Title

Suboptimal stimulation by weak agonist epitope variants does not drive dysfunction of HIV-1-specific cytotoxic T lymphocyte clones.

Journal

AIDS, 33(10)

ISSN

0269-9370

Authors

Grossman, Mark A
Hofmann, Christian
Ng, Hwee L
et al.

Publication Date

2019-08-01

DOI

10.1097/qad.0000000000002259

Peer reviewed

HHS Public Access

Author manuscript

AIDS. Author manuscript; available in PMC 2020 February 27.

Published in final edited form as:

AIDS. 2019 August 01; 33(10): 1565–1574. doi:10.1097/QAD.0000000000002259.

Suboptimal Stimulation by Weak Agonist Epitope Variants Does Not Drive Dysfunction of HIV-1-Specific CTL Clones

Mark A. GROSSMAN[1], **Christian HOFMANN**[2], **Hwee L. NG**[2], **Otto O. YANG**[1,2,3]

[1]Department of Microbiology, Immunology & Molecular Genetics, David Geffen School of Medicine, University of California Los Angeles, Los Angeles, CA, USA

[2]Division of Infectious Diseases, Department of Medicine, David Geffen School of Medicine, University of California Los Angeles, Los Angeles, CA, USA

[3]AIDS Healthcare Foundation, Los Angeles, CA, USA

Abstract

Objective: To assess whether weakly recognized epitope variants induce anergy in HIV-1-specific $CD8^+$ T lymphocyte (CTL) clones as a mechanism of dysfunction.

Design: HIV-1-specific CTL clones were exposed to suboptimally recognized epitope variants, and screened for anergy and other T cell dysfunction markers, and subsequent capability to kill target cells bearing index epitope.

Methods: In addition to the optimally recognized index epitope, two suboptimally recognized epitope variants were selected based on titration curves for killing of peptide-labeled target cells by three HIV-1-specific CTL clones targeting the epitopes SLYNTVATL (Gag 77-85, A*02-restricted), RPAEPVPLQL (Rev 66-75, B*07-restricted), and KRWIIMGLNK (Gag 263-272, B*27-restricted). Consequences of suboptimal stimulation were assessed by cytokine secretion, gene expression, and capacity to kill index epitope-labeled target cells upon rechallenge.

Results: Suboptimal recognition of epitope variants reduced cytokine production by CTL similarly to reduction in killing of target cells. Gene expression profiles after suboptimal stimulation demonstrated no patterns consistent with T cell dysfunction due to anergy, exhaustion, or apoptosis. Pre-exposure of CTL to epitope variants had no discernable impact on their subsequent capacity to kill index epitope-bearing target cells.

Conclusions: Our data explore the hypothesis that poorly recognized epitope variants not only facilitate HIV-1 evasion of CTL recognition, but also induce CTL dysfunction through suboptimal signaling causing anergy. However, the results do not suggest that suboptimal signaling induces anergy (or exhaustion or apoptosis), indicating that the major role of CTL epitope variation is likely viral escape.

Corresponding Author: Otto O. Yang, Division of Infectious Diseases, BSRB 173, 615 Charles E Young Drive South, Los Angeles, CA, 90095, USA, oyang@mednet.ucla.edu, Tel: 310-794-9491, Fax: 310-983-1067.

Conflicts of Interest and Sources of Funding: O.O.Y. serves as Director for Scientific Research at AIDS Healthcare Foundation; for the remaining authors no conflicts of interest are declared.

Keywords

CTL; dysfunction; anergy; exhaustion; peripheral T-cell tolerance

INTRODUCTION

HIV-1-specific $CD8^+$ cytotoxic T lymphocytes (CTL) have an important protective role in HIV-1 pathogenesis, but ultimately fail to prevent disease progression in the vast majority of infected persons. The reasons for this failure are unclear but involve both virologic and immunologic factors: HIV-1 evolves to generate escape variants in CTL epitopes, and HIV-1-specific CTL become dysfunctional. While chronic viral persistence is believed to be a major driver of CTL dysfunction by causing ongoing antigenic stimulation leading to exhaustion [1], dysfunction can be seen even during early infection [2], suggesting that there may be other mechanism(s) besides exhaustion.

It is well established that viral evolution *in vivo* directly decreases epitope recognition by CTL [3, 4], presumably facilitating viral persistence via CTL evasion. However, epitope mutation can result in a spectrum of CTL recognition of HIV-1-infected cells, ranging from complete non-recognition to full recognition [5], indicating that some epitope variants could serve as weak ligands for T cell receptor (TCR) signaling. Growing evidence suggests that suboptimal signaling of T cells can result in anergy [6-8], but it is relatively unexplored whether HIV-1 epitope variation contributes to CTL dysfunction through this mechanism in addition to mediating direct escape.

T cell anergy is a state of antigen-induced hyporesponsiveness that is involved in diseases such as cancer and autoimmune diabetes [9, 10]. It is a non-deletional mechanism of peripheral T cell tolerance thought to have evolved to prevent reactivity to self-antigens [11], and has the hallmarks of defective proliferation and IL-2 production [12, 13]. An initial conceptual model was first characterized using murine $CD4^+$ T cell clones given antigenic stimulation in the absence of both IL-2 and CD80/CD86 co-stimulation [14]. More recently it has been demonstrated that T cell anergy can also be induced via suboptimal antigen stimulation by “altered peptide ligands” (APL) with reduced/suboptimal affinity for the TCR, resulting in incomplete signaling and activation [10, 15-18]. While most demonstrations have utilized $CD4^+$ T cells, there is evidence for this phenomenon in $CD8^+$ T cells as well [19-22].

Given prior findings that epitope variants yield a range of recognition by HIV-1-specific CTL clones reported by our group [5] and others [23, 24], we hypothesized that weakly recognized epitope variants might serve as APL that drive anergy as a mechanism of CTL dysfunction. Using HIV-1-specific CTL clones against three epitopes, we investigated the effects of epitope variants on CTL triggering by assessing target cell killing, secretion of cytokines, and expression levels of genes associated with CTL growth/activation, exhaustion, anergy, or regulation of apoptosis. Furthermore, we examined the effects of CTL pre-treatment with suboptimally recognized peptide variants to evaluate whether such suboptimal stimulation subsequently leads to dysfunctional inability to kill target cells with the index epitope. In contrast to observations of T cell anergy induced by APL in other

systems, we found no evidence of this phenomenon, suggesting that epitope variation causing anergy is not a major driver of HIV-1-specific CTL dysfunction.

MATERIALS AND METHODS

HIV-1-specific CTL clones.

CTL clones S00001-SL9-3.23T, S42758-RL10-3.22, and S00076-KK10-10.37 recognize the epitopes SLYNTVATL (SL9, Gag 77-85, A*02-restricted), RPAEPVPLQL (RL10, Rev 66-75, B*07-restricted), and KRWIIMGLNK (KK10, Gag 263-272, B*27-restricted), respectively. These were isolated at limiting dilution and maintained as previously described [5, 25].

Target cell lines.

T1 (HLA-A*02^{+}), Jurkat (HLA-B*07^{+}), and EBV-immortalized B cells from Subject 00076 (HLA-B*27^{+}) were maintained in R10 medium (RPMI 1640 supplemented with HEPES, penicillin/streptomycin, L-glutamine, and 10% heat-inactivated fetal calf serum) as described previously [26]. These cell lines were used as HLA-matched antigen-presenting cells (APC) and target cells for the CTL clones S00001-SL9-3.23T, S42758-RL10-3.22, and S00076-KK10-10.37, respectively, in all chromium release killing assays.

Selection of epitope variants.

Each CTL clone was initially screened against a panel of previously identified variants in HIV-1 subtype B sequences in the Los Alamos HIV Sequence Database (“filtered web alignment” reference sequences, analyzed using https://www.hiv.lanl.gov/content/sequence/QUICK_ALIGNv2/QuickAlign.html) in peptide titration assays as previously described [5]. The “index,” “variant 1,” and “variant 2” sequences were selected as best-recognized, less-recognized, and least-recognized variants respectively (see Results). For CTL clone S00001-SL9-3.23T, these were SLYNTVATL (index), SL**F**NTVA**V**L (variant 1), and SL**F**NT**I**ATL (variant 2). For CTL clone S42758-RL10-3.22, these were RPAEPVPLQL (index), RP**V**EPVPLQL (variant 1), and RP**T**EPVP**FH**L (variant 2). For CTL clone S00076-KK10-10.37, these were KRWIIMGLNK (index), KRWII**L**GLNK (variant 1), and K**K**WII**L**GLNK (variant 2). The subtype B frequencies of these variants in the sequence database for SL9 index, SL9 variant 1, and SL9 variant 2 were 26.7%, 9.0%, and 2.8% respectively. The subtype B frequencies of these variants in the sequence database for RL10 index, RL10 variant 1, and RL10 variant 2 were 22.7%, 1.1%, and 0.3% respectively. The subtype B frequencies of these variants in the sequence database for KK10 index, KK10 variant 1, and KK10 variant 2 were 10.5%, 79.5%, and 0.6% respectively.

Peptides.

Peptides were purchased from Sigma and were >70% pure by standard desalting. Lyophilized peptides were reconstituted at 2 mg/mL in 10% DMSO and stored in aliquots at −80˚C..

Assessment of peptide-specific cytolytic activity.

The cytolytic activity of the CTL clones was determined by standard ^{51}Cr-release assay as previously described [5, 26]. Briefly, target cells were labeled with ^{51}Cr and then seeded in a 96-well U-bottom plate at 10^4 cells per well for coculture with or without 5 x 10^4 CTL and the indicated peptides for 4 hours at 37˚C, followed by measurement of released ^{51}Cr by scintillation counting. Percent specific lysis was calculated by the formula: (observed release – spontaneous release) ÷ (maximum release – spontaneous release) x 100.

CTL stimulation and sample preparation for cytokine and gene expression assays.

2 x 10^5 CTL were seeded per well in 200 μL of R10 medium in a 96-well U-bottom plate. CTL were either left unstimulated (in R10 medium without peptide), or stimulated with serial 5-fold dilutions of the indicated peptides for 6 hours at 37˚C. Following stimulation, the plate was centrifuged at 1,000x g for 5 minutes at room temperature, and 120 μL culture supernatant from each well was collected and frozen at −80˚C until analysis. CTL were lysed by adding 40 μL of working lysis mixture supplemented with proteinase K (RNA QuantiGene 2.0 Plex kit, Affymetrix) to the remaining 80 μL of culture and mixed by thorough pipetting (final total volume 120 μL). Lysates were transferred to sterile PCR strips and then incubated in a thermocycler at 52.5˚C for 30 min to degrade the ribosomes. Lysates were mixed again by pipetting, then frozen at −80˚C until analysis.

Analysis of secreted cytokines.

The concentration of secreted human IFN-γ, TNF-α, IL-2, MIP-1α, and MIP-1β in cell culture supernatant was measured by Luminex (R&D Systems) according to manufacturer's instructions. Wash steps were performed on a hand-held magnetic plate washer (Bio-Rad) according to manufacturer's instructions. Data was acquired on a MAGPIX instrument using xPONENT 4.2 software.

Gene expression analysis.

A custom RNA QuantiGene Plex 2.0 Luminex kit (Affymetrix) was used to quantitate the relative levels of mRNA transcripts for the following human genes: CD25, CD71, CD95, CD98HC, Tim-3, Lag-3, PD-1, CTLA-4, BTLA, TNFR1, TNFR2, Caspase-3, DGK-a, GRAIL, Itch, Cbl-b, $p27^{kip1}$, Bcl-2, Bim, Egr1, Egr2, Egr3, FoxP3, Ikaros, Deltex1, Tsc1, HPRT1, and GAPDH. Each gene-specific probe was designed by the manufacturer to recognize all known isoforms of the mRNA. 80 μL of undiluted CTL lysate (corresponding to 1.33 x10^5 cells) was used as the input amount of sample per well for quantification. The quantitative sensitivity of this assay to ~2,000 transcripts per well thus allowed detection of <1 transcript/cell, and initial control experiments with lysate from 10^5 stimulated CTL/well demonstrated on-scale detection of the most highly expressed transcripts (not shown). All steps for sample hybridization and detection were performed according to manufacturer's instructions. Wash steps were performed on a hand-held magnetic plate washer (Bio-Rad) according to manufacturer's instructions. Data was acquired on a MAGPIX instrument using xPONENT 4.2 software. With exception of the KK10-specific clone, both the cytokine and gene expression data were obtained from the same batch of CTL. Normalized expression levels compared to unstimulated controls were expressed as fold-change (Fold-Δ) when

baseline expression was readily detectable or change in median fluorescence intensity (ΔMFI) when baseline expression was very low or undetectable using “per-well” normalization to the housekeeping genes HPRT1 and GAPDH by the following equations:

$$\text{Fold-}\Delta = (A_1 \times B_2) \div (A_2 \times B_1)$$

or

$$\Delta\text{MFI} = (A_1 \times B_2 \div B_1) - A_2$$

where

A_1= signal of gene of interest in the stimulated sample

B_1= geometric mean of the signals for HPRT1 and GAPDH in the stimulated sample

A_2= signal of gene of interest in the unstimulated sample

B_2= geometric mean of the signals for HPRT1 and GAPDH in the unstimulated sample

Peptide pre-treatment killing assays.

Allogeneic feeder PBMC and antigen-presenting cells (APC) were irradiated with 3,000 and 10,000 rads respectively prior to culture. CTL were pretreated with feeder PBMC and peptide-pulsed APC at a 1:2:2 ratio (4x 10^4 CTL, 8 x 10^4 PBMC, 8 x 10^4 APC per well) in 200 μL R10 per well in a 96-well U-bottom plate for 48 hours at 37˚C. The CTL then were assayed for killing activity by direct addition of 5 x10^3 target cells that had previously been ^{51}Cr-labeled and index peptide-pulsed (SL9 at 10 ng/mL, RL10 at 1 μg/mL, or KK10 at 100 ng/mL as appropriate) in 50 μL per well. After 4 hours coculture at 37˚C, lytic capacity against the index epitope was determined by measurement of released ^{51}Cr as described above.

RESULTS

Definition and selection of epitope variants that are suboptimally recognized by HIV-1-specific CTL in killing assays.

HIV-1-specific CTL clones S00001-SL9-3.23T (recognizing the index epitope SLYNTVATL, Gag 77-85, A*02-restricted), S42758-RL10-3.22 (recognizing the index epitope RPAEPVPLQL, Rev 66-75, B*07-restricted), and S00076-KK10-10.37 (recognizing the index epitope KRWIIMGLNK, Gag 263-272, B*27-restricted) were screened for their functional avidities against epitope variants in peptide titration killing assays (Figure 1). For each clone, epitope variants were selected to span approximately two orders of magnitude of weak agonism, with “variant 1” and “variant 2” selected as less recognized and least recognized variants respectively. For clone S00001-SL9-3.23T (Figure 1A), these included SL**F**NTVA**V**L (variant 1) and SL**F**NT**I**ATL (variant 2). For clone S42758-RL10-3.22 (Figure 1B), these included RP**V**EPVPLQL (variant 1) and RP**T**EPVP**FH**L (variant 2). For clone S00076-KK10-10.37 (Figure 1C), these included KRWII**L**GLNK (variant 1) and

K**K**WII**L**GLNK (variant 2). These data identified weak agonist APL for each CTL clone, as defined by reduced functional avidity for triggering killing of target cells.

Cytokine release by HIV-1-specific CTL in response to varying levels of index and variant epitope stimulation also reflects suboptimal triggering by weak agonists.

To assess the functional impact of suboptimal stimulation by index and variant epitopes in terms of cytokine production, secretion of IFN-γ, TNF-α, IL-2, MIP-1α, and MIP-1β was measured in culture supernatant from CTL stimulated by titrations of index, variant 1, or variant 2 peptides (Figure 2). There was some heterogeneity in cytokine production between the CTL clones, most notably production of IL-2 by the KK10-specific clone (Figure 2C) and not by the SL9- and RL10-specific clones (Figure 2A and B). There also appeared to be small differences between clones in terms of the relationship of peptide doses required to trigger release of different cytokines; for example, SL9- and RL10-specific clones (Figure 2A and B) seemed to require less peptide to produce MIP-1α and MIP-1β compared to IFN-γ and TNF-α, which was not observed for the KK10-specific clone (Figure 2C). Overall, however, the relative efficiencies of CTL triggering of cytokine release by the index and variant peptides mirrored those for triggering target cell killing in Figure 1, further confirming that variants 1 and 2 functioned as APL with successively reduced CTL triggering.

Weak agonism by APL stimulation also reduces CTL triggering in terms of gene expression, but does not induce selective expression of exhaustion, pro-apoptotic, or anergy markers.

To investigate whether suboptimal stimulation leads to expression of markers of dysfunction, RNA transcripts for proteins associated with T cell activation or dysfunction via exhaustion, apoptosis (which has also been linked to APL stimulation [27]), or anergy were quantified in CTL clones stimulated with the indicated peptides (Figures 3 and 4). Changes in expression relative to unstimulated controls were compared for the following genes: activation markers CD25 [8] and Egr1 [28]; metabolic regulators CD71 (transferrin receptor) [29] and CD98HC (heavy chain of CD98, or large neutral amino acid transporter, LAT1) [29]; exhaustion markers CTLA-4 [11, 30], Lag-3 [22], Tim-3 [31], PD-1 [31], and BTLA [31]; apoptosis regulators Bcl-2 [32], Bim [32], CD95 [33], TNFR1 [33], and TNFR2 [33]; and anergy factors Caspase-3 [34], Cbl-b [35], Deltex1 [36], DGKα [37], Egr2 [38], Egr3 [8], FoxP3 [8], GRAIL [39], Ikaros [6], Itch [39], $p27^{kip1}$ [6], and Tsc1 [40].

Generally, changes in gene expression were similar between CTL clones, with reduced triggering of differential gene expression by variant epitopes compared to the index epitope that was dose-dependent. Activation- and metabolism -associated markers mostly increased with greater stimulation (Figure 3 and Figure 4 top row) except for Egr1 in the SL9-specific clone only (Figure 3A). Most exhaustion markers increased with increasing stimulation (Figure 3 and Figure 4 second row), although PD-1 was undetectable (not shown) and BTLA was minimally detected (Figure 3). The apoptosis regulators Bcl-2, Bim (Figure 3 and Figure 4 third row), and TNFR2 (Figure 3) also increased with greater stimulation, while CD95 and TNFR1 decreased (Figure 3). Of note, weak stimuli did not increase anergy-related genes in comparison to the unstimulated controls. Most of the anergy factors

decreased with greater stimulation (Figure 3 and Figure 4 fourth row) except for Egr2 (Figure 3 and Figure 4 last row) and Egr3 (Figure 3), which increased predominantly at higher peptide stimulation conditions; Deltex1 and GRAIL were undetectable across all conditions (not shown). Overall, weak agonism did not selectively trigger transcriptional profiles indicative of CTL dysfunction due to anergy, exhaustion, or apoptosis.

Exposure of HIV-1-specific CTL to weak agonist APL does not ablate their subsequent ability to kill target cells.

To further evaluate whether suboptimal stimulation causes a dysfunctional phenotype, the CTL clones were pre-exposed to varying doses of index or variant epitopes and then challenged for lytic capacity against target cells labeled with the index peptide. CTL pre-exposed to the index and variant peptides exhibited no notable decrease in subsequent killing of index peptide-loaded target cells compared to control CTL that had not been pre-exposed to any peptide (Figure 5). The level of killing remained stable despite pre-exposure to the variant peptides across a wide spectrum of concentrations yielding CTL stimulation ranging from minimally to fully activating. These results demonstrated maintenance of CTL cytolytic function after weak stimulation by APL, further supporting a lack of anergy following suboptimal stimulation by APL.

DISCUSSION

HIV-1-specific CTL dysfunction is thought to play a pivotal role in the pathogenesis of infection, yet the precise factors determining this phenomenon remain incompletely understood. This dysfunction has been observed in assays such as “polyfunctionality” in cytokine production and cytolytic function marker expression [41] and capacity to suppress viral replication [42, 43], both of which correlate to immune containment of infection. A leading candidate mechanism is CTL exhaustion driven by viral persistence causing continuous antigenic stimulation, which is supported by data such as studies showing shortened telomeres in $CD8^+$ T cells [44] and upregulation of cell surface exhaustion markers [1, 45] in HIV-1 infection.

Another mechanism considered here is anergy due to suboptimal signaling by viral epitope variants that serve as weak agonist APL, which has been well documented in other experimental systems primarily involving $CD4^+$ T cells [15-17]. While APL-induced anergy has been shown for an HIV-1 gp120-specific $CD4^+$ T cell clone [46], it has not been clearly defined for HIV-1-specific $CD8^+$ T cells. Early studies have raised the question of $CD8^+$ T cell anergy in HIV-1 infection, but not via APL stimulation, and they defined “anergy” solely based on the criterion of reduced proliferative capacity [47-49], which is nonspecific and insufficient to differentiate anergy from other dysfunctional processes such as exhaustion or senescence. Thus, our study is the first to directly address APL-induced anergy in HIV-1-specific CTL.

Contrary to the strong evidence for APL inducing T cell anergy through suboptimal signaling, we did not observe evidence for anergy when index and weak agonist epitope variants were tested over a range of concentrations that span a spectrum of CTL triggering ranging from none to full agonism. This range of activity was confirmed by measuring target

cell killing and cytokine release. However, anergy-associated genes were not selectively induced by suboptimal stimulation. Indeed, differential transcription compared to unstimulated CTLs (whether up or down with increasing stimulation) demonstrated simple dose-dependence for index and APL epitopes, rather than any pattern of an anergic profile specific to weak stimulation. Furthermore, there was no direct evidence for dysfunction in terms of target cell killing after pre-exposure to APL. These results argue against weak agonist APL-induced anergy being a key mechanism for CTL dysfunction in HIV-1 pathogenesis.

Lack of epitope variation causing anergy is also consistent with findings of Streeck *et al* [50], who examined the functional outcomes of HIV-1-specific CTL responses in a cohort of persons with acute/early infection. They noted that when epitopes remained fixed the CTL targeting those epitopes became dysfunctional, whereas CTL targeting epitopes that exhibited sequence evolution maintained function. These results suggested that exhaustion plays a major role in loss of CTL function even relatively early in infection, because epitope mutation and presumably diminished recognition was associated with preserved CTL function. Consistent with our results, epitope mutation did not drive CTL dysfunction, as would be expected if altered peptide ligand induced anergy.

The current study is indirectly relevant to another proposed avenue of HIV-1-specific CTL dysfunction termed “antagonism,” in which reduced killing of target cells with the index epitope has been observed in the presence of poorly recognized epitope variants [51-53]. The precise mechanism has not been defined, but our data suggest that it is not mediated by anergy. Observations of antagonism have required excess APL concurrently presented with index epitope, which is more supportive of direct competition for TCR engagement. Moreover, prior reports of antagonism have not tested pre-exposure of HIV-1-specific CTL to APL alone, or explored changes in CTL phenotype specifically induced by APL. Thus the potential role for anergy has not been directly addressed in past demonstrations of antagonism, and our complementary data are not consistent with anergy mediating antagonism.

There are technical caveats that should be considered in interpreting our results. The anergy markers selected for this study were based on prior characterizations of anergy mostly using murine $CD4^+$ T cells, and to a lesser extent in murine $CD8^+$ T cells. While anergy has been evaluated in human T cells [11, 16, 17, 20, 21, 46, 54], the mechanisms and molecular signatures are less defined than for their murine counterparts [6], particularly for $CD8^+$ T cells, and thus it is possible that different factors are involved in human CTL anergy. Indeed, most of the T cell function markers have functional overlap outside the processes they were chosen to represent. Also, while defective proliferation and IL-2 production are considered defining hallmarks of anergy in $CD4^+$ T cells [12, 13], the impact of anergy on CTL cytolytic function is less clear [19], although it might be expected to diminish since the putative biologic role of anergy is avoidance of autoimmunity [11]. As for any *in vitro* study of anergy, experimental conditions differed from those *in vivo*, including the use of synthetic peptide epitopes, non-physiologic antigen-presenting cells that could either artificially provide or lack co-stimulatory ligands, and the use of CTL clones that have been selected to proliferate well over repeated rounds of stimulation.

In summary, our study examined the consequences of weak agonist APL stimulation of HIV-1-specific CTL clones. Despite the firm rationale that this could trigger anergy, supported by studies of suboptimal T cell signaling in other systems, our results demonstrated no evidence for a transcriptional profile or loss of cytolytic function that would indicate anergy. With the caveats that the anergy-mediating factors and/or conditions could differ between our experimental system (using human $CD8^+$ T cell clones) and those of other anergy studies (predominately using murine $CD4^+$ T cells) and conditions *in vivo*, these findings do not support anergy as a major mechanism of HIV-1-specific CTL dysfunction.

ACKNOWLEDGMENTS

We are grateful to the persons from whom these CTL clones were previously isolated. This work was supported primarily by PHS grant AI110343 (O.O.Y.) and a grant from the AIDS Healthcare Foundation (O.O.Y.). Additional support was provided by PHS grants DE025166 (O.O.Y.) and AI028697 (UCLA AIDS Institute and Center for AIDS Research), James B. Pendleton Trust, and McCarthy Foundation. Recombinant human interleukin-2 was provided by the NIH AIDS Reagent Repository. We thank Mary Ann Hausner for expert technical assistance.

LITERATURE CITED

1. Day CL, Kaufmann DE, Kiepiela P, Brown JA, Moodley ES, Reddy S, et al. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. Nature 2006,443:350–354. [PubMed: 16921384]
2. Lecuroux C, Girault I, Cheret A, Versmisse P, Nembot G, Meyer L, et al. CD8 T-Cells from Most HIV-Infected Patients Lack Ex Vivo HIV-Suppressive Capacity during Acute and Early Infection. PLoS One 2013,8:e59767. [PubMed: 23555774]
3. Borrow P, Lewicki H, Wei X, Horwitz MS, Peffer N, Meyers H, et al. Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus. Nat Med 1997,3:205–211. [PubMed: 9018240]
4. Phillips RE, Rowland-Jones S, Nixon DF, Gotch FM, Edwards JP, Ogunlesi AO, et al. Human immunodeficiency virus genetic variation that can escape cytotoxic T cell recognition. Nature 1991,354:453–459. [PubMed: 1721107]
5. Bennett MS, Ng HL, Dagarag M, Ali A, Yang OO. Epitope-dependent avidity thresholds for cytotoxic T-lymphocyte clearance of virus-infected cells. J Virol 2007,81:4973–4980. [PubMed: 17329324]
6. Wells AD. New insights into the molecular basis of T cell anergy: anergy factors, avoidance sensors, and epigenetic imprinting. J Immunol 2009,182:7331–7341. [PubMed: 19494254]
7. Sadegh-Nasseri S, Dalai SK, Korb Ferris LC, Mirshahidi S. Suboptimal engagement of the T-cell receptor by a variety of peptide-MHC ligands triggers T-cell anergy. Immunology 2010,129:1–7. [PubMed: 20002785]
8. Kuklina EM. Molecular mechanisms of T-cell anergy. Biochemistry (Mosc) 2013,78:144–156. [PubMed: 23581985]
9. Crespo J, Sun H, Welling TH, Tian Z, Zou W. T cell anergy, exhaustion, senescence, and stemness in the tumor microenvironment. Curr Opin Immunol 2013,25:214–221. [PubMed: 23298609]
10. Edwards LJ, Evavold BD. Destabilization of peptide:MHC interaction induces IL-2 resistant anergy in diabetogenic T cells. J Autoimmun 2013,44:82–90. [PubMed: 23895744]
11. Maeda Y, Nishikawa H, Sugiyama D, Ha D, Hamaguchi M, Saito T, et al. Detection of self-reactive CD8(+) T cells with an anergic phenotype in healthy individuals. Science 2014,346:1536–1540. [PubMed: 25525252]
12. Macian F, Im SH, Garcia-Cozar FJ, Rao A. T-cell anergy. Curr Opin Immunol 2004,16:209–216. [PubMed: 15023415]
13. Schwartz RH. T cell anergy. Annu Rev Immunol 2003,21:305–334. [PubMed: 12471050]

14. Jenkins MK, Schwartz RH. Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness in vitro and in vivo. J Exp Med 1987,165:302–319. [PubMed: 3029267]
15. Madrenas J, Wange RL, Wang JL, Isakov N, Samelson LE, Germain RN. Zeta phosphorylation without ZAP-70 activation induced by TCR antagonists or partial agonists. Science 1995,267:515–518. [PubMed: 7824949]
16. Korb LC, Mirshahidi S, Ramyar K, Sadighi Akha AA, Sadegh-Nasseri S. Induction of T cell anergy by low numbers of agonist ligands. J Immunol 1999,162:6401–6409. [PubMed: 10352253]
17. Mirshahidi S, Ferris LC, Sadegh-Nasseri S. The magnitude of TCR engagement is a critical predictor of T cell anergy or activation. J Immunol 2004,172:5346–5355. [PubMed: 15100274]
18. Ford ML, Evavold BD. Regulation of polyclonal T cell responses by an MHC anchor-substituted variant of myelin oligodendrocyte glycoprotein 35-55. J Immunol 2003,171:1247–1254. [PubMed: 12874212]
19. Otten GR, Germain RN. Split anergy in a CD8+ T cell: receptor-dependent cytolysis in the absence of interleukin-2 production. Science 1991,251:1228–1231. [PubMed: 1900952]
20. Lim DG, Hollsberg P, Hafler DA. Strength of prior stimuli determines the magnitude of secondary responsiveness in CD8+ T cells. Cell Immunol 2002,217:36–46. [PubMed: 12425999]
21. Carrabba MG, Castelli C, Maeurer MJ, Squarcina P, Cova A, Pilla L, et al. Suboptimal activation of CD8(+) T cells by melanoma-derived altered peptide ligands: role of Melan-A/MART-1 optimized analogues. Cancer Res 2003,63:1560–1567. [PubMed: 12670905]
22. Schietinger A, Delrow JJ, Basom RS, Blattman JN, Greenberg PD. Rescued tolerant CD8 T cells are preprogrammed to reestablish the tolerant state. Science 2012,335:723–727. [PubMed: 22267581]
23. Brander C, Hartman KE, Trocha AK, Jones NG, Johnson RP, Korber B, et al. Lack of strong immune selection pressure by the immunodominant, HLA- A*0201-restricted cytotoxic T lymphocyte response in chronic human immunodeficiency virus-1 infection. J Clin Invest 1998,101:2559–2566. [PubMed: 9616227]
24. Jones NA, Wei X, Flower DR, Wong M, Michor F, Saag MS, et al. Determinants of human immunodeficiency virus type 1 escape from the primary CD8+ cytotoxic T lymphocyte response. J Exp Med 2004,200:1243–1256. [PubMed: 15545352]
25. Adnan S, Balamurugan A, Trocha A, Bennett MS, Ng HL, Ali A, et al. Nef interference with HIV-1-specific CTL antiviral activity is epitope specific. Blood 2006,108:3414–3419. [PubMed: 16882705]
26. Yang OO, Kalams SA, Rosenzweig M, Trocha A, Jones N, Koziel M, et al. Efficient lysis of human immunodeficiency virus type 1-infected cells by cytotoxic T lymphocytes. J Virol 1996,70:5799–5806. [PubMed: 8709196]
27. Ream RM, Sun J, Braciale TJ. Stimulation of naive CD8+ T cells by a variant viral epitope induces activation and enhanced apoptosis. J Immunol 2010,184:2401–2409. [PubMed: 20139280]
28. Collins S, Lutz MA, Zarek PE, Anders RA, Kersh GJ, Powell JD. Opposing regulation of T cell function by Egr-1/NAB2 and Egr-2/Egr-3. Eur J Immunol 2008,38:528–536. [PubMed: 18203138]
29. Zheng Y, Delgoffe GM, Meyer CF, Chan W, Powell JD. Anergic T cells are metabolically anergic. J Immunol 2009,183:6095–6101. [PubMed: 19841171]
30. Dalai SK, Mirshahidi S, Morrot A, Zavala F, Sadegh-Nasseri S. Anergy in memory CD4+ T cells is induced by B cells. J Immunol 2008,181:3221–3231. [PubMed: 18713993]
31. Schietinger A, Greenberg PD. Tolerance and exhaustion: defining mechanisms of T cell dysfunction. Trends Immunol 2014,35:51–60. [PubMed: 24210163]
32. Parish IA, Rao S, Smyth GK, Juelich T, Denyer GS, Davey GM, et al. The molecular signature of CD8+ T cells undergoing deletional tolerance. Blood 2009,113:4575–4585. [PubMed: 19204323]
33. Smith CA, Farrah T, Goodwin RG. The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death. Cell 1994,76:959–962. [PubMed: 8137429]
34. Puga I, Rao A, Macian F. Targeted cleavage of signaling proteins by caspase 3 inhibits T cell receptor signaling in anergic T cells. Immunity 2008,29:193–204. [PubMed: 18701083]

35. Jeon MS, Atfield A, Venuprasad K, Krawczyk C, Sarao R, Elly C, et al. Essential role of the E3 ubiquitin ligase Cbl-b in T cell anergy induction. Immunity 2004,21:167–177. [PubMed: 15308098]

36. Hsiao HW, Liu WH, Wang CJ, Lo YH, Wu YH, Jiang ST, et al. Deltex1 is a target of the transcription factor NFAT that promotes T cell anergy. Immunity 2009,31:72–83. [PubMed: 19592273]

37. Zha Y, Marks R, Ho AW, Peterson AC, Janardhan S, Brown I, et al. T cell anergy is reversed by active Ras and is regulated by diacylglycerol kinase-alpha. Nat Immunol 2006,7:1166–1173. [PubMed: 17028589]

38. Zheng Y, Zha Y, Driessens G, Locke F, Gajewski TF. Transcriptional regulator early growth response gene 2 (Egr2) is required for T cell anergy in vitro and in vivo. J Exp Med 2012,209:2157–2163. [PubMed: 23129747]

39. Heissmeyer V, Macian F, Im SH, Varma R, Feske S, Venuprasad K, et al. Calcineurin imposes T cell unresponsiveness through targeted proteolysis of signaling proteins. Nat Immunol 2004,5:255–265. [PubMed: 14973438]

40. Xie DL, Wu J, Lou YL, Zhong XP. Tumor suppressor TSC1 is critical for T-cell anergy. Proc Natl Acad Sci U S A 2012,109:14152–14157. [PubMed: 22891340]

41. Betts MR, Nason MC, West SM, De Rosa SC, Migueles SA, Abraham J, et al. HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells. Blood 2006,107:4781–4789. [PubMed: 16467198]

42. Saez-Cirion A, Lacabaratz C, Lambotte O, Versmisse P, Urrutia A, Boufassa F, et al. HIV controllers exhibit potent CD8 T cell capacity to suppress HIV infection ex vivo and peculiar cytotoxic T lymphocyte activation phenotype. Proc Natl Acad Sci U S A 2007,104:6776–6781. [PubMed: 17428922]

43. Yang H, Wu H, Hancock G, Clutton G, Sande N, Xu X, et al. Antiviral inhibitory capacity of CD8+ T cells predicts the rate of CD4+ T-cell decline in HIV-1 infection. J Infect Dis 2012,206:552–561. [PubMed: 22711904]

44. Effros RB, Allsopp R, Chiu CP, Hausner MA, Hirji K, Wang L, et al. Shortened telomeres in the expanded CD28-CD8+ cell subset in HIV disease implicate replicative senescence in HIV pathogenesis. Aids 1996,10:F17–22. [PubMed: 8828735]

45. Zhang JY, Zhang Z, Wang X, Fu JL, Yao J, Jiao Y, et al. PD-1 up-regulation is correlated with HIV-specific memory CD8+ T-cell exhaustion in typical progressors but not in long-term nonprogressors. Blood 2007,109:4671–4678. [PubMed: 17272504]

46. Bouhdoud L, Villain P, Merzouki A, Arella M, Couture C. T-cell receptor-mediated anergy of a human immunodeficiency virus (HIV) gp120-specific CD4(+) cytotoxic T-cell clone, induced by a natural HIV type 1 variant peptide. J Virol 2000,74:2121–2130. [PubMed: 10666241]

47. Schols D, De Clercq E. Human immunodeficiency virus type 1 gp120 induces anergy in human peripheral blood lymphocytes by inducing interleukin-10 production. J Virol 1996,70:4953–4960. [PubMed: 8764000]

48. Lewis DE, Tang DS, Adu-Oppong A, Schober W, Rodgers JR. Anergy and apoptosis in CD8+ T cells from HIV-infected persons. J Immunol 1994,153:412–420. [PubMed: 7515928]

49. Dadaglio G, Garcia S, Montagnier L, Gougeon ML. Selective anergy of V beta 8+ T cells in human immunodeficiency virus-infected individuals. J Exp Med 1994,179:413–424. [PubMed: 7905016]

50. Streeck H, Brumme ZL, Anastario M, Cohen KW, Jolin JS, Meier A, et al. Antigen load and viral sequence diversification determine the functional profile of HIV-1-specific CD8+ T cells. PLoS Med 2008,5:e100. [PubMed: 18462013]

51. Sewell AK, Harcourt GC, Goulder PJ, Price DA, Phillips RE. Antagonism of cytotoxic T lymphocyte-mediated lysis by natural HIV-1 altered peptide ligands requires simultaneous presentation of agonist and antagonist peptides. Eur J Immunol 1997,27:2323–2329. [PubMed: 9341776]

52. Meier UC, Klenerman P, Griffin P, James W, Koppe B, Larder B, et al. Cytotoxic T lymphocyte lysis inhibited by viable HIV mutants. Science 1995,270:1360–1362. [PubMed: 7481824]

53. Klenerman P, Rowland-Jones S, McAdam S, Edwards J, Daenke S, Lalloo D, et al. Cytotoxic T-cell activity antagonized by naturally occurring HIV-1 Gag variants. Nature 1994,369:403–407. [PubMed: 7515165]

54. Prinz PU, Mendler AN, Masouris I, Durner L, Oberneder R, Noessner E. High DGK-alpha and disabled MAPK pathways cause dysfunction of human tumor-infiltrating CD8+ T cells that is reversible by pharmacologic intervention. J Immunol 2012,188:5990–6000. [PubMed: 22573804]

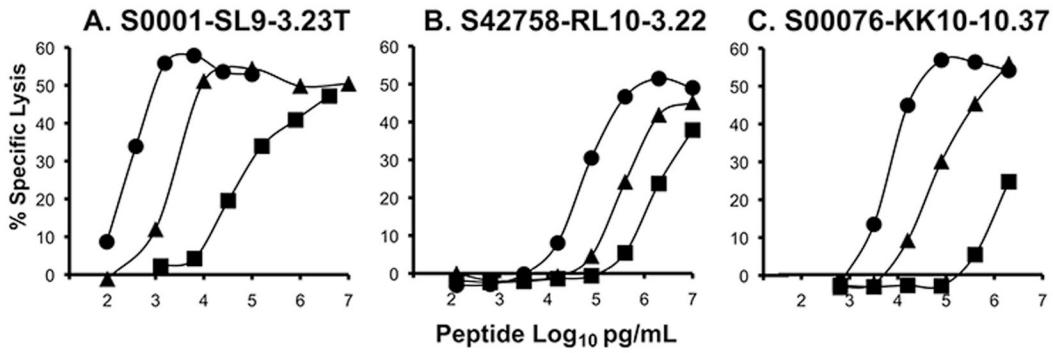

Figure 1. Identification of altered peptide ligand epitopes spanning a range of reduced agonism for triggering cytolysis by CTL clones.

For each CTL clone, two epitope variants were selected to span a broad range of reduced recognition as defined by triggering of target cell killing across varying peptide concentrations. For the remainder of this study these were termed index (

), variant 1 (

), or variant 2 (

), where the index was the best recognized and variant 2 was the least recognized epitope sequence. A. For clone S00001-SL9-3.23T, these were SLYNTVATL (index), SL**F**NTVA**V**L (variant 1), and SL**F**NT**I**ATL (variant 2); results are representative of four independent experiments. B. For clone S42758-RL10-3.22, these were RPAEPVPLQL (index), RP**V**EPVPLQL (variant 1), and RP**T**EPVP**FH**L (variant 2); results are representative of three independent experiments. C. For clone S00076-KK10-10.37, these were KRWIIMGLNK (index), KRWII**L**GLNK (variant 1), and K**K**WII**L**GLNK (variant 2); results are representative of three independent experiments.

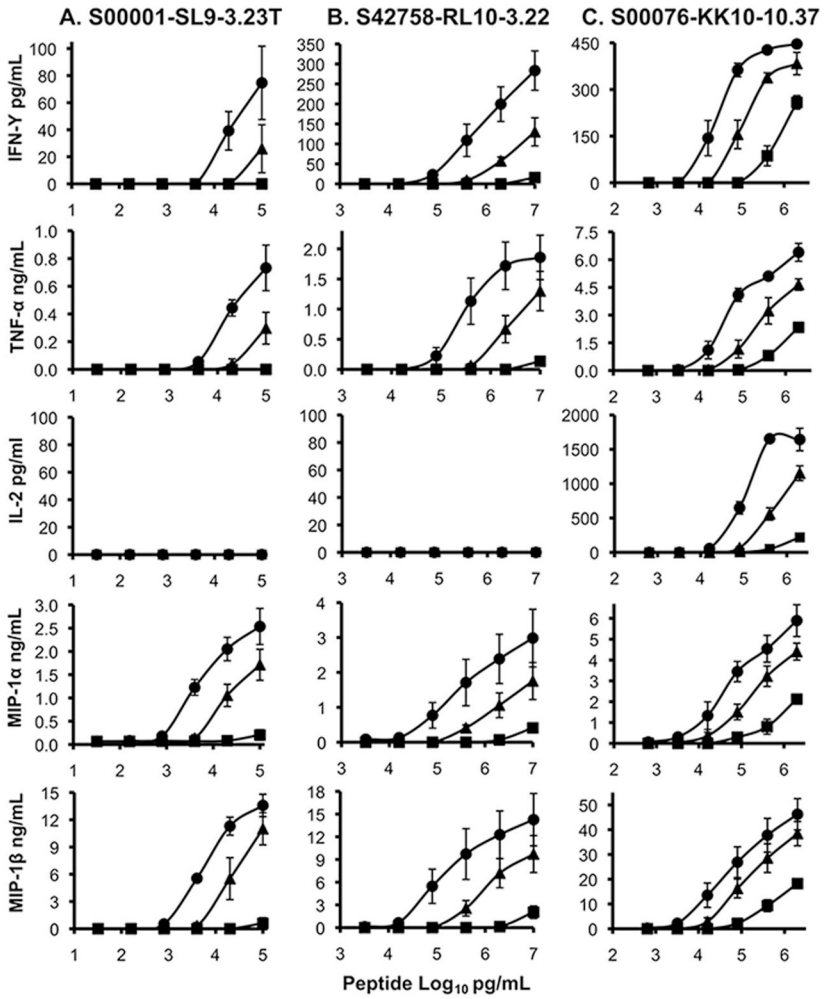

Figure 2. CTL epitope variants that are weak agonists for triggering killing are similarly weak agonists for triggering cytokine release functions.

The concentrations of secreted IFN-γ, TNF-α, IL-2, MIP-1α and MIP-1β after six hours of stimulation with varying concentrations of index (

), variant 1 (

), or variant 2 (

) peptide are shown for CTL clones S00001-SL9-3.23T (A), S42758-RL10-3.22 (B), and S00076-KK10-10.37 (C). The plots indicate average values with error bars representing one standard deviation for three independent experiments.

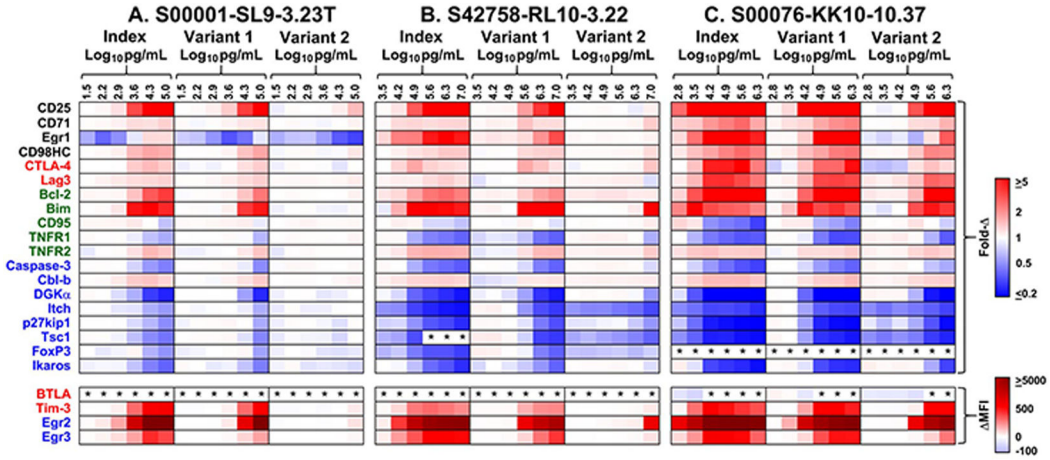

Figure 3. Gene expression changes induced by suboptimal stimulation.

Stimulation-induced differential transcription is expressed as fold-Δ (when baseline expression was readily detectable) or ΔMFI (when baseline expression was very low or undetectable) relative to unstimulated controls after 6 hours of stimulation with varying concentrations of index (

), variant 1 (

), or variant 2 (

) peptide are shown for CTL clones S00001-SL9-3.23T (A), S42758-RL10-3.22 (B), and S00076-KK10-10.37 (C). The indicated relative levels of transcripts for proteins associated with activation/metabolism (black labels), exhaustion (red labels), apoptosis (green labels), and anergy (blue labels) reflect average values from three independent experiments.

* indicates undetectable or minimally detectable expression.

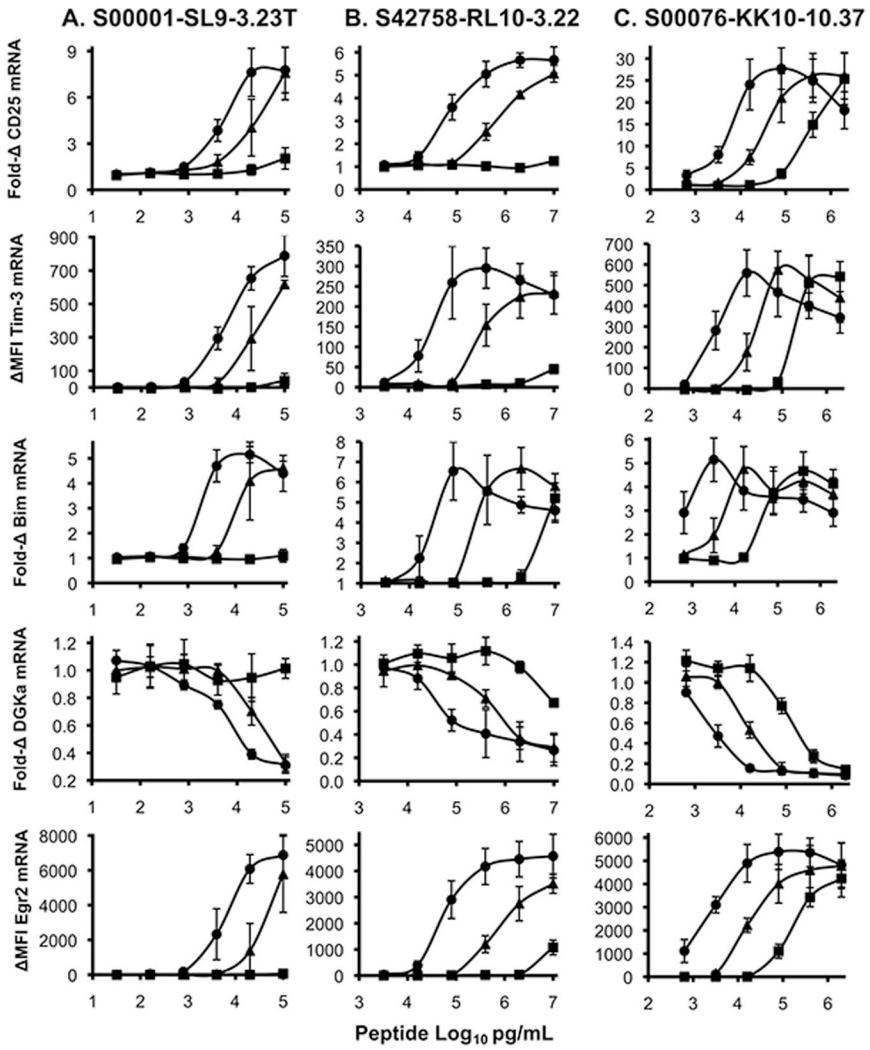

Figure 4. Suboptimal stimulation of CTL does not cause aberrant transcription of markers of T cell dysfunction.

Selected data from Figure 3 are plotted in detail for changes in mRNA levels (relative to unstimulated controls) for CD25, Tim-3, Bim, DGK-α, and Egr2 for CTL clones S00001-SL9-3.23T (A), S42758-RL10-3.22 (B), and S00076-KK10-10.37 (C) for index (

), variant 1 (

), or variant 2 (

) peptides. Plots represent average values with error bars representing one standard deviation for three independent experiments.

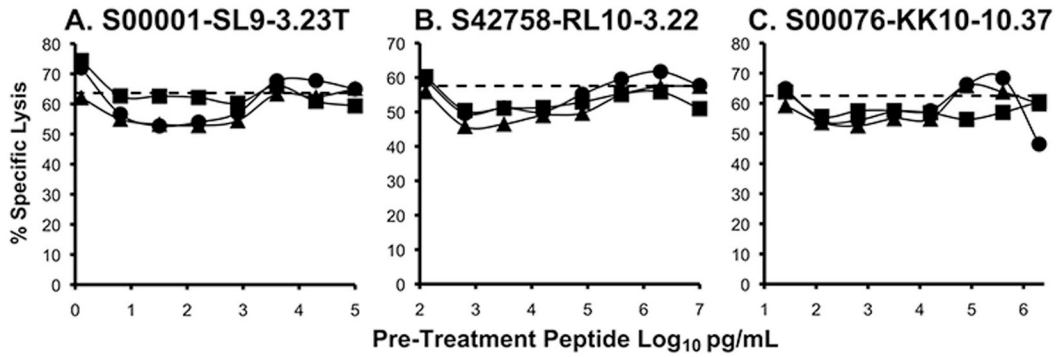

Figure 5. Suboptimal pre-stimulation of HIV-1-specific CTL does not reduce subsequent cytolytic function.

CTL clones S00001-SL9-3.23T (A), S42758-RL10-3.22 (B), and S00076-KK10-10.37 (C) were pre-exposed to the indicated concentrations of index (

), variant 1 (

), or variant 2 (

) peptide presented by irradiated APC matched by the restricting HLA type. After 48 hours, killing of ^{51}Cr-labeled target cells pulsed with the index epitope was assayed. Target cells for clones S00001-SL9-3.23T, S42758-RL10-3.22, and S00076-KK10-10.37 were T1, Jurkat, and autologous EBV-transformed B cells respectively. Dotted horizontal lines represent the levels of killing by control CTL pre-exposed to APC without peptide. Graphs are representative examples from three independent experiments for each CTL clone.