Inefficient Cytotoxic T Lymphocyte–Mediated Killing of HIV-1–Infected Cells In Vivo

Becca Asquith[1,2*], Charles T. T. Edwards[3], Marc Lipsitch[4], Angela R. McLean[1]

1 Department of Zoology, University of Oxford, Oxford, United Kingdom, **2** Department of Immunology, Imperial College London, London, United Kingdom, **3** Nuffield Department of Clinical Medicine, University of Oxford, Oxford, United Kingdom, **4** Departments of Epidemiology and Immunology & Infectious Diseases, Harvard School of Public Health, Boston, Massachusetts, United States of America

Understanding the role of cytotoxic T lymphocytes (CTLs) in controlling HIV-1 infection is vital for vaccine design. However, it is difficult to assess the importance of CTLs in natural infection. Different human leukocyte antigen (HLA) class I alleles are associated with different rates of progression to AIDS, indicating that CTLs play a protective role. Yet virus clearance rates following antiretroviral therapy are not impaired in individuals with advanced HIV disease, suggesting that weakening of the CTL response is not the major underlying cause of disease progression and that CTLs do not have an important protective role. Here we reconcile these apparently conflicting studies. We estimate the selection pressure exerted by CTL responses that drive the emergence of immune escape variants, thereby directly quantifying the efficiency of HIV-1–specific CTLs in vivo. We estimate that only 2% of productively infected $CD4^+$ cell death is attributable to CTLs recognising a single epitope. We suggest that CTLs kill a large number of infected cells (about 10^7) per day but are not responsible for the majority of infected cell death.

Citation: Asquith B, Edwards CTT, Lipsitch M, McLean AR (2006) Inefficient cytotoxic T lymphocyte–mediated killing of HIV-1–infected cells in vivo. PLoS Biol 4(4): e90.

Introduction

Half of all $CD4^+$ T cells that are productively infected with HIV-1 die every 12 h. A productively infected cell is estimated to have a lifespan of about 1 d [1–5], considerably less than the lifespan of $CD4^+$ cells in uninfected individuals [6]. It is not known whether the majority of this cell death is caused by cytotoxic T lymphocyte (CTL)–mediated cytotoxicity or other mechanisms such as viral cytopathicity, activation-induced apoptosis, or complement-mediated lysis.

There is good evidence that the CTL response contributes to the control of HIV-1 infection in vivo [7–11]. However, the magnitude of this contribution is contentious [2,3,12–16]. One of the best pieces of evidence that CTLs exert selective pressure on HIV-1 is the existence of CTL escape mutations. However, these data have never been rigorously quantified. Indeed, it has been argued that CTL escape is a relatively infrequent event, with most CTL clones remaining stable for long periods of time, suggesting either considerable fitness constraints on HIV mutations or weak CTL selection pressure for escape [17]. New data on the in vivo fitness cost of escape variants have recently become available [18–21]. These new data allow analysis of the rate of outgrowth of CTL escape variants that yields estimates of the selective pressure exerted by a single CTL response against HIV-1 in vivo.

The rate at which an escape variant replaces the wild-type, "the rate of escape," is determined by the balance between the efficiency of the CTL clone(s) evaded and the fitness cost of the mutations (Figure 1). The aim of this work was to estimate the efficiency of a single HIV-1–specific CTL response, i.e., the infected cell death rate in vivo attributable to CTL clones recognising one epitope. This was done by quantifying the rate of escape and the fitness cost of CTL escape variants. By comparing the CTL-mediated death rate with the total death rate of HIV-infected cells, the proportion of infected cell death attributable to the CTL response was estimated.

Results

We estimated the rate of escape of 21 reported CTL escape variants using longitudinal data from 12 HIV-1–infected individuals [9,10,19,22–28] (Table 1). In every case we made a "best estimate" of the rate of escape by fitting a simple model to the data (Equation 2 in Materials and Methods). Where possible we also made an "optimistic estimate," which can be considered to be an approximate upper bound on the rate of escape. The fits of the model to the data are shown in Figure 2 and the estimates of the rate of escape are shown in Figure 3 and Table 2. The results were remarkably consistent from one escape variant to the next: in 20 of 21 datasets the rate of escape was less than 0.1 d^{-1}. The median rate of escape was 0.01 d^{-1}, and taking the most optimistic interpretation, the median rate of escape was 0.04 d^{-1}.

It is possible that, as the proportion of cells infected with

Academic Editor: Sarah Rowland-Jones, Weatherall Institute of Molecular Medicine, United Kingdom

Received July 11, 2005; **Accepted** January 23, 2006; **Published** March 14, 2006

DOI: 10.1371/journal.pbio.0040090

Abbreviations: CTL, cytotoxic T lymphocyte; HAART, highly active antiretroviral treatment; HLA, human leukocyte antigen; PBMC, peripheral blood mononuclear cell

* To whom correspondence should be addressed. E-mail: b.asquith@imperial.ac.uk

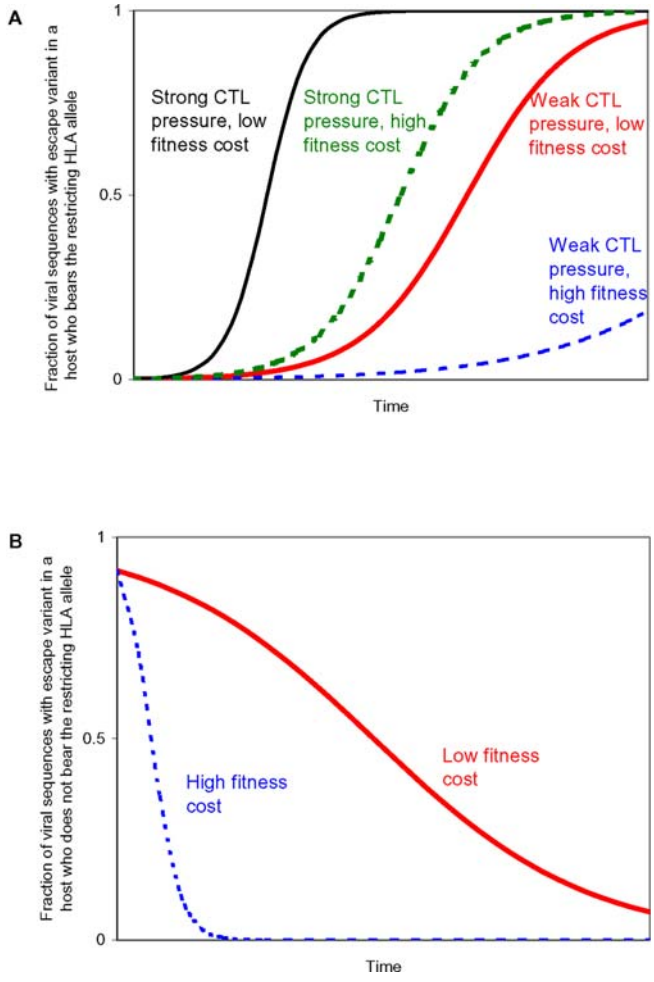

Figure 1. Calculating the Rate of CTL Killing

We need to know how important CTLs are in the control of HIV-1 infection, but it is not possible to measure the rate at which CTLs kill HIV-1–infected cells in vivo using conventional assays. Here we develop an alternative approach. CTL selection pressure drives viral escape, so a surrogate marker of the importance of CTLs is the rate at which CTL escape variants replace the wild-type. (A) In a host bearing the restricting HLA allele a CTL escape variant grows more rapidly than and replaces the wild-type because it is subject to a lower rate of CTL killing. The rate of replacement of the wild-type by the escape variant, "the escape rate," is equal to the difference in growth rate of the wild-type and escape variant. If everything else were equal, the difference in growth rate would be equal to the difference in the CTL killing rate of the wild-type and the escape variant. This would mean that the CTL pressure on the specific epitope that has undergone mutation (the "escape epitope") could be measured by the escape rate. However, everything else is not equal. Many escape variants will carry a fitness cost, which will slow the growth rate of the escape variant and thus decrease the escape rate. This fitness cost is revealed as the reversion rate when the variant is transferred to a host who does not bear the restricting HLA (B). The escape rate will therefore be equal to the rate of lysis by CTLs targeting the escape epitope minus the fitness cost. Expressed in a different way, the rate of lysis of HIV-1–infected cells by CTLs targeting the escape epitope is equal to the escape rate plus the reversion rate. In (A) the relative position of the two lines "strong CTL pressure, high fitness cost" and "weak CTL pressure, low fitness cost" could be interchanged depending on the magnitude of the differences in CTL strength and variant fitness.

DOI: 10.1371/journal.pbio.0040090.g001

wild-type virus declines, the antigenic stimulus declines, causing a loss of epitope-specific CTLs. This would reduce the selection pressure for escape and so would lead to an underestimate of the escape rate that would be observed under continuing CTL pressure. In fact, in the majority of cases where CTL frequency was measured the magnitude of the response to the epitope of interest was reported to be fairly stable through time. However, there were cases where the CTL response did decline or else was not measured, so we quantified the impact of a declining CTL response on the escape-rate estimates. This was done by estimating the half-life of HIV-1–specific CTLs following highly active antiretroviral treatment (HAART) and then assuming that CTLs declined with the same half-life during outgrowth of the variant (see Materials and Methods). Even using this extremely stringent assumption (antigenic stimulus falls considerably more rapidly on commencing HAART than during escape [1,4,8]) the median escape rate was still only 0.055 d^{-1}.

To see if this low rate of escape could be explained by a high fitness cost of CTL escape variants we quantified the rate of reversion of escape variants to wild-type on transmission to individuals who did not possess the human leukocyte antigen (HLA) allele necessary to bind the epitope of interest. We quantified the rate of reversion in seven datasets from five patients [18–21] (Table 3). The fits of the model to the data are shown in Figure 4 and the estimates of the rate of reversion in Figure 5 and Table 4. This analysis showed that the fitness cost of an average CTL escape mutation was very low—about 0.005 d^{-1}. While these low fitness costs were surprising, they are consistent with data from in vitro competition assays [18] and accumulation of escape mutations in the population over time [18,21,29], which both suggest that the fitness cost of some escape variants is close to zero.

Given that escape and reversion rates are consistent across individuals it is reasonable to consider the average rate of escape and reversion of a CTL escape variant. Putting these average estimates together we can estimate the rate of lysis of infected cells by a single CTL response. We found that the rate of lysis was about 0.02 d^{-1} (escape rate + reversion rate = $0.01 + 0.005$ d^{-1}) and was at most 0.06 d^{-1} ($0.055 + 0.005$ d^{-1}). This implies that, using the best estimate, an infected cell that was subject only to lysis by CTLs specific for one epitope would live for about 50 days. A productively infected cell has a lifespan of about 1 d [1–5]; therefore, only about 2% of productively infected cell death is attributable to CTL responses against a single epitope.

On comparing the rate of CTL lysis in individuals with primary and chronic infection we found that the rate of CTL lysis was significantly faster during primary infection than in chronic infection ($p = 0.004$ Wilcoxon–Mann-Whitney two-tailed test, Figure 6).

Discussion

HIV-1–specific CTLs have two known main modes of action: direct killing of infected cells and secretion of soluble antiviral factors. Unless secretion of antiviral factors is highly localised and directed, CTL escape will only reflect escape from CTL-mediated killing since the action of antiviral factors is nonspecific and would therefore give no advantage to escape variants. Our study of the role of CTLs therefore only relates to CTL killing and will not encompass CTL secretion of antiviral factors which may be of considerable significance [11,15].

It would be fascinating to extrapolate these results on the rate of lysis by a single CTL response to estimate the total

Table 1. Escape Datasets Studied

Dataset	Data Source	Class I HLA Type				Disease Stage	Viral Load (Copies/ml)	CD4 Count (Cells/mm^3)	CTL Epitope (HXB2)
		A		B					
1	[27]	0201				Primary	30,000	1,000	Gag p17 77–85
2	[28]	01	03	2705	35	Primary	1,258,925	520	Gag p24 131–140
3	[28]	02	22	2705	35	Asymptomatic	—	330	Gag p24 131–140
4	[23]			27		Late stage	—	250	Gag p24 131–140
5	[9]	01	29	08	44	Primary	146,800	748	Env gp160 31–39
6	[19]	0101	0301	0801	0801	Primary	34,293	490	Gag p17 20–28 and 24–31
7	[19]	0101	0301	0801	0801	Primary	34,293	490	Gag p17 18–26
8	[19]	0101	0301	0801	0801	Primary	34,293	490	Nef 73–82
9	[10]			08		Asymptomatic	—	400	Gag p17 21–35
10	[26]	01	01	07	08	Primary	7,600,000	384	Nef 90–97
11	[25]	03				Asymptomatic	10,000[a]	450	Nef 73–82
12	[22]	03	32	51	15	Asymptomatic	400	900	Env gp41 190–208
13	[22]	03	32	51	15	Asymptomatic	400	1,200	Nef 120–128
14	[22]	03	32	51	15	Asymptomatic	200	1,100	Pol-RT 128–135
15	[24]	2902		1402		Primary	0[b]	—	Env gp160 209–217
16	[24]	2902		1402		Primary	70[b]	—	Env gp41 73–81
17	[24]	2902		1402		Primary	0[b]	—	Gag p17 119–127
18	[24]	2902		1402		Primary	75[b]	—	Tat 24–32
19	[24]	2902		0801	4403	Primary	34,700	972	Env gp160 209–217
20	[24]	2902		0801	4403	Primary	216,400	358	Tat 24–32
21	[24]	1103	2402	1402	1501	Primary	1,000,000	900	Tat 32–41, Tat 36–45, and Tat 39–47

Disease stage (as reported at start of sampling period): primary denotes primary infection and seroconversion, before viral set point was attained; asymptomatic denotes asymptomatic infection (including persistent generalised lympadenopathy); and late stage denotes late-stage infection, CD4 count <200/mm^3.
Viral load and CD4 count are at the start of the sampling period (or where this is not available at the nearest time point).
CTL epitope position is numbered with respect to the HXB2 reference strain.
[a]Escape 11 viral load units are copies per 10^6 CD4$^+$ cells.
[b]Escape 15–18 viral load units are p24 antigen in pg/ml.
— denotes not reported.
DOI: 10.1371/journal.pbio.0040090.t001

extent of CTL-mediated killing. However, to do this it is necessary to know how many of the CTL responses naturally present during HIV-1 infection are of sufficient strength to drive escape. In most but not all reports of CTL escape, the immune response selecting for the variant was immunodominant, suggesting that only a few oligoclonal CTL responses are of sufficient strength to drive escape [24,30,31]. The available literature [19,22,24] suggests that an average individual makes at most 5 CTL responses of such strength (see Materials and Methods). If these five CTL responses are responsible for the majority of CTL lysis then this yields an infected cell death rate of about 0.1 d^{-1}—a tenth of the total infected cell death observed. However, this estimate neglects lysis by the potentially large number of CTL responses of insufficient strength to drive escape. Comprehensive epitope analysis puts the average number of CTL responses in an infected individual at between 14 [32] and 19 [33]. Including lysis by all of these responses increases the proportion of infected cell death attributable to the CTL response to about 20% (see Materials and Methods). So we suggest that although CTL-mediated lysis plays an important role in controlling HIV-1 infection (killing approximately 10%–20% of productively infected CD4$^+$ cells every day), CTLs may not be responsible for the majority of infected cell death. This conclusion, like all scientific conclusions, may change with the advent of new data but it is also consistent with reports of bystander apoptosis [34] and chronic activation-induced cell death [35,36], which indicate that there is considerable CD4$^+$ cell death that cannot be directly attributed to HIV-1–specific CTL-mediated lysis.

Our estimate of the rate of HIV-1–specific CTL lysis totalled across all CTL responses of 0.1 to 0.2 d^{-1} is considerably lower than in vitro estimates (1 d^{-1} [37,38]), possibly reflecting the artificial nature of experiments in which a high density of peptide-pulsed B cells or transformed CD4$^+$ cells infected with a high multiplicity of infection are exposed to CD8$^+$ cells in a homogenous environment. Our estimates are also lower than rates of CTL lysis calculated (albeit using very different methods) for other virus infections which range from 1 d^{-1} for HTLV-I to 12 d^{-1} for acute LCMV [39–42]. Why CTLs kill HIV-infected cells so slowly is not clear. HIV-1–specific CTLs have an unusual phenotype and are low in perforin [15,16,43], and it has been suggested that progressive loss of CD4$^+$ cell help may be important. Consistent with this, we found that the rate of CTL lysis was significantly faster during primary infection than in chronic infection, implying a significant weakening of single CTL responses with long-term infection.

The conclusion that CTL may not be responsible for the majority of infected cell death unifies apparently contradictory results indicating that there is an association between HLA class I type and rate of progression to AIDS despite a lack of association between disease stage and infected cell clearance rate following therapy [2,3]. Infected cell clearance rates are not reduced in subjects with advanced disease; this has been used as an argument that weakening of the CTL response does not accompany disease progression and that

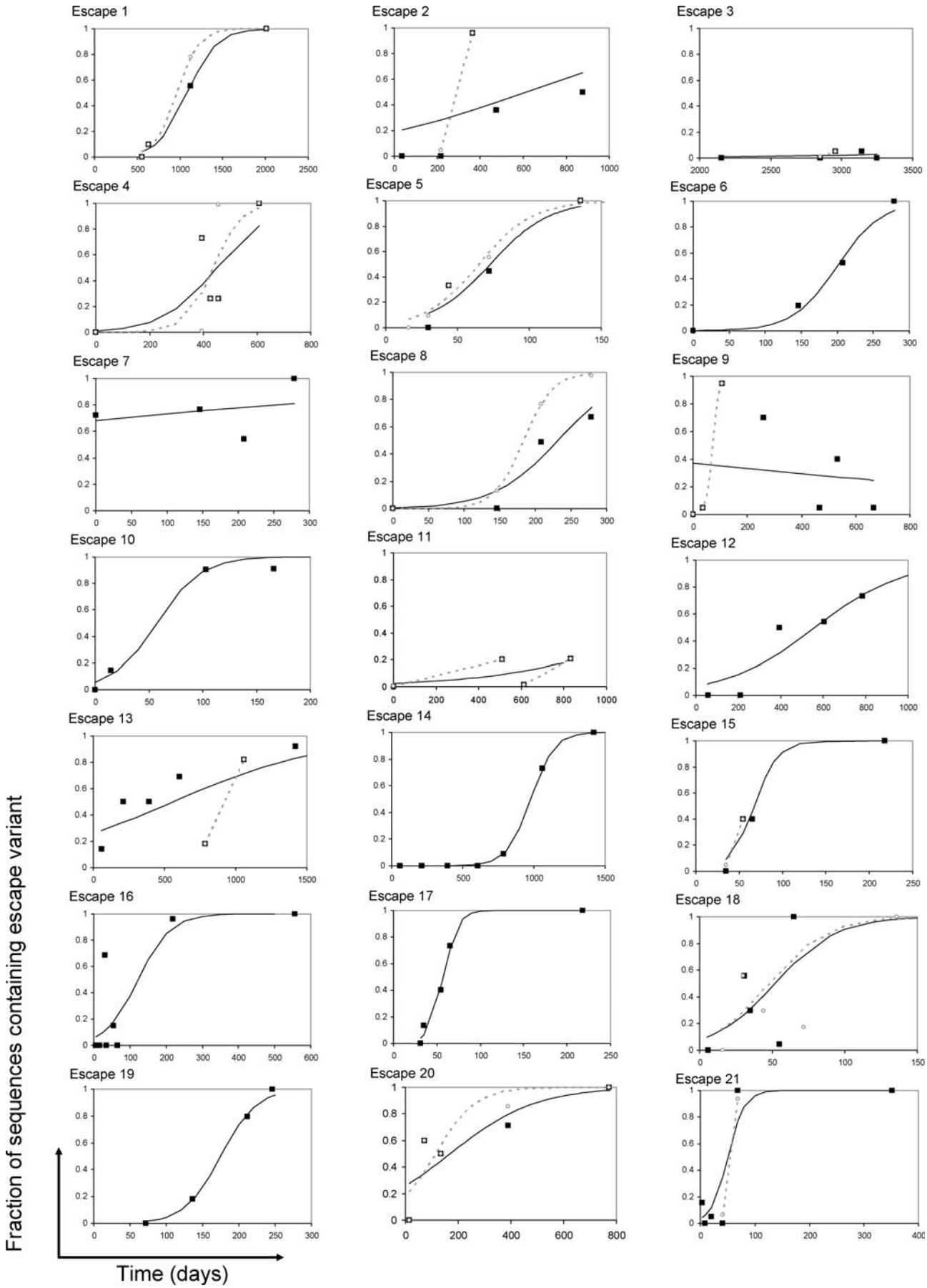
Escape 1
Escape 2
Escape 3
Escape 4
Escape 5
Escape 6
Escape 7
Escape 8
Escape 9
Escape 10
Escape 11
Escape 12
Escape 13
Escape 14
Escape 15
Escape 16
Escape 17
Escape 18
Escape 19
Escape 20
Escape 21
Fraction of sequences containing escape variant
Time (days)

Figure 2. Escape Data and Theoretical Fits

Fit of the model to the experimental data for each of the 21 escape datasets. Best (filled squares) and optimistic (open circles) data and the fit of the model (solid line and dashed line, respectively) to the data. Best estimates of the escape rate of a variant were obtained by fitting the model to the published data. Maximal, "optimistic" estimates of CTL efficiency were also made by omitting data or including mutations that have not been shown to confer escape (e.g., in the case of fluctuating variant frequencies rather than steady outgrowth a more optimistic estimate can often be obtained if some later data are discarded, e.g., Escape 9). These maximal estimates are less accurate but they provide an approximate upper bound on the rate of escape.
DOI: 10.1371/journal.pbio.0040090.g002

CTLs do not play an important protective role. We resolve this issue by suggesting that CTL lysis is small relative to other factors contributing to infected cell death, and therefore between-individual differences in CTL lysis rates (e.g., with disease stage) are unlikely to be detectable by measuring total infected cell clearance rates (see Materials and Methods). However, as we have shown, these small differences can be detected if the death rate attributable to CTLs is measured directly (Figure 6). Furthermore, these small differences in CTL lysis rate translate into large differences in terms of the absolute number of productively infected cells killed, and are likely to be clinically relevant. For example, a difference in lysis rate of 0.1 d^{-1} is equivalent to a difference of about 10^7 productively infected cells killed each day, every day, for several years. Such a large, cumulative difference could well alter the timing of disease progression.

This work has implications for vaccines designed to induce a lytic CTL response. One of the aims of therapeutic, and possibly prophylactic vaccines, is to boost the chronic memory response, so it becomes important to understand why the HIV-1–specific CTL response weakens with time. Furthermore, if it is true that the CTL response kills a minority of infected cells but that this is sufficient to affect clinical outcome, this suggests that, unless the CTL response elicited by vaccines is several-fold more efficient than the natural response, vaccines relying on the lytic pathway are unlikely to prevent infection or to mediate complete viral clearance, but that they may well reduce viral load and lengthen the asymptomatic period.

Materials and Methods

Model of infected cell dynamics. Initially it was assumed that productively infected cell dynamics follow first-order kinetics, as detailed observation of both viral clearance and viral rebound have indicated that this is most appropriate. Later, we relaxed this assumption. Here we refer to the epitope that is no longer recognised due to mutation as the "escape epitope." $CD4^+$ cells productively infected with wild-type virus *(y)* replicate at a rate a (net of all factors except CTL-mediated death), are killed by CTLs recognising epitopes other than the escape epitope at a rate b and are killed by CTLs recognising the escape epitope at a rate c. $CD4^+$ cells productively infected with a CTL escape variant *(x)* replicate at a rate a' (net of all factors except CTL-mediated death) and are killed by CTLs recognising epitopes other than escape epitope at a rate b. Infected cell dynamics are therefore represented by

$$\begin{aligned} \dot{y} &= ay - by - cy \quad \text{wild-type} \\ \dot{x} &= a'x - bx \quad \text{variant} \end{aligned} \tag{1}$$

In a host able to mount the relevant CTL responses (i.e., with the restricting HLA allele), the selective advantage of the escape variant (i.e., the rate of escape) is the difference in growth rate between the escape variant and the wild-type $k = a' - b - (a - b - c) = c - a + a'$.

Many escape mutations will carry a fitness cost that partially offsets the "benefit" to the virus of evading the CTL response. This fitness cost will be the difference in replication rate between the escape variant and the wild-type $\varphi = a - a'$. If φ is greater than zero then the mutation has a deleterious effect on viral replication (in the absence of a CTL response).

Quantification of the rate of escape *(k)*. The rate of escape (i.e., the rate of outgrowth of a CTL escape variant compared to the wild-type [escape variant growth rate − wild-type growth rate]) is determined by the balance between the rate of lysis evaded *(c)* and the fitness cost of

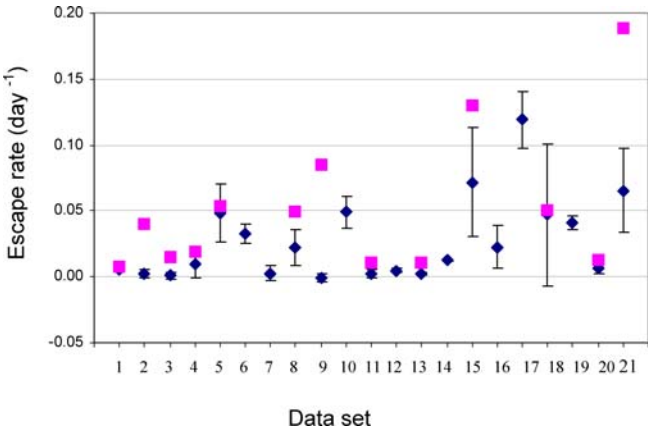

Figure 3. Escape Rate Estimates

Best (filled diamonds) and optimistic (filled squares) estimates of the rate of escape in each of the 21 datasets. Best estimates are shown ±1 standard error.
DOI: 10.1371/journal.pbio.0040090.g003

Table 2. Estimates of the Rate of Escape

Dataset	Best Estimate (d^{-1})	Standard Error	Optimistic Estimate (d^{-1})
1	0.006	0.001	0.008
2	0.002	0.003	0.040
3	0.001	0.003	0.015
4	0.010	0.011	0.019
5	0.048	0.022	0.053
6	0.032	0.008	ND
7	0.002	0.006	ND
8	0.022	0.013	0.050
9	−0.001	0.003	0.085
10	0.049	0.012	ND
11	0.003	0.003	0.011
12	0.005	0.001	ND
13	0.002	0.001	0.012
14	0.012	0.000	ND
15	0.072	0.041	0.130
16	0.023	0.016	ND
17	0.119	0.021	ND
18	0.047	0.054	0.051
19	0.041	0.005	ND
20	0.006	0.004	0.013
21	0.066	0.032	0.189
Median	0.01	—	0.04

The best estimate of the escape rate was obtained by fitting a simple model to the longitudinal escape data using nonlinear least-squares regression. An "optimistic" estimate of the escape rate was made where possible; this can be considered as an approximate upper bound on the rate of escape. Optimistic estimates were often made by omitting later data points; for this reason it was not possible to estimate the standard error for these estimates.
ND, not done (no plausible alternative assumptions giving a more optimistic estimate).
DOI: 10.1371/journal.pbio.0040090.t002

Table 3. Reversion Datasets Studied

Dataset	Data Source	HLA Class I Type				Disease Stage	Viral Load (Copies/ml)	CD4 Count (Cells/mm^3)	CTL Epitope (HXB2)
		A		B					
1	[18]			07	07	Primary	—	—	Gag p24 15–23
2	[20]			07	07	Primary	—	—	Gag p24 108–117
3	[20]			07	07	Primary	—	—	Gag p24 108–117
4	[20]			07	44	Primary	—	—	Gag p24 108–117
5	[19]	0201	3101	3501	3905	Primary	500,000	250	Gag p17 18–26
6	[21]	02	26	51	62	Asymptomatic	13,000	543	Nef 134–143
7	[21]	02	02	39	60	Asymptomatic	72,000	378	Nef 134–143

Disease stage (as reported at start of sampling period): primary denotes primary infection and seroconversion, before viral set point was attained; asymptomatic denotes asymptomatic infection (including persistent generalised lympadenopathy). Viral load and CD4 count are at the start of the sampling period (or where this is not available at the nearest time point). CTL epitope position is numbered with respect to the HXB2 reference strain.

— denotes not reported.

DOI: 10.1371/journal.pbio.0040090.t003

the mutation(s) ($\varphi = a - a'$). The rate of escape ($k = c - \varphi$) was estimated from longitudinal escape data. If $p(t)$ is the proportion of viral sequences that have escape mutations in the epitope of interest at time t then solving Equation 1 we have

$$p(t) = \frac{x(t)}{y(t) + x(t)} = \frac{1}{ge^{-kt} + 1} \quad (2)$$

where $g = y(0)/x(0)$ and $k = c - \varphi$.

This model was fit to longitudinal escape data using nonlinear least-squares regression (Levenberg-Marquardt method) and the rate of escape of the variant ($k = c - \varphi$) estimated. The standard error of the escape rate was estimated using the asymptotic covariance matrix method.

Quantification of the fitness cost of escape variants ($\varphi = a - a'$). If an escape variant is transmitted to an individual who does not have the HLA class I allele to bind the peptide in which the escape mutation has arisen then the selection pressure for the escape mutation will be lost. Both the wild-type and the variant will face the same CTL response, and their relative dynamics will be determined by the difference in their replication rates ($\varphi = a - a'$). If the escape mutation carried a fitness cost then the virus will tend to revert to wild-type. If the mutation was cost neutral then it will tend to be stable over time. The proportion of viral sequences with an escape mutation will still be described by Equation 2 but now there is no CTL response against the escape epitope, so $c = 0$. By fitting the model (Equation 2) with $c = 0$ to longitudinal reversion data the fitness cost of the escape mutation, $\varphi = a - a'$, can be estimated.

Estimate of the average rate of killing of productively infected cells by a CTL response against a single epitope (c). The average rate of escape ($k = c - \varphi$) of a CTL escape variant was estimated from longitudinal escape data. The average fitness cost ($\varphi = a - a'$) of a CTL escape variant was estimated from reversion data. Putting these two estimates together we quantified $c = k + \varphi$ the average rate of killing (per day) of productively infected cells by a CTL response against a single epitope. High levels of multiple infection and recombination [44] ensure that, in cases of simultaneous escape at multiple epitopes, escape at one epitope is independent of escape at another.

Estimate of the average rate of killing of productively infected cells per CTL. We have estimated that a single CTL response kills productively infected cells at a rate of 0.02 d^{-1}. This is an estimate of the cumulative impact of all CD8$^+$ cells recognising that epitope. Many mathematical models express the killing rate of infected cells in units of killing per day per epitope-specific CD8$^+$ cell we therefore re-expressed our estimate in these units to facilitate future development of these models.

In 13 of the escape datasets the proportion of peripheral blood mononuclear cells (PBMCs) responding to the escape epitope was measured by IFNγ ELIspot. In a further two datasets the proportion of CD8$^+$ cells able to bind the escape epitope–presenting allele complex was quantified by major histocompatibility class I tetramer; this figure was converted to the proportion of PBMCs able to bind the escape epitope–presenting allele complex assuming 15% of PBMCs are CD8$^+$ (A. Mosley, personal communication). Using these 15 estimates of specific CD8$^+$ cell frequency in PBMCs (median 0.1%) and an estimate of the number of PBMCs in the body of 1.4×10^{12} [45] we estimated the average rate of killing was 3×10^{-11} per day per epitope-specific CD8$^+$ cell, considerably smaller than the CTL killing rate used in many models [46,47].

Datasets. The rate of escape was quantified in 21 datasets from 12 HIV-1–infected subjects (Table 1). The rate of reversion was quantified in seven datasets from five individuals (Table 3).

The best estimate of the rate of escape (k) was obtained by fitting the model (Equation 2) to the escape data. To estimate the maximal possible efficiency of the CTL response against a particular epitope, "optimistic" estimates were also made where possible. These estimates are less accurate than the best estimates because they involve omitting data or including mutations that have not been shown to confer escape (e.g. in the case of fluctuating variant frequencies rather than steady outgrowth a more optimistic estimate of k can often be obtained if some later data is discarded), but they provide an approximate upper bound on the possible rate of escape. The assumptions made to obtain an optimistic estimate are listed in Supporting Information. For the reversion data optimistic estimates of the rate of reversion were not made because the data were more straightforward—monotonic reversion of a single escape mutation—allowing little scope for alternative assumptions.

To make reliable estimates of the escape rate it was necessary to have at least two data points that were not zero (no escape variants detected) or one (no wild-type sequence detected). All datasets found that matched these criteria were analysed and the results reported here. However, this raised the possibility that faster rates of escape not captured by infrequent sampling protocols were omitted. To avoid this possible bias we also analysed all datasets reporting CTL escape regardless of the number of data points available. The escape rates estimated from these datasets (median $k = 0.01$ d^{-1}) were very similar to those obtained from the full datasets. More importantly there were only ten such datasets (compared to 21 full datasets) despite there being a higher probability of observing them (given equal occurrence), suggesting that the majority of CTL escape is not happening on a much faster scale than that implied by our analysis of the full datasets.

In five of the 21 datasets, sequences were obtained from proviral DNA rather than from viral RNA. There was no significant difference between the rate of escape calculated using DNA and RNA ($p = 0.11$, two-tailed Wilcoxon–Mann-Whitney). In five of the 21 datasets the escape variant was capable of eliciting a partial CTL response in vitro. There was a possibility that incomplete escape would lead to an underestimate of the rate of CTL lysis of infected cells. We took two approaches to quantifying the impact of incomplete escape. First, we simply removed the five cases where escape was incomplete. This yielded a median rate of lysis of 0.013 d^{-1}. Alternatively, we estimated the "escapedness" of the variant in each of the 21 cases considered and adjusted our estimate of the rate of lysis by this factor. Escapedness was defined as the percentage of the CTL response escaped, so an escapedness of 100% means escape was complete. The median escapedness was 100%; the mean was approximately 85%. Clearly, adjusting by the median escapedness does not change our

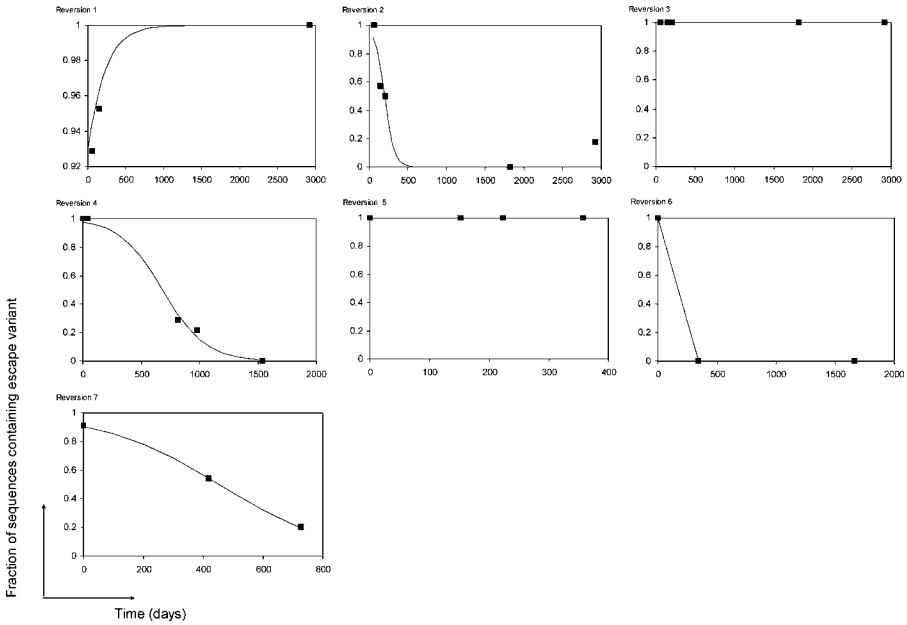

Figure 4. Reversion Data and Theoretical Fits

Fit of the model (solid line) to the experimental data (filled square) for each of the seven reversion datasets.

DOI: 10.1371/journal.pbio.0040090.g004

estimate of the rate of CTL lysis; adjusting by the mean changes it to $(0.01 + 0.005)/0.85 = 0.02$ d^{-1}. Whichever approach is taken, it is clear that incomplete escape has very little impact on our estimate of the rate of CTL lysis (previously 0.015 d^{-1}). Furthermore, the escape rate of the five cases where escape was incomplete was not significantly different from the escape rate of the remaining 16, where escape was between 90%–100% ($p = 0.4$, two-tailed Wilcoxon–Mann-Whitney). One possible explanation for the absence of an association between a slow rate of escape and incomplete escape is that during the in vitro test for escapedness, when very high levels of peptide are added exogenously (often to professional antigen-presenting cells), it is possible to elicit a partial response to a variant that is a genuine complete escape variant in vivo [23].

Quantifying the impact of CTL decline. There is a possibility that as wild-type virus is replaced by the variant the CTL response to the wild-type will decline due to loss of antigen stimulation. This would lead to a reduction in the selection pressure for escape and an underestimate of the escape rate in the presence of continuous selection pressure.

To assess the impact on the estimated rate of escape of a possible decline in the epitope-specific CTL response the model (Equation 1) was adjusted to allow for an exponentially declining CTL response instead of a constant CTL response. So Equation 1 was rewritten as

$$\begin{aligned} \dot{y} &= ay - by - ce^{-mt}y \quad \text{wild-type} \\ \dot{x} &= a'x - bx \quad\quad\quad\quad\ \ \text{variant} \end{aligned} \tag{3}$$

where a, a', b, and c are as before and m is the rate of loss of CTL recognising the epitope in which escape has occurred. So now in Equation 2 g is as before, i.e., $g = y(0)/x(0)$, but k is changed to $k = c(1 - e^{-mt})/(mt) - \varphi$.

The rate of CTL decline (m) was estimated using the rate of HIV-1–specific CTL decline following HAART. Four estimates of the rate of CTL decline have been made: $m = 0.0002$, 0.002, 0.003, and 0.015 d^{-1} [8,48–50]. We used both the mean ($m = 0.005$ d^{-1}) and the maximum ($m = 0.015$ d^{-1}). Following successful HAART viral load falls rapidly and dramatically, by two or more orders of magnitude in a few days [1,4,8]. The loss of antigenic stimulus on commencing HAART is therefore much greater than during CTL escape. Consequently, the estimates of the rate of CTL decline we have used are likely to be too large. These very stringent assumptions mean that the estimates of the escape rate obtained using the model (Equation 3) should be considered to be overestimates. Using $m = 0.005$ d^{-1} we found that the median rate of escape was 0.055 d^{-1}. Using $m = 0.015$ d^{-1} we found that the median rate of escape was 0.086 d^{-1}. In the latter case it was difficult to fit the model (Equation 3) to the escape data, suggesting that the assumptions were too stringent.

Impact of between-individual variation in CTL lysis rate. Following antiretroviral treatment the rate of viral clearance is very consistent between individuals and there is no correlation with disease stage [2–4]. This would seem to imply that differences in the rate of CTL killing are not important predictors of disease progression [38]. However, if the rate of CTL killing is small relative to other factors contributing to the overall rate of viral clearance, it might be very difficult to detect variations in the rate of CTL killing. We have calculated that an average total CTL response kills 10% of productively infected cells per day (lysis rate = 0.1 d^{-1}). We wished to know if between-individual differences in this lysis rate would be manifest in virus clearance estimates.

As an extreme example we considered an individual with a very poor CTL response that is unable to kill any infected cells (total lysis rate = 0 d^{-1}) and an individual with an average CTL response (total lysis rate = 0.1 d^{-1}). This between-individual difference in CTL lysis would lead to a difference in infected cell clearance following HAART of 0.1 d^{-1}. A between-individual difference of 0.1 d^{-1} is well within the range of variation observed (about 0.3–0.7 d^{-1} [4]), despite this range being considered far too small to accommodate between-individual variation in the CTL response [38]. Even the most thorough analysis [4] of very frequent samples (only achieved in a few individuals) yields estimates of the infected cell clearance rate with 95% confidence bounds that are typically ± 0.08 d^{-1}. This raises the possibility that, even in the most favourable experimental settings, it would be difficult to detect even the considerable difference between the very weak and average CTL responder considered here. It is therefore not surprising that less accurate methods have failed to detect an association between infected cell clearance rate and disease stage ($CD4^+$ cell count) of the patient.

The difference between the weak responder and the average responder considered here is equivalent to a difference of about 10^7 productively infected cells killed every day (10% of the productively infected cell pool, which is estimated to contain about 10^8 cells [51]).

Estimating the proportion of productively infected cell death attributable to the total CTL response. We have quantified the rate of lysis of productively infected cells by a single CTL response (i.e., CTL clone[s] targeting one epitope). To make an order of magnitude approximation of the total extent of CTL lysis we needed to estimate how many CTL responses of sufficient strength to drive escape were naturally present during HIV-1 infection. In most, but not all reports of CTL escape, the immune response selecting for the variant was immunodominant, suggesting that only a few oligoclonal CTL responses were able to drive escape [24,30,31]. Three papers

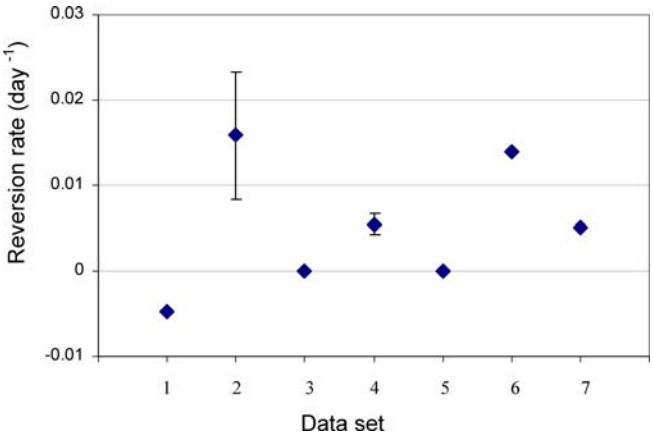

Figure 5. Reversion Rate Estimates

Best (filled diamonds) estimates of the reversion rate in each of the seven datasets. Best estimates are shown ±1 standard error. Escape and reversion may require additional compensatory mutations which will limit the occurrence of escape or reversion and will delay the time until escape or reversion occurs. The effect of such compensatory mutations (even if they occur outside of the epitope) will be fully quantified because their effect will be reflected in the rate of escape/reversion of the variant. It is also possible that compensatory mutations will be acquired after the wild-type has been completely replaced by the escape variant. Interestingly, escape 7 and reversion 5 are "paired." That is, the outgrowth of the escape variant labeled 7 was observed in one individual (the "donor") who transmitted the escape variant to their sexual partner (the "recipient"), and in whom the escape variant then reverted (reversion 5). Outgrowth of the escape variant was observed in the donor during the time at which the recipient was infected. Both the escape and reversion rates are typical and lie within the range of the other observed escape/reversion rates, implying that low reversion rates cannot be attributed to the accumulation of compensatory mutations following escape.

DOI: 10.1371/journal.pbio.0040090.g005

[19,22,24] have estimated the number of CTL responses of sufficient strength to drive escape at any one time. This was done by sequencing either all or the majority of viral genes at multiple time points and looking for evidence of CTL escape. Between them, these three papers studied nine individuals and it was found that, averaging over time, the median number of CTL responses of sufficient strength to drive selection of an escape variant was two (range, zero to five). Although a large number of epitopes were analysed, only one study (Geels et al. [22], who found three such CTL responses) analysed the whole genome (the rest studied three to four genes), so it was a possibility that these figures underestimated the number of CTL responses present at any one time. We therefore

Table 4. Estimates of the Rate of Reversion

Dataset	Best Estimate (d^{-1})	Standard Error
1	−0.005	0.000
2	0.016	0.007
3	0.000	0.000
4	0.005	0.001
5	0.000	0.000
6	−0.014	—
7	0.005	0.000
Median	0.005	

The best estimate of the reversion rate was obtained by fitting a simple model to the longitudinal reversion data using nonlinear least-squares regression. Optimistic estimates of the reversion rates were not made as the data were more straightforward—monotonic reversion of a single escape variant—allowing little scope for alternative assumptions.
— denotes insufficient data to estimate the standard error.

DOI: 10.1371/journal.pbio.0040090.t004

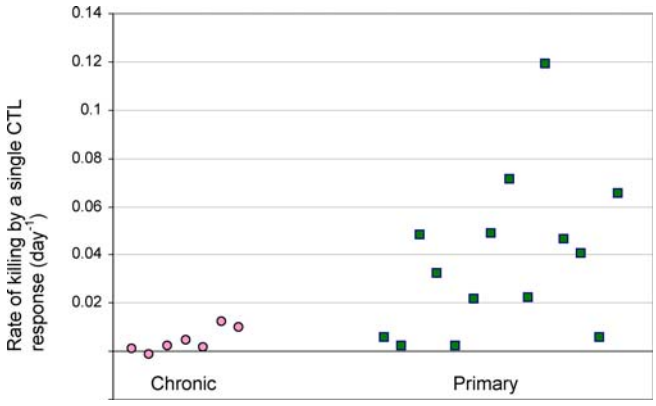

Figure 6. The Rate of CTL Killing Is Significantly Lower in Individuals with Long-Term Infection

Individuals with chronic infection (filled circles) have weaker single CTL responses than individuals with primary infection (filled squares). The median rate of lysis of productively infected cells was 0.008 d^{-1} in chronic infection and 0.04 d^{-1} in primary infection. The rate of CTL lysis was significantly lower in individuals with long-term infection (Wilcoxon–Mann-Whitney $p = 0.004$ two-tailed). Chronic infection was defined as infection in which the viral set point has been attained; primary infection as early infection prior to stabilisation of viral load which will include both seropositive and seronegative individuals.

DOI: 10.1371/journal.pbio.0040090.g006

erred on the side of the importance of CTL and used a higher estimate of five CTL responses of sufficient strength to drive escape. If these five responses accounted for the majority of CTL lysis then the infected cell death attributable to the total CTL response was about $5 \times 0.02 = 0.1$ d^{-1}, i.e., a tenth of the productively infected cell death observed. However, this neglected CTL responses below the "threshold of detection" of our method. It has been found [32,33] that an average HIV-1–infected individual mounts CTL responses against 14–19 epitopes, suggesting that on average nine to 14 CTL responses are below the threshold of detection (too inefficient to drive escape). We wished to estimate the maximum possible contribution to infected cell lysis of these weaker responses by estimating the threshold of detection of our method. From Table 2 it can be seen that the threshold was about 0.002 d^{-1} (two responses weaker than this were measured but they appeared to be artificially deflated by reversion of the variant to wild-type). From Table 4 it can be seen that the average fitness cost was 0.005 d^{-1}. The nine to 14 inefficient responses were therefore estimated to have an efficiency of $0.002 + 0.005 = 0.007$ d^{-1} or less. So the maximum rate of lysis of productively infected cells was $5 \times 0.02 + 9 \times 0.007$ to $5 \times 0.02 + 14 \times 0.007 = 0.16$ to 0.20 d^{-1}; i.e., accounting for the extra inefficient CTL responses increased the proportion of productively infected cell death attributable to the CTL response to about 20%. In order for 50% of productively infected cell death to be attributable to the CTL response it would be necessary to have 60 CTL responses at the detection threshold (in addition to the five above the detection threshold). Even if we considered an extremely strong immune response in which all 14–19 responses were of sufficient strength to drive CTL escape, then the proportion of infected cells killed was still only between 28% and 38% (0.02×14, 0.02×19). It is thought that CTL responses driving escape are of average strength or higher. If CTL responses driving escape were atypically weak (i.e., weaker than the average CTL response) then this calculation would tend to underestimate the importance of CTL.

Robustness of results to model changes. In the basic model (Equation 1) and consequently in the solution (Equation 2) we have assumed that both viral clearance and viral production follow first-order kinetics, i.e., that infected cells die at a constant rate and grow at a constant rate. Following HAART viral load declines exponentially for at least three orders of magnitude [4]. Furthermore, ex vivo studies have indicated that CTL lysis causes an exponential decrease of infected cells [52]. This suggests that viral clearance follows first order kinetics. Changes to this assumption have already been considered above. Similarly, during viral rebound (on stopping HAART or developing drug resistance mutations), viral load rises exponentially for three, sometime four orders of magnitude [53], indicating that viral production also follows first order kinetics.

However, during untreated asymptomatic infection, when viral load is high, it could be argued that target cell limitation becomes important. This will not alter our results, as target cell limitation will act to decrease the growth rate of escape variants and wild-type virus equally, and we are only interested in the difference between these growth rates.

An alternative method of calculating the optimistic estimate was also performed for each of the 21 escape datasets. The rate of escape was calculated from the greatest rate of change in frequency of the variant (between two time points) in the dataset. This was done by fitting a straight line to the transformed data $ln(1/p - 1)$ where p is the frequency of the escape variant. $ln(1/p - 1)$ is ill-defined for $p = 0$ or $p = 1$ so, where the frequency of the variant was $0/n$ this was approximated by $1/n + 1$ and where the frequency was n/n this was approximated by $n/n + 1$. The median rate of escape using this method was 0.05 d^{-1}, which was very close to the median optimistic estimate (0.04 d^{-1}). In cases where the CTL selection pressure for escape declines with time, either due to loss of the CTL response against the wild-type epitope or due to emergence of a new CTL response to the escape variant (when mutation has reduced T cell receptor recognition rather than MHC binding), it would be expected that initial rapid escape would be slowed or even reversed at later time points. This alternative method of calculating the optimistic estimate will reflect the most rapid period of escape and will not be influenced by later reduced selection pressure. Estimates made using this method can therefore be considered to be alternatives to the best estimates with correction for CTL decline or to the optimistic estimates.

Statistical analysis. The rate of CTL killing in primary infection ($n = 14$) was compared with the rate of CTL killing in chronic infection ($n = 7$) using the nonparametric exact Wilcoxon–Mann-Whitney two-tailed test.

Supporting Information

Protocol S1. Fit of Model to the Data

Found at DOI: 10.1371/journal.pbio.0040090.sd001 (66 KB DOC).

Acknowledgments

We thank Mark Geels and Jaap Goudsmit for providing the raw data underlying their published graphs and Rodney Phillips for helpful discussions. We also thank Charles Bangham, Graham Taylor, Angelina Mosley, Sara Marshall, and Kirin Meekings for constructive comments regarding the manuscript.

Author contributions. BA, ML, and ARM conceived and designed the experiments. BA performed the experiments, and analysed the data. BA and ARM wrote the paper. CTTE contributed unpublished escape data.

Funding. BA is grateful to the Leverhulme Trust for financial support.

Competing interests. The authors have declared that no competing interests exist. ■

References

1. Markowitz M, Louie M, Hurley A, Sun E, Di Mascio M, et al. (2003) A novel antiviral intervention results in more accurate assessment of human immunodeficiency virus type 1 replication dynamics and T-cell decay in vivo. J Virol 77: 5037–5038.
2. Wei X, Ghosh SK, Taylor ME, Johnson VA, Emini EA, et al. (1995) Viral dynamics in human immunodeficiency virus type 1 infection. Nature 373: 117–122.
3. Ho DD, Neumann AU, Perelson AS, Chen W, Leonard JM, et al. (1995) Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. Nature 373: 123–126.
4. Perelson AS, Neumann AU, Markowitz M, Leonard JM, Ho DD (1996) HIV-1 dynamics in vivo: Virion clearance rate, infected cell life-span, and viral generation time. Science 271: 1582–1586.
5. Rodrigo AG, Shpaer EG, Delwart EL, Iversen AK, Gallo MV, et al. (1999) Coalescent estimates of HIV-1 generation time in vivo. Proc Natl Acad Sci U S A 96: 2187–2191.
6. Macallan DC, Asquith B, Irvine AJ, Wallace DL, Worth A, et al. (2003) Measurement and modeling of human T cell kinetics. Eur J Immunol 33: 2316–2326.
7. Carrington M, O'Brien SJ (2003) The influence of HLA genotype on AIDS. Annu Rev Med 54: 535–551.
8. Kalams SA, Goulder PJ, Shea AK, Jones NG, Trocha AK, et al. (1999) Levels of human immunodeficiency virus type 1-specific cytotoxic T-lymphocyte effector and memory responses decline after suppression of viremia with highly active antiretroviral therapy. J Virol 73: 6721–6728.
9. Borrow P, Lewicki H, Wei X, Horwitz MS, Peffer N, et al. (1997) Antiviral pressure exerted by HIV-1–specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus. Nat Med 3: 205–211.
10. Phillips RE, Rowland-Jones S, Nixon DF, Gotch FM, Edwards JP, et al. (1991) Human immunodeficiency virus genetic variation that can escape cytotoxic T cell recognition. Nature 354: 453–459.
11. Jin X, Bauer DE, Tuttleton SE, Lewin S, Gettie A, et al. (1999) Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus–infected macaques. J Exp Med 189: 991–998.
12. Feinberg MB, McLean AR (1997) AIDS: Decline and fall of immune surveillance? Curr Biol 7: R136–R140.
13. Van Rompay KK, Singh RP, Pahar B, Sodora DL, Wingfield C, et al. (2004) $CD8^+$-cell-mediated suppression of virulent simian immunodeficiency virus during tenofovir treatment. J Virol 78: 5324–5337.
14. Arnaout RA, Nowak MA, Wodarz D (2000) HIV-1 dynamics revisited: Biphasic decay by cytotoxic T lymphocyte killing? Proc Biol Sci 267: 1347–1354.
15. Appay V, Nixon DF, Donahoe SM, Gillespie GM, Dong T, et al. (2000) HIV-specific CD8(+) T cells produce antiviral cytokines but are impaired in cytolytic function. J Exp Med 192: 63–75.
16. Zhang D, Shankar P, Xu Z, Harnisch B, Chen G, et al. (2003) Most antiviral CD8 T cells during chronic viral infection do not express high levels of perforin and are not directly cytotoxic. Blood 101: 226–235.
17. Bevan MJ, Braciale TJ (1995) Why can't cytotoxic T cells handle HIV? Proc Natl Acad Sci U S A 92: 5765–5767.
18. Draenert R, Le Gall S, Pfafferott KJ, Leslie AJ, Chetty P, et al. (2004) Immune selection for altered antigen processing leads to cytotoxic T lymphocyte escape in chronic HIV-1 infection. J Exp Med 199: 905–915.
19. Milicic A, Edwards CT, Hue S, Fox J, Brown H, et al. (2005) Sexual transmission of single human immunodeficiency virus type 1 virions encoding highly polymorphic multisite cytotoxic T-lymphocyte escape variants. J Virol 79: 13953–13962.
20. Leslie AJ, Pfafferott KJ, Chetty P, Draenert R, Addo MM, et al. (2004) HIV evolution: CTL escape mutation and reversion after transmission. Nat Med 10: 282–289.
21. Furutsuki T, Hosoya N, Kawana-Tachikawa A, Tomizawa M, Odawara T, et al. (2004) Frequent transmission of cytotoxic-T-lymphocyte escape mutants of human immunodeficiency virus type 1 in the highly HLA-A24-positive Japanese population. J Virol 78: 8437–8445.
22. Geels MJ, Cornelissen M, Schuitemaker H, Anderson K, Kwa D, et al. (2003) Identification of sequential viral escape mutants associated with altered T-cell responses in a human immunodeficiency virus type 1-infected individual. J Virol 77: 12430–12440.
23. Goulder PJ, Phillips RE, Colbert RA, McAdam S, Ogg G, et al. (1997) Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS. Nat Med 3: 212–217.
24. Jones NA, Wei X, Flower DR, Wong M, Michor F, et al. (2004) Determinants of human immunodeficiency virus type 1 escape from the primary $CD8^+$ cytotoxic T lymphocyte response. J Exp Med 200: 1243–1256.
25. Koenig S, Conley AJ, Brewah YA, Jones GM, Leath S, et al. (1995) Transfer of HIV-1–specific cytotoxic T lymphocytes to an AIDS patient leads to selection for mutant HIV variants and subsequent disease progression. Nat Med 1: 330–336.
26. Price DA, Goulder PJ, Klenerman P, Sewell AK, Easterbrook PJ, et al. (1997) Positive selection of HIV-1 cytotoxic T lymphocyte escape variants during primary infection. Proc Natl Acad Sci U S A 94: 1890–1895.
27. Jamieson BD, Yang OO, Hultin L, Hausner MA, Hultin P, et al. (2003) Epitope escape mutation and decay of human immunodeficiency virus type 1–specific CTL responses. J Immunol 171: 5372–5379.
28. Kelleher AD, Long C, Holmes EC, Allen RL, Wilson J, et al. (2001) Clustered mutations in HIV-1 gag are consistently required for escape from HLA-B27–restricted cytotoxic T lymphocyte responses. J Exp Med 193: 375–386.
29. Goulder PJ, Brander C, Tang Y, Tremblay C, Colbert RA, et al. (2001) Evolution and transmission of stable CTL escape mutations in HIV infection. Nature 412: 334–338.
30. McMichael AJ, Phillips RE (1997) Escape of human immunodeficiency virus from immune control. Annu Rev Immunol 15: 271–296.
31. Klenerman P, Wu Y, Phillips R (2002) HIV: Current opinion in escapology. Curr Opin Microbiol 5: 408–413.
32. Addo MM, Yu XG, Rathod A, Cohen D, Eldridge RL, et al. (2003) Comprehensive epitope analysis of human immunodeficiency virus type 1 (HIV-1)–specific T-cell responses directed against the entire expressed HIV-1 genome demonstrate broadly directed responses, but no correlation to viral load. J Virol 77: 2081–2092.
33. Frahm N, Korber BT, Adams CM, Szinger JJ, Draenert R, et al. (2004) Consistent cytotoxic-T-lymphocyte targeting of immunodominant regions in human immunodeficiency virus across multiple ethnicities. J Virol 78: 2187–2200.

34. Finkel TH, Tudor-Williams G, Banda NK, Cotton MF, Curiel T, et al. (1995) Apoptosis occurs predominantly in bystander cells and not in productively infected cells of HIV- and SIV-infected lymph nodes. Nat Med 1: 129–134.
35. Sousa AE, Carneiro J, Meier-Schellersheim M, Grossman Z, Victorino RM (2002) CD4 T cell depletion is linked directly to immune activation in the pathogenesis of HIV-1 and HIV-2 but only indirectly to the viral load. J Immunol 169: 3400–3406.
36. Silvestri G, Feinberg MB (2003) Turnover of lymphocytes and conceptual paradigms in HIV infection. J Clin Invest 112: 821–824.
37. Yang OO, Kalams SA, Rosenzweig M, Trocha A, Jones N, et al. (1996) Efficient lysis of human immunodeficiency virus type 1–infected cells by cytotoxic T lymphocytes. J Virol 70: 5799–5806.
38. Klenerman P, Phillips RE, Rinaldo CR, Wahl LM, Ogg G, et al. (1996) Cytotoxic T lymphocytes and viral turnover in HIV type 1 infection. Proc Natl Acad Sci U S A 93: 15323–15328.
39. Barchet W, Oehen S, Klenerman P, Wodarz D, Bocharov G, et al. (2000) Direct quantitation of rapid elimination of viral antigen-positive lymphocytes by antiviral CD8(+) T cells in vivo. Eur J Immunol 30: 1356–1363.
40. Fernandez CS, Stratov I, De Rose R, Walsh K, Dale CJ, et al. (2005) Rapid viral escape at an immunodominant simian-human immunodeficiency virus cytotoxic T-lymphocyte epitope exacts a dramatic fitness cost. J Virol 79: 5721–5731.
41. Asquith B, Mosley AJ, Barfield A, Marshall SE, Heaps A, et al. (2005) A functional $CD8^+$ cell assay reveals individual variation in $CD8^+$ cell antiviral efficacy and explains differences in human T-lymphotropic virus type 1 proviral load. J Gen Virol 86: 1515–1523.
42. Regoes RR, Antia R, Garber DA, Silvestri G, Feinberg MB, et al. (2004) Roles of target cells and virus-specific cellular immunity in primary simian immunodeficiency virus infection. J Virol 78: 4866–4875.
43. Appay V, Dunbar PR, Callan M, Klenerman P, Gillespie GM, et al. (2002) Memory $CD8^+$ T cells vary in differentiation phenotype in different persistent virus infections. Nat Med 8: 379–385.
44. Jetzt AE, Yu H, Klarmann GJ, Ron Y, Preston BD, et al. (2000) High rate of recombination throughout the human immunodeficiency virus type 1 genome. J Virol 74: 1234–1240.
45. Trepel F (1974) Number and distribution of lymphocytes in man: A critical analysis. Klin Wochenschr 52: 511–515.
46. Nowak MA, May RM, Phillips RE, Rowland-Jones S, Lalloo DG, et al. (1995) Antigenic oscillations and shifting immunodominance in HIV-1 infections. Nature 375: 606–611.
47. Nowak MA, Bangham CR (1996) Population dynamics of immune responses to persistent viruses. Science 272: 74–79.
48. Ogg GS, Jin X, Bonhoeffer S, Moss P, Nowak MA, et al. (1999) Decay kinetics of human immunodeficiency virus–specific effector cytotoxic T lymphocytes after combination antiretroviral therapy. J Virol 73: 797–800.
49. Altfeld M, Rosenberg ES, Shankarappa R, Mukherjee JS, Hecht FM, et al. (2001) Cellular immune responses and viral diversity in individuals treated during acute and early HIV-1 infection. J Exp Med 193: 169–180.
50. Casazza JP, Betts MR, Picker LJ, Koup RA (2001) Decay kinetics of human immunodeficiency virus-specific $CD8^+$ T cells in peripheral blood after initiation of highly active antiretroviral therapy. J Virol 75: 6508–6516.
51. Haase AT, Henry K, Zupancic M, Sedgewick G, Faust RA, et al. (1996) Quantitative image analysis of HIV-1 infection in lymphoid tissue. Science 274: 985–989.
52. Grimm EA, Thoma JA, Bonavida B (1979) Mechanism of cell-mediated cytotoxicity at the single cell level. II. Evidence for first-order kinetics of T cell–mediated cytolysis and for heterogeneity of lytic rate. J Immunol 123: 2870–2877.
53. Oxenius A, McLean AR, Fischer M, Price DA, Dawson SJ, et al. (2002) Human immunodeficiency virus-specific CD8(+) T-cell responses do not predict viral growth and clearance rates during structured intermittent antiretroviral therapy. J Virol 76: 10169–10176.