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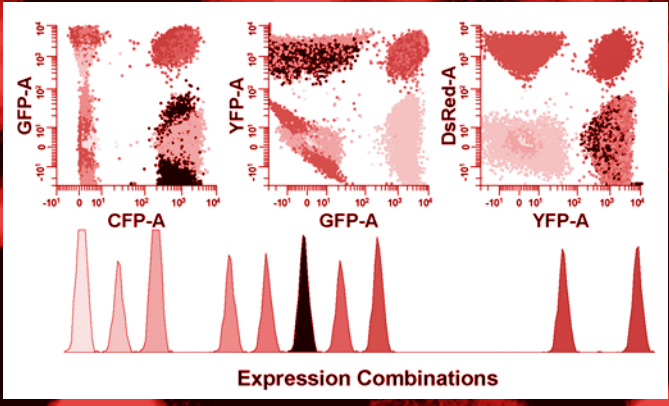

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Assessment of Lymphocyte-Mediated Cytotoxicity Using Flow Cytometry

Luzheng Liu, Beverly Z. Packard, Martin J. Brown, Akira Komoriya, and Mark B. Feinberg

Summary

Cytotoxic lymphocytes, including cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells, kill target cells by releasing granules containing perforin and granzymes, and/or via Fas–Fas ligand interactions. Both pathways lead to prompt activation within target cells of caspase cascades responsible for apoptosis induction and cell death. We have utilized cell-permeable fluorogenic caspase substrates and multiparameter flow cytometry to detect caspase activation in target cells, and applied these tools to quantify and visualize cytotoxic lymphocyte activities. This novel assay, referred to as the flow cytometric cytotoxicity (FCC) assay, is a nonradioactive single-cell-based assay that provides a more rapid, biologically informative, and sensitive approach to measure cytotoxic lymphocyte activity when compared to other assays such as the ^{51}chromium (^{51}Cr) release assay. In addition, the FCC assay can be used to study CTL-mediated killing of primary target cells of different cell lineages that are frequently not amenable to study by the ^{51}Cr release assay. Furthermore, the FCC assay enables evaluation of the phenotype and fate of both target and effector cells, and as such, provides a useful new approach to illuminate the biology of cytotoxic lymphocytes.

Key Words

Apoptosis, caspase, caspase substrate, cytotoxic T lymphocyte, cytotoxicity, flow cytometry, flow cytometric cytotoxicity.

1. Introduction

1.1. Target Cell Killing Mediated by Cytotoxic Lymphocytes

Cytotoxic T lymphocyte (CTL) activity is triggered by T-cell receptor recognition of an antigenic peptide/major histocompatibility complex (MHC), while natural killer (NK) cell-mediated killing is based on inhibitory or stimulatory receptor recognition of specific ligands on target cells ***(1,2)***. Although

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they recognize target cells through different mechanisms, both CTL and NK cells induce target cell apoptosis through either the directed exocytosis of perforin and granzymes or via ligation of "death receptors" in the Fas–Fas ligand (FasL) pathway. Both pathways of cellular cytotoxicity are mediated through the interaction of specific proteins that result in the activation of a cascade of proteases; the latter are characterized by a cysteine residue in the active site and a specificity for cleavage at an aspartic acid in the P_1 position (caspases) ***(1)***. Caspases exist within nonapoptotic target cells in inactive forms. Once activated, they cleave a large variety of substrates that are responsible for the subsequent morphological (cytoskeletal, nuclear, and plasma membrane breakdown) and biochemical (DNA laddering) changes associated with apoptosis. These events ultimately lead to target cell lysis, the end point of the cytotoxic killing process.

1.2. Limitations of the Conventional CTL Assay— ^{51}Chromium (^{51}Cr) Release Assay

Over the past three decades, the ^{51}Cr release assay has been widely used to quantitate cytotoxic lymphocyte activity ***(3)***. In this assay, target cells labeled with radioactive ^{51}Cr are incubated with immune effector cells for 4–6 h. Target cell lysis is then measured by detecting radioactivity released into the culture supernatant.

Although the ^{51}Cr release assay has been widely and productively employed in numerous studies of cellular cytotoxicity, its utility is limited by a number of disadvantages ***(4,5)***. First, ^{51}Cr release assay measures bulk CTL activity using "lytic unit" calculations that do not quantitate target cell death at the single-cell level. Within the context of bulk cell populations used in the ^{51}Cr release assay, variations in the phenotype and function of effector and target cells cannot be assessed directly. Second, CTL killing of primary host target cells often cannot be studied directly, as only certain types of cells, mostly immortalized cell lines, can be efficiently labeled with ^{51}Cr ***(6,7)***. Third, the sensitivity of the ^{51}Cr release assay is further limited by the fact that it measures only end point target cell death. Fourth, measurement of ^{51}Cr release does not permit monitoring the physiology or fate of effector cells as they initiate and execute the killing process. Finally, radioactive materials require special licensing, handling, storage, and disposal, the combination of which substantially increases the total cost of the assay. In the hope of overcoming the limitations of available CTL assays, we sought to take advantage of the power of multiparameter flow cytometry and the advent of fluorogenic caspase substrates to develop a simple, more facile, and more informative strategy to quantify the levels and study the mechanisms of cell-mediated cytotoxicity. This chapter describes the materials and methods employed in the flow cytometric cytotoxicity (FCC) assay resulting from these efforts.

1.3. Recent Development of Flow Cytometry-Based Assays Study Cell-Mediated Immune Response

Recently developed immunologic methods including major histocompatibility complex (MHC) tetramers, intracellular cytokine detection, and enzyme-linked immunospot (ELISPOT) assays have greatly improved sensitivity to enumerate antigen-specific T cells; however, they do not assess the cytolytic function of antigen-specific CTLs ***(8–10)***. Given emerging data indicating that antigen-specific $CD8^+$ T cells may be present in certain chronic infections (e.g., human immunodeficiency virus [HIV]) or malignancies (such as melanoma), but blocked in their ability to produce cytokines or to lyse target cells, assays that measure the complete spectrum of immune effector cell functions at the single-cell level are needed ***(11–14)***. Such assays will be essential for the development of new strategies to maximize immune responses to vaccine antigens, and to manipulate favorably host immune responses in a number of important diseases.

The advantages of utilizing flow cytometric approaches to characterize and quantitate CTL killing has also been apparent to others. Toward this end, other groups have developed flow cytometry-based CTL assays wherein target cell death is assessed based on the amount of fluorochrome released from or retained in the prelabeled target cells ***(15)***, or by detection of the late stages of target cell death using intercalative DNA dyes ***(16)***. However, although these assays provide information that cannot be gleaned from standard ^{51}Cr release assays, none of them reveals the fundamental processes responsible for the initiation and execution of target cell killing. Furthermore, none of these alternative flow cytometric CTL assays has yet been tested and compared with the ^{51}Cr release assay for their ability to quantitate sensitively and accurately CTL responses generated in the course of an in vivo exposure to a defined antigen.

1.4. Fluorogenic Caspase Substrates Detect Cytotoxic Lymphocyte-Induced Target Cell Apoptosis

This chapter describes a flow cytometry assay that detects effector cell-induced caspase activation within individual target cells using a novel and unique class of cell-permeable fluorogenic caspase substrates ***(17–19)***. These reagents (developed by OncoImmunin, Inc., Gaithersburg, MD) are composed of two fluorophores covalently linked to 18-amino-acid peptides containing the proteolytic cleavage sites for individual caspases. In the uncleaved substrates, fluorescence is quenched owing to the formation of intramolecular excitonic dimers. On cleavage of the peptides by specific caspases, the fluorophore–fluorophore interaction is abolished, leading to an increase in fluoresence that can be detected by flow cytometry. In the FCC assay, first target cells are fluorescently labeled (to distinguish them from effector cells *[see* **Subheading 3.1.***]*) and then coincubated with cyto-

toxic effector cells. At the desired time point, medium is removed from samples and replaced with a solution containing a fluorogenic caspase substrate; the latter has spectroscopic properties complementary to those of the target cell label. Following incubation and washing, samples may be analyzed by flow cytometry or fluorescence microscopy. Cleavage of the substrate results in increased fluorescence in dying cells. This chapter uses the following model systems to illustrate the basic principles of the FCC assay: viral antigen-specific mouse CTL killing of target cells and human NK killing of both suspension and adherent human tumor cells. (Additional applications and further characterization of these probes as flow cytometry reagents can be found in Chapter 8 by Telford et al., this volume.)

1.5. Advantages of the FCC Assay Compared to the Conventional ^{51}Cr Release Assay

This assay has been shown to provide a single-cell-based, rapid, quantitative, and sensitive assay to detect lymphocyte-mediated target cell killing in various animal models ***(17)***. Unlike conventional chromium release assays, the FCC assay enables monitoring of cellular immune responses in real time and at the single-cell level using diverse fluorescence detection methods such as flow cytometry and fluorescence and confocal microscopy. Importantly, the assay can be used to study CTL-mediated killing of primary host target cells, and enables assessment of important biological details of the killing process, as well as the fate of immune effector cells during the killing process. These features should enable direct determination of whether specific subpopulations of cells can resist CTL-mediated lysis (e.g., tumor cells or certain virus-infected cells) ***(20,21)*** or alternatively, induce apoptotic deletion of the CTL effectors themselves (e.g., through expression of FasL on specific tumors or immunologically privileged tissues, or as an immune evasion strategy employed by immunodeficiency viruses) ***(22,23)***. Although developed using the murine lymphocytic choriomeningitis virus (LCMV) infection model, this novel approach is readily applicable to other models such as HIV, SIV, Epstein–Barr virus (EBV) and cytomegalovirus (CMV) infections (data not shown). In all, the favorable attributes of the FCC assay may permit new insights into the pathogenesis of important infectious, malignant, and immunologic diseases that have been experimentally unapproachable previously, and provide a practical and useful method to quantitate CTL activity in basic and applied studies of cellular immune responses.

2. Materials

2.1. Preparation of Target Cells

1. Target cells: Either tumor cell lines or primary cells such as mouse splenocytes can be used to prepare a single-cell suspension. For example, EL4 cells (American Type

Culture Collection [ATCC], Manassas, VA, cat. no. TIB-39) or A20 cells (ATCC, cat. no. TIB-208) can be used in mouse models with H-2^b haplotype or H-2^d haplotype, respectively. Other examples include Jurkat cells and MDA-MB-468 (ATCC, cat. no. HTB-132).

2. Complete media (*see* **Note 1**):
   a. RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum ([FBS], HyClone, Logan, VT), 100 U of penicillin G, 100 µg/mL of streptomycin sulfate, and 2 m*M* L-glutamine (100X stock, all from Mediatech, Herndon, VA). EL4, A20 and Jurkat cells can be maintained in this medium.
   b. Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS. Most adherent cell lines such as MDA-MB-468 can be maintained in this medium.
3. Trypan blue (Mediatech).
4. Target cell labeling reagents can be used to label target cells prior to introduction of effectors (two examples are listed below):
   a. Celltracker™ orange CMTMR ([CTO], Molecular Probes, Eugene, OR). λ_{ex} = 540 nm and λ_{em} = 566 nm. It is light sensitive. On receipt, it should be stored desiccated at –20°C until use. Avoid repeated freezing and thawing. Dissolve the lyophilized product in high-quality, anhydrous dimethyl sulfoxide ([DMSO], Sigma Chemical Co., St. Louis, MO) to a final concentration of 10 m*M*. Store small aliquots frozen at –20°C, desiccated, and protected from light. Avoid repeated freezing and thawing. When stored properly, both the solids and the stock solutions are stable for at least 6 mo.
   b. TFL2 (OncoImmunin, Inc.). On receipt, it should be stored desiccated at –20°C until use.
5. For viral antigen-specific mouse CTL killing, synthetic peptides containing the viral CTL epitope of interest are used. An irrelevant peptide should also be employed as a negative control. High-purity peptides can be ordered from American Peptide Company, Inc. (Sunnyvale, CA).
6. Staurosporine *Streptomyces* sp. (Sigma Chemical Co.). Store at 4°C.
7. 15-mL Falcon polypropylene conical tubes with screw caps (BD Biosciences Discovery Labware, Bedford, MA).

2.2. Preparation of Effector Cells

1. Effector cells: Lymphocytes from the spleen or lymph nodes of mice acutely infected with viruses (d 5–8 postinfection) ***(17)***, the NK92 cell line (ATCC, cat. no. CRL-2407), or a CTL cell line.
2. Red blood cell (RBC) lysing buffer (Sigma Chemical Co.).
3. 50-mL Falcon polypropylene conical tubes with screw caps, 70-µm Falcon cell strainer, and 3-mL syringe (all from BD Biosciences).

2.3. Detection of Target Cell Apoptosis by Caspase Substrates

1. PhiPhiLux-G_1D_2 ([caspase 3-like substrate], OncoImmunin Inc.). λ_{ex} = 505 nm and λ_{em} = 530 nm. It is light sensitive, but is stable in the dark at 4°C for up to 2 mo. Freeze at –20°C for longer-term storage. Keep the reagent sterile. *See* **Note 2**.

2. Washing buffer: Dulbecco's phosphate-buffered saline ([PBS], Invitrogen) supplemented with 2% FBS.
3. 96-well U- or V-bottom tissue culture plate with lid, and 5-mL Falcon polypropylene round-bottom tubes (fluorescence-activated cell sorter [FACS] tubes, both from BD Biosciences).

2.4. Flow Cytometry (see Note 3)

1. A flow cytometer such as the FACSCalibur (BD Biosciences Immunocytometry Systems, San Jose, CA) or Epics® XL (Beckman Coulter, Miami, FL).

2.5. Determine Cytotoxic Activity

1. FACS data analysis software such as CellQuest (BD Biosciences), FlowJo (Tree Star, San Carlos, CA), or WinMDI (Dr. Joseph Trotter, Scripps Institute, La Jolla, CA, and BD Biosciences).

3. Methods

3.1. Preparation of Target Cells (see Note 4)

1. Suspend target cells in complete RPMI 1640 medium in a 15-mL conical tube.
2. Count viable cells with the Trypan blue exclusion method. Adjust cell concentration to 2×10^6/mL.
3. Add a 0.5-mL aliquot of target cells into a FACS tube: tube A. Leave tube A on ice until sample acquisition by flow cytometer. (This will serve as the unlabeled target cell control for instrument setting of the flow cytometer.)
4. Add a 0.5-mL aliquot of target cells into a new 15-mL conical tube: tube B. Induce apoptosis by adding an apoptogen such as staurosporine at a final concentration of 1 μ*M*. Incubate in a 37°C 5% CO_2 incubator until **Subheading 3.3., step 5**. Conditions for apoptosis induction will vary with target cell type and apoptogen, but a good starting point is staurosporine at 1 μ*M* for 3–5 h. (Tube B will serve as FL1 channel control for flow cytometer setting.)
5. Divide the rest of target cell suspension equally into two 15-mL conical tubes (tubes *a* and *b*). Add CTO/TFL2 into the tubes at a final concentration of 3/2 μ*M*. Antigenic peptide is added into tube *a* at final concentration of 1 μ*M*, irrelevant control peptide is added into tube *b*.
6. Incubate tubes *a* and *b* at 37°C 5% CO_2 for 1 h with caps loosened. During this time, prepare effector cells (*see* **Subheading 3.2.**).
7. After the 1-h incubation, wash target cells at least once in at least 10-fold volume of complete RPMI 1640.
8. Resuspend cells at 2×10^6/mL in complete RPMI 1640. Leave the cells on ice until effector cells are ready.
9. Take a 0.5-mL aliquot from each of tube *a* and *b* into two FACS tubes (tubes C and D). Leave tube C on ice until sample acquisition. (This tube will serve as FL2 channel control for flow cytometer setting.) Leave tube D on ice until **Subheading 3.3., step 3**. (This tube will be used to measure base line target cell apoptosis.)

3.2. Preparation of Effector Cells From Spleen or Lymph Nodes (see Notes 4 and 5)

1. Harvest spleen or/and lymph nodes on d 5–8 post-viral infection.
2. Place a 70-µm Falcon cell strainer on top of a 50-mL Falcon tube. Place freshly removed organs into the cell strainer.
3. Press the organs against bottom of the cell strainer with plunger of a 3-mL syringe until mostly fibrous tissue remains.
4. Rinse cells through the cell strainer with 20 mL of complete RPMI 1640 medium. Discard the cell strainer.
5. Centrifuge at 250*g* for 10 min and discard supernatant.
6. Remove RBC in spleen samples by resuspending spleen cell pellet in RBC lysing buffer (5 mL/spleen). Incubate at room temperature for 5 min. Add RPMI 1640 to fill the tube, centrifuge at 250*g* for 10 min. Discard the supernatant.
7. Resuspend the cell pellet in the appropriate volume of complete RPMI 1640. Count cells by trypan blue exclusion.
8. Adjust the cell concentration to 5×10^7/mL (if maximal E/T ratio is 25:1).

3.3. Detection of Target Cell Apoptosis by Caspase Substrates

1. Prepare serial dilutions of effector cells in 96-well U-bottomed plate for the desired E/T ratios. For example, to obtain E/T ratios as 25:1, 12.5:1, and 6.25:1, 100 µL/well complete RPMI 1640 is added into wells of rows B and C of the plate. Add 200 µL/well of effector cells (5×10^7/mL) into row A of the plate. Use a multichannel pipet to transfer 100 µL/well of cell suspension from row A into the corresponding wells in row B, mix by pipetting three to five times. Change tips, then transfer 100 µL/well cell suspension from row B into the corresponding wells in row C, mix well, and discard 100 µL/well cell suspension from row C.
2. Add 100 µL/well (2×10^5 cell/well) of target cell suspension into wells of the 96-well plate. Mix target cells and effector cells well by pipetting.
3. Add 200 µL of target cells in tube D (*see* **Subheading 3.1., step 9**) into one well of the 96-well plate.
4. Incubate the plate in a 37°C 5% CO_2 incubator for 1–3 h (*see* **Note 6**).
5. Pellet cells by centrifuging the plate and tube B (*see* **Subheading 3.1., step 4**) at 250*g*. Discard the supernatant by flicking the plate and vacuum aspiration from the tube, respectively.
6. Add 75 µL/sample of caspase substrate PhiPhiLux-G_1D_2 and mix by gentle pipetting. From this point on, do not vortex-mix samples, as apoptotic cells can be "fragile."
7. Incubate cells in a 37°C 5% CO_2 incubator for 30 min (*see* **Note 7**).
8. Wash cells twice by adding 200 µL/sample of ice-cold washing buffer.
9. After the final wash, resuspend the cell pellets into 300 µL/sample of washing buffer.

10. Transfer cells into FACS tubes. Label the tube containing staurosporine-treated target cells as tube B.

3.4. Flow Cytometry

1. Use tube A (unlabeled target cells) to set FL1 and FL2 channels initially, so that the fluorescence intensities of the majority of the events are between 10^0 and 10^1 on each axis.
2. Use tube B (PhiPhiLux-G_1D_2-labeled apoptotic target cells) to set up FL2 channel compensation.
3. Use tube C (CTO/TFL2-labeled target cells) to set up FL1 channel compensation.
4. Run the remaining samples.

3.5. Determine Cytotoxic Activity

Data are analyzed by CellQuest, FlowJo, or WinMDI software to obtain quadrant statistics of distinct cell populations. The total target cell apoptosis is calculated as {(% CTO/TFL2$^+$ caspase$^+$ cells)/[(% CTO/TFL2$^+$ caspase$^+$ cells) + (% CTO/TFL2$^+$ caspase$^-$ cells)]} × 100%. The following are a panel of representative experiments highlighting the broad applications and advantages of the FCC assay.

Typical FCC data measuring antigen-specific CTL activity are shown in **Fig. 1A,B** *(17)*. Spleen cells from C57BL/6 mice acutely infected with LCMV were used as effectors, and target EL4 cells were either loaded with the dominant LCMV H-2^b-restricted CTL epitope $NP_{396–404}$ **(Fig. 1A)** or an irrelevant control peptide **(Fig. 1B)**. Significant target cell apoptosis was detected with the FCC assay only when target EL4 cells were pulsed with the LCMV epitope $NP_{396–404}$, but not with the control epitope. The numbers at the upper right corners are the calculated percentages of target cell apoptosis. **Figure 1C,D** shows the direct comparison of the FCC assay with the ^{51}Cr release assay. CTL activities against a panel of LCMV peptides were measured using the two methods in parallel. D 8 splenocytes were incubated with EL4 target cells pulsed with different peptides at various E/T ratios for 3 h (FCC assay) or 5 h (^{51}Cr release assay). The two methods detected an identical pattern of dominance hierarchy of the CTL activities specific for different peptides. Importantly, the FCC assay was more sensitive than the ^{51}Cr release assay in detecting the CTL response specific for the subdominant epitope $NP_{205–212}$. Similarly, the FCC assay can be used to detect human CTL activity against viral antigens. In **Fig. 2**, a human CTL line was used as effector cells in the FCC assay. Strong killing of BLCL targets pulsed with the HIV A2-restricted epitope QR9 but not the control peptide was detected at an E/T ratio of 5 : 1.

The FCC assay also provides a rapid and sensitive measurement for NK cell-mediated cytotoxicity. Jurkat cell death induced by the NK cell line NK92

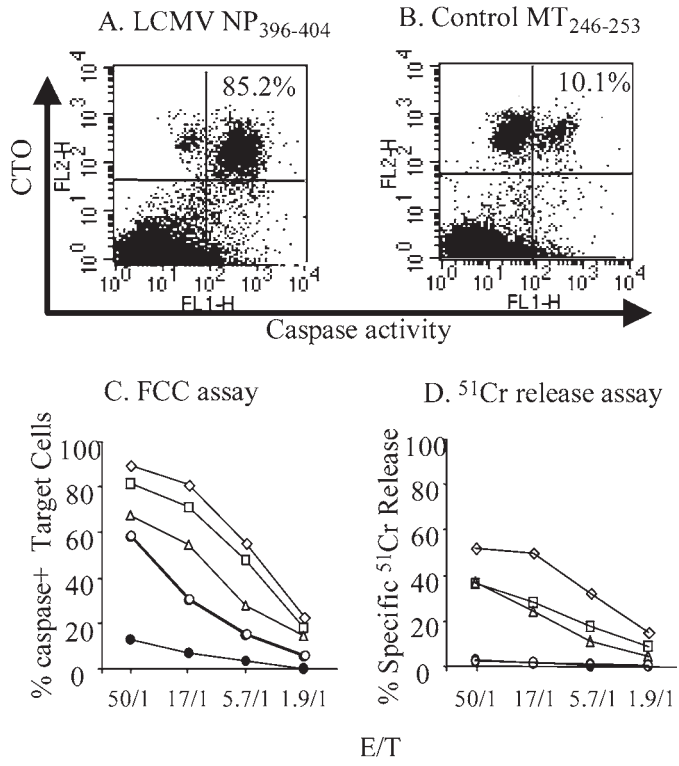

Fig. 1. Detection of LCMV antigen-specific CTL activity *(17)* using the FCC assay. **(A,B)** Spleen cells from d 8 LCMV-infected C57BL/6 were cocultured for 3 h with EL4 cells that were loaded with either the dominant LCMV CTL epitope $NP_{396–404}$ **(A)** or an irrelevant control peptide **(B)**. The E/T ratio was 50:1. Subsequent incubation with PhiPhiLux detected strong caspase activity in target cells pulsed with the LCMV epitope $NP_{396–404}$, but not with the control peptide. The numbers at the *upper right corners* are the percentage of target cell apoptosis. **(C,D)** Comparison of CTL activities specific for a panel of LCMV epitopes measured by FCC and ^{51}Cr release assays. CTO or ^{51}Cr-labeled EL4 cells were pulsed with LCMV peptides $NP_{396–404}$ (◇), $GP_{33–42}$ (□), $GP_{276–286}$ (△), $NP_{205–212}$ (○), or irrelevant peptide $MT_{246–253}$ (●) and then cocultured with the same effectors as in **(A)** and **(B)** for 3 h (FCC assay) or 5 h (^{51}Cr release assay). Target cell death was then assessed by either cleavage of the PhiPhiLux **(C)** or ^{51}Cr release **(D)**. Note that the curves representing CTL response specific for $NP_{205–212}$ and $MT_{246–253}$ overlapped in **D**.

was kinetically measured with a modified "rapid" protocol where the coincubation of targets and effectors was carried out in the caspase substrate solution. This modified protocol allows the detection of the onset of target cell death at considerably earlier time points. As shown in **Fig. 3**, significant target

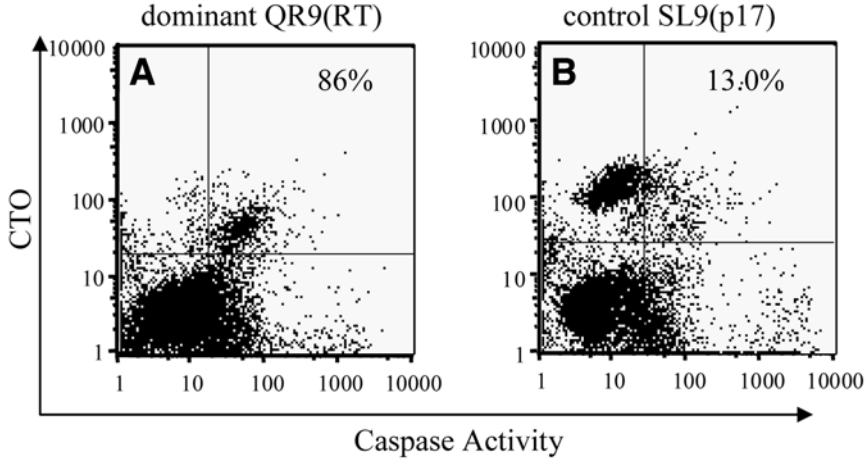

Fig. 2. Strong anti-HIV CTL activity was detected with the FCC assay. An HIV-specific T cell line was generated by stimulating PBMCs from an HLA $A2^+$ HIV^+ donor in vitro with the HIV peptide QR9. These T-cells were then coincubated with a CTO-labeled $A2^+$ B-cell line bearing either the same peptide QR9 **(A)** or a control HIV peptide SL9 **(B)** at 5:1 ratios. Strong QR9-specific CTL activity was detected by the FCC assay.

cell death (50%) was detected as early as 20 min after the coincubation of effectors and targets at a 5:1 E/T ratio. At the end of a 2-h coincubation, 90% of the target cells were shown to be apoptotic.

In addition, the FCC assay is readily convertible to a fluorescence microscopic assay to visualize the lymphocyte-mediated cytotoxic action *(8)*. **Figure 4** demonstrates the detection of NK92-mediated killing of breast carcinoma target cell line MDA-MB-468 cells using both flow cytometry **(Fig. 4A,B)** and confocal microscopy (**Fig. 4C,D**; nonapoptotic target cells are red; apoptotic target cells have both red and green fluorescence signals and therefore appear yellow). Thus, the FCC assay enables monitoring of cellular immune responses in real time and at the single-cell level using diverse fluorescence detection methods.

4. Notes

1. The character of the FBS used in cell culture can be a critical element in obtaining good results with the FCC assay protocol. It may be necessary to screen multiple lots of FBS to obtain an optimal lot that supports high effector cell activity and low target cell spontaneous apoptosis.
2. An alternative caspase substrate (for caspase 6) is used in the CyToxiLux cytotoxicity assay kit (OncoImmunin Inc.) to detect target cell apoptosis (http://www.phiphilux.com/cytotox.htm). Similar results can be obtained using the CyToxiLux kit and the protocol described in this chapter.

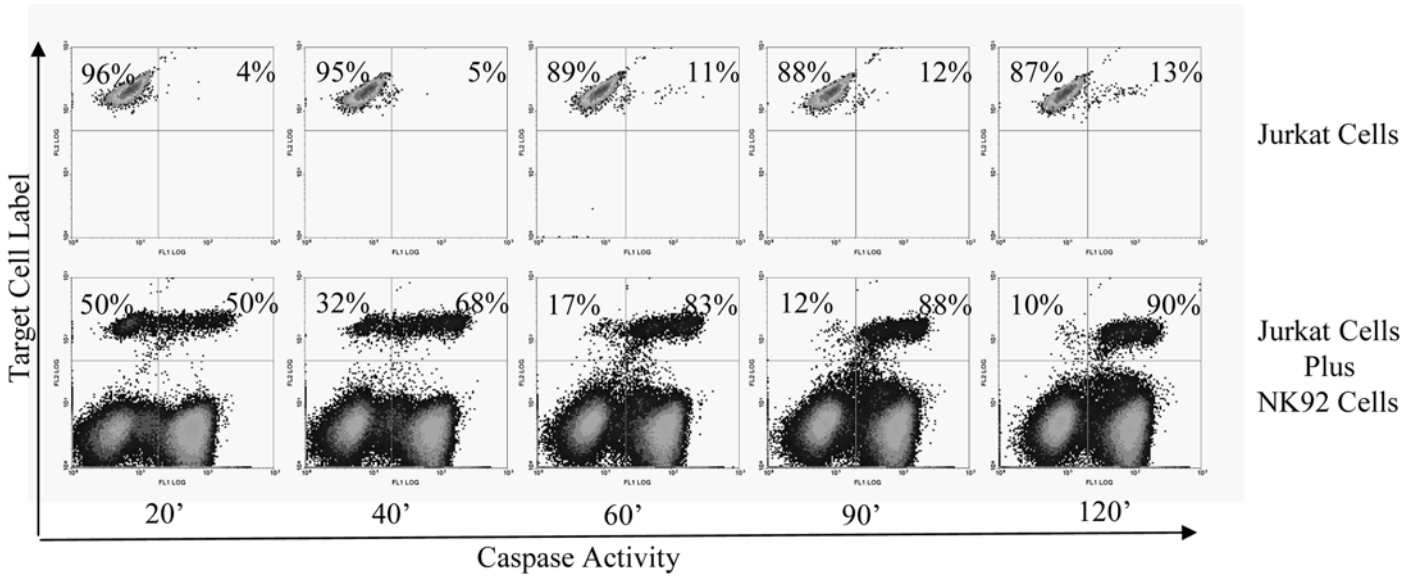

Fig. 3. The FCC assay provides rapid and sensitive detection of NK cell cytotoxicity. TFL2-labeled Jurkat cells were incubated with the NK cell line NK92 at an E/T ratio of 5:1 for the time indicated in the presence of the caspase 6 substrate. Significant cytotoxicity (50%) was detected as early as 20 min.

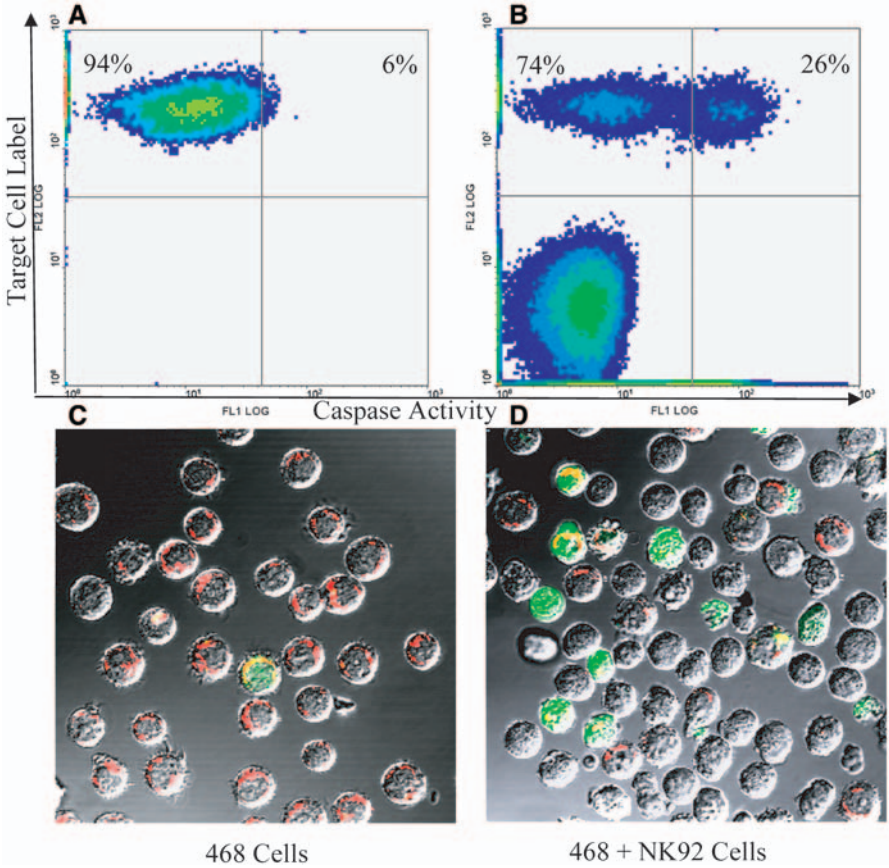

Fig. 4. The detection of NK92-mediated killing of breast carcinoma target cell line MDA-MB-468 cells using both flow cytometry **(A,B)** and confocal microscopy **(C,D)**. Target cells were labeled with TFL2 and incubated without **(A,C)** or with **(B,D)** NK92 cells for 2 h in the presence of caspase 6 substrate at an E/T ratio of 5 : 1. Nonapoptotic target cells are red; apoptotic target cells have both red and green fluorescence signals and therefore appear yellow.

3. Instrument settings of flow cytometry-based assays are critical for quantitation accuracy. Because the fluorescence emissions from PhiPhiLux-G_1D_2 and CTO/TFL2 overlap to some extent, compensation is necessary. A significant amount of orange fluorescence is present in the PhiPhiLux-G_1D_2 emission, and compensation for the FL2 channel may require subtraction of 80% or more from FL1 (FL2 minus ≥80% FL1). A new version of the FCC which uses the same principles described herein but does not require compensation is now available from OncoImmunin, Inc. The only additional requirement is a flow cytometer equipped with both 488-nm and 633/635-nm laser lines.

4. Viability of both target cells and effector cells prior to coincubation is critical for the sensitivity of the assay. Target cells should be maintained in complete medium at optimal concentration, so that baseline cell apoptosis is <15%. For primary cell targets, they should be cocultured with effector cells within 2 h after harvesting to prevent increased spontaneous apoptosis ex vivo. Increased baseline target cell death reduces sensitivity of the assay. Cells should be left on ice whenever applicable. If necessary, nonviable cells can be removed by Ficoll-Paque density gradient centrifugation.
5. In vitro stimulated CTL precursors (CTLp) from previously primed animals can be used as effector cells in the FCC assay. A detailed protocol for CTLp stimulation in vitro is beyond the scope of this chapter and has been described elsewhere ***(13)***. In brief, stimulator cells are prepared by irradiating naïve spleen cells (γ-irradiation using 2000 rad) and pulse them with 1–10 μ*M* antigenic peptide. The stimulator cell suspension is adjusted to $1–6 \times 10^6$/mL. The concentration of the responder cells should be $2–10 \times 10^6$/mL. Optimal concentrations of stimulator and responder cells may vary with the nature of the antigenic differences. Titration of both cell types may be required to identify optimal concentrations. Add 1 mL each of responder and stimulator cells to the wells of a 24-well tissue culture plate (BD Biosciences). Set up at least six wells for each concentration of responder and stimulator cells. Culture cells for 5 d in a 37°C 5% CO_2 incubator. Harvest effector cells into a 15-mL conical tube, centrifuge at 250*g* for 5 min, and resuspend the pellet in complete RPMI 1640 at the desired concentration. Process with the FCC protocol described in **Subheading 3.3.**
6. If coincubation of effector cells and target cells is longer than 3 h, some apoptotic target cells may be lysed and become "invisible" by flow cytometry. Thus, the percentage of CTO/TFL2-positive cells in the total cell population at the end of the assay would be significantly less than at the beginning of the assay. For example, if the E/T ratio is 25:1, target cells (CTO/TFL2-positive) account for only 3.8% of total cells; by the end of the assay, only 1% of CTO^+ cells may remain. This target cell loss has been shown to be antigen specific and effector cell dependent, and therefore, the portion of lost target cells should be considered in the calculation of total target cell death to avoid underestimation of cytotoxic activity.
7. Additional surface marker staining of target and/or effector cells can be done following caspase substrate incubation. Do not fix or permeablize cells that have been incubated with the caspase substrate for antibody labeling. Fixation will cause caspase labeling to be lost. Samples can be stored at 4°C overnight without significant loss of fluorescence signals.

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