, suggesting that this response might be important in the initial containment of acute infection. Because wild-type virus could not be detected in most Mamu-A*01-positive animals by eight weeks post-infection (p.i.) and there were few other amino acid changes in the entire virus in the two animals examined[13], we reasoned that this CTL response effectively eliminated all infected cells that presented epitopes derived from the wild-type virus. In striking contrast to the $Tat_{28–35}$SL8 response, a second Mamu-A*01-restricted CTL response that recognizes the $Gag_{181–189}$CM9 epitope does not generally select for escape variants during early infection, leading to the speculation that SIV can replicate despite this durable, dominant CTL response.

Understanding why $Tat_{28–35}$SL8-specific CTLs are fundamentally different from most CTLs that are elicited during SIV infection will be key to identifying similar rapidly escaping responses in HIV-infected individuals. Possible explanations for the rapid rate of variant selection within the $Tat_{28–35}$SL8 epitope include increased efficacy of CTLs directed against early viral proteins, immunodominant CTL activity against abundant viral proteins, preferential processing of $Tat_{28–35}$SL8 peptides by the proteolytic machinery, or greater ability of $Tat_{28–35}$SL8-specific CTLs to recognize lower concentrations of peptide than other CTL responses. 'Functional avidity' is a recently described term for the capacity of different CTLs to be sensitized by low concentrations of pep-

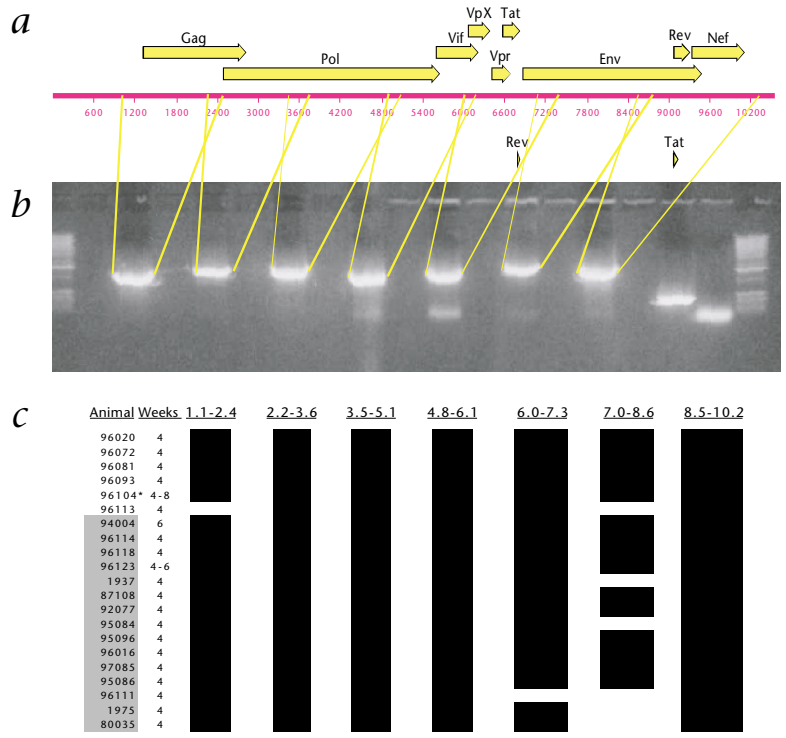

Fig. 1 Viruses from 21 SIV-infected macaques were screened for mixed-base heterogeneity during acute infection. ***a***, Genomic organization of SIVmac239. The locations of the 9 viral open reading frames are shown above the sequence. ***b***, Representative amplification of whole genome from 7 overlapping cDNA fragments. The location of each fragment is shown using lines drawn to *a*. The first and last lanes are size standards, the eighth lane is a positive control for RT-PCR amplification and the ninth lane is a negative control for reaction contamination. ***c***, Viruses from 21 SIVmac239-infected macaques were sequenced during early infection. Black boxes indicate viral amplicons that were directly sequenced and screened for mixed-base heterogeneity. Sample limitations precluded the complete viral genome analysis at 4 wk p.i. in some macaques. Macaques highlighted in gray are Mamu-A*01-positive.

tide[27–29]. CTLs that respond to low peptide concentrations (high functional avidity) are thought to be more effective than CTLs that require higher concentrations of peptide for sensitization[27].

We reasoned that CTLs that exert selective pressure on the viral population coincident with the resolution of acute viremia might be more effective than CTLs that do not select for amino-acid replacements in the acute phase. To identify additional effective CTLs that control wild-type virus during the acute phase, we conducted a cross-sectional analysis of viral variation at four weeks p.i. in 21 animals infected with molecularly cloned SIVmac239 by sequencing entire viral genomes after the emergence of the strong acute phase CTL responses. This approach identified six new CTL responses that select for viral variation in the acute phase and showed that acute phase escape occurred from at least one CTL response in 19 of 21 animals infected with SIVmac239. Notably, the CTLs that selected for escape variants during the acute phase were stimulated by low peptide concentrations, suggesting that they may be CTL responses of particularly high functional avidity.

22 sites of nucleotide heterogeneity at 4 weeks post-infection

To identify CTL responses that control wild-type viral replication during the acute phase, we directly sequenced complete or near-complete SIV genomes from 15 Mamu-A*01-positive and 6 Mamu-A*01-negative macaques at 4 weeks p.i. (Fig. 1). In 14 of the 15 Mamu-A*01-positive animals, mixed-base heterogeneity was identified within the $Tat_{28–35}$SL8 epitope by 8 weeks p.i. (data not shown).

In addition to $Tat_{28–35}$SL8 variation, only one additional site of mixed-base heterogeneity was consistently detected in the Mamu-A*01-positive animals. This substitution, encoding a non-synonymous variant in Rev/Env at nucleotide position 9,110 (site 17 in Fig. 2), was seen in 12 Mamu-A*01-positive and 4 Mamu-A*01-negative macaques. The wild-type sequence at this site is believed to be one of several suboptimal nucleotide sites in molecularly cloned SIVmac239 that frequently reverts to an alternate sequence *in vivo*[30].

In addition to variation at these two sites, 22 other unique sites with variation were identified by 4 weeks p.i. in our analysis of 21 macaques (Fig. 2). This includes 2 sites that were identical in viruses from each of 2 animals (sites 2 and 6 in Fig. 2). Viral clones spanning each of these sites were then analyzed to verify the direct sequencing results. In most cases, the sites identified as mixed bases were located within viral regions that contained variation as determined by clonal analysis. For 13 of the 22 unique sites, more than 50% of the clones were variant within 25 nucleotides of the mixed base (data not shown).

Six CD8 responses against regions of sequence variability

To identify the most likely candidates for regions of the virus evolving rapidly under CTL-mediated pressure, we focused on sites that encoded non-synonymous amino-acid substitutions and sites where more than 50% of the sequenced clones were variant. Eight of the 22 sites met both criteria (highlighted in yellow in Fig. 2), and the variants in these 8 regions were tightly clustered within 8–10 amino-acid (24–30 nucleotides) windows (Fig. 3). Acute phase cellular immune responses (4–8 wk p.i.) were detected against 3 of the 8 regions using an interferon-γ (IFN-γ) ELISPOT assay from frozen peripheral blood mononuclear cells (PBMCs) (sites 2, 6 and 7 in Fig. 3; Supplemental Fig. A online).

Of the remaining five regions of sequence heterogeneity, three contained CTL epitopes using *in vitro* restimulated PBMCs (sites 4, 5 and 8 in Figs. 2 and 3; CTL data not shown). In summary, 6 of the 8 sites containing sequence variation were located within regions targeted by CD8 responses.

We selected the three responses that could be detected directly *ex vivo* for further analysis and determined the minimal optimal CTL epitopes for each of these responses. The three epitopes were fine mapped to the amino-acid sequences YTSGPGIRY ($Nef_{159–167}$YY9) (site 7 in Figs. 2 and 3), GLDKGLSSL ($Nef_{45–53}$GL9) (site 6 in Fig. 2) and Vpr RGGCIHSR ($Vpr_{74–81}$RR8) (site 2 in Fig. 2; Supplemental Fig. B)

The Nef YY9 epitope is restricted by the major histocompatibility complex (MHC) class I molecule Mamu-A*02 (ref. 31 and T.U.V., unpublished data) and virus from Mamu-A*01- and A*02-positive macaque 95084 contains both $Nef_{159–167}$YY9 and $Tat_{28–35}$SL8 escape mutations by four weeks p.i. The same $Nef_{159–167}$YY9 Mamu-A*02-restricted response was mounted by

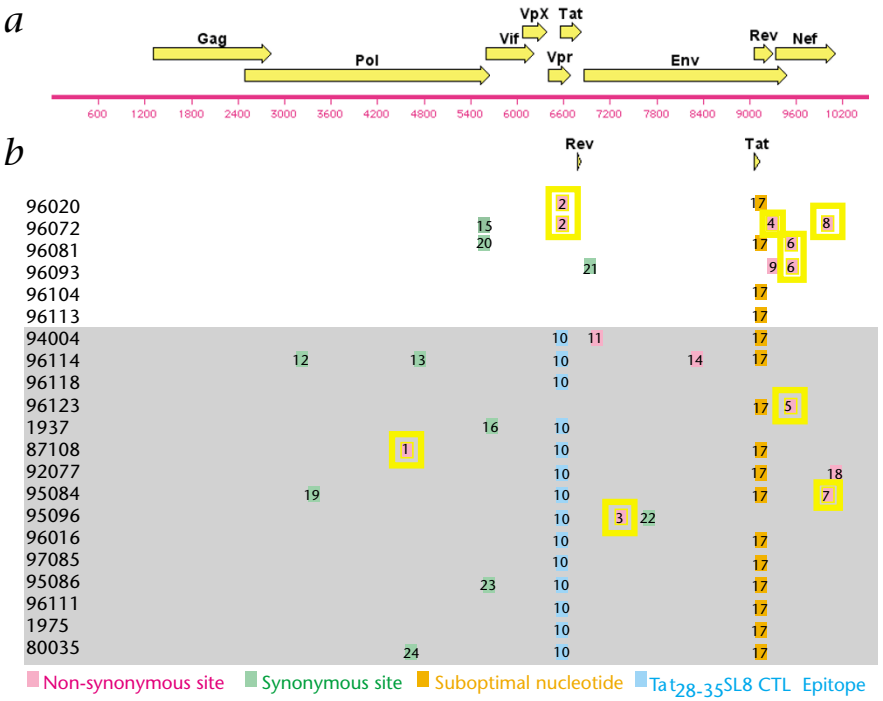

Fig. 2 Twenty-two unique sites of mixed-base heterogeneity identified at 4 wk p.i. with molecularly cloned SIVmac239. ***a***, Genomic organization of SIVmac239. ***b***, Schematic representation of 24 mixed-base sites. The sequence changes observed in each animal are color coded as synonymous, non-synonymous, suboptimal nucleotides, or within the Tat_{28-35}SL8 CTL epitope. Non-synonymous sites where > 50% of clones contain variant sequence (excluding Tat_{28-35}SL8) are boxed in yellow. The positioning of the sequence changes is shown relative to the genome of SIVmac239 in *a*. Animals with gray highlight are Mamu-A*01 positive. ***c***, Table of the 24 variant sites (22 unique + suboptimal Rev/Env + Tat_{28-35}SL8). The 8 sites that accumulated non-synonymous nucleotide variation present in greater than 50% of sequenced clones (excluding Tat_{28-35}SL8) are highlighted in yellow. The sequence changes observed in each animal are color coded as synonymous, non-synonymous, suboptimal nucleotides, or within the Tat_{28-35}SL8 epitopes (all variation in Tat_{28-35}SL8 epitope in Mamu-A*01 positive animals is presumed to be linked to selection against this epitope).

c

Number	Site	Protein	CTL
1	4506	Pol	No
2	6639	Vpr	Yes
3	7238	Env	No
4	9312	Env	Yes
5	9440	Env	Yes
6	9466	Nef	Yes
7	9814	Nef	Yes
8	9826	Nef	Yes
9	9292	Rev	N/T
10	6639:62	Tat	Yes
11	7058	Env	N/T
12	3170	Pol	Syn
13	4766	Pol	Syn
14	8363	Env	No
15	5585	Pol	Syn
16	5776	Vif	Syn
17	9110	Env/Rev	N/T
18	10047	Nef	N/T
19	3404	Pol	Syn
20	5588	Pol	Syn
21	6982	Env	Syn
22	7570	Env	Syn
23	5727	Vif	Syn
24	4586	Pol	Syn

macaque 96072, which also had variation within this epitope (site 8 in Figs. 2 and 3).

Macaque 97073, an animal not included in the original study, also recognized the Vpr_{74-81}RR8 epitope and accumulated viral variation in this epitope (data not shown) and other Mamu-A*02-positive macaques including macaque 95061 consistently targeted the $Nef_{159-167}$YY9 epitope (data not shown). Samples from these macaques were used to characterize the Vpr_{74-81}RR8 and $Nef_{159-167}$YY9 responses in cases where samples from macaques 96072 and 95084 were unavailable.

Table 1 Rate of escape of tested CTL epitopes

CTL epitope / rate of escape	MHC-restriction	Earliest escape detected	Functional avidity* (range (nM))
Acute			
Tat SL8 (STPESANL)	A*01	4 wk	0.18 (0.08–0.21)
Nef GL9 (GLDKGLSSL)	n.d.	4 wk	0.56 (0.19–2.1)
Nef YY9 (YTSGPGIRY)	A*02	4 wk	4.8 (0.75–68)
Vpr RR8 (RGGCIHSR)	n.d.	4 wk	3.0 (2.8–3.25)
Chronic			
Nef AL11(ARRHRILDIYL)	B*03	> 6 mo	23.6 (17–33)
Gag CM9 (CTPYDINQM)	A*01	> 12 mo	13.3 (2.2–30)
Env GI8 (GDYKLVEI)	A*11	> 12 mo	30.7 (21–60)
Env KL9 (KRQQELLRL)	B*03	> 25 mo	17.9 (10–32)
Nef IW9 (IRYPKTFGW)	B*17	> 4 mo	188 (31–580)
Time of death (TOD)			
Gag QI9 (QNPIPVGNI)	A*01	Not detected	574 (150–1400)
Vif QA9 (QVPSLQYLA)	A*01	Not detected	419 (110–1400)

*, geometric mean of all tests to determine peptide concentration needed to induce 50% maximal IFN-γ production. n.d., not determined.

CTLs select for amino-acid variants

To determine whether these newly identified CTL responses selected for escape variants, we examined the nature of the nucleotide mutations in and around the viral sequence encoding the CTL epitopes. In the epitope regions, the number of nonsynonymous substitutions per nonsynonymous site (d_N) exceeded the number of synonymous substitutions per synonymous site (d_S) in all comparisons, and the difference was statistically significant ($P < 0.05$) in the case of Nef GL9 of macaque 96081 (Supplemental Table A). No such pattern was seen in the remainder of the sequenced portions of the gene, and mean dN in the epitopes regions was significantly elevated in comparison with that in the remainder ($P < 0.05$) (Supplemental Table A). Therefore, there was evidence for positive selection in the regions containing the newly identified epitopes, strongly implicating the variation in escape from cellular immune responses.

Epitope variants are poorly recognized by CTLs

We then determined whether epitope variation reduced recognition of the MHC class I peptide complex by the T-cell receptor (TCR). Variant peptides for each epitope were tested against wild-type peptide at various concentrations using CTL lines specific for each epitope. Most of the peptides encoding epitope variants decreased the ability of CTLs to elaborate intracellular IFN-γ, particularly at lower concentrations of peptide (data not shown).

CTL functional avidity seems to influence viral escape

Because the 3 new CTL responses against epitopes in Nef and Vpr exerted selective pressure similar to $Tat_{28–35}$SL8-specific CTLs, we investigated whether these four responses shared any characteristics or qualities that were not seen in responses that did not rapidly select for escape variants. It has recently been shown that T-cell responsiveness to peptide (defined as functional avidity) increases substantially during acute lymphocytic choriomeningitis virus (LCMV) infection[27]. CTL responses that selected for escape variants during acute infection might be of high functional avidity. SIV-specific CTLs that respond effectively to low concentrations of peptide may have greater *in vivo* potency than those that require high peptide concentrations for stimulation, as has been previously observed in CTL responses to other viral infections[29,32–34].

To measure functional avidity, we tested the ability of T cells to elaborate intracellular IFN-γ in response to stimulation with different peptide concentrations. Ten-fold dilutions of peptides were tested over a range of 5 pM to 5 μM and the concentration of peptide that yielded 50% of the maximal IFN-γ response was determined and termed the $1/2_{max}$ response. For each epitope, at least two samples were tested and functional avidity was reported as the geometric mean of the individual $1/2_{max}$ responses.

First we compared the functional avidities of the well-described $Tat_{28–35}$SL8- and $Gag_{181–189}$CM9-specific CTL responses. Although the functional avidity of each response was relatively constant over time and in multiple animals (Supplemental Fig. C), we observed a consistent difference between the functional avidities of $Tat_{28–35}$SL8 and $Gag_{181–189}$CM9 responses.

We then tested 5 previously defined SIV CTL epitopes that demonstrated intermediate escape kinetics (approximately 1 y p.i.)[22,23], 2 previously defined Mamu-A*01-restricted CTL epitopes[35] that do not select for CTL escape variants even at the time of death in multiple animals (data not shown), and the 4 CTL epitopes that select for escape variants during early infection. These epitopes are grouped in Table 1 according to their relative rates of escape, defined as acute (< 16 wk p.i.), chronic (> 16–52 wk p.i.) or time of death (unable to escape even at time of death (TOD)).

The 11 CTL epitopes had a wide range of avidity values (Fig. 4). The epitopes generally fell within 3 groupings: those with geometric mean $1/2_{max}$ responses less than 5.0 nM, those between 5.0 and 50 nM and finally those greater than 50 nM. Those epitopes that did not escape, even at the time of death, demonstrated low avidity values (high $1/2_{max}$) (Fig. 4 and Table 1). In contrast, 5 of the 6 chronically escaping epitopes grouped tightly and displayed moderate avidity values, only the $Nef_{165–173}$IW9 response exhibited low avidity (Fig. 4). Finally, of those epitopes escaping rapidly, 4 out of 4 demonstrated high functional avidity (Fig. 4). Therefore, for 10 out of the 11 epitopes investigated there was a clear association between the avidity of a CD8 response with its ability to induce rapid viral escape in SIV.

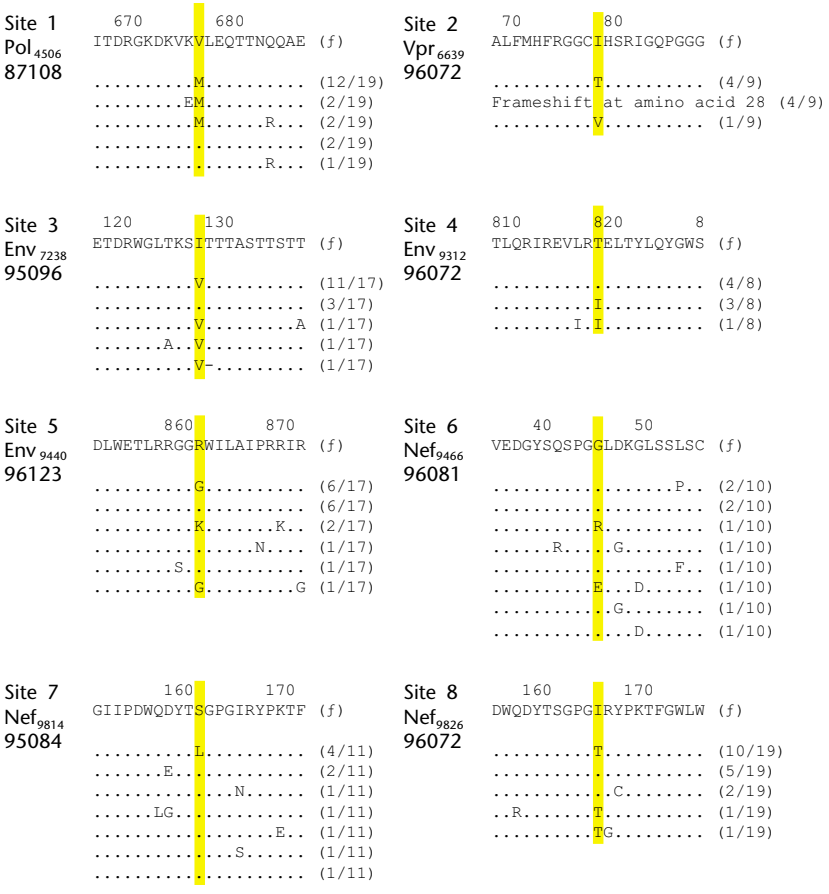

Fig. 3 Eight viral regions exhibited variation consistent with CTL escape by 4 wk p.i. The predicted amino-acid sequence is shown for each DNA sequence. The amino-acid position containing the site of variability identified by direct sequencing is highlighted in yellow. 10 flanking amino acids on either side of the variable site are shown. The numbers in parentheses reflect the number of clones containing a given sequence divided by the total number of clones examined. The numbers above the wild-type sequences show the amino-acid positions of the shown residues within each viral protein.

Discussion

Viruses from 19 of 21 animals escaped from at least one CTL response by 8 weeks p.i., indicating that CTL escape is a hallmark of acute SIV infection. Given the similarities between acute HIV and SIV infections, our results with SIV-infected monkeys likely mirror the events that occur during acute HIV infection. Between two and eight weeks p.i., the plasma virus concentration in most macaques typically declines by at least two orders of magnitude coincident with the development of strong CTL responses. Although some of this viral load reduction can likely be attributed to factors such as depletion of available target cells and the development of immune responses other than CTLs, it is tempting to speculate that the CTL responses that select for escape variants during acute infection are primarily responsible for the initial reduction in

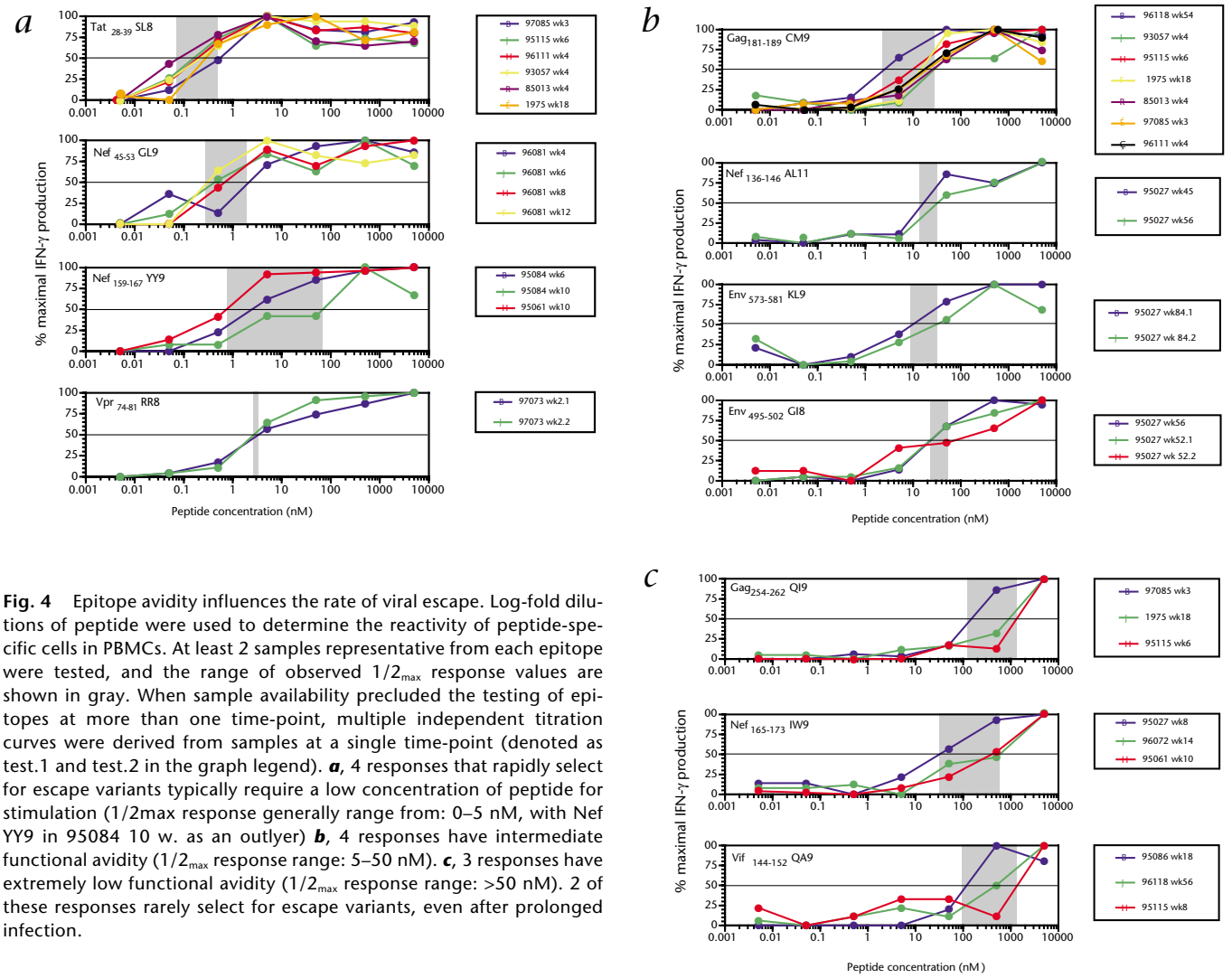

Fig. 4 Epitope avidity influences the rate of viral escape. Log-fold dilutions of peptide were used to determine the reactivity of peptide-specific cells in PBMCs. At least 2 samples representative from each epitope were tested, and the range of observed $1/2_{max}$ response values are shown in gray. When sample availability precluded the testing of epitopes at more than one time-point, multiple independent titration curves were derived from samples at a single time-point (denoted as test.1 and test.2 in the graph legend). ***a***, 4 responses that rapidly select for escape variants typically require a low concentration of peptide for stimulation (1/2max response generally range from: 0–5 nM, with Nef YY9 in 95084 10 w. as an outlyer) ***b***, 4 responses have intermediate functional avidity ($1/2_{max}$ response range: 5–50 nM). ***c***, 3 responses have extremely low functional avidity ($1/2_{max}$ response range: >50 nM). 2 of these responses rarely select for escape variants, even after prolonged infection.

plasma virus concentration. The inability of CTLs to resolve viremia during chronic infection may be due to the loss of the most potent and effective CTL specificities during the first weeks of infection. Because the concentration of HIV in the plasma after seroconversion predicts the rate of disease progression[36–38], effective resolution of acute infection may influence chronic disease progression. The presence of a single acute CTL response that selects for escape variants may be analogous to treatment with a single antiretroviral drug, where initial success at reducing viral replication is rapidly lost as the viral population becomes resistant. The effect of multiple acute CTL responses, then, may be analogous to multidrug therapy; delaying the emergence of escape variants, reducing viral burden, and possibly enabling a broadening of the immune response by preserving the functional capacity of the $CD4^+$ T-lymphocyte population. In this way, long-term nonprogression of infected individuals may be potentiated by events that occur during the first weeks of infection.

As it is unlikely that all virus-specific CTL responses mounted by an individual are equally effective, understanding the contribution of each response to the control of viral replication will be critical to defining the correlates of protection. Although dominant responses can be identified by enumerating antigen-specific cells that react to stimulation with a particular peptide, it is unlikely that immunodominance and CTL efficacy are correlated[33,39]. Identifying responses that actively select for viral variants (implying that the selecting CTLs eliminate wild-type virus) using CTL escape analyses is currently our only measure of CTL efficacy *in vivo*. Our study is the first comprehensive, cross-sectional analysis of CTL escape during acute SIV infection. Most of the prior studies on CTL escape have examined short regions of the virus or have analyzed well-defined CTL epitopes, largely because it has been difficult to simultaneously examine large numbers of CTL responses and sequence complete viral genomes. Screening entire viral genomes for evidence of CTL escape may be necessary to determine which of the many acute phase cellular immune responses are effective.

In spite of the challenges involved in analyzing immune responses and viral evolution in HIV-infected patients, identifying analogous HIV-specific CTL responses that select for HIV escape variants during early infection may be important for HIV vaccine design since these CTLs may be fundamentally different from those that do not select for escape variants during early infection. Our results suggest that there may be at least two different methods to identify these potentially important responses. Similar to

this study, prospective viral sequencing can be used to look for rapidly evolving regions of the virus that may represent sites targeted by CTLs. A second approach might be to examine the functional avidity of HIV-specific responses. We have shown that each of the four CTL responses studied that select for escape variants during acute SIV infection can be stimulated with low concentrations of peptide, suggesting that these CTLs are of high functional avidity. This raises the possibility that CTLs that select for escape variants in the acute phase could be identified on the basis of high functional avidity during chronic infection, bypassing some of the difficulties associated with studying acute infection.

It is likely that the functional avidity of a particular CTL response is not the only determinant of the rate of viral escape. Examination of more epitopes might reveal additional responses with high functional avidity that do not select for escape variants during acute infection. Epitopes within conserved regions of the virus that do not easily tolerate variation but have high functional avidity were not considered in this study, as we biased our analysis exclusively towards those epitopes that rapidly escape. Evaluating whether slowly escaping, high-functional avidity CTLs exist in SIV infection may prove useful for vaccine design. It is also possible that high CTL functional avidity is necessary, but not singly sufficient, for a response to select for escape variants during acute infection. Additional factors, such as the kinetics of viral protein expression, the abundance of processed epitope on the cell surface, and the functional and structural constraints on particular viral regions may also influence the rate of CTL escape. Certain responses, however, consistently select for escape variants during acute infection of macaques that express the appropriate restricting MHC class I allele. This underscores the reproducibility of escape and implies that there may be a 'biological determinism' that governs cellular immune reactivity to SIV.

These findings may also have implications for global HIV diversity. A recent report suggests that CTL escape mutations, like antiviral drug resistance mutations, can be transmitted among individuals and stably maintained even in the absence of the original selective pressure[40]. Given that CTL escape mutations that accumulate during acute infection persist throughout infection, it is likely that these escape mutations are frequently transmitted between individuals. This raises the possibility that global HIV diversity may be partially attributable to variation that arises early during infection and that acutely escaping CTL epitopes in HIV may be lost as the epidemic progresses.

By examining the acute phase of SIVmac239 infection in 21 animals, we have shown that CTL escape is a hallmark of SIV infection and that the rate of CTL escape may be linked to the functional avidity of CTL responses. The emergence of similar escape mutants in HIV may explain the failure of initial immune responses to eliminate circulating virus and may contribute to the establishment of chronic infection.

Methods

Animals, viruses and infections. Rhesus macaques used in this study were identified as Mamu-A*01-, -A*02-, -A*11-, -B*03- or -B*17-positive, by PCR-SSP and direct sequencing as described[41]. All macaques used in this study were Mamu-A*01-positive, with the exception of macaques 97073, 96020, 96072, 96081, 96093, 95027, 96104 and 96113. The vaccination and challenge regimens of macaques involved in this study have been published[13,22,42,43]. The sequences of all viruses used in this report have been reported[44,45]. SIV-infected macaques were cared for according to an experimental protocol approved by the University of Wisconsin Research Animal Resource Committee.

Amplification of viral RNA from plasma. Cell-free plasma was obtained by centrifugation of EDTA anti-coagulated whole blood on a Ficoll density gradient. Virus was extracted from the plasma using the QIAGEN QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, California) according the manufacturer's instructions for large volume samples. For each viral RNA sample, 7 RT-PCR reactions were performed with the QIAGEN One Step RT-PCR kit, generating overlapping amplicons spanning the open reading frames of SIVmac239. Each of the amplicons spanned 1.2–1.7 kb of the genome, overlapping with each adjacent amplicon by at least 100 base pairs (Fig. 1*a* and *b*). The primer pairs for RT-PCR included the following: 1) 1151-F and 2445-R; 2) 2227-F and 3748-R; 3) 3518-F and 5184-R; 4) 4881-F and 6139-R; 5) 6007-F and 7341-R; 6) 6966-F and 8612-R; and 7) 8534-F and 10203-R. The RT-PCR conditions for amplicons 1, 4, 5, 6 and 7 were as follows: 50 °C for 1 h, 95 °C for 15 min, 45 cycles of 94 °C for 30 s, 59 °C for 30 s and 72 °C for 120 s, and 68 °C for 20 min. The melting characteristics of the primers used in amplicons 2 and 3 required a 55 °C annealing temperature, all other parameters were unchanged.

Mixed base sequence detection. The amplified cDNA was purified using a commercially available kit (QIAGEN PCR Purification Kit). Both strands of each amplicon were sequenced on ABI 377 automated sequencers (Applied Biosystems, Foster City, California) using a panel of either 8 or 10 sequencing primers. The sequences and positioning of these oligos have been described[13].

Factura v.2.2 (Applied Biosystems) was used to process the raw sequence data, automatically identifying all nucleotide sites where the ratio of the highest signal peak to the second highest signal peak was less than 2:1. These edited files were then imported into AutoAssembler v2.1 (Applied Biosystems) and assembled into contiguous sequences spanning the amplicons. After manual proofreading, the edited sequences for each amplicon were added to a new AutoAssembler project and assembled into a larger contiguous fragment spanning the entire viral genome. The viral sequences were then compared to the Genbank SIVmac239 sequence using the ClustalW alignment function in MacVector 7.0 Trial Version (Oxford Molecular, Oxford, UK).

Generation of viral clones. PCR products containing sites of interest were directly cloned into the commercially available vector pCR2.1-TOPO using the TOPO-TA Cloning Kit (Invitrogen, Carlsbad, California). Individual clones were obtained using the QIAprep Spin Miniprep Kit (QIAGEN). At least 10 clones spanning each nucleotide site of interest were sequenced using a subset of the sequencing primers described above.

Isolation of PBMCs and generation of *in vitro*-cultured CTL effector cells. PBMCs were isolated from EDTA or heparin-treated whole blood using Ficoll/diatrioate gradient centrifugation as described[22]. CTL cultures were established from EDTA or heparinized-treated peripheral blood samples as described[46].

IFN-γ ELISPOT assay. IFN-γ ELISPOT was performed on approximately 2×10^5 frozen PBMCs as described[47], and each sample was tested in triplicate. Responses significantly higher than background levels (twice the spot forming cells (SFCs) from untreated PBMCs plus 5 spots) and significant according to Student's *t*-test ($P < 0.05$) were considered positive.

Statistical analysis of sequence data. Numbers of synonymous nucleotide substitutions per synonymous site (d_S) and numbers of nonsynonymous substitutions per nonsynonymous site (d_N) were estimated by Nei and Gojobori's (1986) method[48]. Standard errors of means of d_S and d_N for sets of pairwise comparisons were estimated as described[49]. 100 base pairs of flanking sequence in each direction were analyzed to provide data for the remainder.

Intracellular cytokine staining avidity assay. Intracellular cytokine staining (ICS) for IFN-γ production by *in vitro*-cultured CTL effector cells was performed as previously described. To determine the functional avidity of each response, 10-fold dilutions of peptides ranging from 5 pM to 5 μM were tested with fresh and frozen PBMCs in ICS assays. Each epitope was analyzed in at least 2 individual experiments. The IFN-γ responses to peptide dilutions were expressed as a percentage of the maximal IFN-γ response seen in each

particular assay. Functional avidity was calculated as the peptide concentration capable of eliciting 50% of the maximal IFN-γ response in each individual assay[27]. In order to graphically compare the $1/2_{max}$ IFN-γ responses between assays, the amount of IFN-γ produced by a CD8 response at the various peptide concentrations was represented as a percentage. The percentage of IFN-γ produced was calculated using the following formula: % IFN-γ production = (experimental production at each peptide concentration – minimum production) / (maximum production – minimum production). The values for the maximum and minimum IFN-γ production were independently determined from the range of peptide concentrations tested in each assay. The geometric mean of the individual $1/2_{max}$ IFN-γ responses for a given CD8 response was then calculated.

Note: Supplementary information is available on the Nature Medicine *website.*

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Competing interests statement

The authors declare they have no competing financial interests.

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