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Research paper

A versatile flow-based assay for immunocyte-mediated cytotoxicity☆

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ABSTRACT

Cell-mediated cytotoxicity is a critical function of the immune system in mounting defense against pathogens and cancers. Current methods that allow direct evaluation of cell-mediated cytotoxicity suffer from a wide-range of drawbacks. Here, we present a novel strategy to measure cytotoxicity that is direct, sensitive, rapid, and highly adaptable. Moreover, it allows accurate measurement of viability of both target and effector cells. Target cells are fluorescently labeled with a non-toxic, cell-permeable dye that covalently binds to cell proteins, including nuclear proteins. The labeled target cells are incubated with effector cells to begin killing. Following the killing reaction, the cell mixture is incubated with another dye that specifically stains proteins of dead cells, including nuclear proteins. In the final step, cell nuclei are released by Triton X-100, and analyzed by flow cytometry. This results in four nuclear staining patterns that separate target and effector nuclei as well as nuclei of live and dead cells. Analyzing nuclei, instead of cells, greatly reduces flow cytometry errors caused by the presence of target-effector cell aggregates. Target killing time can often be reduced to 2 h and the assay can be done in a high throughput format. We have successfully validated this assay in a variety of cytotoxicity scenarios including those mediated by NK-92 cells, Chimeric Antigen Receptor (CAR)-T cells, and Tumor Infiltrating Lymphocytes (TIL). Therefore, this technique is broadly applicable, highly sensitive and easily administered, making it a powerful tool to assess immunotherapy-based, cell-mediated cytotoxicity.

1. Introduction

Cell-mediated cytotoxicity is an essential element of the immune response. Cytotoxic lymphocytes (CTLs), like T cells and NK cells, accomplish this task through direct killing of target cells. Effector-target cell engagement results in the production and secretion of inflammatory cytokines, as well as direct target cell lysis by the delivery of toxins like perforin and granzyme, and/or death signals like Fas-L and TRAIL (Martinez-Lostao et al., 2015). Accurate measurement of cell-mediated cytotoxicity is a critical in vitro method to evaluate the killing ability of effector cells.

Various cytotoxicity assays have been developed, each with their own limitations. Among methods that use radioactive isotopes, the chromium release assay has been the most useful. Developed in the late 1960s, it involves the loading of target cells with Chromium-51 radionuclide, which is released into the supernatant upon cell lysis and measured (Brunner et al., 1968). Although an accurate reflection of the relative degree of cell death, radioactive assays do not provide data from individual cells and raise safety concerns.

Non-radioactive cytotoxic assays may be based on loading target cells with compounds that change fluorescence after killing, like time-resolved fluorescence resonance energy transfer (TR-FRET) probes and calcein (Granberg et al., 1988; Lichtenfels et al., 1994; Somanchi et al., 2015). However, these methods often have high levels of spontaneous release and variable cell labeling, which is dependent on intracellular esterase activity. Another set of assays, which measures the release of constitutively expressed molecules like lactose dehydrogenase, adenylate kinase, and glyceraldehyde 3-phosphate dehydrogenase, often exhibits poor sensitivity and does not distinguish between death of target and effector cells (Corey et al., 1997; Decker and Lohmann-Matthes,

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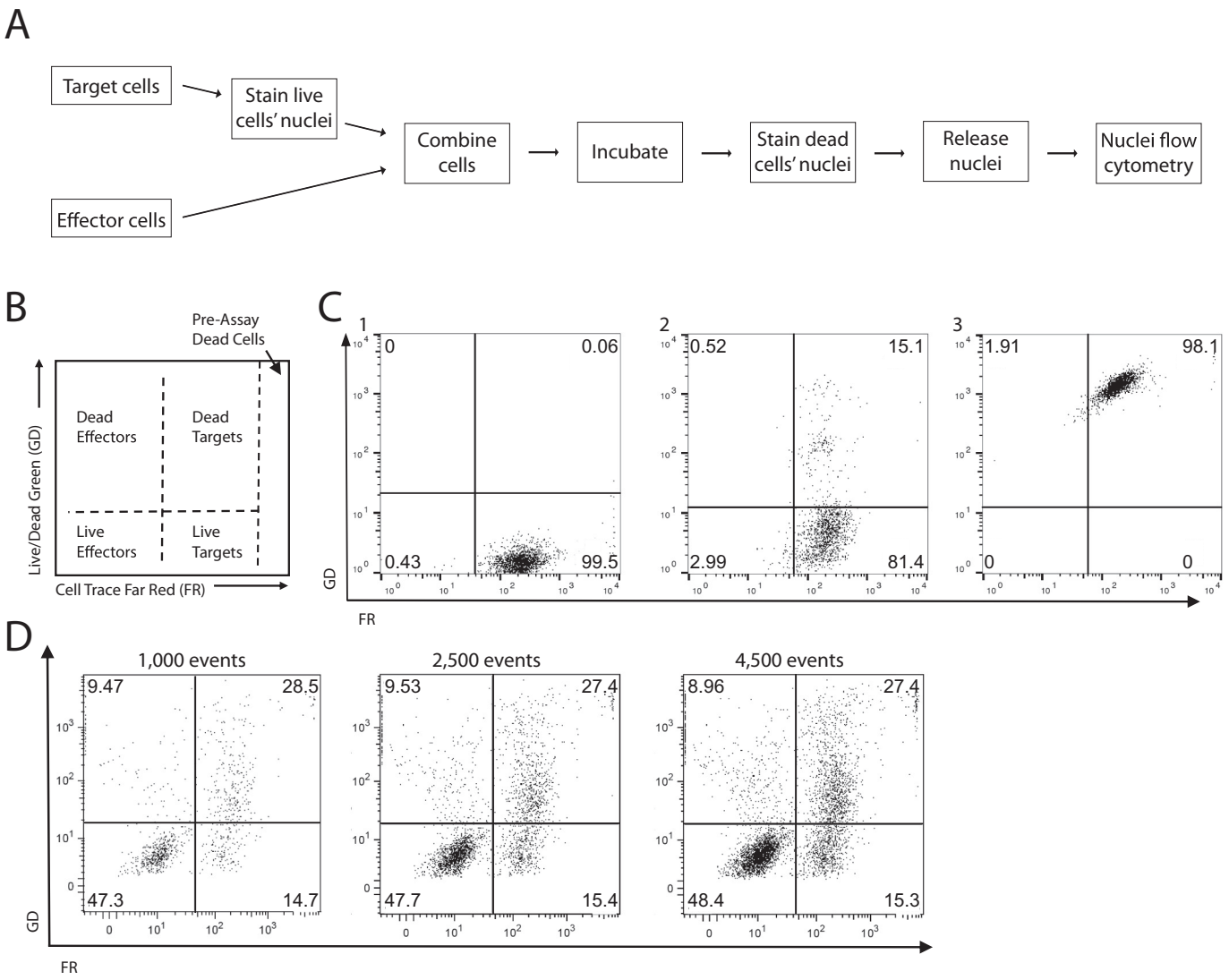

Fig. 1. Cytotoxicity assay scheme, development and reproducibility. (A) Flow chart of main parameters of the cytotoxicity assay. (B) Schematic showing that FR staining (red fluorescence) of target nuclei vs. unstained effector nuclei on the x-axis. Dead cells can be distinguished by GD (green fluorescence) on the y-axis. Any target cells that start already dead before the cytotoxicity assay (pre-assay dead cells) accumulate additional FR dye that places them in the far right column. (C) Target Daudi nuclei stained only with FR, with FR and then GD or with FR and then GD and Triton-X-100. (D) Flow cytometry analysis of a 2-hr cytotoxicity assay (NK-92 and Daudi) using increasing amounts of measured nuclei (left to right), shows target killing ratios of 64.7%, 62.7% and 62.4%. Representative data of at least 3 experiments is shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

1988; Olsson et al., 1983). Measurement of luciferase released from target cells is highly sensitive, but requires the target cells to be genetically manipulated to express the luciferase (Matta et al., 2018).

Indirect methods such as gamma interferon secretion by T cells allow fast and high throughput analysis of effector cell activation, but do not register target killing (Lalvani and Pareek, 2010). Recently developed impedance-based measurement of target cell killing allows fast and accurate measurement, but needs a sophisticated and expensive device. It relies on the measurement of the impedance of a monolayer of target cells attached to a chip (Peper et al., 2014), and is not useful for cells with low adhesiveness, such as hematopoietic targets.

All of the aforementioned methods analyze cell populations, and may not adequately represent the actual killing events. For example, Cr-51 released from killed target cells is not uniform, but rather varies for each cell. Therefore, the total quantity of chromium release may not adequately represent the actual proportion of dead cells in the population.

Flow cytometric analysis detects the killing on an individual cell basis, which is quite desirable, because it allows the measurement of the exact portion of a target population that is damaged, and flow cytometers can be used with multi-well plates in a high throughput fashion. However, target-effector aggregation, as an integral part of cell-mediated killing, can dramatically impede cell analysis, and lead to high levels of inconsistency.

In the approach described here, instead of whole cells, we analyze the nuclei of the cells obtained after a cytotoxicity reaction. This approach allows for the analysis of individual nuclei, free of aggregates. The nuclei are differentially labeled based on whether they originated from targets or effectors and whether they were in cells that were alive or dead at the conclusion of the assay. In addition, it is versatile for numerous killing assays involving cell lines, primary cells, cells growing as adherent or suspension culture, without the need for genetic modification of the targets, and is sensitive enough to use on small cell numbers, low E/T ratios, and short incubation times. Here we provide the examples of measuring in vitro specific cytotoxicity as follows: cell line target killing by NK-92 cells, CAR-mediated killing using human or murine T cells, and melanoma cell killing by TILs.

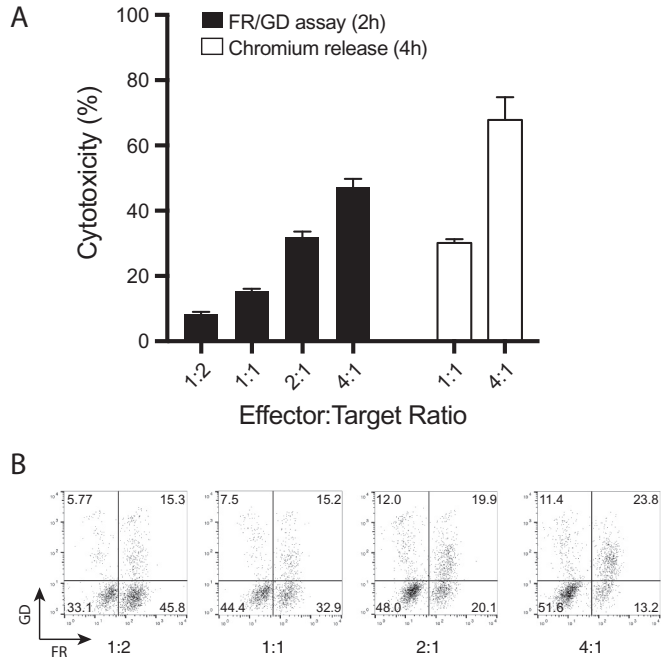

Fig. 2. Detection of NK-92 killing Daudi cells. (A) Calculated Daudi cell killing at the listed E/T ratios. Clear bars are at the indicated E:T ratio for the standard 4 hour chromium release assay. Comparison of different E:T ratios by FR/GD assay shows a $p < .0001$ by one-way ANOVA. (B) Flow cytometry plots of NK-92 cells mixed with FR-stained Daudi cells for 2 h at 1:2, 1:1, 2:1 and 4:1.

2. Materials and methods

2.1. Cells

Daudi, U937, and NK-92 cells were obtained from the American Type Culture Collection (ATCC). Primary mouse CD8+ T cells were isolated from lymph nodes and spleens of adult C57BL/6 males by negative selection (Stem Cell Technologies, Catalogue No. 19853), and CD19-positive B cells by positive selection (Stem Cell Technologies, Catalogue No. 18954). All cells except mouse B cells were grown in RPMI 1640 with phenol-red pH indicator, supplemented with 10% heat-inactivated FBS (Medium A). Primary mouse B cells were used immediately. Primary human cells were obtained from healthy donors, after informed consent was given according to a protocol approved by the institutional review board. Primary human CD3+ T cells were grown in Medium A as previously described (Rabinovich et al., 2009). TILs were grown in X Vivo 15 media supplemented with 10% AB serum, while the primary melanoma cells were grown in Opti-Mem media supplemented with 5% FCS. CD3+ T cells, TILs and NK-92 cells were grown with 100 U/ml of human recombinant IL-2.

2.2. RNA modification of T cells

Mouse anti-CD19 CAR was kindly provided by J.N. Kochenderfer (Kochenderfer et al., 2010). Human anti-CD33 CAR was obtained from Creative Biosciences. mRNA synthesis and transfection were performed as previously described (Rabinovich et al., 2006, 2009; Rabinovich and Weissman, 2013).

2.3. Antibodies

To block the interaction between TILs and melanoma cells two antibodies were used: α-HLA-ABC (Cat.#16-9983-85) from Thermofisher, and α-CD3 (OKT3) (Cat.# 317326) from Biolegend. An isotype control, IgG2a, κ (Cat.#16-4724-82) from Thermofisher was also used. To measure CD33 expression two antibodies were used: α-human CD33 (Cat.#303407) and an isotype control, IgG1, κ (Cat.#400119) from Biolegend. All antibodies were used in accordance with the manufacture's recommendation.

2.4. Chromium release assay

The chromium release assay was performed as previously described (Rabinovich et al., 2006, 2009). Cells were grown in RPMI, loaded with 50 μCi of chromium, and lysed with 1% Triton X-100.

2.5. Cytotoxicity assay

Cell labelling stock solutions of target cell staining, non-toxic cell permeable far-red dye (FR) (Invitrogen C34564) and dead cell indicator, non-permeable for living cells Green-Dead dye (GD) (Invitrogen L23101) in DMSO were made as recommended by the vendor. Cell labelling was performed using the vendor's standard

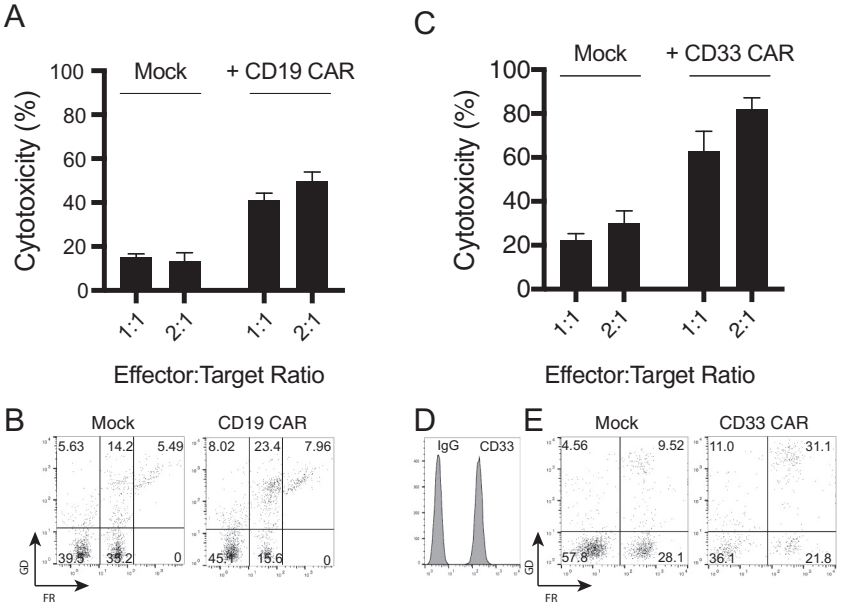

Fig. 3. Detection of CAR-T cell cytotoxicity. (A) Calculated cytotoxicity of C57BL/6 murine CD8+ T cells (effectors) mock electroporated or electroporated with CD19 CAR mRNA, mixed with murine CD19+ B cells (targets) at 1:1 and 2:1 (E:T) for 2 h. 2way ANOVA shows a statistically significant increase in CAR cytotoxicity compared to mock electroporated cells ($p < .0001$). (B) Flow cytometry plots of mock or CD19 CAR electroporated murine T cells mixed with FR-stained murine B cells for 2 h at 1:1. Pre-assay dead B cells accumulated additional FR dye and are visible in the far right column (also see Fig. 1B schematic). (C) Calculated cytotoxicity of primary CD3+ ex vivo activated human T cells mock electroporated or electroporated with CD33 CAR mRNA, mixed with the U937 AML cell line at 1:1 or 2:1 for 2 h. 2way ANOVA shows a statistically significant increase in CAR cytotoxicity compared to mock electroporated cells ($p < .001$). (D) Flow cytometry plots of CD33 expression on U937 cells with peaks from IgG or CD33 antibody staining labeled. (E) Flow cytometry plots of mock or CD33 CAR electroporated T cells mixed with U937 cells for 2 h at 1:1.

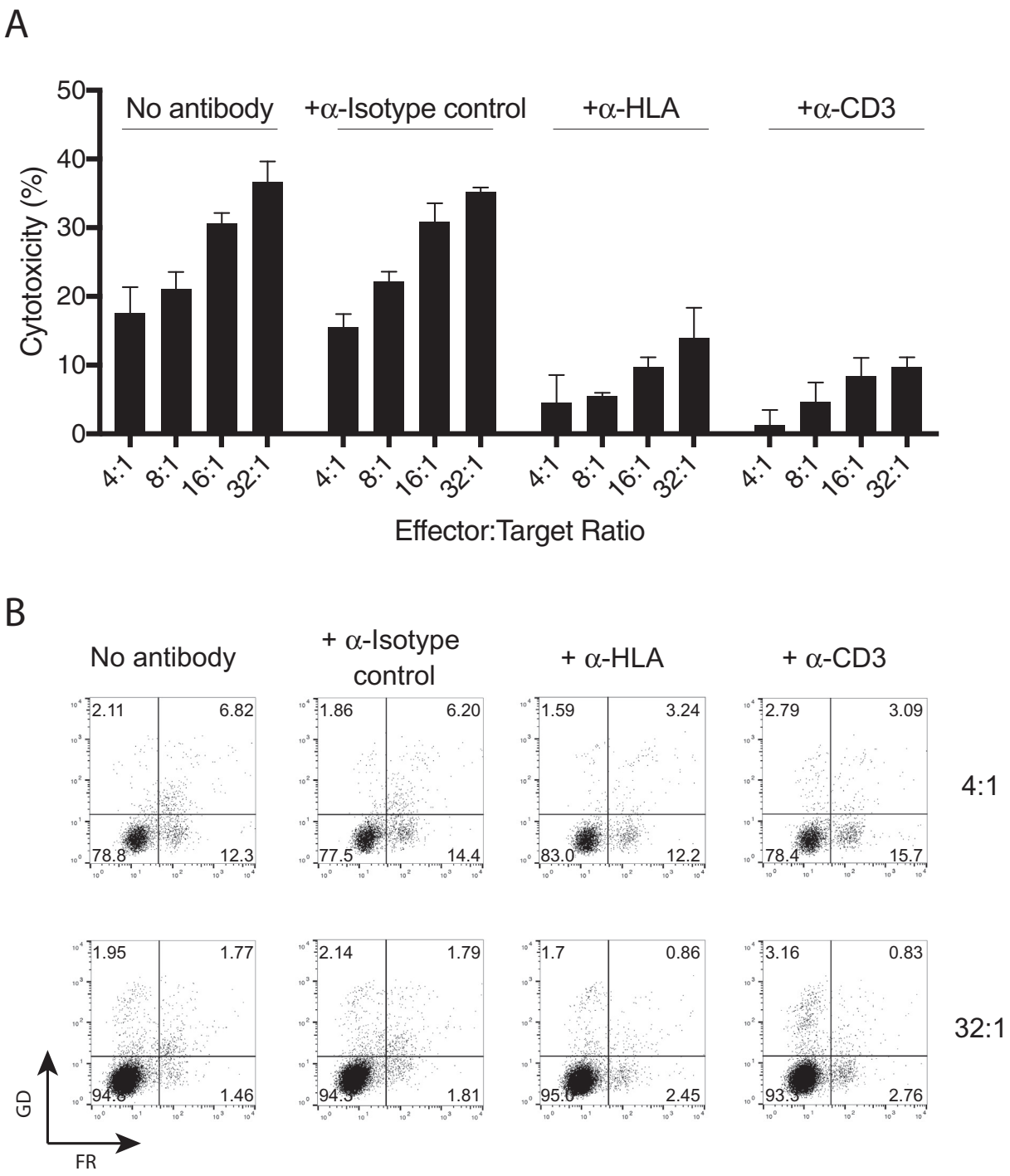

Fig. 4. Detection of cytotoxicity of melanoma tumor infiltrating lymphocytes (TILs). (A) Measurement of cytotoxicity of autologous melanoma by the patient's expanded primary TILs at several TIL:tumor ratios for 3 h and with the addition of two blocking antibodies: α-HLA and α-CD3. 2way ANOVA shows statistically significant increases with increasing E:T ratios for no antibody and α-isotype control antibody ($p < .0001$), statistically significant differences at each E:T ratio between no antibody or α-isotype control and α-HLA or α-CD3 ($p < .0001$), and no statistically significant differences at each E:T ratio between no antibody and α-isotype control or α-HLA and α-CD3. (B) Flow cytometry plots of TILs mixed with FR-stained primary melanoma cells for 3 h at 4:1 and 32:1.

protocols with some modifications. Target cells were resuspended at 1×10^6 cells/ml in PBS buffer containing calcium and magnesium (PBS + CaMg) (Thermofisher 14040133). FR stain was added for a final concentration of 0.5 μM (1/2000 dilution of the dye stock), and < 5 ml per horizontally placed polypropylene tube (Corning 352070) was left at room temperature in the dark for 20 min. An equal volume of RPMI medium with 40% BSA (American BIO, #01088-00100), was then added to inactivate free dye and the cells were incubated for an additional 5 min at room temperature in the dark before being centrifuged and resuspended in fresh Medium A and left in a vertically placed, open 50 ml Falcone tube in the CO_2 incubator for at least 30 min. For the killing assay, usually, target cells were plated in a 96 round bottom well plate (Nunc Cat.# 163320) with 25,000 to 100,000 cells per well. Then, effector cells were added in the wells to achieve a desired E/T ratio in a final volume of 150 μl/well. Plates were mixed at 530 rpm for 20 s on a Fisher Plate Vortex (S02216101), spun at 250*g* for

3 min and incubated in the CO_2 incubator for the times indicated in the figures (typically 2 h). After incubation, cells were spun again at 250 *g* for 3 min, medium was aspirated and cells were resuspended in 100 µl of PBS + CaMg with GD (1/2000 dilution from the stock), mixed on a Fisher Plate Vortex and incubated for 30 min in the dark. Cells were spun, medium was aspirated and 100 µl PBS + with 5% BSA was then added to inactivate free dye. The cells were mixed and incubated for 5 min at room temperature in the dark. Cells were then spun again, resuspended in PBS with 0.5% albumin and 0.2% Triton X-100, mixed and incubated for 10 min at room temperature in the dark. Nuclei were then immediately analyzed or fixed with 2% formaldehyde (final concentration) prior to flow cytometry. Two important target cell controls were always set up: (Martinez-Lostao et al., 2015) target cells without effector cells as a baseline no killing value, and (Brunner et al., 1968) target cells as in control (Martinez-Lostao et al., 2015), but the second staining with GD was made in the presence of 0.2% Triton X-100 as a 100% killing value. The samples of free nuclei in control (Brunner et al., 1968) after GD staining were not aspirated and used immediately or after formaldehyde fixation.

In some cases, when higher E/T ratios and/or longer incubation times were preferable, the incubation volume was increased to prevent medium acidification. This volume was pre-determined based on visual inspection of the pH indicator in the medium in reference to a colorimetric pH scale. For example, effectors with low metabolic activity, like TILs, the volume was adjusted to 200 µl/well, whereas for effectors with high metabolic activity, like NK-92 cells, the volume was increased to 500 ul/well, when working with > 3:1 or > 2 h. When performing the cytotoxicity assay in a total volume of 200 µl we used the 96-well round bottom plates (Nunc Cat.#163320) and mixed each well by pipetman, whereas for volumes of 500 µl we used 2 ml Eppendorf tubes and each tube was mixed individually.

2.6. Flow cytometry

Flow cytometric analysis was performed at the Yale Cancer Center Flow Cytometry Shared Resource (New Haven, CT) using a Stratedigm 13-color flow cytometer (Stratedigm). Samples were either read in 96-well format with a plate reader or in flow tubes with microtiter inserts (Fisherscientific Cat. # 02-681-376) to accommodate the small volumes. Data were analyzed using FlowJo (Version 10.2) software. Nuclei were gated by FSC-H/SSC-H and then single nuclei (typically > 80% of the events) were collected FSC-H/FSC-A analysis.

2.7. Data analysis

All assays were performed in triplicate with data analysis in Excel and GraphPad Prism 7. For each experiment, target cells without any effector cells were used as a baseline viability and target cells incubated with Triton X-100 during staining with GD were used as a maximum cell killing value. The percent killing was calculated as a ratio of the dead to total events for the TG cells: Percent killing = % cells positive for both FR and GD/ % total FR positive cells × 100. Specific lysis was then calculated by: Specific lysis (%) = (Percent Killing – Spontaneous Death)/(Maximum Killing – Spontaneous Death) × 100. Groups were compared using *t*-ANOVA.

3. Results

3.1. Assay design and optimization

To develop a highly adaptable cytotoxicity assay we used two types of widely available non-toxic protein-labeling dyes. First, prior to the cytotoxicity reaction, target cells were stained with non-toxic CellTrace dyes (ThermoFisher) that penetrate membranes of live cells and covalently bind to amines throughout the cell including nuclei, without detrimental effect to cell viability. To label dead cells at the end of the assay, we optimized use of a LIVE/DEAD dye (Thermofisher), which also covalently binds to amines, but only penetrates cells with a damaged cell membrane. To effectively read the double staining pattern of nuclei we chose dyes with substantially separated fluorescent profiles. After preliminary testing, we found that "Celltrace™ Far Red" (FR) (excitation at 630 nm and emission at 661 nm) and green LIVE/DEAD™ Fixable Dead Cell Stain (GD) (excitation at 495 nm and emission at 520 nm) were an optimal combination (Fig. 1).

To determine the ideal working conditions of the two dyes we stained Daudi cells with different concentrations of the dyes to obtain nuclear staining that allowed a clear separation of stained and unstained nuclei (Fig. 1C). We found that the optimal concentration for nuclear staining is 0.5 µM (1/2000 dilution of the dye stock). To find conditions that prevent dye leakage from stained to unstained nuclei, FR-stained target cells were resuspended in Medium A and incubated for 0,10, 30 and 90 min in the CO_2 incubator. Then, stained cells were mixed with an equal number of unstained cells, lysed with Triton X-100 and analyzed by FACS to evaluate the dye leakage (data not shown). For all subsequent experiments, a 30-minute incubation in medium A before use of the FR-stained target cells was used, because this was the minimum time necessary to prevent FR leakage. For GD, we found that a 1/2000 dilution of the dye stock is optimal for the staining, and a 5 min incubation in Medium A with 5% albumin before cell lysis is sufficient to prevent the leakage of GD. Thus, unlabeled effector cells could be mixed with FR-labeled target cells and their nuclei easily distinguished (Fig. 1C).

Importantly, this method displays a high precision of measuring cytotoxicity, with equivalent results whether measuring only 1000 target nuclei or as many as 4500 (Fig. 1D). Thus, variations in cell count can be accommodated, as the only key measurement is the ratio of live to dead target cells, which is independent of their absolute number.

3.2. Killing Daudi cells by NK-92 cells

FR-stained Daudi cells were mixed with NK-92 cells at various E/T ratios. Cells were analyzed after a 2 hr incubation, stained with GD, and nuclei were analyzed. FR-positive Daudi nuclei (targets) and FR-negative NK-92 nuclei (effectors) are clearly distinguishable (Fig. 2A and B). Likewise, GD-positive Daudi and NK-92 nuclei from dead cells can be distinguished from GD-negative nuclei. Incubation with triton X-100 was used as a positive control for 100% cell death. Increasing the E/T ratio results in increased killing (Fig. 2A and B). A standard 4-hour chromium release assay with NK-92 and Daudi cells was performed to compare cytotoxicity to the FR/GD assay (Fig. 2A).

3.3. Cytotoxicity of CAR-T cells

Next, we tested the ability to measure CAR-mediated killing. Primary murine T cells were electroporated with 3 µg of murine anti-CD19 CAR mRNA, and incubated with autologous B cells, selected with anti-CD19 magnetic beads. We performed the killing reaction for 2 h at E:T ratios of 1:1 and 2:1. The assay detected specific CAR-mediated cytotoxicity in primary T cells in a dose dependent fashion (Fig. 3A,B). The primary murine B cells exhibited high pre-assay death, which appeared as brighter FR positive cells in the column to the far right and were excluded from the analysis. We next tested primary human T cells electroporated with a CD33 CAR mRNA to kill the U937 Acute Myeloid Leukemia (AML) cell line. We first confirmed that the U937 AML cell line expressed CD33 (Fig. 3D). The CD33 CAR T cells exhibited high levels of dose dependent cytotoxicity (Fig. 3C,E).

3.4. Killing primary melanoma cells with primary Tumor Infiltrating Lymphocytes (TILs)

Finally, we used the new assay to detect the cytotoxicity of tumor infiltrating lymphocytes derived from a patient's metastatic melanoma.

TILs and melanoma cells were isolated from the same patient with metastatic melanoma and grown separately. The melanoma cells were stained with FR and mixed with TILs at varying TIL:tumor cell ratios which demonstrate a dose-dependent increase in cytotoxicity by this assay (Fig. 4). Addition of CD3 or HLA antibodies blocking TCR-MHC binding reduced cytotoxicity, while an isotype control antibody did not.

4. Discussion

In this study, we developed a novel assay to measure cell-mediated cytotoxicity that has several advantages over other assays. The use of relatively inexpensive and commercially available non-toxic dyes that can be measured by flow cytometry makes this a widely available assay to most laboratories. By using these dyes, we can specifically mark the target cell population and avoid misinterpreting effector cell death as seen with LDH or other endogenous molecule release assays. They also avoid the lengthy time necessary to label target cells with luciferase expressing constructs as well as the regulatory hurdles, safety hazards, high spontaneous leakage and population averaging seen with the chromium release assay. We performed staining in PBS + CaMg, because divalent ions can increase viability of some cells without compromising staining efficiency, but PBS without Ca and Mg can be used as well. By using flow cytometry, the assay is able to measure individual cell death events rather than averaging over the entire population of cells. Importantly, by isolating and measuring only the nuclei, the assay is able to avoid complications arising by specific target-effector aggregation in the whole cell analysis. Such aggregation may include a considerable amount of the target cells, especially at high E/T ratios, and complicate the analysis. Although free nuclei can also stick to each other, especially after formaldehyde fixation, that binding is non-specific, and can be easily corrected by analyzing singlets. Whereas we used Triton X-100 to release the nuclei, other non-ionic detergents also may be used. Finally, the assay is highly versatile, compatible with multiple different types of targets, growing either as adherent cells or suspension cells, as well as multiple different effector cells (e.g. cell lines and primary cells).

Although we have found this assay very robust there are a few caveats regarding its application. First, the amount of dye, time to preload the target cells, and time to incubate after pre-loading should be experimentally adjusted for each target cell to obtain optimal nuclei staining and to avoid dye leakage during the experiment. Second, as with all cytotoxicity assays, ensuring high starting viability of the target cell population is important to increase specificity and the signal to noise ratio. As seen, a relatively short incubation with a low E/T ratio (e.g. 1:1 and 1:2) is sufficient for many cases, although both of these parameters had to be increased for the TILs and melanoma. In comparison, a standard chromium assay often involves a 4-hr incubation with similar or higher E:T ratios. It is difficult to directly compare absolute values among different assays; however, this assay is highly reproducible between replicates and shows a proportional increase in cytotoxicity with increasing E:T ratios. Finally, since the targets need to be incubated with the dye, the assay is suitable for *in vitro* but not *in vivo* applications. It is worth to note that prior to the assay, cell growth is monitored in assay conditions with effector cells to assure that the time of incubation is short enough and the volume of medium is abundant enough to avoid metabolic exhaustion. In practice, preventing excessive acidification, visualized by phenol-red in reference to a colorimetric pH scale, is sufficient.

Future improvements in the assay may be made with the use of different staining dyes. For example, dye leakage may vary among different dyes and it might be possible to identify a target cell dye with less leakage than FR, which would require less post-staining incubation. In addition, while we only apply two color flow cytometry in the use of this assay, one can easily envision expansion of the assay to include additional, separately labeled cell populations by various dyes. Thus, a simple extension of this assay can measure cell killing in complex cell mixtures of multiple types of effector, target, and helper cells.

Respective contributions

P.R. designed and executed experiments and edited the paper. B.C. isolated and electroporated primary mouse T and B cells. S.R.K. and J.Z. performed chromium release assays and edited the paper. M.K. isolated and prepared human T cells and TILs. A.B. and D.K. designed T cell and TIL activation and propagation. M.H., D.K. and S.M.W. provided critical experimental review and edited the paper. S.G.K. designed and reviewed experiments and wrote the paper.

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