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New flow cytometric assays for monitoring cell-mediated cytotoxicity

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Abstract

The exact immunologic responses after vaccination that result in effective antitumor immunity have not yet been fully elucidated and the data from *ex vivo* T-cell assays have not yet defined adequate surrogate markers for clinical efficacy. A more detailed knowledge of the specific immune responses that correlate with positive clinical outcomes should help to develop better or novel strategies to effectively activate the immune system against tumors. Furthermore, clinically relevant material is often limited and, thus, precludes the ability to perform multiple assays. The two main assays currently used to monitor lymphocyte-mediated cytotoxicity in cancer patients are the ⁵¹Cr-release assay and IFN- γ ELISpot assay. The former has a number of disadvantages, including low sensitivity, poor labeling and high spontaneous release of isotope from some tumor target cells. Additional problems with the ⁵¹Cr-release assay include difficulty in obtaining autologous tumor targets, and biohazard and disposal problems for the isotope. The ELISpot assays do not directly measure cytotoxic activity and are, therefore, a surrogate marker of cytotoxic capacity of effector T cells. Furthermore, they do not assess cytotoxicity mediated by the production of the TNF family of death ligands by the cytotoxic cells. Therefore, assays that allow for the simultaneous measurement of several parameters may be more advantageous for clinical monitoring. In this respect, multifactor flow cytometry-based assays are a valid addition to the currently available immunologic monitoring assays. Use of these assays will enable detection and enumeration of tumor-specific cytotoxic T lymphocytes and their specific effector functions and any correlations with clinical responses. Comprehensive, multifactor analysis of effector cell responses after vaccination may help to detect factors that determine the success or failure of a vaccine and its immunological potency.

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Keywords

cell-mediated cytotoxicity; cytotoxic T lymphocyte; multiparameter flow cytometry; natural killer cell; vaccine

Immune response profiling and monitoring are key elements in the development of new biotherapies, particularly in the areas of autoimmune disorders, infectious diseases, cancer, transplantation, allergies and vaccines. Thus, selection of *ex vivo* monitoring methods that provide the best measure of cell-mediated cytotoxicity, the main immune mechanism of protection from various pathogens and cancer, is important in determining correlations between clinical and immunologic responses to specific immunotherapy, and to determine the optimal vaccine strategy.

Mechanisms of cell-mediated cytotoxicity

There is now ample evidence that CD8⁺ cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells are key players in innate and adaptive immune response [1]. Cell-contact-dependent cytotoxicity is the hallmark of CD8⁺ T-cell and NK-cell function. *In vitro* cytotoxicity assays have shown the presence of two major contact-dependent cytotoxic pathways. First, the exocytosis of lytic granules by cytotoxic effector cells, comprising a pore-forming toxin, perforin, and pro-apoptotic serine proteases, granzymes, which synergistically kill target cells by activating various lytic pathways [2]. Second, the production by the effector cells of the TNF family members, such as TNF- α , Fas ligand (FasL) or TRAIL, which induce multimerization of their cognate receptors on target cells resulting in apoptosis induction.

Natural killer cells are the first line of defense against virus-infected and malignant cells, as they detect the expression of atypical or the absence of classical MHC class I molecules on the target cells. By contrast, CTLs are the effector cells of the adaptive immune system. They require at least three signals – T-cell receptor ligation with specific peptide presented on MHC class I molecule, costimulation and cytokine signals – to become activated. Despite these differences in their recognition and cell signaling pathways, the cytotoxic effector pathways used by NK cells and CTLs are quite similar. Both NK cells and CTLs contain specific lytic granules. However, naive CD8⁺ T cells, in contrast to NK cells, do not constitutively express these lytic organelles; both perforin and granzymes are expressed by CD8⁺ T cells only following their activation and clonal expansion. Furthermore, both NK cells and T cells can produce TNF, FasL and TRAIL upon appropriate recognition of target cells.

Over the last 15 years, there have been major advances in understanding the molecular basis of direct cell-mediated cytotoxicity, which was supported by both the *in vitro* and *in vivo* studies. Perforin seems to be essential for granule-mediated cytotoxicity and the only known function of perforin to date is in promoting disruption of target cell membranes. There has been a general consensus that certain granzymes, particularly granzyme B (GrB), are important killer serine proteases of the secretory granules. On transfer into target cells, GrB can activate target cell caspases that results in apoptotic death of target cells.

In addition to perforin-dependent cytotoxicity, cytotoxic effector cells use several members of the TNF family of proteins, such as TNF- α , FasL or TRAIL, for their cytolytic function [3,4]. Binding of these proteins to their appropriate receptors on target cells can trigger activation of the extrinsic apoptosis signaling pathway in the target cells. One benefit of two distinct cytotoxic effector pathways may be to increase the spectrum of target cells that can

be lysed by cytotoxic effector cells, as well as providing an alternative lytic pathway in situations where one pathway is blocked or ineffective.

Concerning the biological significance of these direct cell-mediated cytotoxic pathways, it is well established that the perforin-dependent pathway is important in the immune response to certain viruses and tumor cells [1]. In some instances, Fas/FasL-mediated cytotoxicity may replace perforin in causing tumor rejection. It has been shown in adoptive transfer experiments with various CD8⁺ T cells that in addition to perforin and FasL, TNF- α , lymphotoxin- α , IFN- γ and the activation of the cytotoxic effector function of macrophages could all contribute to tumor rejection [5–9]. Furthermore, in the majority of adoptive T-cell transfer studies, IFN- γ was crucial for efficient tumor rejection [10]. IFN- γ can enhance MHC class I and Fas levels on tumor cells, thus increasing their sensitivity to CTL-mediated killing. However, in many cases, tumor rejection did not require IFN- γ responsiveness of tumor cells. Furthermore, various cytokines released by NK cells and CTL in the tumor milieu, can attenuate tumor angiogenesis by affecting not only tumor cells directly, but also by regulating activity of vascular endothelial cells, as well as tumor-associated fibro-blasts, myeloid-derived suppressor cells and alternatively activated M2 macrophages. Therefore, NK cells and CTLs may function in multiple ways to cause tumor rejection, either by directly killing tumor cells or by changing the microenvironment of the tumor in a way that is hostile to further tumor growth and development. In the future, a fuller understanding of the mechanisms by which NK cells and CTLs can reject tumors *in vivo* may form a rational basis for significantly improving cancer immunotherapy.

Analyzing cell-mediated cytotoxicity

The most popular assay for evaluating cell-mediated cytotoxicity is the radioactive chromium (⁵¹Cr)-release assay. It was first developed in 1968 by Brunner *et al.* [11], and continues to be the most specific assay of its kind. It is based upon the passive internalization and binding of ⁵¹Cr from sodium chromate by target cells. Lysis of the target cells by effector cells results in the release of the radioactive probe into the cell culture supernatant, which can be detected by a γ -counter. Although several alternatives were proposed (Box 1), this assay is considered to be the ‘gold standard’ to measure cell-mediated cytotoxicity. However, while it has benefits of being reproducible and relatively easy to perform, it has several drawbacks: it provides only semiquantitative data unless it incorporates a limiting dilution component [12]; it has a relatively low level of sensitivity; very often there is a need to stimulate cytotoxic cells several times before testing their lytic activity, which may distort the composition and activity of the T-cell populations from their original state; it lacks information about behavior of single cells; there is poor labeling of some target cell lines; a high spontaneous release from some target cell lines occurs; since an autologous tumor is difficult to obtain, other targets must be used that may not reflect the actual ability to lyse autologous tumor cells *in vivo*; its inter-assay variability is considerable; and there are biohazard and disposal problems associated with radioisotope usage. For these reasons, a search for other methods that could replace the ⁵¹Cr-release assay has been ongoing.

Box 1

Alternative to chromium-release cytotoxicity assays

Assays based on using radioactive compounds other than ⁵¹Cr

- ¹²⁵I- or ³H-labeling of target DNA to test ‘bulk’ DNA degradation [71–73]
- JAM test: detection of apoptotic cleavage of ³H-labeled target DNA [74]

Assays based on release of nonradioactive compounds from target cells measured by fluorometry

- Europium- and samarium-release assays [75–77]
- CFDA- and BCECF-based assays [78–80]

Assays based on detection of enzymatic activity in target cells

- Measurement of endogenous alkaline phosphatase activity [81]
- LDH: enzyme-release assay [82,83]
- Fluorometric method based on hydrolysis of MUH [84,85]
- Calcein-AM-based assays [86,87]
- MTT assay – based on detection of cleavage of tetrazolium salts [88]

Assays based on analysis of target cells transfected with β -gal or other foreign enzyme genes

- Release of firefly luciferase or bacterial β -gal: colorimetric or luminometric methods [89–91]
- LysisSpot assay: combination of β -gal transfection of targets and ELISpot [92]
- Biophotonic cytotoxicity assay [22]
- Bicistronic vector-based assay [93]

‘Bulk’ assays based on release of nonradioactive compounds from effector cells

- BLT assay – to detect esterase activity of serine proteases in granules [94–96]
- ELISA (e.g., IFN- γ and GrB) [97]

Single-cell-based assays to detect early activation of effector cells

- Calcium flux assay [98]
- Phosphorylation of signal transduction protein-based assays [99–100]

Quantification of CTL precursors

- LDA [12,101,102]

AM: Acetoxymethyl; BCECF: 2'-7'-biscarboxyethyl-5(6)-carboxyfluorescein; BLT: Benzoyloxycarbonyl-L-lysine thiobenzyl ester; CFDA: Carboxyfluorescein diacetate; CTL: Cytotoxic T lymphocyte; ELISpot: Enzyme-linked immunospot assay; GrB: Granzyme B; JAM: Just another method; LDA: Limited dilutions assay; LDH: Lactate dehydrogenase; MTT: Methylthiazolyldiphenyl-tetrazolium bromide; MUH: 4-methylumbelliferyl heptanoate.

Assays that can monitor both CTL frequency and function, such as the IFN- γ enzyme-linked immunospot assay (ELISpot), have gained increasing popularity for monitoring clinical trials and in basic research [13]. The ELISpot assays enumerate antigen-specific lymphocyte frequency by measuring secretion of specific immune proteins engaged in the specific pathway utilized to mediate lysis of target cells. ELISpot assays detect locally secreted cytokine molecules by means of antibody-coated plastic plates or nitrocellulose membranes to capture the secreted cytokine derived from the productive interaction of the effector cell and its target cell. There are numerous advantages to utilizing the ELISpot assays over the standard ^{51}Cr -release assay: the ELISpot assays enumerate antigen-specific lymphocyte frequency by measuring secretion of a specific immune protein and, as such, the ELISpot

assays are both qualitative and quantitative; the ELISpot assays use a lower number of effector cells to accurately assess activity, which is beneficial for monitoring clinical trials with limited numbers of patients' cells available; the high sensitivity and specificity of the ELISpot assays are beneficial for clinical monitoring; in addition, the problems associated with the labeling efficiency of targets are not a concern with the ELISpot assays. Accordingly, it was proven that IFN- γ ELISpot correlates well with CTL frequencies determined by ^{51}Cr -release assay and a variant of the limited dilutions assay, the multiple microculture assay [14]. Furthermore, results from various clinical trials, including peptide and whole-tumor cell vaccination and cytokine treatment, showed the suitability of the ELISpot assay for monitoring T-cell responses [15]. However, using the IFN- γ ELISpot assay alone may not be sufficient because noncytotoxic cells can secrete IFN- γ whereas CTLs with cytotoxic activity do not always secrete IFN- γ [16]. The GrB ELISpot assay may be a more direct measure of cell-mediated cytotoxicity compared with the IFN- γ ELISpot, since GrB is a key mediator of target cell death via the granule-mediated pathway [17]. Therefore, the release of GrB by cytotoxic lymphocytes upon effector–target interaction may be a more specific indicator of CTL and NK cytotoxic ability than IFN- γ secretion. Both the GrB and IFN- γ ELISpot assays are superior alternatives to the ^{51}Cr -release assay to test CTL response. Our research with samples from melanoma patients vaccinated with gp100:209M peptide suggests that the GrB ELISpot assay may be successfully applied to evaluate CTL precursor frequency. Reactivity in the GrB ELISpot was more closely associated with cytotoxicity in the ^{51}Cr -release assay than the tetramer or IFN- γ ELISpot assays [18].

It is important to emphasize that ^{51}Cr -release and the GrB ELISpot assays measure different aspects of cell-mediated cytotoxicity – target cell death and effector cell function, respectively. One of the limitations of the GrB ELISpot assay is that it provides no information on the cells that manifested that function. It also measures degranulation, but not direct target cell lysis. As such, degranulation may not always equate to cell death if target cells contain serpin proteinase inhibitor 9, a protein that inhibits the proteolytic activity of GrB, or if effector cells are perforin deficient. The GrB ELISpot assay also does not account for cytotoxicity mediated by the FasL pathway. Therefore, when appropriate, the two assays should be used in concert to obtain more detailed knowledge of the specific immune responses that correlate with positive clinical outcomes. However, clinically relevant material is often limited and, thus, this restricts the ability to perform multiple assays. Assays that allow for the simultaneous measurement of several parameters may be more advantageous for clinical monitoring. In this respect, the flow cytometric assays that will enable detection and enumeration of tumor-specific CTLs and their specific effector functions provide new insights on the mechanism of cell-mediated killing and will help to improve cancer vaccine design and evaluation in the future.

Flow cytometric analysis of antigen-specific T cells

Flow cytometry assays are a powerful tool for analyzing antigen specific T-cell responses in a quantitative manner. Flow cytometry rapidly measures the fluorescence and other optical properties of individual particles including cells. In a typical flow cytometer, the sample is carried in a stream of liquid through a laser beam and three primary measurements are made: forward light scatter (cell size), side light scatter (cell refractivity or granularity) and excited fluorescent dyes emission. The fluorescence components can be correlated with the size, structure and other physical aspects of cells. Suspension cell systems such as blood and bone marrow cells and tumor single-cell suspension are ideal for flow cytometric analysis. It is easy to obtain high-quality information by labeling cells with polyclonal or monoclonal antibodies against surface antigens or receptors. The more complex instruments, known as

cell sorters, can recover specific cells after measurement for further cell culture and biochemical analyses.

The biggest advantage of flow cytometry is the combination of multiparameter measurements and high-speed analysis. Owing to recent advances in laser and fluorochrome technology, commercially available systems allow detection of up to 18 colors at analysis rates of more than 20,000 events per second (70,000 theoretically). The tremendous amount of data allows analysis of multiple characteristics of such rare cells as antigen-specific T lymphocytes, including their phenotype and functional activity. Flow cytometric assays are currently widely used for enumeration and phenotypic characterization of lymphocytes, measuring cytokines and other secreted molecules, intracellular signaling, function and cell proliferation [19–21].

Numerous attempts have been made to develop flow cytometry-based methods to overcome some of the limitations of the ^{51}Cr -release and recently introduced ELISpot assays. Flow cytometric cytotoxicity assays provide several advantages, including: the avoidance of radioactive compounds; the detection of cytotoxicity at the single-cell level; evaluation of all stages of the cytotoxic process; and the possibility of characterizing the phenotype of involved cells. A number of flow cytometric cytotoxicity assays have been recently offered and evaluated (Figure 1).

Flow cytometric monitoring of cell-mediated target cell death

Although numerous flow cytometric assays for the evaluation of cell-mediated target cell death have been developed (Figure 1), the most popular approaches are based on detection of caspase activation; annexin V binding to apoptotic cells; and uptake of propidium iodine (PI) or 7-amino-actinomycin D (7-AAD) by dead or dying target cells.

As it was discussed earlier, following T-cell receptor recognition of antigenic peptide–MHC class I complexes on the surface of target cells, CTLs induce target cell apoptosis either through directed exocytosis of perforin and granzymes or by the Fas/FasL signaling pathway. An immediate event following both types of cytotoxic signaling is the activation of the caspase cascade in targeted cells. He *et al.* described a highly sensitive flow cytometry-based CTL assay to measure the cleavage of caspase-3 in target cells [22]. The assay involved labeling of P815, EL4 and T2 lymphoma cells with a cell tracker dye DDAO-SE and staining permeabilized cells with antibody against cleaved (activated) caspase-3. This assay proved to be robust and reliable in evaluating antigen-specific CTL activity in a number of human and murine systems, including mixed lymphocyte responses, human peptide-specific T-cell responses induced *in vitro*, and CTL responses following immunization of mice with viral and peptide vaccines. The data obtained were comparable with the results of the ^{51}Cr -release assay, but displayed higher sensitivity. Targets can also be labeled with Paul Karl Horan (PKH)26 [23] and after incubation with effectors can be stained with antibody highly specific for the cleaved form of caspase-3. Liu *et al.* developed a flow cytometry CTL (FCC) assay based on measurement of CTL-induced caspase activation in target cells through the detection of specific cleavage of fluorogenic caspase substrates [24,25]. The authors have shown that this assay reliably detected antigen-specific CTL-mediated killing of target cells. The assay provided a more sensitive, informative and safer alternative to the standard ^{51}Cr -release assay. According to Chahroudi *et al.*, the caspase-based FCC assay that measures the CTL-induced caspase activation in target cells using cell permeable fluorogenic caspase substrates is faster and safer than standard ^{51}Cr -release assay, more sensitive (since it detects the early marker of apoptosis), allows characterization of a single cell and evaluation of primary target cells, and also allows the sorting experiments to be performed for additional analyses [26].

Another approach, based on the availability of live cell fluorescent GrB substrate, was recently introduced. The appearance of GrB activity in the cytoplasm of target cells can be used at the single-cell level to quantitate and visualize cellular attack by effector cells [27]. In the early study, Packard *et al.* combined the GrB substrate with a second fluorogenic substrate selective for caspase-3 to perform both flow cytometry and fluorescence confocal microscopy studies of cytotoxicity [28].

Another very popular approach for the evaluation of cell-mediated cytotoxicity is based on annexin V binding to apoptotic cells. Annexin V has a high affinity for phosphatidylserine (PS), a phospholipid normally found in the inner leaflet of the plasma membrane. Upon induction of apoptosis, PS is externalized, resulting in its accessibility to exogenous annexin V. This, therefore, provides a convenient *in vitro* tool to measure apoptotic cell death. For example, an assay based on two-color flow cytometry has been developed by Goldberg *et al.* to measure CTL- and NK cell-mediated cytotoxicity [29]. After effector/target cell incubation, CTL or NK populations were stained with an effector cell-specific phycoerythrin (PE)-conjugated antibody, and annexin V–fluorescein isothiocyanate (FITC) was added to detect cells with PS on the surface. Target cells were gated upon as PE negative. A strong correlation between cytotoxicity measured with the proposed assay and a standard ^{51}Cr -release assay was revealed. The flow cytometry-based assay showed increased sensitivity at early time points after incubation and provided an opportunity for analysis of target cells at the single cell level. A similar strategy was used by others, when targets and effectors were stained with specific monoclonal antibodies and a combination of annexin V and PI was used to identify apoptotic/dead cells [30].

Light scatter alone is usually inadequate for differentiating dead from live cells. Probably the most widely used approaches for the evaluation of target cell death are based on uptake of DNA intercalating fluorescent agents such as PI due to increased cell membrane permeability of dying target cells. PI carries two positive charges, which prevents it from entering intact (live) cells. When complexed to DNA, PI is excited by 488 nm to give a bright signal. Since effector cells may also undergo apoptosis, it is important to differentiate targets and effectors when evaluating target cell death. For this purpose, different effector or target cell labeling procedures can be utilized, especially those that employ the PKH family of lipophilic dyes. Several assays have been described in which a combination of fluorescent dyes and DNA-intercalating dyes was used to directly visualize dying cells. For instance, Karawajev *et al.* have used FITC to directly label the membranes of target cells and PI to discriminate between live and dead cells [31]. Mattis *et al.* [32] and Piriou *et al.* [33] utilized DiOC18₍₃₎ target cell labeling in combination with PI staining of dead cells. Flieger *et al.* used differential staining employing PKH2 to label targets and PKH26 to label effectors, and used PI for live/dead cell discrimination [34]. Hatam *et al.* demonstrated the effectiveness of using PKH26 to label the K562 target cells and PI intercalation into killed target cell DNA to determine the percentage of target cells killed by effector NK cells [35]. This method compares favorably with the ^{51}Cr -release assay, and is quicker and easier to perform. Comparing a standard ^{51}Cr -release assay with DiOC18₍₃₎/PI-based flow cytometry assay for evaluating NK cell cytotoxicity, Motzer *et al.* reported that intra-assay variability between methods differed only at the lowest effector–target ratios evaluated [36]; inter-assay variability was wide but did not differ between methods. However, with strong a correlation between methods, cytotoxicity detected by ^{51}Cr release was higher than that detected by flow cytometry for all tested subjects. In conclusion, the authors found no compelling reason to adopt NK cell cytotoxicity by flow cytometry over ^{51}Cr release [36]. In the *In Vitro/In Vivo* Technique for Assessing Lysis (VITAL) assay [37], a novel approach has been developed incorporating the use of multiple target cell populations labeled with different dyes (5- and 6-carboxyfluorescein diacetate succinimidyl ester [CFSE] or chloromethyl-benzoyl-aminotetramethyl-rhodamine), so that cytotoxicity can be assessed against titrated

doses of a given antigen, or against a range of different antigens simultaneously; live/dead discrimination is based on PI-negative gating. Target-cell death within the PKH26-positive target cell population can also be assessed by the addition of the viability probe, TO-PRO-3 iodide (He-Ne laser-excitable fluorescent dye) [38,39]. Ozdemir *et al.* described a flow cytometric approach for determination of *in vitro* cell-mediated cytotoxicity utilizing three-color flow cytometric assay [40]. The basic strategy involves labeling effector or target cells with specific fluorochrome-conjugated antibodies, in addition to staining with annexin V-FITC and PI to identify apoptotic/dead cells. The effector and target cell populations, as well as conjugates, were clearly and easily identified. Significant correlation was found between cytotoxicity calculated by this technique and ^{51}Cr -release assay results [40]. A four-color flow cytometric assay based on differential staining of both target and effector cells with monoclonal antibodies conjugated with up to four different fluorochromes was developed by Zimmerman *et al.* [41]. Differential immunostaining of both target (CD10, CD34, CD13 or CD33) and effector cells (CD3 or CD56) was performed prior to the reaction. Cytotoxicity of NK and T-cell activity against leukemic cells was calculated by the specific loss of target cells. This assay was optimized later, introducing a novel five-color flow cytometric assay for the evaluation of NK cell activity against adherent tumor cells [42]. Besides an enhanced cytotoxic activity corresponding to increasing effector/target ratios, the authors could demonstrate an increasing cytotoxicity in a time-dependent manner over a time period of 8 h. Thus, this novel flow cytometric cytotoxicity assay enabled efficient quantification of the phenotype of both effector and adherent target tumor cells, and therefore represents a useful tool for research on immunotherapies that rely on cytotoxic effector cells.

It is important to notice that the most commonly used alternative to PI is 7-AAD. It intercalates into double-stranded nucleic acids and, thus, it is excluded by viable cells but can penetrate the cell membranes of dying or dead cells. Fluorescent molecules of 7-AAD can be excited by 488 nm argon laser and emit in the far red range of the spectrum ($\lambda_{\text{em,max}}$: 655 nm). Its spectral emission can be effectively separated from the emissions of PE ($\lambda_{\text{em,max}}$: 578 nm) and PKH67 ($\lambda_{\text{em,max}}$: 502 nm). This distinguishes 7-AAD from PI ($\lambda_{\text{em,max}}$: 625 nm), which has significant spectral overlap with PE. We found that the levels of uptake of 7-AAD and PI by dead target cells were virtually identical [Derby E, Malyguine A, Unpublished Data]. Measurement of annexin V binding, executed simultaneously with the PI or 7-AAD uptake, provides an excellent way to detect apoptotic cells and to discriminate between different stages of apoptosis at the single-cell level. Aubry *et al.* used PKH26 for labeling of effector cells, employed Annexin V-FITC, which allows for the direct evaluation of early apoptotic cells and PI, which distinguishes late apoptotic cells with compromised membranes [43]. By excluding PKH26-positive effector cells from the analysis, the authors estimated the percentage of PKH26-negative (target) cells in the early stages of apoptosis as well as late apoptotic and necrotic cells. Lecoeur *et al.* also approached effector/target discrimination by labeling effectors [44]. CFSE was used to distinguish effector cells, while the annexin V/7AAD combination was used to determine early and late apoptosis and necrosis in the CFSE-negative target cell population. We have used PKH67 to label target cell membranes and annexin V-PE and 7-AAD to estimate cell death [45]. A strong direct correlation has been found between the percentage of dead target cells in the flow cytometry assay and the results of ^{51}Cr -release assay when human lymphokine-activated killer cells and CTLs were used as a model system. We have shown also that both NK cells and CTLs kill tumor cells mostly by granule-mediated mechanisms, as lysis was blocked by a perforin inhibitor concanamycin A (folimycin), but was significantly less sensitive to carbobenzoxy-valyl-alanyl-aspartyl-[0-methyl]-fluoromethylketone caspase (zVAD-FMK) inhibitor. This method allows a reliable discrimination of target and effector cells while at the same time providing a fine difference between the two major events, early apoptosis and the increase in membrane permeability. We have also examined mechanisms of target cell lysis by effector T cells from gene-

targeted and mutant mice using this flow cytometry assay [46]. Target cells were labeled with PKH67 dye. Cell death was estimated by 7-AAD inclusion and annexin V-PE binding. A direct correlation has been found between the percentage of dead target cells in flow cytometry assay and the results of indium (^{111}In)-release cytotoxicity assay when effector T cells from either perforin-knockout ($Pfp^{-/-}$) or $FasL^{gld}$ (nonfunctional FasL) mice were used. As was revealed, the granule-mediated mechanism was utilized by T cells from $FasL^{gld}$ mice; by contrast, T cells from $Pfp^{-/-}$ mice used death receptor-mediated lysis [46]. Therefore, the flow cytometric assay could be applied irrespective of which cytotoxic effector pathway is involved.

Consequently, evaluating target cell death by flow cytometric assays has clear advantages over the standard ^{51}Cr -release assay, since they are safer, usually more sensitive and provide a fine distinction between the early apoptosis and the increased membrane permeability during early and later events in cell death.

Flow cytometric monitoring of effector-cell frequency & activity

Several methods have been developed to identify antigen-specific CD8^+ T cells. These include, most notably, MHC class I tetrameric complexes [47–49], intracellular cytokine staining [50–53] and a combination of these methods [54,55]. Both of these techniques can provide valuable information regarding the frequency, phenotype and/or the functionality of antigen-specific T cells, and can be effectively applied to clinical studies. However, these assays do not examine the ability of the CD8^+ T cells to elicit cytotoxic activity since it is well documented that many tetramer-positive cells do not produce detectable levels of $\text{IFN-}\gamma$ after direct *ex vivo* stimulation with cognate peptide; CD8^+ T cells that produce cytokine after stimulation are not always cytotoxic; and tetramer-positive cells occasionally fail to kill targets expressing the specific epitope [56]. Furthermore, cytotoxic lymphocytes can mediate cell death by Fas–FasL interaction and by the secretion of cytotoxic molecules including GrB and perforin. GrB and perforin are released from cytotoxic lymphocytes by the exocytosis of cytoplasmic granules, which are membrane-enveloped lysosomes within cytotoxic effector cells. The lipid bilayer surrounding cytotoxic granules contains lysosomal-associated membrane glycoproteins (LAMPs) including CD107a (LAMP-1), CD107b (LAMP-2), and CD63 (LAMP-3). Under appropriate stimulation of the cytotoxic effector cell, degranulation occurs when microtubules are mobilized by polarization and transport the granules toward the synapse formed between the effector and target cell [57]. Once the granules reach the plasma membrane of the cytotoxic cell, the membranes fuse, allowing the lysosome to release its contents (granzymes and perforin) into the synapse. The result is the eventual death of the target cell. As a consequence of the fusion of lysosomal and cellular membranes during the degranulation process, glycoproteins once exclusive to the lysosomal membrane are now expressed on the cell surface. With the advent of antibodies specific for CD107a and CD107b, this process can be assessed using flow cytometry techniques. Anti-CD107 antibodies will attach to the surface of any effector cell that is expressing CD107 due to degranulation.

Betts *et al.* presented a novel technique to enumerate antigen-specific CD8^+ T cells based on visualization of exposure of CD107a and CD107b onto the cell surface as a result of degranulation [58,59]. This exposure was associated with loss of intracellular perforin and was inhibited by colchicine and, therefore, was dependent on degranulation. CD107-expressing CD8^+ T cells were shown to mediate cytotoxic activity in an antigen-specific manner. Measurement of CD107a and CD107b expression can also be combined with MHC class I tetramer labeling and intracellular cytokine staining. It has also been shown that CD107 expression on *ex vivo*-activated CD8^+ T cells provides a more accurate assessment of degranulation than CD63 expression does. The latter has been previously used as a

positive marker associated with the deposition of FasL on the cell surface during degranulation in ionomycin-stimulated CD4⁺ and CD8⁺ T-cell clones. Flow cytometry-based detection of CD107a and b finds more and more recognition as a reliable assay in clinical monitoring. For example normal, HIV- and vaccinia-infected patients were tested by CD107a and intracellular cytokine staining by Casazza *et al.* [60]. The expression of CD107a can be used to isolate tumor-cytolytic T cells from blood samples of cancer patients [61]. Authors combined CD107a mobilization with peptide–MHC tetramer staining to directly correlate antigen specificity and cytolytic ability at a single-cell level. It was demonstrated that tumor-cytolytic T cells with high recognition efficiency represent a minority of peptide-specific T cells, which could be expanded *ex vivo* while maintaining their cytolytic potential. The ability to rapidly identify and isolate tumor-specific cytolytic T cells might open novel opportunities to improve the efficacy of cancer immunotherapy approaches. Patients with metastatic renal cell carcinoma were evaluated by Kaufman *et al.* [62]. Phenotypic characterization was done by four-color flow cytometry analysis of peripheral blood mononuclear cells (PBMCs) using different antibodies, including CD107a, anti-GrB and anti-perforin. Walker *et al.* characterized the phenotype and function of gp100 peptide-specific memory CD8⁺ T cells in melanoma patients after primary gp100 (209–2M) immunization [63]. Gp100 peptide-induced *in vitro* functional avidity and proliferation responses and melanoma-stimulated T-cell CD107 mobilization were compared for cells from all time points for multiple patients.

Interestingly, the CD107a degranulation assay is also applicable for evaluation of NK cell activity. For example, NK cells from HIV-1-infected subjects with detectable viral loads expressed significantly higher levels of CD107a [64]. CD107a expression correlated with NK cell-mediated cytokine secretion and cytolytic activity, suggesting that CD107a represents a good marker for the functional activity of NK cells [65]. Tomescu *et al.* have observed that sorted CD107a⁺ NK cells exhibited continued lytic potential against a wide variety of target cells, including tumor and virally infected target cells [66]. CD107a-positive and CD107a-negative sorted NK cells displayed similar long-term viability, killing potential and response to inflammatory cytokines.

Flow cytometric monitoring of cell-mediated target cell death & effector-cell frequency & activity simultaneously

For more accurate monitoring of cell-mediated immunity, an ideal assay should evaluate both the target cell death and effector cell function simultaneously. Several approaches were used to address this issue (Table 1). Kim *et al.* developed a new four-color FCC assay to simultaneously measure NK cell cytotoxicity and NK cell phenotype [67]. Target K562 or Daudi cells were labeled with cell tracker orange prior to the addition of effector cells; after coincubation, 7-AAD was added to measure death of target cells. The phenotype of effectors, viability of targets, the formation of tumor–effector cell conjugates and absolute numbers of all cells were measured. In this study, cell tracker orange reflected the number of remaining targets; 7-AAD indicated cell death by necrosis and late apoptosis; anti-CD3, anti-CD16 and anti-CD56 antibodies detected phenotypes of the effector cells; anti-CD69 antibody picked early activation marker indicating effector cell function; anti-GrB antibody measured proapoptotic protease marker for target cell lysis and the number of effector–target conjugates estimated the ability of effector cells to bind to the targets. The proposed FCC assay is more sensitive than the ⁵¹Cr-release assay and reliably measures NK cell-mediated killing of target cells in normal controls and subjects with cancer [67].

A highly sensitive and reproducible multiparametric flow cytometry-based cytotoxicity assay utilizing low numbers of antigen-specific T cells has been recently described by Devevre *et al.* [68]. Peptide/MHC multimer-positive CD8⁺ T cells were purified by

fluorescence-activated cell sorting from PBMCs of healthy donors, cloned by limiting dilution and periodically expanded. Purified T cells were tested for CD107a cell surface expression and their cytotoxicity evaluated based on the frequency of dead cells in chloromethyl-benzoyl-aminotetramethyl-rhodamine-labeled target cell populations using 4', 6-diamidino-2-phenylindole dihydrochloride. This LiveCount assay proved to be more sensitive than the ^{51}Cr -release assay allowing the measurement of cytotoxic activity at low lymphocyte to target cell ratios and the characterization of effector cells. However, one limiting factor is the requirement for sufficient lymphocyte cell numbers after coincubation with target cells to allow post-lytic activity characterization of the CD8^+ T cells involved. Another limitation is a necessity for purification of the antigen-specific CD8^+ T-cell population by fluorescence-activated cell sorting. To date, the LiveCount assay represents one of a very few possibilities of assessing the *ex vivo* cytolytic activity of low-frequency antigen-specific CTLs from patient material.

Previously, we developed a three-color flow cytometric assay to measure cell-mediated cytotoxicity [45]. With the objective of simultaneously evaluating target cell death and effector cell frequency, we later combined the measuring of the expression of the degranulation marker CD107a by effector cells with the apoptosis marker annexin V binding to target cells. Using human cytotoxic antipeptide as well as allogeneic CTLs, we found a significant incubation time-dependent increase of surface CD107a expression on effector cells due to degranulation. A parallel increase of annexin V binding to target cells due to target cell apoptosis was also found. These two parameters have shown excellent correlation in all effector/target cell systems studied. To find possible connections between effector cell degranulation (CD107a expression), GrB secretion and target cell death (annexin V binding), the GrB ELISpot assay and flow cytometric assay were performed and excellent cross-correlation was found between all three parameters. The specificity of the assay was also demonstrated [69].

Next, we further optimized the assay by adding CD8 staining as an additional parameter to determine CD107a expression only within the CD8^+ population of effector cells to ensure that its expression is reflective of antigen-specific degranulation (Figure 2) [70]. Although our previously described assay was developed by using *in vitro*-stimulated CTLs generated from normal donor PBMCs with known reactivity to viral peptides, the end point application of the current assay was to evaluate clinical samples from patients vaccinated with different peptides. To this end, we used cryopreserved samples of PBMCs from vaccinated melanoma patients with known response to the gp100:209 in IFN- γ and GrB ELISpot assays. Samples were kindly provided by Steven Rosenberg of the US NIH. In the initial ELISpot assays, these PBMCs were thawed and stimulated *ex vivo*. Owing to the high sensitivity of the ELISpot assays, use of PBMCs was appropriate and immune responses were detected. Activity against the relevant g209 and the control g280 peptide measured in the flow cytometry assay was directly compared with results obtained from the GrB ELISpot assay and the standard ^{51}Cr -release assay run in tandem. However, *ex vivo* responses to melanoma antigens were not detected in the flow cytometry assay, probably due to the fact that the level of antigen-specific precursors within the samples was below the level of the assay's sensitivity. To enhance the precursor frequency within the PBMC samples, *in vitro* stimulation of the clinical samples was performed. Nonpulsed target cells incubated with specific CTLs were used as background control for cell loss. On the basis of our findings from the direct comparison of GrB and IFN- γ secretion by patient PBMCs and their stimulated CTL cultures, we concluded that this stimulation did not artificially generate antigen-specific CTLs [70]. Therefore, the use of *in vitro*-stimulated antigen-specific CTLs is appropriate to measure immune responsiveness to vaccination by the flow cytometric assay. When 7-day CTLs were used, little or no effector cell degranulation or cytotoxicity was measured flow cytometry in prevaccination samples. After vaccination, an increase in

both degranulation and target cell death could be determined when target cells were pulsed with g209. No or very low reactivity was found against g280 at any time point. Our findings exhibited an excellent correlation between CD107a expression and GrB secretion in ELISpot and also annexin V binding to target cells and specific lysis measured in the ^{51}Cr -release assay. Results obtained with the flow cytometry assay were highly reproducible. Further testing of additional clinical material is warranted to substantiate the reproducibility and to validate the assay. Regardless, based on our initial findings, flow cytometry can be applied for monitoring of clinical samples for CTL effector reactivity. The proposed assay measures the cytolytic effector cell activation, frequency and phenotype, and also target cell death in the same sample. This format allows for a more rapid and efficient acquisition of both tumor target cell cytotoxicity and CTL activation while saving labor and valuable patient material. In addition, analyzing multiple parameters in one sample helps to avoid inter-sample variations [70].

Expert commentary

The therapeutic use of the immune system to attack tumor cells has been a longstanding vision among tumor immunologists and clinicians. However, evaluation and comparison of immunotherapeutic clinical trials require a proper analysis of immune responsiveness in treated patients. To date, unfortunately, the proper immunologic responses induced by vaccination that result in effective tumor rejection have not been elucidated. One significant unresolved issue in modern immunotherapy is the observation that the tumor-specific cellular immune response that follows the course of immunotherapy does not always lead to clinically proven cancer regression. This is despite the ability of patients' T cells to produce immunostimulatory cytokines and the generation of tumor-specific cytotoxic lymphocytes able to recognize and efficiently kill tumor cells *ex vivo*. Based on large amounts of clinical data, appreciable numbers of treated patients exhibited the tumor-specific T-cell response, while only a small percentage of these patients experienced clinically noticeable tumor regression. This devastating lack of a correlation between the tumor-specific cytotoxic immune responses and clinical efficacy of immunotherapy may be explained, among other reasons, by the lack of accepted uniform standards for immunological monitoring of clinical trials. Furthermore, it is very unlikely that the analysis of any single immunological parameter is appropriate to provide clinically feasible information regarding extremely complex interactions between different cell subsets in the tumor milieu. The past few years have been productive in terms of advancing our understanding of the cellular and molecular mechanisms involved in the initiation of efficient antitumor immunity and the breaking of immune tolerance to tumor cells. Based on this knowledge, it is conceivable that assays that allow simultaneous measurement of several immunological parameters may be more advantageous for clinical monitoring of patients in clinical trials. The capability of current flow cytometry-based techniques to deliver multiparameter data enables a new biomarker-based approach for monitoring various characteristics of immune reactions in cancer patients.

A fuller understanding of the mechanisms by which NK cells and CTLs eliminate or control tumors should form a rational basis for significant improvement of the efficacy and comparability of immunotherapeutic approaches to cancer. One of the most utilized assays available for evaluation of cell-mediated cytotoxicity is the ^{51}Cr -release assay; while being highly reproducible and relatively easy to perform, it has several serious drawbacks limiting its further development and utilization. The ELISpot assay has recently gained increasing popularity for immunomonitoring of clinical trials. It can reliably assess both CTL frequency and function, but cannot provide any information about direct lysis of target cells. Similarly, although MHC class I tetramer assays, intracellular cytokine analyses and the combinations of these methods are quite popular, they do not allow measurement of

cytotoxicity directly and the results of these assays do not always correlate with cellular cytotoxicity.

Numerous attempts have been made to develop flow cytometry-based methods to overcome limitations of the currently available cytotoxicity assays. The flow cytometry technique, combining multiparameter measurements and high-speed analysis, allows the measurement of several characteristics of relatively rare antigen-specific T lymphocytes, including their numbers, phenotype, activity and functionality. Modern flow cytometric cytotoxicity assays provide significant advantages over other methodologies, which include the absence of radioactive compounds, detection of cytotoxicity at the single-cell level, evaluation of all stages of the cytolytic process and the opportunity to characterize multiple subsets of involved cells.

The most promising approaches for the assessment of cell-mediated cytotoxicity by flow cytometry are based on the detection of at least three groups of parameters: activation markers on the effector cells, particularly CD107a; markers of induction of apoptotic pathways on target cells (e.g., caspase activation and annexin V binding); and markers of cell membrane permeability (PI and 7-AAD). Importantly, evaluation of target cell death by flow cytometry has clear advantages over the standard ^{51}Cr -release assay, including safety, higher sensitivity, and the opportunity to distinguish between the early apoptotic events and an increase in membrane permeability, which reflects the later events in cell death. There are a few flow cytometry-based cytotoxicity assays that simultaneously measure the cytolytic effector cell activation, frequency and phenotype, as well as target cell death. In spite of the fact that these assays are not yet widely utilized in ongoing clinical trials, the format of these assays supports their growing popularity, allowing a rapid and efficient acquisition of both tumor target cell cytolysis and cytotoxic cell activation, while saving labor and valuable patient material. Wide utilization of flow cytometry-based assays will enable researchers to readdress the issues of positive correlation between the level and functional activity of tumor-specific CTLs and patients' clinical responses to immunotherapeutic intervention. Undoubtedly, these data will help significantly improve the design of cancer vaccines and, consequently, the efficacy of cancer clinical protocols in the near future.

Five-year view

Monitoring of immunotherapeutic clinical trials has undergone a considerable change in the last decade, resulting in a general agreement that immune monitoring should guide the development of cancer vaccines. The emphasis on immune cell functions and quantitation of antigen-specific T cells has been playing a major role in attempts to establish correlations between therapy-induced alterations in immune responses and clinical end points. Based on current knowledge, the cytotoxic immune response to vaccination represents an extremely complex array of interactions between immune, tumor and stromal cells and, therefore, cannot be realistically evaluated based on a single immunological parameter or cell type. By contrast, a comprehensive systemic approach is required for improving the quality of serial monitoring to ensure that it adequately and reliably measures changes induced in patients by administered vaccines or immunomodulators. Many novel tools are now available to detect immune responses against known and unknown tumor antigens, including MHC tetramers, ELISpot assays and cytokine release/catch assays. However, very often no correlation was observed between the reactivity against specific tumor antigens and the clinical outcome.

Over the last decade, our knowledge of the immune system has greatly increased, partly due to the development of flow cytometry, and today, multicolor flow cytometry is one of the most powerful tools for the study of the immune system. Although developing a reliable multicolor panel is time consuming and requires a number of validation trials, the amount of

information provided by such a panel, compared with two- to four-color assays, aids in our understanding of the immune system, potentially defining cell subsets that are crucial for the appropriate evaluation of patients' responses to therapy. Flow cytometry-based cytotoxicity assays for immunomonitoring are still in their infancy, but a number of variables are recognized that can contribute to the exploitation of the assays' full potential. The ability of current flow cytometry techniques to deliver multiparameter data enables a new biomarker based approach for monitoring variable parameters of immune reactivity and immune response development. Flow cytometry assays, which are capable of registering the cytotoxic effector cell activation, frequency and phenotype, in addition to assessing target cell death in the same sample, will provide valuable information while saving labor and consumables. In addition, using multicolor flow cytometry can decrease the amount of blood needed for immunomonitoring, which is often limited, especially in clinical protocols. This could be an important addition to the available repertoire of tests used for monitoring patients' responses in immunotherapeutic cancer trials. Further development and improvement of flow cytometric equipment, reagents, software, data analysis, as well as the introduction of new approaches and biomarkers, will hopefully enhance our understanding of immune reactions associated with successful immunotherapy.

Creating centralized Clinical Improvement Act/Good Laboratory Practice-certified immunomonitoring/flow cytometry laboratories and international networks seems to be essential to perform comprehensive multiparameter monitoring of cell-mediated immune responses, and to standardize and validate the assays to be used. Validation of newly developed assays by several reference laboratories should help to accelerate incorporation of feasible standardized cytotoxicity assays into current clinical practice. Another major breakthrough in the near future might be the application of 12–15-color flow cytometry that evaluates other effector and regulatory immune cells in addition to cytotoxic lymphocytes, as well as their potential interaction with targeted tumor cells. This has now proven to be effective and informative for characterizing blood cell subsets in patients and, in comparison to many *ex vivo* functional assays, is much more straightforward. Finally, strengthening interactions between the clinical investigator, the clinical immunologist and the biostatistician seems to be crucial for successful introduction, validation and use of novel or improved immune monitoring methods in clinical studies.

Key issues

- Despite substantial evidence suggesting that cell-mediated immune responses are imperative in controlling tumor growth in cancer patients, multiple clinical trials have revealed that the immune responses do not very often reflect the clinical outcome in patients with cancer.
- The exact immunologic responses induced by vaccination and resulting in effective antitumor immunity in cancer patients have not been fully elucidated. An understanding of the mechanisms by which natural killer cells and cytotoxic T lymphocytes can kill tumors may form a rational basis for significantly improving the efficacy of cancer immunotherapy.
- Numerous attempts have been made to develop flow cytometry-based methods to overcome the many limitations of currently available cytotoxicity assays. Flow cytometric cytotoxicity assays provide several advantages, including the avoidance of radioactive compounds, the detection of cytotoxicity at the single-cell level, evaluation of all stages of the cytolytic process and the possibility of widely characterizing involved cells. They proved to be reliable, sensitive, specific and highly efficient. Flow cytometry combines measurements of multiple cellular parameters and high-speed analysis, allowing researchers to

analyze crucial characteristics of such rare cells as antigen-specific T lymphocytes, including their phenotype and functional activity.

- There is a good correlation between the results obtained from flow cytometry-based cytotoxicity assays and the results of the ^{51}Cr -release and ELISpot assays.
- The most popular approaches for the evaluation of cell-mediated cytotoxicity by flow cytometry are based on detection of activation markers on effector cells and signs of activation of apoptotic pathways in targets cells, as well as an increase of target cell permeability and lysis.
- Several flow cytometric cytotoxicity assays that simultaneously measure the cytotoxic effector cell activation, cell frequency and phenotype, as well as target cell death, in the same sample are currently available.
- Although these assay have, to date, limited clinical application, this format allows for a more rapid and efficient acquisition of both tumor target cell lysis and cytotoxic cell activation, while saving labor and valuable patient materials.
- Further development and improvement of flow cytometric cytotoxicity assays, which enable detection and enumeration of tumor-specific cytotoxic T cells and their specific effector functions as well as target cell killing, will be crucial for improving both cancer vaccine design and immunomonitoring of patients in the future.

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Figure 1. Cytotoxicity assays based on flow cytometry

(1) Labeling cells with fluorescent dyes, transfection with GFP or fluorescent antigens. Labeling can be done using: fluorescein isothiocyanate labeling [31]; diiodoacetylcarboxycyanine perchlorate₍₃₎ (DiOC18₍₃₎) [32,33,103]; calcein acetoxymethyl ester [104]; 2',7'-bis-(2-carboxyethyl)-5 (and-6)-carboxyfluorescein (BCECF) [105]; carboxyfluorescein succinimidyl ester or chloromethyl-benzoyl-aminotetramethyl-rhodamine [37,44,67,106]; PKH dyes [34,38,39,43,45,46,70,107]; transfection, transduction with GFP or fluorescent antigens [108–112]. (2) Detection of caspase activation and/or GrB substrate cleavage in target cells, via staining with mAb to activated caspases [23,113,114]; detection of cleaved fluorogenic caspase substrate [24–26,28] or detection of cleaved fluorogenic GrB substrate [27,28]. (3) Detection of target apoptosis by annexin V binding to 'flipped' phosphatidylserine [29,40,43,45,46,69,70,105,107,115–118]. (4) Uptake of DNA intercalating fluorescent agents due to increased cell membrane permeability of dying target cells. Fluorescent agents are propidium iodide [31–34,37,41,105,107,108]; TO-PRO[®]3 [38,39] or 7AAD [44–46,67,118]. (5) Analysis of effector cells degranulation with anti-CD107 mAb staining [19,58–61,68–70,98,119–121]. (6) Cytokine flow cytometry. Provides valuable information regarding the functionality of antigen-specific T cells, but does not accurately examine the ability of the CD8⁺ T cells to elicit cytotoxic activity [12,20,50,51,54,55,122–125]. (7) Effectors staining with MHC tetramers. Provides valuable information regarding the frequency and phenotype of antigen-specific T cells, but does not

accurately examine the ability of the CD8⁺ T cells to elicit cytotoxic activity [47,48,55,59,⁶¹,⁶⁸,119,120,126–128]. **(8)** Phenotypic and activation markers staining [29,40,41,56,67,97,129]. **(9)** Detection of uptake of target membrane or antigens by effector cells [130–133]. **(10)** Detection of effector–target conjugate formation. Conjugate formation between target cells and cytotoxic cells while evaluates effector frequency has been shown to occur independently of killing [67,134,135]. 7AAD: 7-Aminoactinomycin D; GFP: Green fluorescent protein; GrB: Granzyme B; mAb: Monoclonal antibody; PKH: Paul Karl Horan; PS: Phosphatidylserine; TNF: Tumor necrosis factor.

Figure 2. Application of a flow cytometric cytotoxicity assay for monitoring a cancer vaccine trial

Target cell death is measured using annexin V binding to PKH67-positive cells and CTL degranulation determined by CD107a staining on the CD8-positive cells. The gp100:209-specific CTLs were generated by incubation of melanoma patients' peripheral blood mononuclear cells for 1 week with g209 peptide in the presence of IL-7 and IL-2.

APC: Antigen-presenting cell; CTL: Cytotoxic T lymphocyte; GrB: Granzyme B; PE: Phycoerythrin; PKH: Paul Karl Horan; PS: Phosphatidylserine.

Table 1

Flow cytometric assays for monitoring cell-mediated target cell death, and effector cell frequency and activity, simultaneously.

Study (year)	Target cell analysis		Effector cell analysis		Effector–target conjugates		Correlation with ⁵¹ Cr release	Correlation with GrB ELISpot	Clinical application shown	Ref.
	Fluorochrome-labeled target cells enumeration/gating	Early apoptosis	Cell permeability	Phenotype/ gating	Activation markers	Degradulation				
Devevre <i>et al.</i> (2006)	CMTMR	NT	DAPI	CD8/MHC tetramer (sorting)	NT	CD107a	Yes	NT	No	[68]
Burkett <i>et al.</i> (2005)	PKH67	Annexin V	NT	No	NT	CD107a	Yes	Yes	No	[69]
Kim <i>et al.</i> (2007)	CMTMR	NT	7-AAD	CD3, CD16, CD56	CD69 and ICS with GrB mAb	NT	Yes	NT	No	[67]
Zaritskaya <i>et al.</i> (2009)	PKH67	Annexin V	NT	CD8	NT	CD107a	Yes	Yes	Yes	[70] (Figure 2)

7-AAD: 7-aminoactinomycin D; CMTMR: Chloromethyl-benzoyl-aminotetramethyl-rhodamine; DAPI: 4', 6-diamidino-2-phenylindole dihydrochloride; ELISpot: Enzyme-linked immunospot assay; GrB: Granzyme B; ICS: Intracellular cytokine staining; mAb: Monoclonal antibody; NT: Not tested; PKH: Paul Karl Horan.