Video Article

Cell-based Flow Cytometry Assay to Measure Cytotoxic Activity

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Abstract

Cytolytic activity of CD8+ T cells is rarely evaluated. We describe here a new cell-based assay to measure the capacity of antigen-specific CD8+ T cells to kill CD4+ T cells loaded with their cognate peptide. Target CD4+ T cells are divided into two populations, labeled with two different concentrations of CFSE. One population is pulsed with the peptide of interest (CFSE-low) while the other remains un-pulsed (CFSE-high). Pulsed and un-pulsed CD4+ T cells are mixed at an equal ratio and incubated with an increasing number of purified CD8+ T cells. The specific killing of autologous target CD4+ T cells is analyzed by flow cytometry after coculture with CD8+ T cells containing the antigen-specific effector CD8+ T cells detected by peptide/MHCI tetramer staining. The specific lysis of target CD4+ T cells measured at different effector versus target ratios, allows for the calculation of lytic units, $LU_{30}/10^6$ cells. This simple and straightforward assay allows for the accurate measurement of the intrinsic capacity of CD8+ T cells to kill target CD4+ T cells.

Video Link

The video component of this article can be found at http://www.jove.com/video/51105/

Introduction

Cytolytic activity is the major function of CD8+ T cells but is still rarely measured as assays used for this purpose are cumbersome and have been difficult to standardize. Accurate measurement of this function is of paramount importance when characterizing effector functions of CD8+ T cells, as no reliable predictors of effective cell-mediated cytotoxicity have been described yet[1,2]. Here, we propose a new functional assay to measure the cytotoxic activity of antigen-specific CD8+ T cells on target CD4+ T cells. Several approaches have been developed to provide alternatives to the gold standard, chromium release assay. We present here a cell-based assay that reveals the entire process of killing as it measures the death of live target cells. This method was derived from protocols of flow cytometry-based cytotoxic assays *in vivo* in mice[4,5] and *in vitro* in humans[6]. In this protocol, the antigen-specific CD8+ T cells contained in the total CD8+ T cell population are used as effector cells and autologous CD4+ T cells are used as target cells. Effector CD8+ T cells of interest are enumerated using MHCI/peptide tetramers[7]. Death of target cells is calculated by the ratio between peptide loaded/nonloaded CD4+ T cells. We have previously shown that this method was reproducible, sensitive, specific and did not depend on the number of effector cells within the total CD8+ T cell population[8]. By enumerating both the number of effector and target cells in the coculture assay, the intrinsic capacity of CD8+ T cells to kill target cells can be calculated and expressed in lytic units[9].

Protocol

1. Preparation of Effector CD8+ T Cells

1. Thaw autologous cryopreserved PBMCs (2-3 vials of 50 x 10^6 cells) by transferring the cryovial from liquid nitrogen to a 37 °C water bath.
2. Wash the cells by filling the tube to 50 ml with complete RPMI (4 mM L-glutamine, and 100 U/ml penicillin and streptomycin, supplemented with 10% FBS). Count PBMCs and resuspend cells at a concentration of 5 x 10^6/ml in complete RPMI.
3. Add specific peptide (5 µg/ml) and IL-2 (10 ng/ml) to PBMCs.
4. Set-up culture in 96 deep well plate; seed 1 ml of cell suspension to each well.
5. After 3 days of culture, replace half of the cell culture medium with fresh complete RPMI.
6. After 6 days of culture, collect all PBMCs with multichannel pipette and transfer cells in sterile reservoir.
7. Count, wash, and resuspend PBMCs at 5 x 10^7/ml in the recommended separation buffer in 14 ml round bottom tubes. Add human CD8+ T cell enrichment cocktail at 50 µl/ml cells. Mix and incubate at room temperature for 10 min. Add magnetic particles at 150 µl/ml and incubate for 5 min.
8. Bring the cell suspension up to 7 ml by adding the separation buffer. Proceed to immunomagnetic isolation of untouched CD8+ T cell by placing the tube into the magnet. After 5 min, with the tube still in the magnet, pour the cells of interest into a new 15 ml conical tube. Take a small aliquot and stain the cells with antibodies against CD3 and CD8 in 1X PBS-2% FBS for 30 min at 4 °C. The purity of the CD8+ T cells can then be measured via flow cytometry with an expected purity of 95% or higher.

9. Resuspend CD8+ T cells in 450 µl with complete RPMI.
10. Add 225 µl of complete RPMI into 5 screw cap tubes.
11. Prepare serial dilutions (from 1:2 to 1:32) by transferring 225 µl of CD8+ to the next tube (*see* **Figure 1** *upper panel*).

2. Preparation of Target CD4+ T Cells

1. Thaw autologous cryopreserved PBMCs (1 vial of 50 x 10^6 cells) by transferring the cryovial from liquid nitrogen to a 37 °C water bath.
2. Transfer the cell suspension to a 50 ml conical tube containing 10 ml of RPMI.
3. Wash cells and resuspend PBMCs at 5 x 10^7/ml with separation buffer in 14 ml round bottom tubes. Add human CD4+ T cell enrichment cocktail at 50 µl/ml cells. Mix and incubate at room temperature for 10 min. Add magnetic particles at 100 µl/ml and incubate for 5 min at room temperature.
4. Bring the cell suspension up to 7 ml by adding separation buffer. Proceed to the isolation of CD4+ T cells by placing the tube into the magnet. After 5 min, with the tube still within the magnet, pour the desired cells into a new 15 ml conical tube. Take a small aliquot and stain the cells with antibodies against CD3 and CD4 in 1x PBS-2% FBS for 30 min at 4 °C. The purity of the CD4+ T cells can then be measured via flow cytometry with an expected purity of 95% or higher.
5. Count CD4+ T cells, split them into two 15 ml conical tubes and wash in warm 1x PBS.
6. Prepare a 0.4 µM CFSE-high working solution in warm PBS.
7. Prepare a 0.04 µM CFSE-low working solution by diluting a portion of the 10x CFSE high working solution in warm PBS.
8. Resuspend cells at 20 x 10^6/ml of PBS (if the number of CD4+ T cells is less than 10 x 10^6/ml resuspend cells in 0.5 ml of PBS) and stain half of the CD4+ T cells by adding one equal volume of 0.4 µM CFSE-high working solution (final concentration 0.2 µM). For example, for 1 ml of cells, add 1 ml of the CFSE working solution. Stain the remaining half of the CD4+ by adding one equal volume of 0.04 µM CFSE-low working solution (final concentration 0.02 µM). Stain cells for 15 min at 37 °C in 5% CO_2 atmosphere.
9. After the incubation, pellet cells at 1,500 rpm for 5 min and resuspend in 1 ml of warm complete RPMI to quench the labeling reaction.
10. Pulse CFSE-low CD4+ T cells by adding the peptide of interest at a final concentration of 5 µg/ml in the complete RPMI and incubate for 45 min in 5% CO_2 atmosphere at 37 °C.
11. Wash CFSE-high and CFSE-low CD4+ T cells in complete RPMI. Repeat for a total of two washes. Resuspend at a concentration of 2 x 10^5 cells/ml each in complete RPMI.
12. Mix the two target populations at a ratio of 1:1 (CFSE-High:CFSE-Low) (*see* **Figure 1** *lower panel*).

3. Coculture

1. From each dilution of CD8+ T cells, seed 100 µl in duplicate to round-bottom 96-well plate.
2. Add the mixed target CD4+ T cells (100 µl) to each dilution of effector cells for a final volume of 200 µl.
3. To measure the basal apoptosis, seed 3 wells with targets cells alone.
4. Incubate coculture for 6 hr in 5% CO_2 atmosphere at 37 °C.

4. Flow Cytometry Staining and Acquisition

1. Transfer cells to a V-bottom 96-well plate and stain with PE-conjugated pMHC tetramers for 15 min in 5% CO_2 atmosphere at 37 °C.
2. Wash in 1x PBS-2% FBS and stain cells with Far Red LIVE/DEAD fixable dead cell, αCD3-Alexa700, αCD8-Percp, αCD4-Brilliant Violet 650 for 30 min at 4 °C, protected from light.
3. Wash cells once with 1x PBS-2% FBS and resuspend in 100 µl with 2% formaldehyde in 1x PBS.
4. Acquire cells on flow cytometer.

Representative Results

The schematic in **Figure 1** summarizes the assay. Purified CD8+ T cells (effector cells) were resuspended in 450 µl of complete RPMI and serial dilutions were performed by adding 225 µl of CD8+ T cells to the next tube containing 225 µl of media (upper panel). Purified CD4+ T cells (target cells) were counted, split into two tubes and stained with two different concentrations of CFSE (high and low) as described in the protocol. CFSE-low CD4+ T cells were pulsed with the peptide of interest and CFSE-high CD4+ T cells remained unloaded as a control. CFSE-high and CSFE-low CD4+ T cells were resuspended at 2 x 10^5/ml and mixed at a ratio of 1:1 (lower panel). The mixed CD4+ T cells were seeded at 100 µl in each well. Three wells containing the mixed CD4+ T cells were used for triplicate of CD4+ T cells alone (wells in red). 100 µl of each CD8+ T cell dilution was added in duplicate to the remaining wells containing the mixed CD4+ T cells (wells in green). The killing of target cells was assessed by flow cytometry after 6 hr of coculture.

After incubation, cells were analyzed and gated as in **Figure 2**. The ratio of viable CFSE-high versus CFSE-low target cells, un-pulsed or peptide pulsed was analyzed from a small FSC-A/SSC-A gate. Live cells were gated on LIVE/DEAD negative. CD4+ T cells were plotted on CFSE in order to analyze the ratio CFSE-low versus CFSE-high cells. A larger FSC-A/SSC-A gate was then created to enumerate the total number of effector and target cells after 6 hr incubation, either dead or alive. Tetramer+ CD8+ T cells and CFSE-low CD4+ T cells were plotted in order to analyze the ratio effector versus target cells. The total number of effector CD8+ T cells was determined by gating on CD8+ and tetramer+ cells. The total number of target cells was determined by gating on CD4+ T cells CFSE-low and LIVE/DEAD positive and negative cells.

The results presented in **Figure 3** show that the ratio of CFSE-low versus CFSE-high cells (target ratio) was 1:1 when CD4+ target cells were cultured in absence of effector cells. However, the ratio of CFSE-low versus CFSE-high cells was drastically decreased when CFSE-low cells pulsed with peptide were incubated with the total CD8+ T cell population containing antigen-specific CD8+ effector T cells. As the ratio decreased, the CFSE-low CD4+ pulsed target cells became LIVE/DEAD positive. The dilution of the effector cells (right panel) resulted in restoration of the CFSE ratio and decreased the number of LIVE/DEAD positive peptide-pulsed CFSE-low CD4+ target T cells.

For each well, the ratio between pulsed and un-pulsed live CD4+ target T cells (CFSE-low/CFSE-high) in the presence of different dilutions of effector CD8+ T cells was measured by calculating the ratio between antigen-pulsed CFSE-low CD4+ T cells and un-pulsed CFSE-high CD4+ T cells after 6 hr of culture with effector CD8+ T cells. The percentage of specific lysis for each well was calculated as follows: % specific lysis = 100 - ((CFSE low/CFSE high) in the presence of effector cells / mean of the three wells containing target cells alone in the absence of effector cells) x100. To determine the intrinsic cytolytic capacity of antigen-specific CD8+ T cells and compare results between donors, the cytolytic activity was expressed in lytic units as previously reported[8]. The effector versus target ratio (E/T) was measured by calculating the ratio between total tetramer+ CD8+ T cells and total CFSE-low CD4+ T cells for each well. The percentage of specific lysis was plotted in function of the E/T ratio in a log scale as illustrated in **Figure 4**. A linear regression was calculated from the plot and the equation of the trendline was used to calculate the lytic units $LU_{30}/10^6$ cells (*i.e.* the number of CD8+ effectors T cells required to kill 30% of 10^6 target CD4+ T cells).

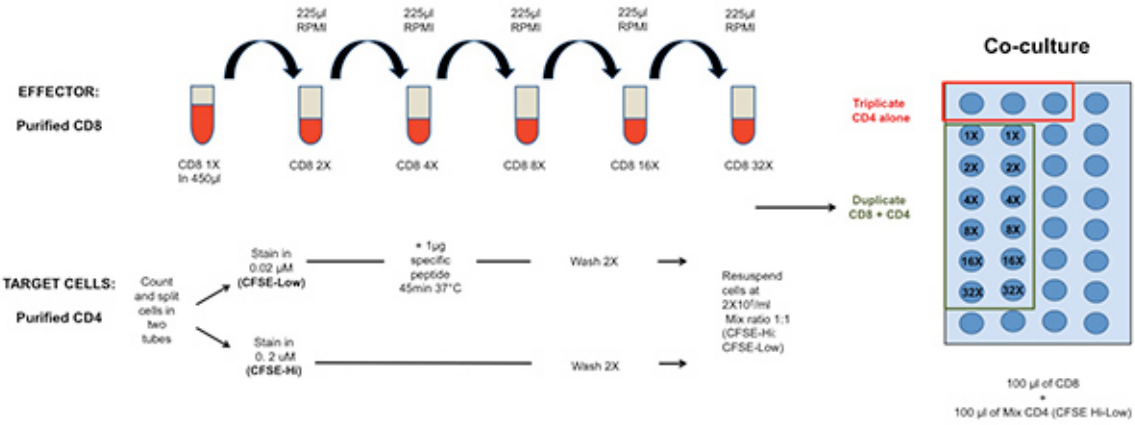

Figure 1. Schematic representation of the cytotoxic assay coculture. Purified CD8+ T cells were resuspended in 450 µl of complete RPMI and 5 serial dilutions were performed. Purified CD4+ T cells were counted, split into two tubes and stained with two concentrations of CFSE (0.02 µM CFSE-low and 0.2 µM CFSE-high). Only CFSE low CD4+ T cells were pulsed with 5 µg of specific peptide. After 45 min, both CFSE low and high CD4+ T cells were washed 2x, resuspended at 2 x 10^5/ml and mixed at a ratio of 1:1. 100 µl of mixed CD4+ were seeded in a 96-well plate in the presence or absence of 100 µl of each CD8+ T cell dilution for 6 hr. Click here to view larger image.

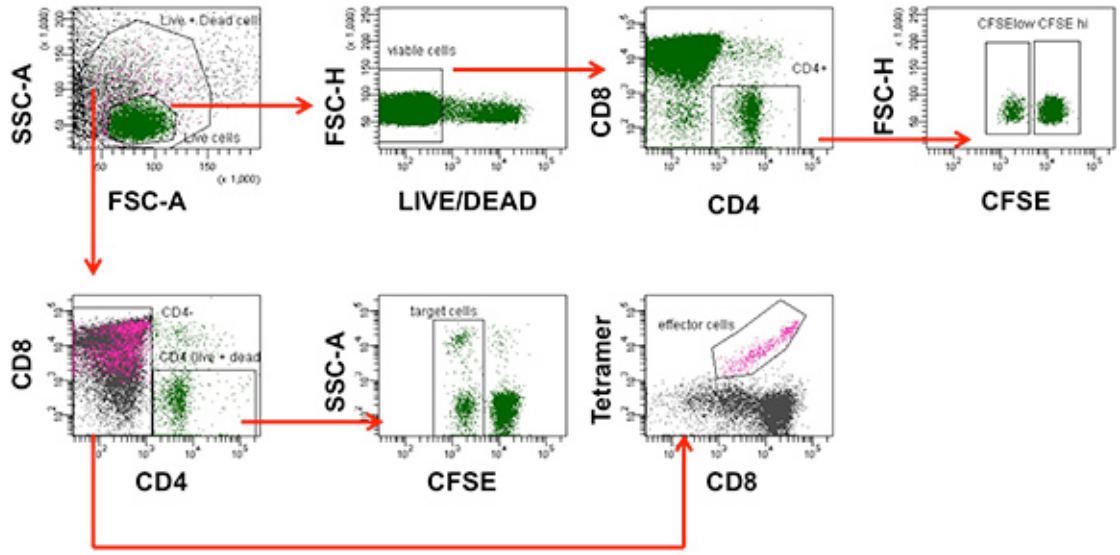

Figure 2. Gating strategy of the cytotoxic assay. From a smaller FSC-A/SSC-A gate, the ratio of CFSE-low versus CFSE-high cells was analyzed within the CD4+ LIVE/DEAD negative cells. From a larger FSC-A/SSC-A gate, effector cells were analyzed by gating on CD8+ T cells and tetramer+ cells. The number of target cells was analyzed by gating on CD4+ T cells, CFSE-low and LIVE/DEAD positive and negative cells. Click here to view larger image.

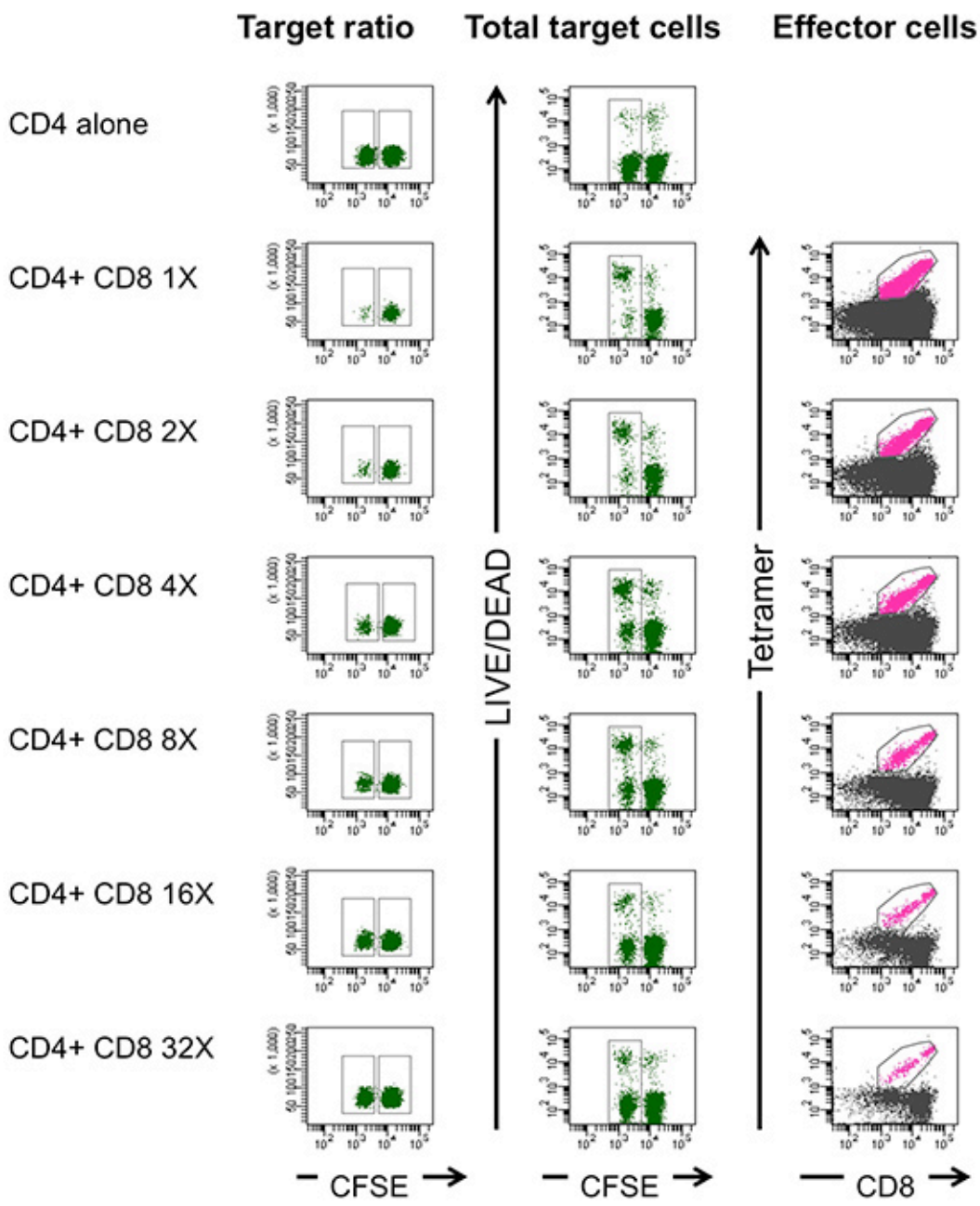

Figure 3. Representative results of FACS staining. CFSE-low/CFSE-high ratio (left panels), target cells (middle panels) and effector cells (right panels) in the different coculture conditions. Click here to view larger image.

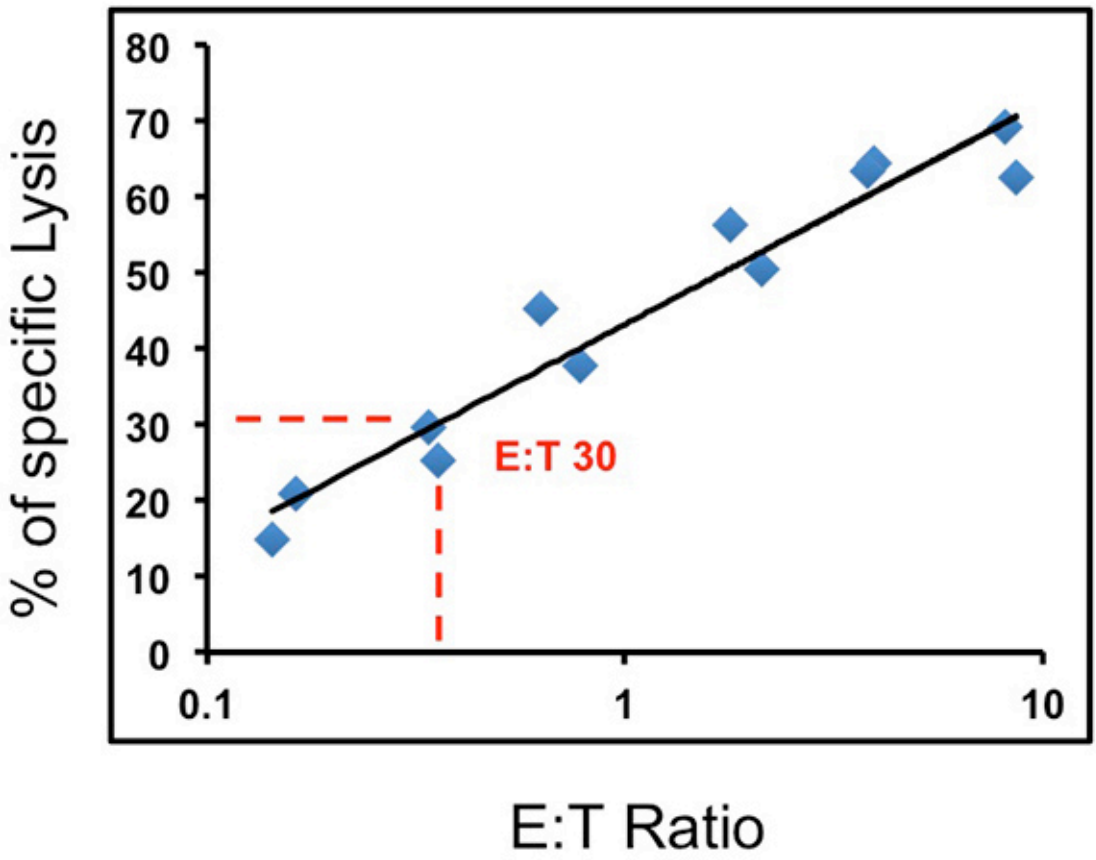

Figure 4. Linear regression of specific lysis in function of the effector/target ratio. The equation of the trendline is used to calculate the lytic units $LU_{30}/10^6$ cells. Click here to view larger image.

Discussion

The assay described here allows for the quantification of the principal function of CD8+ T cells: the cytolytic activity. The accurate measurement of this function is of paramount importance when characterizing effector functions of CD8+ T cells, as previous studies reported discordance between this function and the cytokine secretion of antigen-specific CD8+ T cells[10,11]. Due to the short coculture incubation time (6 hr) and the use of autologous target cells, there is low rate of nonspecific killing of target cells. This assay is therefore highly specific and reliable. This assay can also be easily modified to analyze different cell subsets. The effector CD8+ T cells can be replaced by other cell types, such as CD4+ T cells as recently described in the *in vivo* killing assay[12]. The target CD4+ T cells can also be replaced by other cell types, as we previously described the use of autologous B cells as target cells[8]. In this system, the origin of the antigen can be replaced as well. For example, target cells could be infected with a virus instead of pulsed with peptides. This protocol provides an accurate method to quantify the cytolytic activity of antigen-specific CD8+ T cells. As this method is versatile and easy to perform, measuring the killing capacity of effector cells might be performed more often in the quantification of CD8+ T cell functions.

Disclosures

The authors declare that they have no competing financial interests.

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References

1. Appay, V., Van Lier, R. A. W., Sallusto, F. & Roederer, M. in *Cytometry*. **73A**, 975-983 (2008).
2. Betts, M. R. & Harari, A. Phenotype and function of protective T cell immune responses in HIV. *Curr. Opin. HIV AIDS*. **3**, 349-355, doi:10.1097/COH.0b013e3282fbaa81 (2008).

3. Roder, J. C. *et al.* A new immunodeficiency disorder in humans involving NK cells. *Nature.* **284**, 553-555 (1980).
4. Barber, D. L., Wherry, E. J. & Ahmed, R. Cutting edge: rapid *in vivo* killing by memory CD8 T cells. *J. Immunol.* **171**, 27-31 (2003).
5. Byers, A. M., Kemball, C. C., Moser, J. M. & Lukacher, A. E. Cutting edge: rapid *in vivo* CTL activity by polyoma virus-specific effector and memory CD8+ T cells. *J. Immunol.* **171**, 17-21 (2003).
6. Devevre, E., Romero, P. & Mahnke, Y. D. LiveCount Assay: concomitant measurement of cytolytic activity and phenotypic characterisation of CD8(+) T-cells by flow cytometry. *J. Immunol. Methods.* **311**, 31-46, doi:10.1016/j.jim.2006.01.011 (2006).
7. Altman, J. D. *et al.* Phenotypic analysis of antigen-specific T lymphocytes. *Science.* **274**, 94-96 (1996).
8. Mbitikon-Kobo, F. M., Bonneville, M., Sekaly, R. P. & Trautmann, L. Ex vivo measurement of the cytotoxic capacity of human primary antigen-specific CD8 T cells. *J. Immunol. Methods.* **375**, 252-257, doi:10.1016/j.jim.2011.09.008 (2012).
9. Bryant, J., Day, R., Whiteside, T. L. & Herberman, R. B. Calculation of lytic units for the expression of cell-mediated cytotoxicity. *J. Immunol. Methods.* **146**, 91-103 (1992).
10. Varadarajan, N. *et al.* A high-throughput single-cell analysis of human CD8(+) T cell functions reveals discordance for cytokine secretion and cytolysis. *J. Clin. Invest.* **121**, 4322-4331, doi:10.1172/JCI58653 (2011).
11. Trautmann, L. *et al.* Profound metabolic, functional, and cytolytic differences characterize HIV-specific CD8 T cells in primary and chronic HIV infection. *Blood.* **120**, 3466-3477, doi:10.1182/blood-2012-04-422550 (2012).
12. Hildemann, S. K. *et al.* High Efficiency of Antiviral CD4(+) Killer T Cells. *PLoS One.* **8**, e60420, doi:10.1371/journal.pone.0060420 (2013).

