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A real-time killing assay to follow viral epitope presentation to CD8 T cells

Pauline Gourdain, Julie Boucau, Georgio Kourjian, Nicole Lai, Ellen Duong, and Sylvie Le Gall

Ragon Institute of MGH, MIT and Harvard, Massachusetts General Hospital, Harvard Medical School, Cambridge, MA, USA

Abstract

The ability of cytotoxic T lymphocytes (CTL) to clear virus-infected cells requires the presentation of viral peptides intracellularly processed and displayed by major histocompatibility complex class I. Assays to measure CTL-mediated killing often use peptides exogenously added onto target cells –which does not account for epitope processing- or follow killing of infected cells at a single time point. In this study we established a real-time fluorogenic cytotoxic assay that measures the release of the Glucose-6-phosphate-dehydrogenase by dying target cells every 5 minutes after addition of CTL. It has comparable sensitivity to ⁵¹chromium-based killing assay with the additional advantage of incorporating the kinetics of epitope presentation. We showed that HIV infection of immortalized or primary CD4 T cells leads to asynchronous killing by two CTL clones specific for epitopes located in different proteins. Real-time monitoring of killing of virus-infected cells will enable identification of immune responses efficiently preventing virus dissemination.

Keywords

HIV; antigen processing; CD8 T cells; real-time killing assay; cytotoxicity; kinetics

1. Introduction

Cytotoxic T lymphocytes (CTL) are one of the key components of host defense against viral infection or tumor cell elimination. Virus-specific CTL responses are elicited in most viral infections, including Influenza, HIV, EBV, CMV and HCV (Bangham, 2009). HIV-specific CTL play a critical role in containing HIV viremia in acute infection or in situation of spontaneous control (Hersperger et al., 2011). However in chronic infections such as HIV and HCV the virus is not cleared despite the presence of CTL responses (Migueles and Connors, 2001; Rowland-Jones et al., 2001). The identification of criteria defining protective immune responses is paramount to the design of vaccine immunogens (Burton et al., 2012). Several parameters including peptide sequence and avidity for MHC and T cell receptor (TCR), proliferative capacity, production of cytokines and cytolytic granule release

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Corresponding author: Sylvie Le Gall, PhD., Ragon Institute of MGH, MIT and Harvard, Massachusetts General Hospital, Harvard Medical School, 400 Technology Square, Cambridge, MA 02139, USA, Tel: 001 857 268 7010, Fax: 001 857 268 7142, sylvie_legal@hms.harvard.edu.

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contribute to the antiviral capacity of the CTL responses (Almeida et al., 2007; Migueles et al., 2008; Hersperger et al., 2010; Ndhlovu et al., 2013). However one parameter often disregarded in the identification of efficacious immune responses is the timing of epitope presentation.

The killing of an infected cell by epitope-specific T cells is the culminating event of a multistep process. MHC-I epitopes displayed in the surface of cells come from the degradation of proteins in a multistep pathway involving peptidases located in the cytosol, in the endoplasmic reticulum, and for exogenous antigens peptidases from the endo-lysosomal pathway (Neefjes et al., 2011). The presentation of MHC-I-epitope is a prerequisite to the interaction between target and effector cells, activation of T cells, release of cytolytic granules content by T cells, and eventually disintegration of the target cell.

Multiple assays have been developed to assess killing of target cells or activation of effector T cells at a given time point following infection (Shacklett, 2002; Lemonnier, 2013). Radioactive isotope ^{51}Cr or tritium (^3H) killing assays are the traditional methods to assess virus-specific T cell-mediated cytotoxicity (Brunner et al., 1968; Usharauli et al., 2006). They are based on generating infected target cell populations and labeling them with ^{51}Cr or ^3H . The addition of virus-specific T cells leads to killing of target cells and release of radioactivity into the culture supernatant. Quantification of ^{51}Cr - or ^3H -release provides a measure of the specificity and the cytotoxic capacity of T cells in the effector cell population at a single time point typically measured several hours after addition of CTL.

Several non-radioactive techniques have been developed to assess the killing of target cells (Cholujova et al., 2008) or apoptosis in various culture conditions (McMillian et al., 2002). Dyes to label target cells include carboxyfluorescein succinimidyl ester (CFSE) (Wierda et al., 1989), MitoTrackerGreen (MTG), calceinacetoxymethylester (CAM), Vybrant DiO (DiO), 7-amino-actinomycin D (7-AAD) (Lecoeur et al., 2001; Sheehy et al., 2001) or caspase-3 substrates (Liu et al., 2002; He et al., 2005). They measure cell apoptosis (7-AAD; caspase-3 substrates), disintegration of dying cells after recognition by T cells (release of CFSE or MTG), the proportion of live/dead cells with two substrates such as CFSE/PKH26 (Lee-MacAry et al., 2001; Sheehy et al., 2001), or track surviving cells (CAM; Roden et al., 1999). All these dyes are intracellular markers which may leak out of the cells and can be toxic to primary cells such as dendritic cells. Extracellular substrates cleaved by enzymes released by dying cells such as resazurin cleaved by glucose 6-phosphate dehydrogenase (G6PDH) have been used to measure apoptosis and drug-induced cytotoxicity but not to follow T cell-mediated lysis of target cells (Batchelor and Zhou, 2004).

Indirect assays to assess recognition of target cells by effector T cells measure CD8 T cell activation by measuring the production of cytokines such as interferon gamma or Interleukin 2 (Miyahira et al., 1995; Maino and Picker, 1998; Mwau et al., 2002; Janetzki et al., 2005; Nomura et al., 2008), the release of cytolytic granules through exposure of CD107a (Betts et al., 2003) or cytotoxic molecules (perforin and granzymes) (Snyder et al., 2003; Snyder-Cappione et al., 2006; Hersperger et al., 2010). However these parameters do not address whether killing and clearance of infected cells occurred, a critical issue in chronic infections such as HIV and HCV where T cells can be partly functionally impaired (Hersperger et al., 2011).

Despite their advantages, all these approaches provide limited information about the timing of presentation of an epitope to its cognate CTL, a factor critical to ensure efficient clearance of infected cells –that is independent of the antiviral function of T cells-. Here we present a new non-radioactive cytotoxic assay with a low toxicity and a low cell number

requirement that allows us to sensitively measure the killing of target cells in real-time after addition of CTL, and to compare the kinetics of presentation of endogenously processed HIV epitopes to HIV-specific CTL.

2. Material and Methods

2.1 Study participants

HIV-negative and HIV-infected donors were recruited at Massachusetts General Hospital (MGH) in Boston. Partners Human Research Committee (Boston, MA) approved the use of anonymous buffy coats under protocol 2005P001218, the use of samples from HIV-negative coded donors under protocol 2010P002121 and the use of coded HIV-infected samples under protocols 2010P002463 and 2003P001894. All participants provided written informed consent for participation in the study.

2.2 Peptides

Highly purified peptides (>98% pure) were purchased from MGH peptide core facility.

2.3 Cell culture

EBV-immortalized B cells were maintained in RPMI 10% FCS. KF11-, TW10-, ATK9- and RK9-specific CTL clones were isolated by limiting dilution and maintained in the presence of 50U/ml IL-2 (R10-IL2) using the CD3-specific mAb 12F6 and irradiated PBMC as stimulus for T cell proliferation (Le Gall et al., 2007).

2.4 Cell sorting

CD4 T cells were enriched from freshly isolated peripheral blood mononuclear cells (PBMCs) by magnetic immunodepletion of cells expressing CD8, CD14, CD16, CD19, CD20, CD36, CD56, CD66b, CD123, TCR α / β , glycophorin A and dextran-coated magnetic particles, according to the manufacturer's instructions (StemCell). The percentage of CD4 T cells assessed by flow cytometry was >90%. PHA-stimulated CD4 T cells were obtained by incubating CD4 T cells at 1×10^6 /ml in R10-IL2 with 0.25 μ g/ml PHA for 4–6 days.

2.5 HIV infection

VSV-G–pseudotyped viral stocks were prepared by cotransfection of 293T cells with NL4-3 proviral DNA (or with a GFP-encoding provirus) along with a CMV-VSV-G plasmid and titrated as previously described (Miura et al., 2009). B cells or PHA-activated CD4 T cells were harvested and incubated at 2×10^6 /well in R10 in 24-well plates. VSV-G–pseudotyped virus was added at a concentration of 100ng p24 per well for 5 hours. Cells were washed twice and plated again. Every other day until day 10, cells were monitored for infection rate and used as targets in real-time or ^{51}Cr killing assays.

2.6 Flow cytometry

Cells were washed and incubated with CD4-PE and HLA-DR-APC antibody (pre-titrated volume, BD Pharmingen). Cells were then permeabilized with the Cytofix/Cytoperm Plus kit (BD Biosciences) for 20 min following manufacturer's instructions and stained for intracellular p24 with p24-FITC antibody (pre-titrated volume, Santa Cruz Biotech) for 30 min. Cells were fixed and acquired on a two-laser Calibur flow cytometer using CellQuestPro software (BD Biosciences) and data were analyzed using FlowJo software (TreeStar).

2.7 Real-time fluorogenic killing assay

Target cells (B cells or PHA-activated CD4 T cells) were harvested, suspended in fresh medium (Complete RPMI without serum, R⁺) and plated at 20×10^3 cells/75 μ l per well in dark 96-well plates. Peptides were added in 75 μ l of RPMI without serum (R⁺) at different concentrations (0.4 μ g/ml; 0.04 μ g/ml; 0.004 μ g/ml). Plates were incubated at 37°C, 5% CO₂ for 20 min. Plates were washed once with 150 μ l of RPMI 10% serum (R10) per well and spun 30 sec, 2500 rpm. 225 μ l of media was aspirated at the lowest speed with an automatic multichannel pipette. Plates were washed for the second time with 225 μ l of R10 per well and spun 30 sec at 2500 rpm. 225 μ l of media was aspirated at the lowest speed with an automatic multichannel pipette. A third wash was performed before CTL were added at 40×10^3 cells/75 μ l per well in R10 serum. 50 μ l of resazurin/reaction mixture was added to each well (Vibrant Cytotoxicity Assay Kit Molecular Probes; (Batchelor and Zhou, 2004)). For a positive control, 5 μ l of 100X Triton cell lysis buffer was added. Wells were mixed with the automatic multichannel pipette and fluorescence emission (Ex: 530–560/ Em: 580–600) was recorded every 5 min for 4h on a Victor3 plate reader (PerkinElmer). Spontaneous fluorescence is defined as fluorescence emission by uninfected B cells and CTL without exogenously added peptide. Maximum fluorescence is defined as fluorescence emission by B cells mixed with CTL and cell lysis buffer. Specific fluorescence was calculated as [(fluorescence in the presence of target cells and CTL – spontaneous fluorescence)/(maximum fluorescence – spontaneous fluorescence)] x 100. Target cells with or without peptides were incubated with substrate in order to check the basal level of G6PDH released by targets (fluorescence levels below to spontaneous release).

2.8 ⁵¹Cr-based killing Assay

⁵¹Cr-labeled B cells were pulsed with different peptide doses (0.4 μ g/ml, 0.04 μ g/ml or 0.004 μ g/ml of the optimal peptide and 0.4 μ g/ml of the mutated one) diluted in R⁺ for 30 minutes at 37°C. KF11-specific, TW10-specific, RK9-specific, or ATK9-specific CTL clones were added at a 4:1 ratio and incubated for 4 hours with target cells. Radioactivity was measured in 30 μ l of culture supernatant. Cell lysis was calculated as [(⁵¹Cr release due to peptide – spontaneous release)/(total release – spontaneous release)] x 100 as in (Le Gall et al., 2007).

2.9 Statistical analysis

Maximum slope and area under curve (AUC) were calculated with Microsoft Excel. Data were analyzed using GraphPad Prism software (version 5).

3. Results

3.1 A fluorogenic assay to measure real-time CTL-mediated killing of target cells

We aimed to design a killing assay that allows us to follow the lysis of target cells after addition of CTL in real-time. We adapted a fluorogenic assay measuring the activity of glucose 6-phosphate dehydrogenase (G6PDH) during apoptosis and drug-induced cytotoxicity (Batchelor and Zhou, 2004), an enzyme released by dying cells (Zhang et al., 2000). The membrane-impermeable substrate is added in the culture medium -which avoids intracellular toxicity and leakage observed with intracellular substrates. The release of G6PDH by dying cells leads to the cleavage of a G6PDH-specific fluorogenic substrate and fluorescence emission (figure 1A). To measure CTL-mediated cell killing HLA-B57⁺ B cells were pulsed with variable amounts of cognate peptide (HLA-B57-restricted KF11, KAFSPEVIPMF, aa 30–40 in Gag p24) or with a mutated KF11 peptide (KF11-K89 KAFSPEVIKKF) of low functional avidity (i.e low binding affinity for HLA-B57 and/or TCR of CD8 T cells) (Lazaro et al., 2011). Spontaneous release of G6PDH was measured with B cells and CTL without peptides (or with irrelevant peptide) and maximum release

was measured with B cells and CTL mixed with Triton lysis buffer. Fluorescence emission was measured every 5 minutes from the moment CTL (or detergent) were mixed with target cells. Fluorescence emission increased over time and with the amount of KF11 peptide used to pulse target cells (figure 1B). At each reading time point we calculated a specific fluorescence –equivalent to a specific lysis %– as [(fluorescence in the presence of peptide-pulsed cells–spontaneous fluorescence)/ (maximum fluorescence –spontaneous fluorescence)]x100 (figure 1C). In the presence of KF11-pulsed B cells lysis was detectable as early as 1 hour after addition of CTL and increased over time. The lysis % was proportional to the amount of KF11 peptide used to pulse cells. In the presence of a mutated peptide KF11-K89 lysis remained low (figure 1C). To validate the assay we performed in parallel a fluorogenic and a ^{51}Cr -based killing assay on HLA-A11⁺ B cells pulsed with increasing amounts of HLA-A11-restricted B cell line and HLA-A11-restricted ATK9 peptide (AIFQSSMTK, aa 158–166 in reverse transcriptase of HIV-1 polymerase (Walker et al., 1989)). The specific lysis % measured at 4 hours after addition of the CTL similarly increased with the amount of peptides in both assays (figure 1D). We expanded the comparison of the two killing assays to two target cell lines, 2 CTL clones and 6 different peptide concentrations ranging from 0 to 0.2ug/ml: HLA-B57⁺ B cells pulsed with KF11 or HLA-A3⁺A11⁺ B cells pulsed with or A11-ATK9 peptide. Lysis % measured in parallel by real-time fluorogenic or ^{51}Cr -based assays for each peptide-clone combination were compared 4 hours after addition of CTL clones. We found a significant correlation between the lysis % obtained with the two different assays ($p < 0.0001$ $r = 0.55$ Spearman test; figure 1E), indicating that target cell lysis as measured by real-time killing assay accurately corresponded to the one measured by classical ^{51}Cr release assay with the advantage of following target cell lysis in real-time.

3.2 Real-time detection of epitopes presented by HIV-infected cells to virus-specific CTL

We aimed to assess whether the assay is sensitive enough to measure the killing of HIV-infected cells, where viral antigens are endogenously processed and presented at the cell surface rather than exogenously pulsed onto cells (figure 2). HLA-B57⁺ B cells were incubated for 5 h with VSV-G-pseudotyped HIV and then extensively washed. Cells were collected at 10 h, 24 h, and 48 h after infection and used as targets in a real-time killing assay with CTL specific for two HLA-B57-restricted p24 epitopes KF11 and TW10 (epitope TSTLQEQIGW, aa 108–117 in Gag p24) (Llano A, 2009). As shown in figure 2A, the percentage of target cell lysis increased with time starting at 10% after 10h post infection until 50–60% after 48h. These results indicate that the presence of KF11 and TW10 epitopes displayed with MHC-I at the surface of B cell increased during the first 48 h of infection. As a positive control, target cells loaded with 0.4μg/mL of KF11 or TW10 peptide were evenly lysed at 60–70% at each time point. By comparing the lysis of HIV-infected B cells with that of B cells pulsed with various amounts of optimal epitopes KF11 or TW10 as in (Le Gall et al., 2007), we estimated that the antigenic peptide equivalent displayed at the surface of B cells between 10 and 48 h after infection increased from 0.16 to 27nM for KF11, and 3.4 and from 86nM for TW10. We compared the killing of target B cells at 10 to 48 h post-infection with the corresponding percentage of infected B cells determined by intracellular p24 staining. Infection was measured by flow cytometry after intracellular staining of B cells with a p24 antibody and ranged in average between 3 and 43% depending on the time point. For both KF11- and TW10-specific CTL clones (figure 2B), we found a strong association between lysis % and % of p24-positive cells ($p < 0.0001$ $r = 0.857$, $p < 0.0001$ $r = 0.901$ Spearman test, respectively fig 2B), indicating that target cell lysis as measured by real-time killing assay reflected the percentage of HIV-infected cells processing and presenting HIV epitopes. In addition, HLA-B57⁺ or HLA-A03⁺11⁺ B cell lines were infected with a HIV-derived lentiviral vector expressing GFP and pseudotyped with VSV-G. Infection rates were measured by GFP expression and ranged between 6 and 62%. HLA-

B57⁺ B cells were used as targets with KF11- and TW10-specific CTL, HLA-A03⁺A11⁺ B cell lines were used as targets with p17 A03-RK9 (RLRPGGKKK, aa 20–28 in p17 Gag (Harrer et al., 1996; Yu et al., 2002)) and A11-ATK9 specific CTL. The killing of two different target cell lines by four different clones correlates with the % of GFP-expressing cells ($R=0.6716$; $p=0.0003$), indicating that the real-time CTL killing assay allows the detection of a wide range of infected cells presenting various amounts of HIV epitopes.

3.3 Monitoring differential epitope presentation by HIV-infected cells

We then assessed whether the real-time killing assay was adequate to compare the kinetics of presentation of several HIV epitopes during HIV infection (figure 3). In order to specifically compare epitope presentation and killing by different CTL clones without confounding factors we chose epitopes for which the functional avidity of the epitopes for MHC-I and the TCR of CTL was equivalent. Building on previous measurements done by ⁵¹Cr-release assay (Le Gall et al., 2007; Zhang et al., 2012) we compared the fluorescence-based killing of B cells expressing HLA-B57 and/or HLA-A11 pulsed with increasing amounts of peptides 4 hours after addition of CTL (figure 3A). Similar lysis % of target cells by the two clones were observed at various peptide concentrations, showing similar functional avidity of the two clones. We then compared the endogenous presentation of HLA-B57-restricted KF11 epitope located in Gag p24 and a HLA-A03/11-restricted ATK9 epitope from reverse transcriptase, a protein expressed later in the viral cycle. HLA-B57⁺A11⁺ B cells were infected with VSV-G-pseudotyped HIV-1. Infection rate with this VSV-G non-replicative virus ranged between 10–75% between days 1 to 4 and diminished after 4 days as non-infected cells grew faster. Lysis of target cells 3 days post-infection in one representative experiment was monitored over 4 hours after addition of KF11- and ATK9-specific CTL (figure 3B). It showed that killing of infected cells by KF11-specific CTL started earlier after addition of CTL and reached higher specific lysis compared to ATK9 (32% vs 9%). Considering that the 2 epitopes have similar functional avidity this difference in lysis is likely due to differences in the amount of KF11 and ATK9 peptides displayed by HIV-infected cells. Target cell lysis at 4 hours after CTL addition was monitored at day 2, 3, 4, 5, 7 and 10 post-infection (figure 3C). The killing of HIV-infected B cells by KF11-specific CTL occurred as early as day 2 whereas killing by ATK9-specific CTL began at day 3. Alternatively HLA-B57⁺A11⁺ primary CD4 T cells stimulated with PHA were infected with replicative HIV-1 NL4-3. Killing of CD4 T cells by epitope-specific CTL was monitored during 4 hours at day 2, 3, 4, 5, 7 and 10 post-infection. Similarly to the kinetics of killing of HIV-infected B cells, the killing of HIV-infected CD4 T cells by ATK9-specific CTL was delayed (day 4) compared to killing by KF11-specific CTL (day 2). These results suggest that delayed and lower expression of Pol compared to Gag and/or less efficient processing of this RT epitope leads to a lesser epitope presentation and delayed killing of HIV-infected CD4 T cells by the ATK9-specific CTL. In conclusion, the real-time killing assay allows us to indirectly evaluate the amount of viral epitope presented by infected cells and to identify viral epitopes detectable by CTL early in the course of infection.

4. Discussion

CD8 T cells play a major role in the clearance of many viral infections and constitute a critical arm of immune responses elicited by vaccines. It is therefore important to develop assays identifying immune responses able to efficiently kill virus-infected cells. The efficiency of killing of infected cells is defined not only by the intrinsic capacity of T cells to secrete cytokines and granzymes, but also by their capacity to rapidly recognize their targets. CTL-mediated killing of infected cells requires the degradation of a pathogen protein into epitopes, loading onto MHC-I and display of the epitope by MHC-I at the cell surface in sufficient amount to trigger activation of CTL. This sequence of events is dynamic, yet

current killing assays do not permit multiple measurements after infection as they typically monitor a single time point.

Here we developed a fluorogenic killing assay to monitor in real-time the killing of HIV-infected cells by CTL. The assay makes use of an extracellular G6PDH-specific substrate – that limits toxicity and leakage and allows us to follow target cell lysis every 5 minutes or as frequently as needed after addition of CTL. It allowed parallel detection of the killing of immortalized or primary target cells by several epitope-specific T cell clones. This assay gives similar lysis % to classical ^{51}Cr -release assay, uses fewer cells, is throughput and sensitive enough to detect cells pulsed with low concentration peptides, as well as HIV-infected cells displaying peptides endogenously processed during infection. Although HIV infection was the focus of this study, this assay could be used with any primary cells infected with any virus or viral vector and to measure the killing mediated by various immune cells with cytolytic capacities such as CD8 T cells, cytolytic CD4 T cells or NK cells.

A major application of this assay is to be able to monitor epitope presentation after viral infection. We can compare the presentation of epitopes from viral proteins expressed at various stages of the viral life cycle or compare the presentation of epitopes within a given protein. We showed in this study that the killing of HIV-infected B cell lines as well as the killing of HIV-infected primary CD4 T cells by CTL recognizing a Pol epitope ATK9 is delayed compared to that triggered by a Gag-specific CTL (KF11) despite equivalent functional avidity of the two clones. This delay in recognition of infected cells could be due to the 20-fold higher amount of Gag compared to Pol in HIV virions. Pol is synthesized as part of a Gag-Pol polyprotein by a ribosome frameshift near the 3' end of *gag* and requires further cleavage of Gag-Pol by HIV protease. This process results in lower and later production of Pol compared to Gag (Jacks et al., 1988; Louis et al., 1994) and may lead to a lower and delayed presentation of the Pol epitope to epitope-specific CTL. Another difference in Gag and Pol epitope presentation could stem from differences in the efficiency and timing of epitope production. We previously showed that the endogenous expression of Gag p17 protein with a C-terminal tag containing a fragment of Pol including ATK9 leads to 4-fold less killing (measured by ^{51}Cr release assay) by Pol ATK9-specific CTL than by the Gag-specific RK9 CTL, demonstrating that even a synchronized expression of Gag-Pol did not erase the difference in CTL killing efficiency (Le Gall et al., 2007). In support of this difference in epitope presentation we demonstrated that the cytosolic degradation of Gag p17 RK9-containing peptides lead to earlier and higher production of epitope RK9 than that of Pol ATK9-containing peptide (Le Gall et al., 2007). We showed that differences in HIV epitope production and presentation are driven by motifs within (Lazaro et al., 2011) and outside (Draenert et al., 2004; Zhang et al., 2012) of the viral epitope and will affect recognition by CTL ((Lazaro et al., 2011) and additional unpublished data). Altogether these results suggest that differences in the timing and amount of epitopes produced inside infected cells and the timing of their display at the cell surface is defined by the efficiency of epitope processing as much as by the kinetics and level of expression of viral proteins during the virus life cycle.

5. Conclusion

We developed a throughput real-time CTL killing assay that integrates kinetics of viral life cycle, efficiency of degradation of viral proteins into epitopes and timing of presentation of viral epitopes to monitor killing of virus-infected cells by various virus-specific CD8 T cells (and potentially by any other immune cells with cytolytic function). This assay, adaptable to any virus infection or target cells, will allow to easily identify immune responses able to

recognize infected cells early and efficiently –which is integral to preventing viral spread and therefore of high interest for vaccine design.

Acknowledgments

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Abbreviations

CFSE	carboxyfluorescein succinimidyl ester
MTG	MitoTrackerGreen
CAM	calceinacetoxymethylester
7-AAD	7-amino-actinomycin D
G6PDH	glucose 6-phosphate dehydrogenase

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Figure 1. A fluorogenic assay to measure real-time CTL-mediated cell killing

A. Principle of the coupled enzymatic assay for detection of glucose 6-phosphate dehydrogenase activity. Oxidation of glucose 6-phosphate by glucose 6-phosphate dehydrogenase results in the generation of NADPH, which in turn leads to the reduction of resazurin by diaphorase to yield fluorescent resorufin (Batchelor and Zhou, 2004). B. HLA-B57⁺ B cells were incubated with increasing amounts of B57-restricted KF11 optimal peptide (0.1ug/ml black squares; 0.04ug/ml black triangles; 0.01ug/ml black circles; 0.004ug/ml black diamonds) or a mutated version of KF11 KF11-K89 (open circles) poorly recognized by KF11-specific CTL clones. Spontaneous cleavage of substrate (Spont; X) was calculated at each time point as the fluorescence released by B cell + KF11-specific CTL point without peptide. Maximum (Max; +) is calculated as the fluorescence released by B cell in the presence of KF11-specific CTL and Triton 0.5%. Fluorescence emission was measured every 5 minutes for 4 hours from the time the CTL were added to target cells. Autofluorescence of substrate in the presence of PBS is subtracted at each time point. C. Specific fluorescence in the presence of increasing amount of KF11 or KF11-89 is measured at each point as $[(\text{Fluorescence} - \text{Spont}) / (\text{Max} - \text{Spont})] \times 100$. D. ⁵¹Cr-labeled or unlabeled HLA-B57⁺ B cells were incubated with increasing amounts of KF11 peptide and used as targets in a ⁵¹Cr-based or fluorogenic killing assay with KF11-specific CTL (E:T 4:1). Lysis percentage of target cells by Cr-release assay (black squares) or fluorescence assay (black circles) was measured after a 4-hour incubation. E. The comparison of killing of target cells pulsed with increasing amounts of peptides was expanded to 4 different CTL clones (A11-ATK9, A03-RK9, B57-KF11, B57-TW10). Correlation calculated with Spearman test.

Figure 2. Detection of HIV-infected cells by real-time killing assay

A. HLA-B57⁺ B cells were infected with VSV-G-pseudotyped HIV. Lysis of HIV-infected (white bars) or peptide-pulsed (grey bars) target cells by HLA-B57-restricted Gag KF11-specific (left) or TW10-specific (right) CTL was monitored at 10, 24 or 48 hours post-infection. Lysis % corresponds to a 4-hour time point after addition of CTL. B. Correlation between target cell lysis by KF11- (black circles) or TW10-specific (black triangles) CTL clones and % infected cells of measured by p24-specific intracellular staining. C. HLA-B57 or HLA-A03/11 B cells were infected with a VSV-G pseudotyped lentiviral vector expressing GFP. Correlation between HLA-B57 or HLA-A03/11 target cells lysis by HLA-B57-restricted ISW9- (black inverted triangles), KF11- or TW10-specific CTL clones or by A3-RK9- (black squares) or A11-ATK9-specific (black diamonds) respectively and % of infected cells monitored by GFP staining. Correlation by Spearman test.

Figure 3. Real-time epitope presentation by HIV-infected cells to CTL

A. HLA-A11⁺B57⁺ B cells were pulsed with increasing amounts of B57-restricted KF11 or A11-restricted ATK9 and used as targets in a fluorescence killing assay with KF11-specific (open circles) or ATK9-specific (black squares) CTL. Average of 3 experiments. B. HLA-A11⁺B57⁺ B cells were infected with VSV-G pseudotyped HIV. At 3 days post-infection cells were used as targets with KF11- (black circles) or ATK9-specific CTL (black triangles). Specific fluorescence was measured every 5 minutes for 4 hours after addition of epitope-specific CTL. C. Lysis of infected B cells by KF11-specific (dark grey) or ATK9-specific (light grey) CTL was measured 2 to 10 days post-infection. Average of 4 experiments. D. HLA-A11⁺B57⁺ PHA-activated primary CD4 T cells were infected with replicative HIV-1 NL4-3 and lysis of infected cells by KF11-specific (dark grey) or ATK9-specific (light grey) was measured 2 to 10 days post-infection. Lysis % in panels C and D correspond to the 4-hour time point after addition of CTL.