Epitope-Dependent Avidity Thresholds for Cytotoxic T-Lymphocyte Clearance of Virus-Infected Cells

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Cytotoxic T lymphocytes (CTLs) are crucial for immune control of viral infections. “Functional avidity,” defined by the sensitizing dose of exogenously added epitope yielding half-maximal CTL triggering against uninfected target cells (SD$_{50}$), has been utilized extensively as a measure of antiviral efficiency. However, CTLs recognize infected cells via endogenously produced epitopes, and the relationship of SD$_{50}$ to antiviral activity has never been directly revealed. We elucidate this relationship by comparing CTL killing of cells infected with panels of epitope-variant viruses to the corresponding SD$_{50}$ for the variant epitopes. This reveals a steeply sigmoid relationship between avidity and infected cell killing, with avidity thresholds (defined as the SD$_{50}$ required for CTL to achieve 50% efficiency of infected cell killing [KE$_{50}$], below which infected cell killing rapidly drops to none and above which killing efficiency rapidly plateaus. Three CTL clones recognizing the same viral epitope show the same KE$_{50}$ despite differential recognition of individual epitope variants, while CTLs recognizing another epitope show a 10-fold-higher KE$_{50}$ demonstrating epitope dependence of KE$_{50}$. Finally, the ability of CTLs to suppress viral replication depends on the same threshold KE$_{50}$. Thus, defining KE$_{50}$ values is required to interpret the significance of functional avidity measurements and predict CTL efficacy against virus-infected cells in pathogenesis and vaccine studies.
efficiency of killing cells infected with whole HIV-1 containing the same epitope variants. The results reveal narrow avidity thresholds separating efficient CTL antiviral activity from inability to recognize infected cells.

MATERIALS AND METHODS

Target cell lines. The HIV-1-permissive T1 and Jurkat cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, l-glutamine, HEPES, and penicillin-streptomycin (R10) as previously described (21). These were utilized as MHC-matched target cells for A*02- and B*07-restricted CTL clones, respectively.

HIV-1 stocks. Site-directed mutagenesis was performed on HIV-1 molecular clone NL4-3 by overlapping PCR as previously described for the variants of Gag amino acids (aa) 77 to 85 (23) (HXB2 numbering system) and using the QuickChange mutagenesis system (Stratagene) for the others. The p83-2.1 plasmid (23) (containing the gag-pol portion of the genome up to a unique EcoRI site) was utilized as the backbone for the mutations in Gag p17 aa 77 to 85 (SL9) and reverse transcriptase (RT) aa 309 to 317 (IV9). A modified version of the p83-10 plasmid (containing the env-nef portion beyond the EcoRI site) containing a methionine-to-alanine mutation at aa 20 of Nef (2) (p83-10.M20A) was utilized for the Rev aa 66 to 75 (RL10) mutations. After mutagenesis, sequences were confirmed. Final HIV-1 stocks were produced by coelectroporation of the appropriate p83-2.1 variant with the appropriate p83-10.M20A variant, followed by expansion, harvesting, cryopreservation, and titer determination as previously described (2). All viruses used for this study thus contained the Nef M20A mutation to avoid the confounding effects of Nef-mediated downregulation of HLA class I (2).

HIV-1-specific CTL clones. CTL clones were derived from the peripheral blood mononuclear cells of HIV-1-infected individuals by limiting-dilution cloning and were maintained with periodic restimulations (anti-C3D antibody and irradiated allogeneic peripheral blood mononuclear cells) in R10 supplemented with 50 μM interleukin-2 (R10-50) (21–23). Clone 68a62 was the generous gift of Bruce D. Walker.

Chromium release assays. Target cell killing by CTL clones was assessed by standard chromium release assays (21). Briefly, target cells (uninfected or infected) were 51Cr labeled (in the presence or absence of 10 μM synthetic epitope [Sigma] and incubated with or without CTL for 4 hours at an excess effector-to-target cell ratio of 5:1 (5 × 104 CTL and 104 target cells per well in 96-well U-bottom plates). Supernatants were then harvested for measurement of released 51Cr by scintillation counting (LumaPlate [Packard] and Microbeta [Wallac]). Specific lysis was calculated by subtracting the control spontaneous release from the test release and dividing that sum by the difference of the control maximum release minus the spontaneous release: specific lysis = (observed chromium release − spontaneous chromium release) ÷ (maximal chromium release − spontaneous chromium release).

Functional avidity measurements. Functional avidity of CTL clones was determined by standard peptide titration chromium release assays (21, 23). Briefly, the chromium-labeled target cells were preincubated with serial dilutions of the cognate peptide (Sigma) before the chromium release assay. Functional avidity was measured as the concentration of peptide yielding 50% of maximal CTL killing (SD50).

Determinations of killing efficiency. The killing efficiency of CTL clones against specific HIV-1 strains was determined by infecting T1 or Jurkat target cells with the appropriate HIV-1 stock at excess multiplicity (>2 tissue culture infectious doses per cell) for 4 days. These were then utilized in standard chromium release assays as target cells for the CTL clones. The efficiency of infected cell killing was calculated from the observed specific lysis adjusted for the maximal activity of the CTL against the maximally recognized peptide-labeled control and the percentage of infected cells (observed killing divided by the theoretical maximal killing): killing efficiency = specific lysis of infected cells × (best specific lysis of peptide-labeled uninfected cells × percentage of infection). For example, a CTL clone demonstrating 80% specific lysis of peptide-labeled control uninfected cells, exposed to target cells that were 70% infected, could be expected to kill at most 80% × 70% = 56% of the cells. If this clone was to effect 28% specific lysis of the infected cell culture, the killing efficiency would be determined to be 28 × 56 = 50%.

Virul inhibition assays. HIV-1 growth inhibition by CTL clones was assessed by coculturing CTLs with acutely infected cells as previously described, (22, 23), with the following modifications. Target T1 or Jurkat cells were acutely infected with the indicated HIV-1 strain at a multiplicity of infection of 10−2 and cocultured with the specified CTL clone at an effector-to-target ratio of 0.25:1 (1.25 × 104 CTLs with 5 × 104 target cells per well in a 96-well flat-bottom plate in 200 μl R10-50 medium), or with no CTLs (control), in triplicate wells. On days 2, 4, and 6 postinfection, 100 μl supernatant was removed for quantitative p24 antigen enzyme-linked immunosorbent assay (DuPont, Boston, MA) and replaced with fresh R10-50. Virus suppression was calculated as follows: inhibition efficiency = (log p24 without CTL − log p24 with CTL) ÷ log p24 without CTL.

RESULTS

Virus-specific CTL clones recognize epitope mutants with functional avidities varying over orders of magnitude. The functional avidities (SD50 values) of HIV-1-specific CTL clones for the cognate epitope and panels of epitope variants were evaluated according to standard assays. These clones had defined epitope specificities, targeting A*02-restricted epitopes in Gag (SLYNTVATL [SL9], aa 77 to 85 of Gag [strain HXB2 numbering]) and RT (ILKEPVHGV [IV9], aa 309 to 317 of RT) and a B*07-restricted epitope in Rev (RPAEVPVLO [RL10], aa 66 to 75 of Rev). The SD50 values of these CTLs were measured by standard titration of exogenously added synthetic epitopes on HLA-matched cells in killing assays (Fig. 1A and B). Additionally, parallel SD50 values were assessed using variant peptides to determine the functional avidity of the CTLs for panels of epitope variants (Fig. 1C to E). The avidities of the clones for these panels of epitope variants spanned orders of magnitude, and single amino acid substitutions were sufficient to markedly alter SD50, confirming the important influence of epitope sequence variation on CTL recognition.

Cells infected with viruses containing epitope mutations are recognized with varying efficiency by CTLs. To manipulate the avidity of CTL interaction with virus-infected cells, HIV-1 molecular clones were genetically constructed to produce whole infectious viruses expressing epitopes corresponding to those tested for SD50 with synthetic peptides. The CTL clones were then tested for their efficiency of killing HLA-matched cells that were acutely infected with these viruses. CTL clearance of infected cells varied from no killing to 100% efficiency for the different epitope variants (Fig. 2A to C). This indicated a profound impact of epitope sequence variation on the ability of CTLs to recognize and clear infected cells in the context of whole viruses.

CTL killing efficiency against infected cells demonstrates a steeply sigmoid relationship to functional avidity for epitope. When killing efficiency (determined with whole virus-infected cells) was plotted against functional avidity (determined with synthetic peptides), all tested clones exhibited a narrow (approximately 1 log) threshold of functional avidity, above which killing of infected cells was maximal and below which there was little or no killing (Fig. 3A to E). Thus, the relationship between CTL functional avidity and killing of infected cells was revealed to be sigmoid, and the SD50 required for 50% killing efficiency (KE50) (below which killing rapidly dropped to none and above which killing rapidly plateaued) could be defined. Furthermore, the KE50 was equivalent for the three Gag SL9-specific CTL clones, while that for the Rev RL10-specific CTL clone was clearly more than 10-fold higher, demonstrating that the KE50 varies by epitope (Fig. 3F). The higher KE50 for Rev RL10-specific CTLs indicated that less avidity is required for killing of infected cells versus the Gag SL9-specific CTLs.
FIG. 1. Antiviral CTLs vary widely in their functional avidity for cognate epitope and epitope variants. (A and B) HIV-1-specific CTL clones recognizing epitopes in Gag (SLYNTVATL, SL9), Pol (ILKEPVHGV, IV9), and Rev (RPAEPVPLQL, RL10) were screened for their functional avidity against their cognate peptides and variant peptides using standard peptide titration chromium release assays. Selected peptides are depicted to demonstrate the range of avidities for each clone. (C to E) The functional avidities (peptide concentration yielding 50% of maximal lysis [SD50]) for the listed CTL clones against panels of peptides were determined as described above. The mean SD50 values are plotted; error bars represent standard deviations when repeated determinations were performed (n = 4 for CTL clone S1-SL9-3.23T except for the ----L--V- variant, n = 3 for CTL clone S36-SL9-10.18T except for the ----L--V- variant, and n = 3 for CTL clone S42758-RL10-3.22).
Overall, these data defined epitope-dependent KE\textsubscript{50} thresholds for efficient CTL killing of virus-infected cells. Epitope variation has a significant impact on inhibition of viral replication by CTLs. Because suppression of viral replication by CTLs can also be mediated by a noncytolytic pathway via cytokine release (18, 22) or can be affected by factors such as efficiency of infected cell killing over time rather than just absolute levels of killing (3), CTL clones were directly evaluated for their ability to suppress HIV-1 replication in coculture assays (Fig. 4A to D). Similarly to the killing efficiency data, the suppression of viral replication varied greatly between viruses with different epitope variants, ranging from none to several orders of magnitude (Fig. 4E and F). These results again confirmed the marked impact of epitope variation on antiviral CTL activity.

The KE\textsubscript{50} also defines the threshold for inhibition of viral replication by CTLs. When the ability of CTLs to suppress viral replication was compared to killing efficiency for cells infected with the same epitope variants, there was a high degree of correlation between these parameters (Fig. 5). This agreed with prior observations that both cytolytic and noncytolytic pathways of CTL activity are mediated by TCR triggering and that killing is the dominant mechanism of HIV-1 inhibition (18, 22). Comparison of functional avidity versus virus suppression revealed the same sigmoid relationship as that for functional avidity versus infected cell killing efficiency, with the same threshold requirements for SD\textsubscript{50} (Fig. 6A to C). Again, comparison of Gag SL9-specific CTLs and Rev RL10-specific CTLs revealed that the latter require less avidity (higher SD\textsubscript{50}) for efficient antiviral activity (Fig. 6D). These data demonstrated that the KE\textsubscript{50} threshold also accurately reflects the avidity required for CTLs to suppress viral replication.

**DISCUSSION**

Given the critical role of CTLs in controlling viral infections, there has been intense interest in devising assays that predict their antiviral activity (20). Because measuring the antiviral activity of CTLs against autologous target cells infected with the pathogen of interest usually is not feasible (due to technical issues such as unavailability of virus-permissive autologous cells and poor in vitro viral growth), the vast majority of proposed techniques have utilized synthetic peptides as a surrogate for endogenously produced viral epitopes. Assays of cytokine production after exposure to peptides or binding to peptide/MHC tetramers have served as surrogates for detecting antiviral activity of CTLs (20). However, such assays only detect the presence of virus epitope-specific CTLs and do not measure their interaction with virus-infected cells.

In an attempt to quantify antiviral activity, the concept of virus-infected cells, using panels of viruses containing epitope variants corresponding to those in Fig. 1. The cells were generally infected at \(>50\%\) (data not shown). The killing efficiency is plotted for each virus; error bars represent standard deviations when repeated determinations were performed \((n = 5\) for CTL clone S1-SL9-3.23T, \(n = 3\) for CTL clone S36-SL9-10.18T, \(n = 3\) for CTL clone 93b-SL9-1.9, and \(n = 2\) for CTL clone S42758-RL10-3.22).
“functional avidity” or measurement of the quantity of synthetic peptide required for CTL triggering has been developed. This approach has been utilized for two major inferences about antiviral CTLs: estimating the antiviral efficiency of CTLs (1, 9, 14) (assuming that CTLs requiring less peptide are more efficient) and evaluating the status of evolving epitope polymorphisms as either recognized or escape variants (5, 6, 11, 14, 15, 23) (comparing the SD50 of the index epitope to those of variants). CTL adoptive transfer data in murine models have demonstrated a role of avidity in efficacy of the antiviral activity of CTLs, demonstrating that higher-avidity CTLs are more protective against viral infections in vivo (1, 9). However, the precise relationship of avidity and antiviral activity has not been previously measured directly.

A limitation to functional avidity measurements is that they entirely bypass the factors determining the quantity of viral epitopes presented on the cell surface during natural infection (Fig. 7). Numerous factors affect the efficiency of epitope presentation, including levels of viral protein expression, efficiency of epitope processing in the proteasome and transport by the transporter associated with antigen processing, and epitope affinity for MHC-I. Only the last of these factors is included in SD50 measurements, which reveal concentrations of exogenously added peptide (which in turn correlate to concentrations of cell surface epitope–MHC-I complexes) needed for TCR triggering. However, such data are difficult to interpret unless they can be related to the amount of epitope generated by a virus-infected cell; for example, CTLs with low avidity but recognizing a copiously produced viral epitope could clear infected cells just as efficiently as CTLs with high avidity recognizing a weakly produced epitope.

Our data address this issue directly, by comparing the functional avidity of CTLs against panels of epitope variants to killing of virus-infected cells bearing the same epitope variants. The findings reveal sharp thresholds of functional avidity (which can be quantified as the SD50 needed for 50% efficiency of infected cell killing, or the KE50) separating nonrecognition from fully efficient recognition of infected cells. As might be expected, these thresholds vary according to different epitopes. Clearly, there are differences in the expression levels, processing, and transport of epitopes from different proteins. In the case of HIV-1, it has been shown that Gag is expressed at a 20-fold excess compared to polymerase (12), and individual epitopes from these proteins can vary greatly in their processing and transport (8). Thus, the magnitude of the KE50 for any given epitope depends on variable factors that are not generally measured or defined in standard assays for virus-specific CTLs.

There are some caveats to the interpretation of this study. A technical point is that the measurement of KE50 depends on the accuracy and comparability of CTL killing of cells infected with different viruses, measured as “killing efficiency.” This measurement assumes that observed specific lysis of peptide-pulsed uninfected cells is a reflection of maximal killing ability of the CTLs against infected cells and that observed specific lysis of virus-infected cells accurately reflects the percentage of cells that become susceptible to killing. Data from a prior study (21) of HIV-1-infected cells and their clearance by CTLs indirectly support these technical assumptions; that study showed that the highest observed killing of fully infected cells (100% infection by intracellular p24 staining) closely matched levels of killing of peptide-pulsed uninfected cells. The use of cloned CTLs that have been selected and passaged in vitro is also a potential limitation; however, a clonal population with uniform avidity is required for meaningful comparisons. Finally, a more general caveat is the correlation of in vitro killing assays to CTL killing in vivo. While this is difficult to demonstrate in this human system, murine data comparing an in vivo killing assay.
to in vitro killing suggest a correlation (13). Overall, the clear and consistent relationships we observe with multiple CTL clones and epitopes are convincing support for the concept of epitope-dependent, narrow-avidity thresholds for killing of infected cells.

Our findings have important implications for the interpretation of SD50 measurements. It has been suggested that higher-avidity CTLs may have greater antiviral activity, but in light of these data, it is the relationship of CTL avidity to the KE50 for its target epitope that determines whether killing occurs efficiently, and greater avidity beyond this threshold does not equate to better activity. This is consistent with the role of the TCR functioning as a trigger. A theoretical advantage for higher avidity beyond the threshold, however, is a greater margin of safety for tolerating epitope mutation. SD50 measurements have been utilized frequently as a reflection of whether or not epitope mutation represents CTL escape, by quantitating the difference in SD50 between index and variant epitopes.
Again, the results of the current study shed light on this approach. It becomes clear that the degree of change in functional avidity is not directly relevant, but rather the key factor is whether the change moves the SD50 beyond the KE50 threshold. CTL clones recognizing the same epitope can vary by several orders of magnitude in their SD50; thus, a small change in avidity for a clone near the threshold could completely ablate antiviral activity, whereas even a large change in avidity for another clone further from the threshold could have no effect.

By example, our data define KE50 values for three HIV-1 epitopes, including the well-studied SL9 epitope (SLYNTVATL, Gag p17 77 to 85). Notably, Brander et al. published a detailed study of SD50 values of CTLs for SL9 and common epitope variants (6). In many cases, the avidities of CTLs for epitope variants were lower by only 1 or 2 log units, while to others fell by up to 6 log units, which was suggested to indicate that these variants could differ in their efficiency of escape. However, our data reveal that the KE50 for SL9 is approximately 3.2 log10 pg/ml, and in nearly every case the shift in SD50 for the SL9 variants crossed this threshold; thus, these variants probably do represent CTL escape mutations, and the degree of SD50 shift between epitope variants is functionally irrelevant.

Having data on KE50 for individual epitopes suggests a possible novel approach to experimentally estimate the quantity of epitope presentation on the surface of virus-infected cells. Past work in this area has relied on acid elution of massive numbers of infected cells to isolate and quantitate cell surface epitopes (17). The KE50 is defined as the functional avidity (SD50) required for a CTL clone to achieve 50% killing efficiency of infected cells expressing endogenous epitope, and the SD50 is defined as the concentration of exogenously added epitope allowing 50% efficiency of killing of uninfected cells. Thus, choosing an epitope variant for which the SD50 and KE50 are equal and adding that epitope to uninfected cells at its SD50 concentration results in cell surface labeling that approximates endogenous expression from viral infection. If the exogenously added epitope can be tagged to allow detection, the number of molecules bound per cell could then be estimated.

Understanding and predicting KE50 values are valuable for studying viral immunopathogenesis and vaccine development. Beyond measuring SD50 values for virus-specific CTL responses in virus-infected subjects or vaccinees, knowing the KE50 values for those epitopes is the key to interpreting whether CTLs recognizing those epitopes have antiviral potential and whether epitope variants are recognized or escape mutations. While a vaccine could induce CTL responses that are detectable under the excess peptide conditions of assays such as ELISPOT or tetramer binding, those responses could lack sufficient avidity to clear virus-infected cells, rendering the vaccine-generated response incapable of viral clearance. Only measurement of SD50 values and knowledge of the KE50 thresholds for these responses would reveal this problem. This may be especially relevant to predicting the ability of CTLs to cross-recognize a pathogen with varying sequence. Directly relevant to vaccine development for HIV-1 (which exists as several genetic clades), CTL recognition of cross-clade epitopes presented via recombinant vaccinia virus vectors (4, 7) or synthetic peptides (10, 19) has been proposed to indicate promising cross-clade CTL efficacy. However, these CTL responses may not be adequate if their functional avidity against the clade variant epitopes is insufficient to clear infected cells, even though cross-reactive CTL responses can be detected.

![FIG. 6. CTL suppression of viral replication is subject to the same avidity thresholds as killing of infected cells.](image-url)

![FIG. 7. Limitations in predicting CTL killing of virus-infected cells using functional avidity measurements.](image-url)
using excess epitope presentation via vaccinia virus or synthetic peptides.

Our empirical approach for determining KE50 values clearly is not feasible on a large scale. However, algorithms for predicting epitope processing and HLA binding are available and with improvement could theoretically provide the information needed to predict these thresholds for specific epitopes. Hopefully, determining KE50 for more epitopes combined with future advances in technology can provide a validated approach for predicting the efficacy of virus-specific CTLs for pathogenesis and vaccine studies.

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