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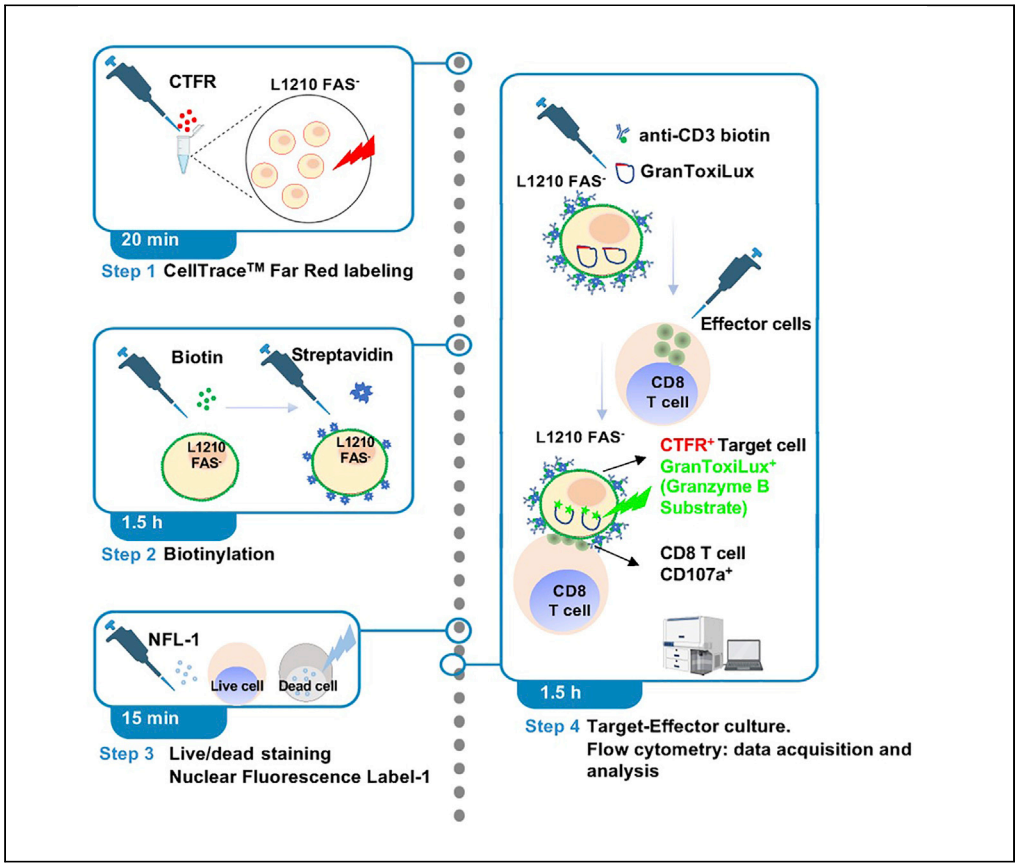

CD8 T and NK cells mediate killing by delivery of perforin and granzyme B (GZB) stored in lysosome-like granules. We present a flow-cytometry-based protocol combined with a redirected killing assay to evaluate granule exocytosis and the cytotoxic potential of human CD8 T cells and NK cells. We describe the assessment of the delivered GZB inside the target cells. We then detail the detection of lysosome membrane protein CD107a exposed on the cell surface of the effector cells upon degranulation.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Flow cytometry protocol to evaluate GZB delivery and activity inside the target cells

Redirected killing assay to assess the full cytotoxic potential of CD8 T cells

Protocol for the evaluation of the *ex vivo* cytotoxicity of human NK cells

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A flow-cytometry-based assay to assess granule exocytosis and GZB delivery by human CD8 T cells and NK cells

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SUMMARY

CD8 T and NK cells mediate killing by delivery of perforin and granzyme B (GZB) stored in lysosome-like granules. We present a flow-cytometry-based protocol combined with a redirected killing assay to evaluate granule exocytosis and the cytotoxic potential of human CD8 T cells and NK cells. We describe the assessment of the delivered GZB inside the target cells. We then detail the detection of lysosome membrane protein CD107a exposed on the cell surface of the effector cells upon degranulation.

For complete details on the use and execution of this protocol, please refer to Chen et al. (2021).[1]

BEFORE YOU BEGIN

This protocol describes the steps to determine CD8 T cell cytotoxic potential mediated by the granule exocytosis pathway using a redirected killing assay.[2,3] Using this assay, we evaluated the role of the Protease Activated Receptor-1 in the cytotoxic function of human CD8 T cells, and PAR1 deficient murine virus-specific CD8 T cells.[1]

The granule exocytosis pathway involves the delivery of preformed cytotoxic molecules stored in lysosome-like granules. Upon TCR stimulation, secretory lysosomes containing perforin and the serine proteases granzyme A, B and others, release their content at the immunological synapse formed by the effector and the target cell.[4–8] The delivered Granzyme B (GZB) into the target cells triggers a cascade of events involving caspase activation and target cell death by apoptosis.[9–11]

To evaluate the target cell killing by the granule exocytosis pathway, GZB delivery and activity inside the targets is detected with a cell permeable substrate that contains a GZB cleavage site.[11] GZB-dependent cleavage of the quenched substrate is detected by a fluorescent signal.[11] In addition, using a FAS deficient target cells, substrates that detect both GZB and caspase 8 activation can also be used for evaluation of the granule exocytosis pathway.[1,11–13]

The protocol describes the redirected killing, an antigen independent assay that allows the evaluation of the cytotoxic potential of polyclonally activated human CD8 T cells. Using this flow cytometry protocol two important cellular events can be detected, the delivery and activity of GZB inside the live target cells before undergoing cell death by apoptosis, and the degranulation of CD8 T cells.[1]

We also present an additional application of this assay for evaluation of the *ex-vivo* cytotoxicity of human NK cells from PBMCs using a human immortalized myelogenous leukemia cell line K562 as target cells.

This protocol can be extended to other experimental approaches that aim to measure the granule exocytosis pathway by CD8 and NK cells.

Institutional permissions

Healthy controls were obtained from the MedStar Georgetown University Hospital and the NIH Blood Bank under both institutional review board approved protocol. Healthy volunteers signed informed consent.

Isolation of human CD8 T cells from peripheral blood

Timing: 1.5 h

This section describes the isolation of Peripheral Blood Mononuclear cells (PBMCs) from whole blood for CD8 T cell purification.

30 mL of whole blood was collected in BD Vacutainer Blood Collection with EDTA.

Note: Approximately 30 × 10^6 total PBMCs can be isolated from 30 mL fresh blood from a healthy donor. The yield of CD8 T cells will depend on the frequency of CD8 T cells in total PBMCs. It is expected that between 0.5 to 1 × 10^6 total CD8 T cells will be recovered after magnetic isolation from 10 × 10^6 PBMCs from healthy volunteers. Note that in clinical conditions such as HIV infection (these individuals have inverted CD4/CD8 T cell ratio) and the number of CD8 T cells recovered will be higher from 10 × 10^6 PBMCs.

1. Prepare Blood Dilution Buffer:
   a. PBS (without Ca^{++}/Mg^{++}) with 2% heat-inactivated Fetal Bovine Serum (FBS).
2. Prepare 50 mL tubes with 15 mL Ficoll-Paque in each tube.

Note: For smaller volume of blood is recommended to use 15 mL tubes with 3 mL Ficoll-Paque and 7 mL of diluted blood.

3. Mix the blood by gently inverting the BD Vacutainer Blood Collection tubes or using a 10 mL pipette and slowly pipetting up and down the blood.
4. Carefully aspirate with a 10 mL pipette and transfer 15 mL of whole blood from BD Vacutainer Blood Collection tubes into 2 tubes of 50 mL (use the electronic pipettor set at low speed for discharging the blood into the tubes).
5. Dilute the 15 mL of whole blood with 15 mL Blood Dilution Buffer (1:1).
6. Mix very gently up and down the blood using a 10 mL pipette and transfer the diluted blood on the top of the Ficoll-Paque until transferred the 30 mL of blood (Figure 1).

⚠ CRITICAL: To avoid mixing the diluted blood sample with Ficoll-Paque solution discharge the blood slowly on the top of the Ficoll-Paque using a 10 mL pipette (set up the speed of the electronic pipettor to minimum speed). It is critical to add the blood at steady speed to avoid disrupting the interface with the Ficoll-Paque.

Optional: LeucoSep Tubes (with a barrier) can be used as an alternative for PBMCs isolation.

7. Centrifuge the tubes at 400 × *g* for 30 min at room temperature (25°C). Set the deceleration to 0.

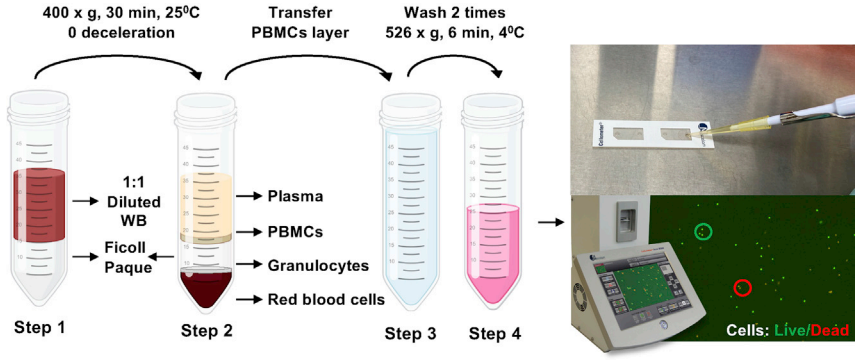

Figure 1. The schematic diagram of PBMCs isolation procedure, cell counting and viability

8. Collect carefully the interface containing the peripheral mononuclear cells layer (PBMC) located between plasma and Ficoll-Paque with a 10 mL pipette and transfer to new 50 mL tubes (see Figure 1).

   Note: Collection of the interface can be performed removing the plasma first and collecting the interface with 5 mL pipette.

9. Add Blood Dilution Buffer up to total 50 mL and centrifuge the tubes at 526 × *g* for 6 min at 4°C.
10. Repeat the wash step once more.
11. Resuspend the cell pellet by finger flick the tube before adding 5 mL of X-VIVO™ 15 media and count the viable cells.
12. To count viable cells, vortex the cell suspension and take 20 µL of the cell suspension and dilute with 20 µL of AO/PI (Acridine Orange/Propidium Iodide, Nexcelom).
13. Count the cells using automatic cell counter (Cellometer Auto 2000, Nexcelom), Figure 1.

    Note: The nuclei dual fluorescence labeling discriminate viable cells (green, AO) and dead cells (red, PI). Measurement of the viability was performed using an automatic cell counter. Viability can be also performed manually using fluorescent dyes (AO/PI) or Trypan Blue in a hemocytometer.

    Note: Freshly isolated PBMCs can be used for NK cells *ex-vivo* cytotoxicity assay please go to section Redirected cytotoxicity assay step 25 (NFL1 staining). The granzyme B activity-based assay protocol was optimized using total PBMCs that has the advantage to test NK cell activity without the step of NK cell isolation from PBMCs that it may require higher number of PBMCs. Isolation of NK cells can be used in those specific functional assays that it may require purified NK cells.

14. Separate the number of PBMCs necessary for the isolation of CD8 T cells (go to step 16). The rest of cells can be frozen for later use as follow:
    a. Prepare cryogenic tubes and make appropriate labels.
    b. Pellet the cells at 526 × *g* for 6 min at 4°C.
    c. Resuspend the pellet by finger flick the tube and add 1 mL of Cell Culture Freezing Medium (Gibco) and further mix by pipetting up and down with 1 mL pipette. The cell concentration at 20 × 10^6 cells/mL.
    d. Transfer 500 µL of cells into each cryogenic tube (10 × 10^6 PBMCs per tube).
    e. Place the cryogenic tubes in the freezing container containing cold isopropyl alcohol (Mr Frosty™, ThermoFisher) and store at −80°C freezer.
    f. After 12–16 h transfer the cryogenic tubes into liquid nitrogen (−180°C, vapor phase) for final storage.

Note: In addition, frozen PBMCs after an overnight resting period (see below step 15) can be used for the isolation of CD8 T cells and to perform *ex-vivo* cytotoxicity assay of NK cells.

15. Frozen PBMCs can be used for isolation of CD8 T cells. Thawing and resting of PBMCs as follow (example 1 vial of 10×10^6 PBMC):
    a. Prepare thawing media by adding 16 μL of Benzonase (25 U/μL, Novagen) in 8 mL of X-VIVO™ 15 media (final concentration 0.044 U/μL).

    Note: Benzonase nuclease prevents the clumping of PBMCs by digesting DNA and RNA released from the dying cells during the thawing improving the cell recovery.

    b. Warm up the PBMCs vials in a 37°C water bath for 20–30 s (only thaw partially) and add 500 μL of the X-VIVO™ 15 media containing Benzonase (step a).
    c. Gently pipette up and down (1 mL tip) to thaw the cells and transfer into the 15 mL tube containing the media with Benzonase.
    d. Repeat this step by adding more media until everything is thaw.
    e. Centrifuge the cell suspension at 526 × *g* for 6 min at 4°C and wash once more with X-VIVO™ 15 media.
    f. Resuspend the cells in 2 mL and count viable cells (as described in step 12) and adjust to a cell concentration of 2×10^6 cells/mL.
    g. Transfer to a 50 mL conical tube with caps loosened and place in the incubator at 37°C and 5% CO2 for 14–16 h.
    h. After the resting period, centrifuge the cell suspension at 526 × *g* for 6 min at 4°C and wash once with X-VIVO™ 15 media and count viable cells.
16. Isolation of CD8 T cells from PBMCs by magnetic negative selection.

    Note: *In vitro* expansion of CD8 T cells by polyclonal stimulation with cross-linked anti-CD3 and anti-CD28 mAbs could lead to approximately 3–6 times expansion of the initial number of cells after 7-day culture.

    a. Prepare 500 mL of the Magnetic Cell Separation buffer (MACS buffer) following the manufacturer' instructions (https://www.miltenyibiotec.com/upload/assets/IM0001984.PDF):
        i. PBS (without Ca^{++}/Mg^{++}) with 0.5% of bovine serum albumin (BSA) and 2 mM of EDTA (see preparation at the materials and equipment Section).
    b. Freshly isolated or frozen and rested (see step 15) PBMCs are washed once in the MACS buffer by centrifugation at 526 × *g* for 6 min at 4°C.
    c. Proceed with the isolation protocol (https://www.miltenyibiotec.com/upload/assets/IM0001984.PDF) provided by the manufacturer for the human CD8 T cell isolation kit (See the key resources table).
    d. Resuspend purified CD8 T cells in 2 mL of X-VIVO™ 15 media and count viable cells as described in step 12.
    e. Adjust the cell concentration to 1×10^6 cells/mL with X-VIVO™ 15 media.
    f. Take a small aliquot (5×10^4 cells/mL) of purified CD8 T cells to check the purity by flow cytometry.

In vitro polyclonal expansion of human CD8 T cells (effector cells)

Timing: 10–15 days

This section describes the *in vitro* polyclonal TCR stimulation of human CD8 T cells.

17. Pre-coat a 24 well plate with 0.25 mL/well of the mAbs mix (10 μg/mL of anti-CD3 and 5 μg/mL anti-CD28 mAbs diluted in PBS).
18. Incubate the plate overnight (12–16 h) at 4°C or 2 h at 37°C.

Optional: anti-CD28 mAb (5 μg/mL) can be added soluble to the T cell culture.

19. After the incubation period aspirate the mAbs solution and wash the wells by adding 2 mL of sterile PBS.
20. Repeat this step three times to ensure that the mAbs solution is completely removed (avoid the plate to dry by adding PBS until culturing the cells).
21. Culture isolated CD8 T cells at step 16 at 2 × 10^6 cells per well in X-VIVO™ 15 media in the pre-coated plates for 3 days at 37°C and 5% CO_2.

Note: If the protocol requires expansion of larger numbers of CD8 T cells use larger plates. Pre-coat 12 and 6 well plates with (anti-CD3 and anti-CD28 mAbs) using 0.5 mL and 1 mL respectively at the same concentration as step 17. Culture isolated CD8 T cells at 4–5 × 10^6 cells/well CD8 T cells and 8–10 × 10^6 cells/well in 12 well plate and 6 well plates respectively.

22. On day 3. Resuspend the cells in the wells and count viable cells.
23. Preparation of X-VIVO™ 15 media with rhIL-2 for the expansion of CD8 T cells:
    a. Thaw on ice 10 μL aliquot of the stock (1 × 10^6 U/mL) and resuspend in X-VIVO™ 15 media with 990 μL to obtained a working concentration of 1 × 10^4 U/mL.
    b. The rhIL-2 (1 × 10^4 U/mL) working solution can be stored at 4°C for 4 weeks.

Note: rhIL-2 stock (1 × 10^6 U/mL) is stored at −80°C. Preparation of smaller volumes of rhIL-2 working solution is recommended in cases that rhIL-2 is not used often.

    c. Pre-warm X-VIVO™ 15 media in 37°C in the water bath.
    d. Prepare 10 mL of X-VIVO™ 15 media containing 50 U/mL of rhIL-2 by adding 50 μL of rhIL-2 (1 × 10^4 U/mL) to 10 mL of pre-warm X-VIVO™ 15 media.
24. Adjust the cell concentration to 2 × 10^6 cells/mL with X-VIVO™ 15 media and transfer 1 mL of the cell suspension into a new plate (2 × 10^6 cells/well).

⚠ CRITICAL: Transferring CD8 T cells to a new uncoated plate will allow maximum re-expression of surface CD3 molecules which is critical for the redirected killing assay with the anti-CD3 mAb.

25. Add 1 mL of the X-VIVO™ 15 media containing rhIL-2 50 U/mL (prepared at 23d) to each well. The final concentration of rhIL-2 for the expansion is 25 U/mL.

Note: When adjusting the cell concentration avoid washing the T cells. T cells secrete growth factors that favor their expansion. Alternatively, in step 24, when transferring the cells to a new plate cells can be adjusted to 1 × 10^6 cells/mL and transfer to 2 mL/well of the cell suspension; and rhIL-2 can be directly added to each well. In this case, 5 μL/well of rhIL-2 (1 × 10^4 U/mL) is added to the 2 mL/well.

26. Culture T cells for an additional 2–3 days at 37°C and 5% CO_2 to allow further expansion.
27. At day 7–10. CD8 T cells are ready to use in the redirected killing assay.

Note: CD8 T cells can be maintained at a cell concentration of 1–2 × 10^6 cell/mL using X-VIVO™ 15 media supplemented with 25 U/mL of rhIL-2. To dilute the CD8 T cells in culture

count the viable cells and calculate approximately the amount of media necessary to dilute the cells. For example, supplement 10 mL of pre-warm X-VIVO™ 15 media with 25 μL of rhIL-2 (1×10^4 U/mL) to have a concentration of 25 U/mL.

⚠ CRITICAL: Human CD8 T cells expanded in these culture conditions should be used as a maximum of 15 days of culture (it may be donor dependent). CD8 T cells cultured for longer periods may lose cytotoxic activity.

Target cells recovery and maintenance

Timing: 1 week

28. The murine lymphoma cell line, L1210-FAS$^-$ was a kind gift of Dr. P. A. Henkart (National Cancer Institute, National Institutes of Health, Bethesda, MD).
29. The human myelogenous leukemia cell line K562 cell line was obtained from American Type Culture Collection (ATCC, USA). The K562 cell line is used for *ex-vivo* cytotoxicity assay of NK cells.
30. Cell lines were maintained in supplemented RPMI 1640 media containing 10% of heat-inactivated Fetal Bovine Serum (FBS), 2 mM of L-Glutamine and 100 U/mL of Penicillin and 100 μg/mL of Streptomycin (See the materials and equipment section for supplemented RPMI 1640 media).
31. K562 and L1210 cell lines was maintained at a cell concentration between 0.5–1 $\times 10^6$ cells/mL in supplemented RPMI 1640 at 37°C and 5% CO_2.

⚠ CRITICAL: The cell lines should be cultured at appropriate cell concentration to prevent cell death induced by overgrowth. Viability of the target cells should be above 90% live cells before performing the cytotoxicity assay. Cell lines were used within 30 passages or cultured no longer than 30 days.

Note: This protocol can be performed using other cell lines human and murine, and primary cells including activated B and T cells as targets. This protocol has been reported for human CD8 T cells polyclonally stimulated and virus-specific CD8 T cells using murine lymphoblast cell line EL4 as targets.[1] Other cell lines can be used in this assay and it will require optimization of the Cell Trace Far Red labeling as described in section CellTrace™ Far Red (CTFR) Labeling of L1201-FAS$^-$ target cells step 1. Alternatively, anti-CD16 or other mAbs can be used to trigger antibody-dependent cell-mediated cytotoxicity (ADCC) by NK cells in this assay. Mouse cell lines including RMA-S (MHC-I-deficient target cell line), the mouse mastocytoma cell line P815 coated with specific antibodies can be used as target cells to measure murine NK cell mediated ADCC.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
BD Pharmingen™ Purified NA/LE Mouse Anti-Human CD3 (working concentration: 10 μg/mL)	BD Bioscience	Cat#567108
BD Pharmingen™ Purified NA/LE Mouse Anti-Human CD28 (working concentration: 5 μg/mL)	BD Bioscience	Cat#555725
eBioscience™ Biotin-Mouse Anti-Human CD3 (UCHT1) (working concentration: 10 μg/mL)	Thermo Fisher Science	Cat#13-0038-82
APC-H7 Mouse Anti-Human CD107a (5 μL/10^6 cells)	BD Bioscience	Cat#561343
BUV395 Mouse Anti-Human CD8 (5 μL/10^6 cells)	BD Bioscience	Cat#563795

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Peripheral blood mononuclear cells (PBMCs) (both male and female human subjects, ages range from 35–70)	Participants were studied under a MedStar Georgetown University Hospital and an NIH Institutional Review Board approved protocol	N/A
Chemicals, peptides, and recombinant proteins		
X-VIVO™ 15 Serum-free Hematopoietic Cell Medium	Lonza	Cat#BE02-060F
Gibco™ RPMI 1640 Medium, no glutamine	Fisher Scientific	Cat#21-870-092
BenchMark™ Fetal Bovine Serum	GeminiBio	Cat#100-106
Gibco™ L-Glutamine (200 mM)	Fisher Scientific	Cat#25-030-164
Gibco™ Penicillin-Streptomycin (10,000 U/mL)	Fisher Scientific	Cat#15-140-163
PBS (1×) without calcium and magnesium	Lonza	Cat#BE17-516F
HBSS without calcium and magnesium, without phenol red	Fisher Scientific	Cat#14-175-095
Quality Biological Inc EDTA (0.5 M, pH 8.0)	Fisher Scientific	Cat#50-983-251
Bovine serum albumin (BSA), Fraction V-Solution 30%	GeminiBio	Cat#700-100
ViaStain™ AOPI Staining Solution, Nexcelom Bioscience	Fisher Scientific	Cat#NC1412892
Recovery™ Cell Culture Freezing Medium (Gibco)	Fisher Scientific	Cat#12-648-010
MilliporeSigma™Novagen™Benzonase™ Nuclease, >99%	Fisher Scientific	Cat#70-664-3
Dead Cell Removal Kit	Miltenyi Biotec	Cat#130-090-101
EasySep™ Dead Cell Removal (Annexin V) Kit	Stemcell	Cat#17899
Invitrogen™ OneComp eBeads™ Compensation Beads	Fisher Scientific	Cat#50-112-9031
rhIL-2	Biological Resources Branch, Frederick National Laboratory, NCI, NIH	N/A
Isopropyl alcohol(2-propanol)	Millipore Sigma	Cat#I9516-1L
Biotin -X-NHS, water-soluble	Millipore Sigma	Cat#203189-50MG
Streptavidin from *Streptomyces avidinii*	Millipore Sigma	Cat#S4762
Benzonase nuclease, Purity > 99%	Millipore Sigma	Cat#70664-3
Cytiva Ficoll-Paque™ PREMIUM, 1.078 g/mL	Fisher Scientific	Cat#45-001-751
Gibco™ HEPES (1 M)	Fisher Scientific	Cat#15-630-106
NaN_3	Millipore Sigma	Cat#S2002-100G
Critical commercial assays		
CellTrace™ Far Red Cell Proliferation Kit	Thermo Fisher Scientific	Cat#C34572
4× GranToxiLux (customized)	OncoImmunin, Inc	Cat#GTL702-8
NFL1	OncoImmunin, Inc	Cat#NFL-1
$CD8^+$ T Cell Isolation Kit, Human	Miltenyi Biotec	Cat#130-096-495
Experimental models: Cell lines		
L1210-FAS^- cells	A gift of Dr. P. A. Henkart (National Cancer Institute, National Institutes of Health, Bethesda, MD).	N/A
K562 cells	ATCC	Cat#CCL-243
Other		
BD Vacutainer™ Plastic Blood Collection Tubes with K_2EDTA	Fisher Scientific	Cat#02-657-32
Falcon™ 50 mL Conical Centrifuge Tubes	Fisher Scientific	Cat#14-432-22
Falcon™ 15 mL Conical Centrifuge Tubes	Fisher Scientific	Cat#05-527-90
Corning™ Costar™ 24-well Flat Bottom Cell Culture Plate	Fisher Scientific	Cat#07-200-84
Corning™ 96-well, Cell Culture-Treated, V- shaped Bottom Microplate	Fisher Scientific	Cat#07-200-96
MilliporeSigma™ Stericup™ Sterile Vacuum Filter Units	Fisher Scientific	Cat#SCGPU05RE
Falcon™ 10 mL Disposable Polystyrene Serological (Corning™) Pipettes	Fisher Scientific	Cat#13-675-20
Automated cell counter Cellometer Auto 2000	Nexelom Bioscience	Cat#Auto2000-201-0549
Freezing container Mr. Frosty™, Thermo Scientific™	Fisher Scientific	Cat#15-350-50

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Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Nunc™ Biobanking and Cell Culture Cryogenic Tubes (Thermo Scientific™)	Fisher Scientific	Cat#12-565-163N
BD FACSymphony™ A5 Cell Analyzer	BD Bioscience	N/A
Software and algorithms		
FlowJo	FlowJo	https://www.flowjo.com/solutions/flowjo

MATERIALS AND EQUIPMENT

Supplemented RPMI 1640 media		
Reagent	Final concentration	Amount
Penicillin-Streptomycin (10,000 U/mL)	Penicillin (100 U/mL) Streptomycin (100 μg/mL)	5 mL
L-Glutamine (200 mM)	L-Glutamine (2 mM)	5 mL
FBS	10%	50 mL
RPMI 1640 Media	N/A	440 mL
Total	**N/A**	**500 mL**
Media is filtered (0.22 μm) and stored at 4°C up to one month.		

Blood dilution buffer		
Reagent	Final concentration	Amount
FBS	2%	10 mL
PBS	N/A	490 mL
Total	**N/A**	**500 mL**
Store at 4°C for a maximum of one month.		

Magnetic Cell Separation buffer (MACS buffer)		
Reagent	Final concentration	Amount
EDTA (0.5 M)	EDTA (2 mM)	2 mL
Bovine serum albumin (BSA, 30%)	BSA (0.5%)	8.3 mL
PBS	N/A	489.7 mL
Total	**N/A**	**500 mL**
Store buffer at 4°C for a maximum of one month.		

Anti-CD3/anti-CD28 mAbs plate coating		
Reagent	Final concentration	Amount
Anti-CD3 mAbs (1 mg/mL)	Anti-CD3 mAbs (10 μg/mL)	10 μL
Anti-CD28 mAbs (1 mg/mL)	Anti-CD28 mAbs (5 μg/mL)	5 μL
PBS	N/A	0.985 mL
Total	**N/A**	**1.0 mL**
Fresh mixture of mAbs should be prepared each time. Coating plate volume: 250 μL/24 well plate.		

CellTrace™ Far Red solution serial dilution

10 μM CellTrace Far Red solution		
Reagent	Final concentration	Amount
CellTrace™ Far Red (1 mM)	CellTrace™ Far Red (10 μM)	1 μL
PBS	N/A	99 μ
Total	**N/A**	**100 μL**
Fresh solution should be prepared before each experiment. For CellTrace™ Far Red labeling of the L1210 cell line, dilute with PBS to 0.1 μM final concentration.		

10× HEPES saline buffer pH= 7.5		
Reagent	Final concentration	Amount
HEPES (1 M)	HEPES (0.1 M)	50 mL
NaCl	NaCl (1.5 M)	43 g
diH_2O	N/A	450 mL
Total	**N/A**	**500 mL**
Adjust pH with NaOH and store at 4°C for up to 2 months.		

1× HEPES + 1% BSA saline buffer		
Reagent	Final concentration	Amount
BSA (30%)	BSA (1%)	1.67 mL
1× HEPES saline buffer	N/A	48.33 mL
Total	**N/A**	**50 mL**
10× HEPES saline buffer is diluted with diH_2O to 1× HEPES saline buffer before use. Fresh solution is prepared before each experiment.		

2 mM Biotin solution		
Reagent	Final concentration	Amount
Biotin-X-NHS	2 mM	1.11 mg
1× HEPES saline buffer	N/A	1 mL
Total	**N/A**	**1 mL**
Fresh solution should be prepared for each experiment. For biotinylation of targets cells dilute with 1× HEPES saline solution to 0.2 mM final concentration.		

20 μg/mL streptavidin (SA)		
Reagent	Final concentration	Amount
Streptavidin (2 mg/mL)	Streptavidin (20 μg/mL)	5 μL
1% BSA HEPES saline buffer	N/A	495 μL
Total	**N/A**	**500 μL**
Stock solution SA 2 mg/mL is stored at −20°C. Fresh solution should be prepared for each experiment.		

40 μg/mL anti-CD3 biotin		
Reagent	Final concentration	Amount
Anti-CD3 biotin (0.5 mg/mL)	Anti-CD3 biotin (40 μg/mL)	2.4 μL
X-VIVO™ 15	N/A	27.6 μL
Total	**N/A**	**30 μL**
Fresh dilution should be prepared for each experiment.		

Flow Cytometry (FC) Staining buffer		
Reagent	Final concentration	Amount
NaN_3 (5% solution)	0.01%	1 mL
BSA (30%)	BSA (0.01%)	167 μL
HBSS (without Ca^{++}/Mg^{++})	N/A	499 mL
Total	**N/A**	**500 mL**
Store solution at 4°C for up to two months.		

Flow cytometer (BD Symphony 5A)		
Reagent	Laser (excitation nm)	Emission filter/nm
CTFR	Red (628)	670/30
NFL1	UV (355)	450/40
GranToxilux	Blue (488)	515/20
CD8	UV (355)	390/20
CD107a	Red (628)	780/60

Alternatives: BD-Symphony 5A was used in this assay and NFL1 was detected in 355 nm excitation (UV) and 450/50 nm bandpass emission filter. Other Flow Cytometers can be used, if there is no UV laser, NFL1 staining can be detected using 405 nm excitation (Violet) and 450 nm bandpass emission filter.

Alternatives: The fluorochromes of the mAbs used for the detection of the degranulation of CD8 T cells can be exchanged depending of the configuration of the instrument.

STEP-BY-STEP METHOD DETAILS

Preparation of the target cells for redirected cytotoxicity assay

This part of the protocol describes the preparation of the target cells for the redirected killing assay using activated human CD8 T cells (Effector T cells). To facilitate CD8 T cell degranulation and delivery of the lysosome granule content at the immunological synapse, proteins from the cell surface of the target cells are biotinylated followed by binding of streptavidin.[2] This process creates binding sites and crosslink of the biotin labeled anti-CD3 mAb on the surface of the target cells. Once targets and effectors are cultured together, the crosslinked anti-CD3 mAb will trigger TCR-dependent degranulation of the effector CD8 T cells leading to the release of the granule content at the immunological synapse. The delivery and activity of the GZB inside the target cells is detected by a quenched substrate that contains the GZB cleavage site and after processing emits a fluorescence signal. The degranulation of the effector CD8 T cells is assessed by exposure of the granule membrane protein CD107a.[14,15] Both events in the target and effector cells are measured by flow cytometry.

In this protocol, we use a FAS deficient cell line L1210 (L1210-FAS$^-$) that allows the evaluation of the granzyme B mediated apoptosis. Because this target is FAS deficient other substrate that detects GZB and caspase 8 activation can be used. The L1210-FAS$^-$ cell line is first labeled with CellTrace™ Far Red (CTFR). This allows the discrimination between the target cells CTFR$^+$ and the effector cells (CTFR$^-$) facilitating the analysis of GZB activity in the target cells. The degranulation event in the effector T cells, is identified by surface staining of CD8 and CD107a mAbs (See gating strategy, Figure 4).

The redirected killing assay determines the cytotoxic potential of effector CD8 T cells independently of antigen recognition.[1] This assay can be used to determine effector CD8 T cell differentiation and cytotoxic function; and for the rapid screening of therapeutic agents designed to improve or inhibit cytotoxic function of effector CD8 T cells. In addition, the flow cytometric cytotoxicity assay can be used to evaluate *in vitro* cytotoxic capacity of antigen-specific T cells such as LCMV-GP33 specific CD8 T cells.[1]

CellTrace™ Far Red (CTFR) labeling of L1210-FAS$^-$ target cells

Timing: 20 min

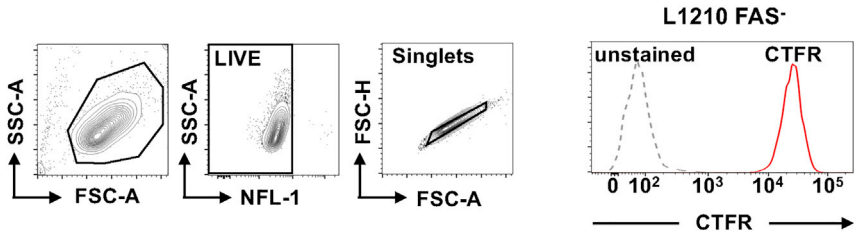

Figure 2. Optimized CTFR staining of L1210 cells

Example of optimal labeling of L1210 cells (target cells) analyzed by flow cytometry. This concentration was chosen by comparison between unstained cells and the signal separation of $CTFR^+$ cells (fluorescence intensity between 10^4 to 10^5). This facilitate the flow cytometry gating analysis of effectors and targets.

This section of the protocol describes the labeling of the cell line L1210 (target) with a cell permeable dye CellTrace™ Far Red (CTFR). The labeling of the target cells allows the cell discrimination from the effector CD8 T cells and the evaluation of granzyme B delivery inside the $CTFR^+$L1210 targets.

1. Prepare CellTrace™ Far Red (CTFR) solution:
   a. Dilute the 10 μM CellTrace™ Far Red to a final concentration of 0.1 μM with PBS (without Ca^{++}/Mg^{++}). Prepare a working concentration of 0.1 μM of CellTrace™ Far Red by diluting 2.5 μL of the 10 μM stock solution with 247.5 μL of PBS (without Ca^{++}/Mg^{++}).

   Note: For *ex-vivo* cytotoxicity of NK cells, labeling of K562 cell line is performed with CellTrace™ Far Red at 0.15 μM final concentration.

   ⚠ CRITICAL: The working concentration of CTFR should be optimized for each target cell line. Label target cells with different concentrations of CTFR as described in steps 6 and 7. The best concentration to label the cells is assessed by flow cytometry. The optimal concentration will be the one that provides the best separation (at least 1 Log difference) between the unstained cells and $CTFR^+$ cells. For example, unstained cells will have an autofluorescence (fluorescence scale between 10^1 to 10^2) and the signal of $CTFR^+$ cells the fluorescence intensity between 10^4 to 10^5 (See Figure 2).

2. Mix the L1210-FAS^- cell line (this cell line grows in suspension) up and down with a 10 mL pipette and take an aliquot to measure cell count and viability as described in section isolation of human CD8 T cells from peripheral blood, step 12.
3. Harvest a volume corresponding to 5–7 × 10^6 targets (L1210-FAS^-) from the flask into 15 mL tubes and pellet the cell suspension by centrifugation at 526 g for 6 min at 20°C.

   Note: Labeling with CTFR cells and the washing steps may lead to losing between 1 to 5% of cells. To achieve less target cell loss the viability should be above 90% before experiment. In the following step 6, an example 5 × 10^6 cells after CTFR labeling is used.

4. Aspirate the supernatant, resuspend the pellet and wash by addition of 10 mL of PBS (without Ca^{++}/Mg^{++}).
5. Centrifuge the cells at 526 g for 6 min at 20°C. Repeat the washing step twice.
6. Aspirate and discard supernatant and resuspend the cell pellet by finger flick the tube.

   Note: This step is critical to eliminate the protein from the supplemented RPMI media to allow better labeling of the cells.

7. Add 250 μL of CTFR 0.1 μM working solution (step 1a) to the L1210-FAS^- cells to a concentration of 20 × 10^6 cells/mL.

8. Resuspend the cells with a 1 mL tip by pipetting up and down.
9. Cover the tubes with aluminum foil and incubate the L1210-FAS$^-$ cells in a water bath at 37°C for 8 min. Mix the cells every 2–3 min.
10. Quench the reaction adding cold filtered Fetal Bovine Serum (1:1 volume) to the cell suspension of L1210-FAS$^-$ cells, vortex and place the tube on ice for 5 min.
11. Wash the cells by addition of 10 mL of PBS (without Ca^{++}/Mg^{++}) and centrifuge the cells at 526 g for 6 min at 4°C. Repeat the washing step twice.
12. For redirect cytotoxicity assay, proceed to biotinylation of target cells step 13. See section biotinylation of target cells for redirect cytotoxicity assay.

Note: For *ex-vivo* cytotoxicity using K562 as targets, CTFR labeled K562 are adjusted to 5 × 10^6 cells/mL in X-Vivo^{TM} 15 media. CTFR labeled K562 cultured alone in the presence of GZB substrate is used as negative control of GZB activity. In addition, CTFR$^+$ L1210-FAS$^-$ mixed with the PBMC can be used as negative control of GZB delivery by NK cells (no recognition). Proceed to step 25, redirected cytotoxicity assay section.

Biotinylation of target cells for redirect cytotoxicity assay

Timing: 1.5 h

This section describes the biotinylation of surface proteins and coating with streptavidin to create binding sites and crosslink of the biotin labeled CD3 mAb (clone UCHT1) as we previously described.[2] The L1210-FAS$^-$ cell line allows to measure the granule exocytosis pathway or GZB mediated apoptosis.

13. Preparation of working solutions of Biotin -X-NHS and streptavidin in HEPES saline buffer:
    a. Prepare 100 mL of 1× the HEPES saline buffer 10× (0.1 M HEPES, 1.5 M NaCl, pH 7.5) with diH_2O.
    b. Prepare 50 mL 1× HEPES saline buffer (0.1 M HEPES, 1.5 M NaCl, pH 7.5) and 1% bovine serum albumin (BSA).
    c. Before weighting the Biotin -X-NHS warm the vial for 5 min at room temperature.
        i. To prepare 2 mM concentration, weight 1.11 mg of Biotin -X-NHS and add 1 mL of 1× HEPES saline buffer.
        ii. When the Biotin -X-NHS is dissolved, dilute 1:10 the 2 mM solution with 1× HEPES saline buffer to obtain a final concentration of 0.2 mM.
    d. Thaw Streptavidin (2 mg/mL stock) and dilute to a working concentration of 20 μg/mL in HEPES + 1% BSA saline buffer.

Note: The 10× HEPES saline solution can be prepared and stored at 4°C up to 2 months.

⚠ CRITICAL: The Biotin -X-NHS 2 mM working solution should be prepared fresh for each experiment.

14. Resuspend the cell pellet of CTFR$^+$ L1210-FAS$^-$ cells with 2 mL of HEPES saline buffer and count and check viability as described in described in section isolation of human CD8 T cells from peripheral blood, step 12.
15. Place the CTFR$^+$ L1210-FAS$^-$ cells (5 × 10^6 cells) into a 15 mL tube and wash by adding 8 mL of HEPES saline buffer to the cells and centrifugate at 526 × *g* for 6 min at 4°C.
16. Repeat this washing step once more.
17. Aspirate the supernatant and resuspend the cell pellet with 1 mL of 0.2 mM Biotin -X-NHS to reach a cell concentration of 5 × 10^6 cells/mL.
18. Vortex and place the tube on ice for 30 min.

19. Wash the cells once with HEPES saline buffer containing 1% BSA by centrifugation at 526 × *g* for 6 min at 4°C.
20. Resuspend cell pellet with 0.5 mL of 20 μg/mL streptavidin (SA) to a cell concentration of 10×10^6 cells/mL and transfer to a new tube.
21. Incubate the tube at room temperature (20°C–25°C) for 30 min.
22. Wash the cells with HEPES saline buffer containing 1% BSA by centrifugation at 526 × *g* for 6 min at 20°C.
23. Aspirate the supernatant and resuspend the cells in 1 mL of X-VIVO™ 15 media.
24. Count the cells and adjust to 5×10^6 cells/mL in X-VIVO™ 15 media.

Note: The biotinylation and washing steps may lead to a cell loss of less than 2% of cells. Separate a small number of cells (1×10^5) to use as the compensation control of CTFR labeled cells.

Redirected cytotoxicity assay

Timing: 1.5 h

This section describes the preparation and plating of effector CD8 T cells and the biotinylated (B)-Streptavidin (SA)-CTFR$^+$L1210-FAS$^-$ target cells (B-SA-CTFR$^+$L1210FAS$^-$) for redirect killing assay. Before the plating the cells both effectors and targets are labeled with a Live/Dead staining using NFL1 (OncoImmunin, Inc). NFL1$^+$ (dead cells) will be excluded from the flow cytometry analysis.

25. Live/dead staining using NFL1 (OncoImmunin, Inc) is performed following manufacturer instructions (https://www.phiphilux.com/assays/Cytotoxicity_Assays/NFL1.pdf).
    a. Resuspend CD8 effector T cells and B-SA-CTFR$^+$L1210FAS$^-$ target cells at a concentration of 5×10^6 cells/mL in X-VIVO™ 15 media in a 15 mL tube.
    b. Dilute 1 μL of NFL1 in 9 μL of X-VIVO™ 15 media (1:10 working solution).
    c. Add 3.3 μL of NFL1 working solution into 1 mL of the cell suspension to achieve 1:3000 final dilution.
    d. Incubate for 15 min at 37°C and 5% CO_2.
26. Wash twice with X-VIVO™ 15 media by centrifugation at 526 × *g* for 6 min at 20°C.
27. Aspirate the supernatant, resuspend in X-VIVO™ 15 media and count the viable CD8 effector T cells and B-SA-CTFR$^+$L1210FAS$^-$ target cells as described in section isolation of human CD8 T cells from peripheral blood, step 12.
28. Preparation of effectors (E) and target (T) cell suspensions for an E:T ratio of 27:1.
    a. Adjust the B-SA-CTFR$^+$L1210FAS$^-$ Target to a cell concentration of 1.6×10^6 cells/mL (4×10^4 cells/well final).
    b. Adjust the effector CD8 T cells to a cell concentration of 43.2×10^6 cells/mL (1.08×10^6 cells/well final).
    c. 25 μL of target cells and effector cells are plated in each well.

Note: The effector to target ratio (E:T ratio) should be optimized for each type of effectors from human, murine CD8 T cells and NK cells. This protocol presents an example using human effector CD8 T cells polyclonally expanded *in vitro*. The E:T ratio was optimized to 27:1.

Note: According to specific E:T ratio, the maximum number of total cells (targets and effectors) per condition should not exceed 1.5×10^6, V-bottom 96 well plate (section redirected cytotoxicity assay step 29). For higher numbers of cells, it is recommended to perform the incubation in a 15 mL conical tubes (with the same volumes). This provides more surface and avoid unspecific cell death.

⚠ CRITICAL: Targets cells should be plated at 5×10^4 and no less than 2.5×10^4 cells per 96 well plate. This range is optimal to collect sufficient events for the flow cytometry analysis.

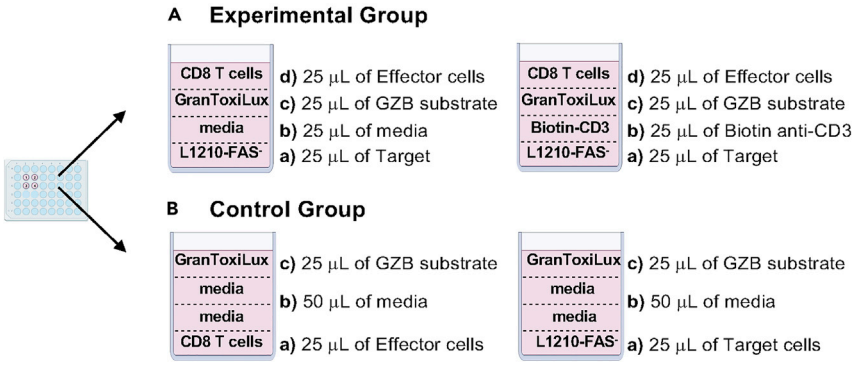

Figure 3. Schematic representation of the culture of Target and Effector cells in a redirected killing assay

(A) Experimental wells: CD8 T cells (Effectors) and L1210-FAS$^-$ targets (B-SA-CTFR$^+$L1210FAS$^-$ targets$^+$) cultured at E:T ratio of 27:1 in the presence of media and biotin labeled anti-CD3 mAb.

(B) Experimental Controls: CD8 T cells and L1210-FAS$^-$ cultured in the presence of GranToxilux: Granzyme B substrate. Effector, target cells and reagents are platted in the order of a to d.

Note: *Ex vivo* cytotoxicity assay of NK cells: Freshly isolated or thaw and rested overnight (14–16 h) PBMCs can be used for ex-vivo NK cell cytotoxicity using K562 cell line at a 60:1 and 10:1 (E:T) ratio. CTFR labeled K562 cultured alone in the presence of GZB substrate is used as negative control of GZB activity in the targets. In addition, CTFR$^+$ L1210-FAS$^-$ mixed with the PBMC can be used as negative control of GZB delivery by NK cells.

29. Plating of the effector and targets into V-bottom 96 well plate (Figure 3):
    a. Experimental group E:T ratio of 27:1:
        i. CD8 T cells + B-SA-CTFR$^+$L1210FAS$^-$ + media + GranToxiLux.
        ii. CD8 T cells + B-SA-CTFR$^+$L1210FAS$^-$ + biotin-CD3 mAb + GranToxiLux.
    b. Control group:
        i. Control Effector alone: CD8 T cells + GranToxiLux + 50 µL media.
        ii. Control Targets alone: B-SA-CTFR$^+$L1210FAS$^-$ + GranToxiLux + 50 µL media.

Note: Total volume of each well is 100 µL. 50 µL X-VIVO^{TM} 15 is added to the Target and Effector alone culture conditions.

30. Centrifuge the plate at 189 × *g* for 3 min at 20°C.
31. Place the plate in the incubator at 37°C and 5% CO_2 for 45 min.
32. To monitor the degranulation of CD8 T cells add 10 µL of a cocktail of mAbs containing CD8 and CD107a mAbs.

Note: Add the mAbs cocktail slowly and centrifuge the plate at 189 × *g* for 30 s at 20°C to allow mixing the mAbs and cells. Alternatively, cut the top of a 200 µL yellow tip and mix up and down very slowly to avoid disrupting the conjugates.

33. Incubate in the incubator at 37°C and 5% CO_2 for an additional 15 min.

Note: For *ex-vivo* cytotoxicity with NK cells a cocktail of CD3, CD19, CD16 and CD56 is used to measure degranulation of the NK cell subsets.

Flow cytometry staining, acquisition and analysis

Timing: 1 h

This section describes the staining of the cell surface of the effector CD8 T cells after the incubation time with the target cells to monitor the degranulation. The degranulation of the effector CD8 T cells is evaluated by surface expression of CD107a, a granule membrane protein that is exposed on the surface of CD8 T cells during delivery of the granzyme B to the target cells ($CD107a^{+}CD8^{+}$ T cells) CD8 T cells This section also describes the preparation of the compensation tubes, flow cytometer setup, acquisition, and analysis including gating strategy to analyze the delivery of GZB and effector degranulation.

34. Flow cytometry (FC) Staining buffer: HBSS (without Ca^{++} and Mg^{++}) + 0.01% of NaN_3 + 0.01% BSA.
35. Prepare compensation tubes:
    a. Unstained: CD8 T cells.
    b. Unstained: L1210-FAS^{-} cells.
    c. CTFR: B-SA-$CTFR^{+}$L1210FAS^{-} targets (section biotinylation of target cells for redirect cytotoxicity assay step 24).
    d. NFL1: NFL1 labeled CD8 T cells (section redirected cytotoxicity assay, step 25).
    e. GranToxiLux: compensation beads incubated with FITC labeled mAb.
    f. CD8 BUV395: compensation beads incubated with CD8 mAb.
    g. CD107a APC-H7: compensation beads incubated with CD107a mAb.
36. Staining of compensation beads:
    a. Prepare FACS tubes containing 100 μL of (FC staining buffer).
    b. Resuspend the compensation beads vial by vortexing and add one drop into the FACS tubes.
    c. Add 5 μL of FITC labeled mAb (anti-CD3 mAb was used as compensation control for the GranToxiLux substrate).
    d. 5 μL of anti-CD8 BUV395 to the compensation tube.
    e. 5 μL anti-CD107 CD107a APC-H7 mAb to the compensation tube.
    f. Incubate compensation beads for 30 min at 4°C.
    g. Wash once with 2 mL FC staining buffer by centrifugation at 526 × *g* for 6 min at 4°C aspirate or decant the supernatant and resuspend the beads in 100 μL.
    h. Keep on ice and protected from the light with aluminum foil until setting up the compensation.
37. Resuspend the cells in the 96 wells gently with 200 μL pipette and transfer the cell suspension into a FACS tube. Wash the cells once by adding 2 mL of cool (4°C) FC staining buffer.
38. Centrifuge the FACS tubes at 526 × *g* for 6 min at 4°C.
39. Aspirate the supernatant and resuspend the cells in 200 μL of cool FC staining buffer. Keep the cell suspension on ice and acquire in the flow cytometer.

⚠ CRITICAL: Set up the flow cytometer in advance before acquisition of the cells (For setup please see materials and equipment). Keep FACS tubes of the samples on ice all the time and protected from the light until acquisition. Cells should be flow immediately and no more than 1 h to avoid losing signal of the granzyme B activity in the target cells.

Flow cytometer (BD Symphony 5A) setup for compensation

Reagent	Detectors/nm
Unstained cells	
B-SA-$CTFR^{+}$L1210FAS^{-}	670/30
CD8 T cell NFL1 (labeled)	450/40
GranToxilux (CD3 FITC)	515/20
CD8 BUV395	390/20
CD107a APC-H7	780/60

Note: CD8 T cells and L1210 has similar autofluorescence. A mixed of both cells can be used as unstained control and perform the automatic compensation calculation by the instrument.

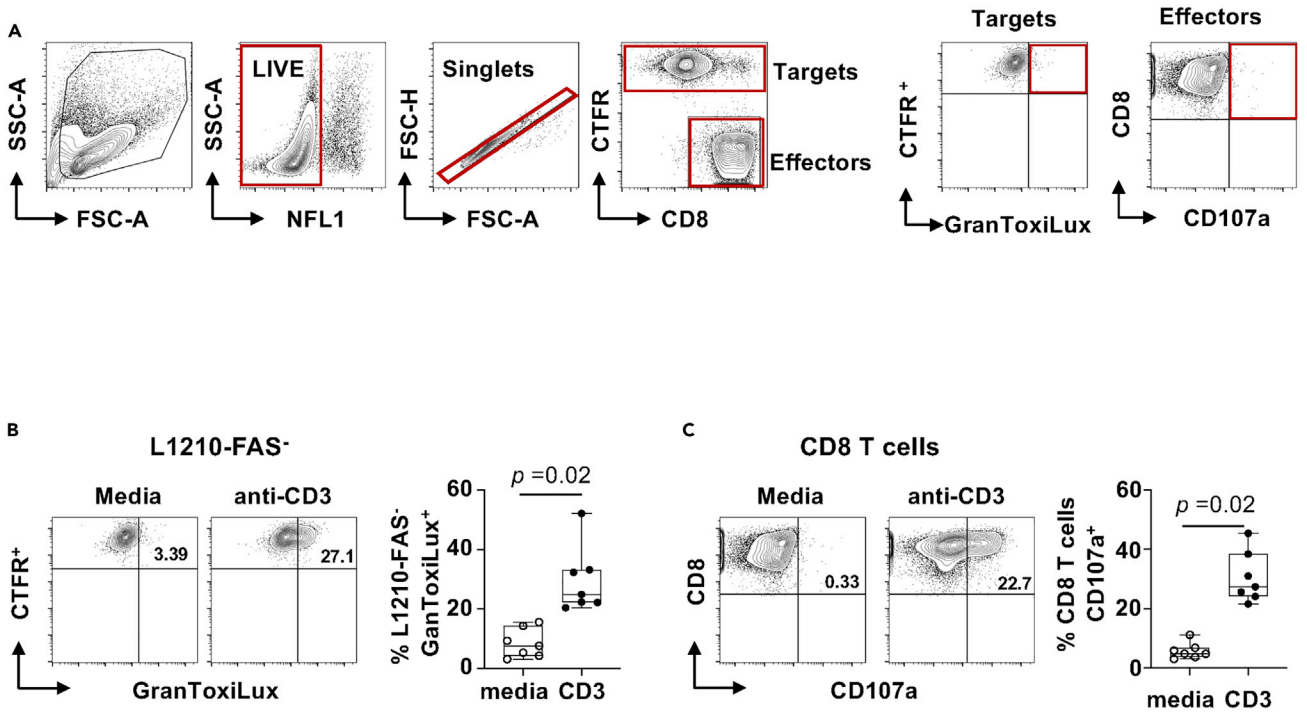

Figure 4. Flow cytometry analysis of GZB delivery and activity in the target cells and degranulation of effector CD8 T cells in redirected killing assay

(A) Gating strategy for the analysis of target cells ($CTFR^+$) and Effector cells ($CTFR^-CD8^+$) T cells. Panel A has been reported in the manuscript related to this protocol.[1]

(B) Left panel: representative gating strategy for the analysis of GZB delivery and activity $GranToxiLux^+$ in the targets ($L1210FAS^-$). Right panel: Compiled data of CD8 T cells from healthy controls (n= 7).

(C) Left panel: representative gating strategy for the degranulation ($CD107a^+$) of CD8 T cells stimulated with CD3 mAb. Right panel: Compiled data of CD8 T cells from healthy controls (n= 7). Comparisons between culture conditions were performed using a non-parametric Wilcoxon test. *P* value < 0.05 was considered significant. The graph is represented by box and whisker showing the median value with first and third quartiles in the box, with whiskers extending to the minimum and maximum values.

For targets and effector with higher autofluorescence between them test compensation matrix individually to evaluate whether this introduce a difference compensation matrix that may impact the analysis.

40. The gating strategy and analysis as shown in Figure 4.
    a. **Total live cells:** cells were gated based on side scatter area (SSC-A) and forward scatter area (FSC-A). A wider gate on the FSC-A was used to include potential cells that are undergoing early steps of apoptosis and will show lower FSC-A as a result of membrane damage.
    b. The FSC-A vs SSC-A gate is followed by exclusion of death cells ($NFL1^+$ cells) both Targets and Effectors.
    c. The LIVE gate ($NFL1^-$ cells) is followed by FSC-A vs FSC-H to exclude doublets.

Note: The redirected killing assay promotes a strong conjugate formation mediated by the CD3 mAb. In assays in which antigen specific T cells are tested, the frequency of conjugates formed by $CTFR^+$ (Targets)-$CD8^+$ (T cells) could be informative and can be analyzed. In this case it is recommended to test different concentration of antigenic peptide to evaluate conjugate formation.

The redirected killing assay was used to evaluate the role of PAR-1 signaling in human CD8 T cell mediated granzyme B delivery, and virus (LCMV-Gp33)-specific CD8 T cells.[1]

d. **Target cells:** $CTFR^+$ cells (B-SA-$CTFR^+$L1210FAS^- $targets^+$) were identified based on $CTFR^+$ to distinguish them from the effector cells $CTFR^-CD8^+$ T cells.
   i. The delivery and activity of the GZB inside the target cells ($CTFR^+$ gate) is evaluated by $CTFR^+substrate^+$(GrantoxiLux) cells.
e. **Effector T cells:** The degranulation of $CD8^+$ T cells is measured by surface expression of CD107a in $CD8^+$ T cells.

EXPECTED OUTCOMES

CD8 T cells incubated with the target cells in the presence of biotin anti-CD3 mAb will trigger TCR-induced degranulation ($CD107a^+$CD8 T cells). The delivery and activity of GZB inside the targets will be detected by the fluorescence signal of the GZB processed substrate ($CTFR^+GranToxiLux^+$) as shown in the Figures 4A and 4B. The percentage of $GranToxiLux^+$ target cells represents the percentage of cells that CD8 T cells delivered the granule content and will be killed. As control, CD8 T cells incubated with the target cells in the absence of biotin anti-CD3 mAb will not degranulate and CD107a will not be exposed on the cell surface. In this case, the target cells ($CTFR^+$) will not present a fluorescent signal of the GZB substrate ($CTFR^+GranToxiLux$).

The *ex vivo* cytotoxicity assay of NK cells, PBMCs were cultured in presence of the K562 cell line, a NK cell sensitive target and two E:T ratios were measured. L1210-FAS^- cell line was used as negative control or GZB delivery. In addition, as negative control K562 alone incubated with substrate will be negative GZB substrate signal. The degranulation of NK cells was monitored by surface expression of CD107a (Figures 5A–5C).

QUANTIFICATION AND STATISTICAL ANALYSIS

GranToxiLux is used to measure the GZB delivery by effector cells inside target cells. The frequency of $GranToxiLux^+$ target cells is a function of the delivery of the cytotoxic granule content and potential killing by effector cells (cytotoxic CD8 effector T cells and NK cells). The frequency of CD8 T cells $CD107a^+$ is a function of degranulation of effectors.

Statistical analysis: Comparisons between culture conditions were performed using a non-parametric Wilcoxon test. P value < 0.05 was considered significant.

> ***Note:*** In the *ex-vivo* cytotoxicity assay of NK cells using PBMCs the E:T ratio should be adjusted to the frequencies of NK cells present in the PBMCs from each donor. This can be assessed by flow cytometry as shows Figure 5.

Adjusted E:T = (#PBMCs × % Total NK cells/100)/ #Target cells.

% GZB delivery/activity = % $GranToxiLux^+$ of the adjusted E:T ratio.

Figure 5 shows an example:

E:T ratio of 60:1 of total PBMCs = 1.8×10^6 PBMCs : 3×10^4 K562 targets.

The PBMCs of this particular donor had a 7.2% of total NK cells ($CD56^+$ lymphocytes) measured by flow cytometry.

The total # of NK cells in the PBMCs = $(1.8 \times 10^6$ cells × 7.2)/100) = 1.29×10^5 NK cells.

Adjusted E:T ratio = (1.29×10^5 cells (effector NK cells) / 3×10^4 (K562 target cells)) = 4.3:1 ratio.

The % GZB delivery/activity in a 4.3: 1 (E:T ratio) = 73% (Figure 5B).

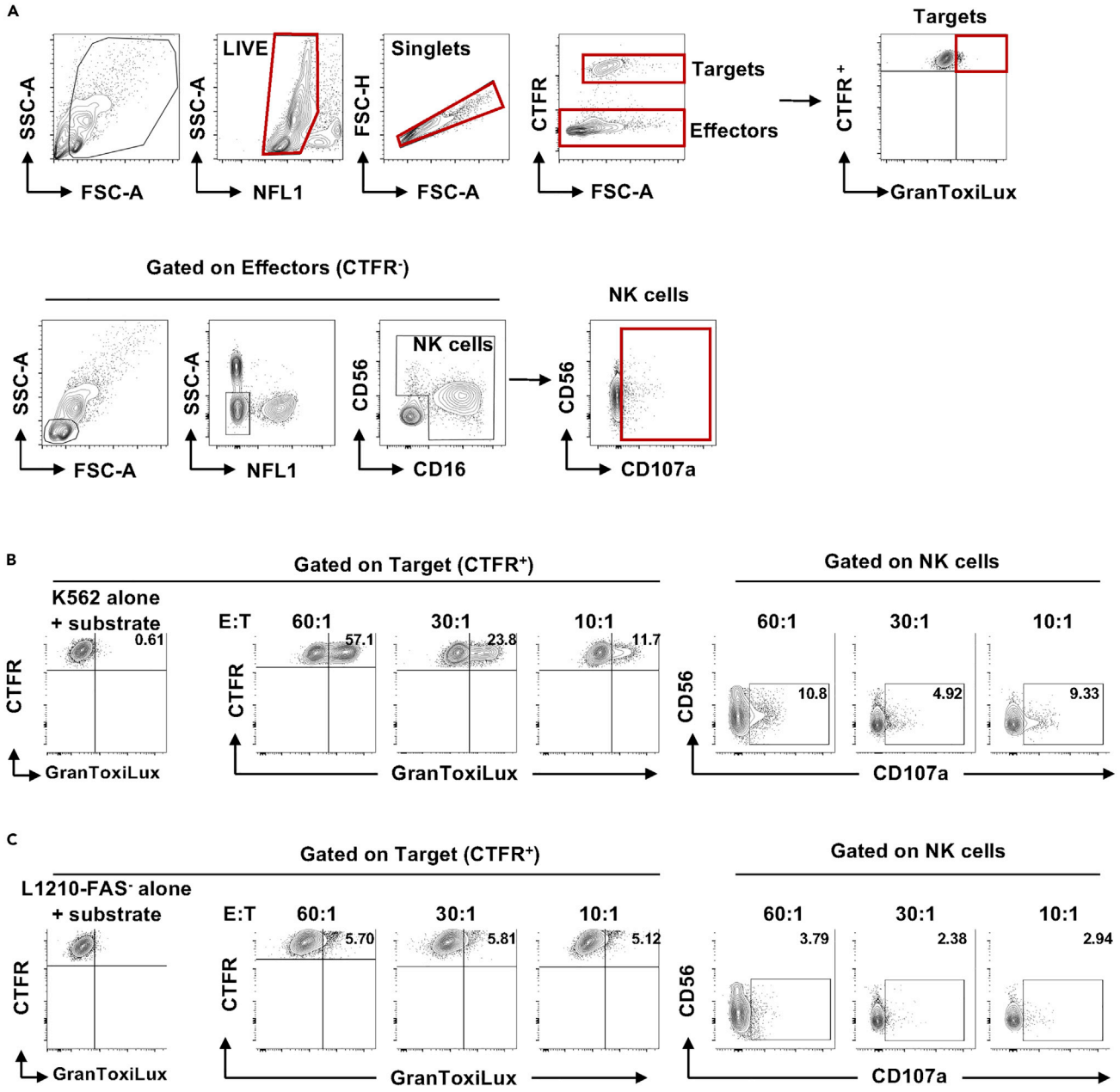

Figure 5. *Ex vivo* cytotoxicity of human NK cells from PBMCs

(A) Gating strategy for the analysis of target cells ($CTFR^+$) and Effector cells (NK cells). NK cells were gated as $CD3^-CD19^-$, $CD56^+$ and $CD16^+$ cells.

(B) PBMCs were cultured with the NK sensitive cell line K562 at E:T ratio of 60:1, 30:1 and 10:1. Left panel: Analysis of GZB delivery and activity ($GranToxiLux^+$); and right panel: degranulation ($CD107a^+$) of NK cells.

(C) Left panel: Negative control of GZB activity and delivery using a murine L1012-FAS^- cell line cultured with PBMC; and right panel: degranulation ($CD107a^+$) of NK cells.

LIMITATIONS

The activity of GZB inside the target cells ($GranToxiLux^+$ Target cells) is highly dependent on the effector function of T cells. Therefore, the E:T ratio should be optimized for each system regardless of whether the system is antigen independent (redirected killing assay) or antigen dependent (virus-specific CD8 T cells).

This protocol measures an early step after GZB delivery and longer incubation periods of effectors and target cells is no recommended because late apoptotic cells with increase membrane permeability may lose the fluorescence signal. Key factors to be considered are the optimization of E:T ratios and incubation time for the particular experimental system using cells from human and mice.

TROUBLESHOOTING

Problem 1

Low viability of the effector cells (section *In vitro* polyclonal stimulation of human CD8 T cells (Effector cells, step 27); and target cells section target cells recovery and maintenance, step 31).

Potential solution

- In the case of low viability, it is recommended that dead cells are eliminated by either Ficoll gradient or other commercial kits (such as Dead Cell Removal kit from Miltenyi Biotec or Stem Cell), one day before the assay and rested during (14–16 h). The viability of the effectors and target cells before the assay should be between 90%–95%.

Problem 2

Low or no GranToxiLux$^+$ cells (section flow cytometry staining, acquisition and analysis step 39).

Potential solution

- This assay is time sensitive. Set up the flow cytometer in advance before acquisition of the cells. It is recommended to acquire the samples as soon as possible and not longer than 1 h after harvesting (*section* flow cytometry staining, acquisition and analysis step 39).
- If any fluorescent antibodies need to be added, make sure the fluorochromes will not have compensation issues with the substrate (GranToxiLux). GranToxiLux Substrate has ex: 488 nm and em: 520 nm.
- Try different E:T ratio and incubation time. The protocol described was optimized for human CD8 T cells in redirected killing assay using the L1210-FAS$^-$ as targets, and to test *ex-vivo* cytotoxicity of NK cells from PBMCs. The protocol was also optimized with murine virus (LCMV-GP33) specific-CD8 T cells, please see Chen et al.[1]
- It is recommended to use the *in vitro* activated human CD8 T cells between day 10–15 days of expansion. Longer *in vitro* cultures may lead to a decline of the cytotoxic function.

Problem 3

Don't have enough effector CD8 T cells (section *In vitro* polyclonal stimulation of human CD8 T cells (Effector cells, step 27).

Potential solution

In the case of low numbers of CD8 effector T cells, to achieve the required E:T ratio reduce the number of target cells per condition. It is not recommended to use less than 2.5×10^4 target cells per condition.

Problem 4

No difference of %GranToxiLux$^+$ target cells among different treatment or donors (section flow cytometry staining, acquisition and analysis step 40).

Potential solution

Optimization of an appropriate E:T ratio for each type effector cells either antigen specific or in redirected killing assay is recommended. The maximum and the minimum of the E:T ratio it will depend on the activity of the particular effector cells (human, murine etc).

- Activated CD8 T cells from healthy controls will have some variability in the potential killing activity. When CD8 T cells are limited, it is recommended that several E:T ratios are tested to evaluate the optimal ratio for each type of effectors (for example 27:1, 9:1, and 3:1) and to cover the range of heterogeneity between donors, particularly human samples. In our experience we found that between 27:1 and 20:1 is optimal for human polyclonally stimulated CD8 T cells. Please, see examples of human CD8 T cells (27:1 E:T ratio) and murine Gp33 specific-T cells (9:1 and 3:1 E:T ratio).[1]
- In the case of no differences of the % substrate$^+$ target cells between treatments/culture conditions and controls consider:
  - It is possible that the assay is at the maximum GZB delivery/activity and reach a plateau for that E:T ratio and no differences are appreciated between the treatments. In this case, it is recommended to test lower E:T ratios to evaluate the effects of treatments.

Example of E:T ratio (Figure 5). PBMCs were cultured at E:T ratio of 60:1 30:1 and 10:1 with K562. K562 alone and L1210-FAS$^-$ (non-sensitive target for NK cells) cells were used as negative control of Granzyme B delivery and activity.

- Avoid adding cytokines the day before to the assay that may lead to an unspecific killing and/or higher background in GZB delivery in the target cells.

Note: Once established the optimal E:T ratio, the assay is reproducible among the donors and the same donor tested in independent experiments.

Problem 5

CTFR staining is dim (section CellTrace™ Far Red (CTFR) Labeling of L1201-FAS$^-$ target cells *step 1).*

Potential solution

Overgrown cell lines may not label well with CTFR. In addition, the yield of the cells and viability maybe low. It is recommended to maintained the cell lines at the corresponding cell concentration and dilute the target cells with fresh media one day before the experiment to assure maximum viability.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Marta Catalfamo (mc2151@georgetown.edu).

Materials availability

This study did not generate new reagent.

Data and code availability

This study did not use any database and code.

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AUTHOR CONTRIBUTIONS

M.C. designed the research. M.S., T.L., M.A., and G.K. performed human CD8 T cell experiments, and T.L. performed the NK cells. T.L., M.S., and M.C. wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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