Ex vivo Natural Killer Cell Cytotoxicity Assay

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[Abstract] Natural Killer (NK) cells are cytotoxic lymphocytes that constitute a major component of the innate immune system. Immunosurveillance of the host by NK cells for malignant and virally-infected cells results in direct cytotoxicity and the production of cytokines to enhance the immune response. This protocol will describe the "gold standard" chromium release assay for measuring the target cell killing capacity of NK cells. Key features of this cytotoxicity assay are that it is performed with sorted NK cells as the effectors and any Major Histocompatibility Class I (MHC-I)-low or deficient tumor cell line can be used as the target cells.

Materials and Reagents

1. Lympholyte-M (Cedarlane, catalog number: CL5035)
2. RPMI 1640 (Hyclone, catalog number: SH300027.01)
3. Chromium-51 (PerkinElmer, catalog number: NEZ030001MC)
4. Poly (I:C) (Sigma-Aldrich, catalog number: P1530)
5. Source of mouse spenocytes: C57Bl/6 mice (Charles Rivers, strain code: 027)
6. NK sensitive target cell lines: YAC-1, ATCC, TIB 160
7. 1x sterile PBS (Hyclone, catalog number: 21-031-CV)
8. Running buffer (Miltenyi Biotech, catalog number: 130-091-221)
9. Washing buffer (Miltenyi Biotech, catalog number: 130-092-987)
10. AutoMACS pro columns (Miltenyi Biotech, catalog number: 130-021-101)
11. AutoMACS buffer (see Recipes)
12. Complete RPMI (see Recipes)
13. NK cell media (see Recipes)

Equipment

1. 50 ml tubes (BD Biosciences, Falcon®, catalog number: 352098)

2. 15 ml tubes (BD Biosciences, Falcon®, catalog number: 352096)
3. 96 V well plates (Corning, Costar®, catalog number: 3894)
4. AutomacsPro Separator (Miltenyi biotech, model: 130-092-545)
5. Gamma counter (PerkinElmer, model: 2470)
6. Incubator (5% CO_2, 37 °C) (Sanyo)
7. Centrifuge (when parameters of brakes are unspecified, maxmal acceleration and deceleration are used) (Thermo Fisher Scientific, model: ST40R)
8. Dissection instruments (small forceps, scissors)
9. Cell strainers 70 µm (Thermo Fisher Scientific, catalog number: 22363548)
10. DX5 (CD49b) microbeads (Miltenyi Biotech, catalog number: 130-052-501)

Procedure

A. *In vivo* stimulation of NK cells

1. NK cells need to be stimulated *in vivo* in order to be able to kill. Poly (I: C) (TLR3 agonist) injection is the gold standard method of activating NK cell killing ability.
2. 3 C57Bl/6 mice (6-8 weeks of age, each weighing approximately 20 g) are usually sufficient in order to get enough NK cells. However, for any *in vivo* treatment (*i.e.* virus infection) that might result in lymphopenia or lymphocyte migration to the periophery, 4-5 mice may be needed.
3. Inject mice intraperitoneal (i.p.) with 150 µg Poly (I: C) (stored at -20 °C, stock is 10 mg/ml) diluted in 1x PBS (15 µl stock + 185 µl 1x PBS) 18 h before euthanizing the mice (*e.g.* inject at 2:00 PM if you plan on euthanizing mice at 8:00 AM the following day).
4. Prepare dissection instruments harvesting splencoytes.

B. Harvest splenocytes

1. 1 h before starting assay, remove Lympholyte from 4 °C. Lympholyte needs to be used at room temperature and protected from light (handle under biosafety cabinet with lights off).
2. Prepare one 50 ml tube and two 15 ml tubes for each spleen. Place a 70 µm cell strainer on each opened 50 ml tube. Prime each strainer with 1 ml of cold 1x PBS. Add 5 ml of Lympholyte into each 15 ml tube (protect tubes from light).
   Note: 1 spleen will need two 15 ml tubes, each containing 5 ml Lympholyte.
3. Euthanize mice by cervical dislocation, remove spleen and place on 70 µM strainer
4. Crush 1 spleen on a cell strainer over 50 ml tube, rinse twice with 10 ml of 1x PBS (I often rinse the underside of the filters as well if visible clumps of red spleen are observed). Filter again with 10 ml of 1x PBS using the same filter if needed. Spin tubes containing splenocytes at 500 *x g*, 5 min, 4 °C.

5. Discard supernatant and resuspend splenocyte pellet in 10 ml 1x PBS. Carefully layer 5 ml of resuspended splenocytes on top of the Lympholyte layer (5 ml of the 1st 15 ml tubes, then the remaining 5 ml on the 2nd 15 ml tube). Spin 1,500 *x g*, 15 min, room temperature, acceleration at 1, deceleration at 2 (minimal speed).
6. Carefully pipet lymphocyte layer (blurry interface layer between Lympholyte at the bottom and PBS on top) and transfer to a new 50 ml tube. Carry-over of small amounts of Lympholyte and PBS layers are acceptable because of washing procedure in steps 7 and 8. You can combine mice treatments here (*i.e.* all the same treatments together).
7. Fill 50 ml tube with 1x PBS (first wash to remove excess Lympholyte). Spin down 500 *x g*, 5 min 4 °C.
8. Discard supernatant. Resuspend pellet in 10 ml AutoMACS buffer. Spin down as in step II-7. During spin, harvest target cells. Discard supernatant.

C. NK cell sort (with DX5 microbeads)

1. Resuspend splenocytes pellet in 300 µl AutoMACS buffer per spleen (we usually pool 3 spleens per tube).
2. Add 100 µl of DX5 microbeads per spleen (manufacturer recommends 100 µl beads volume for 1 x 10^8 cells or less) and mix well. Incubate for 15 min at 4 °C. During incubation, start target cell labeling with chromium.
3. Add 10 ml of AutoMACS buffer to stop DX5 microbead incubation. Spin as in step B-7.
4. Discard supernatant. Resuspend pellet in 500 µl AutoMACS buffer per spleen (*e.g.* 1.5 ml for 3 pool spleens).
5. Proceed to sort. Turn AutoMACS Pro sorter on during last spin and do a rinse before starting.
6. Place tubes (input in row A, negative fraction in row B, positive fraction in row C) of rack holder. The size of the tubes used for sort depends on the AutoMACS rack holder used. Usually a standard 50 ml tube for the 3 holder rack, 15 ml tube for 5 holder rack, and 5 ml flow cytometry tubes for 6 holder rack.
   Note: "Input" = tube which contain cells to be sorted; "negative fraction" = eluate after sort containing $DX5^-$ non-NK cells; "positive fraction" = eluate after sort containing $DX5^+$ NK cells.
7. Select program: *Possel* with a quick rinse (qrinse) between each tube and rinse after the last tube. Start the sort. It will take approximately 5-7 min per sort with a 2 min qrinse in between.
8. After the sort, count the number of cells in the positive fraction tube (2 ml total volume) and determine the cell concentration. Spin down as in step B-7. During spin, prepare 96

V-well plate (add 100 µl NK cell medium to all wells that need it: 3 minimum release wells, and all wells containing 25, 12 and 6 E: T ratios).

9. Resuspend sorted NK cells at concentration of 1.5 x 10^6 cells/ml in NK cell medium.
10. Plate NK effector cells in 100 µl for the top two ratios (50:1 and 25:1), then dilute 2 fold downwards to 12:1 and 6:1 E: T ratios). Scheme: each 50:1 E: T wells contain 1.5 x 10^5 NK cells; each 25:1 E: T wells contain 7.5 x 10^4 NK cells; each 12:1 E: T wells contain 3.75 x 10^4 NK cells; each 6:1 E: T wells contain 1.875 x 10^4 NK cells.
    Note: All treatments and controls are plated in triplicate wells in 100 µl.
11. Overlay effector cells with target cells (as prepared in step IV) from a solution of 30,000 cells/ml (3,000 cells/well) in 100 µl.
12. Add 100 µl of 10x SDS to maximal release wells.
13. Incubate 4 hours in 37 °C, 5% CO_2. Spin plate at 500 *x g* and 4 °C, transfer 100 µl of supernatant to test tubes for CPM counting. Proceed to gamma counter previously calibrated for ^{51}Cr.
14. % Release measurement.
    (experimental release-average of minimal release)/(average of minimal release-average of maximal release) x 100
15. Calculate % for each well.

D. Target cell labeling

1. Harvest YAC-1 target cells during last spin before the addition of the DX5 microbeads.
    Note: YAC-1 target cells are grown in cRPMI and are non-adherent. They should be passaged for 1.5 weeks prior to use in killing assay.
2. Start the ^{51}Cr labeling during the 15 min incubation of the splenocytes with the DX5 microbeads.
3. The 1 h incubation should be done while you are plating your NK effector cells.
4. Wash three times (twice in cRPMI, final wash in NK cell media), count and resuspend cells at a 30,000 cells/ml concentration.

Recipes

1. AutoMACS buffer in 500 ml
    PBS
    2.5 g Bovine Serum Albumin
    2 ml of 5 mM EDTA
2. Complete RPMI in 500 ml
    500 ml of RPMI-1640

50 ml Heat-inactivated Fetal Bovine Serum

5 ml of Pencillin-Streptomycin 10,000 U each/ml

3. NK cell media

   500 ml of cRPMI

   5 ml 1 M HEPES

   5 ml 100 mM Sodium Pyruvate

   5 ml 100x Non-Essential Amino Acids

   0.5 ml of 2-mercaptoethanol for final concentration of 5×10^{-5} M

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