

Lung Clearance Assay

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[Abstract] Lung clearance assay tests the ability of innate immune cells (mainly NK cells) to eradicate *in vivo* cells injected via the tail vein of the mice. This assay helps to elucidate the role played by NK cells and their receptors (if the mice are genetically modified) against various human and mouse targets in an *in vivo* setting (Stern-Ginossar *et al.*, 2008; Halfteck *et al.*, 2009; Tsukerman *et al.*, 2012).

Materials and Reagents

1. Cell labeling dyes
 - a. Vybrant CM-Dil (Life Technologies, Invitrogen™, catalog number: V-22888)
 - b. VybrantDiD (Life Technologies, Invitrogen™, catalog number: V-22887)
 - c. CellTrace CFSE (Life Technologies, Invitrogen™, catalog number: C-34554) (either CM-Dil or CFSE can be used based on the preferred wave length either FL-1 for CFSE or FL-2 for the CM-Dil)
2. Other materials
 - a. Phosphate buffered saline (PBS)
 - b. 1x Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, Invitrogen™, catalog number: 10313-039)
 - c. Fetal bovine serum (Life Technologies, Invitrogen™, catalog number: 11091-148)
 - d. 40 micron cell strainer (BD Biosciences, Falcon®, catalog number: 352340)
 - e. Mice of interest
 - f. NaN₃ (Sodium Azide)
 - g. Erythrocyte lysis buffer (ELB) (see Recipes)
 - h. FACS buffer (see Recipes)

2. Antibodies

To demonstrate that the observed effect is specific we recommend to either blocking the relevant receptors, or by depleting NK cells, by using anti-NK1.1 (in case the receptor is present), or by using anti-asialo GM1.

- a. Anti NK1.1 (eBioscience, catalog number: 14-5941-82)
- b. Anti-Asialo-GM1 (eBioscience, catalog number: 16-6507-39)

- c. Anti-Mouse CD314 (NKG2D) (eBioscience, catalog number: 14-5873-82)

Equipment

1. BD LSR II flow cytometer

Procedure

1. The tail vein injection requires prior practice, mature male mice are easier to inject. We suggest using 8-12 weeks male mice.
2. The depletion of NK cells for a period of one day is obtained by I.P (Intra peritoneal) injection of 150 microgram (in up to 250 μ l PBS) of anti NK1.1 up to 24 h prior to the experiment.
3. Note: Not all cell types are suitable for these experiments, small cells are hard to recover, large cells may clog the lungs, some cells are not labeled well etc. Therefore, preliminary experiments using limited amount of animals are strongly recommended. The lines that we tested are: HeLa, PC-3, DU 145, PD1.6 and YAC-1. Since the tail vein injections may vary between different mice even in the same group there is a need for an internal injection control which is usually cells that are resistant to mouse NK cell killing. Label two cell types (experiment and control) according to the manufacturer instructions for the dyes. If the cells used were not in the manufacturer protocol calibration *in vitro* staining experiments are required. Labeling of HeLa (the control population for the majority of our experiments), Jurkat, 3T3, P3X, PC-3 DU 145 is performed as follows:
 - a. Suspend cells at a density of 1×10^6 /ml in serum-free culture medium (DMEM or RPMI).
 - b. Add 1- 5 μ l (from the stock) of the cell-labeling solution per ml of cell suspension. Mix well by gentle pipetting. Do not exceed 5 ml volume in each labeling tube. The differences between CFSE and Vybrant-CM-Dil are, the emission (FL-1/FL-2) and the range of labeled cells (we recommend working with the Vybrant-CM-Dil when possible).
 - c. Incubate at 37 °C HeLa (8 min), Jurkat (2 min), 3T3 (15 min), P3X (15 min), PC-3 (30 min) DU 145 (30 min). We recommend incubating in 15 ml tubes covered with aluminum foil.
 - d. Centrifuge at 450-515 $\times g$ for 5 min.
 - e. Remove the supernatant and gently resuspend the cells in warm (37 °C) medium.
 - f. Repeat the wash procedure (3-d and 3-e).

4. Resuspend the cells to a final concentration of 10^7 cells per ml these cells are to be injected to the mice, 2×10^6 cells of each cell type (4×10^6 total) are injected via the tail vein per mouse; the final injection volume is 400 μ l.
5. Pass the cells through a 40 μ m cell strainer.
6. Set aside 400 μ l of the cell mixture that will serve as the pre-injection tube. Keep these cells at 37 °C for the duration of the experiment.
7. Inject the cells into the tail vein of the mice.
8. Wait for 5 h.
9. Sacrifice the mice according to ethical regulations, we recommend using CO₂.
10. Harvest the lungs, place them in a 6 well plates with 2 ml ice cold PBS until harvesting the lungs of the entire experiment is completed.
11. Place a 40 μ m cell strainer on top of a 50 ml tube, transfer the lungs into the strainer, transfer the PBS from the 6 well into the tube.
12. Smash the lungs by using the cylinder/pestle of a 5 ml syringe; add more PBS as required.
13. After obtaining a homogenous solution centrifuge the tubes at 450-515 x g for 5 min.
14. Discard supernatant and resuspend cells with 10 ml of ELB (erythrocyte lysis buffer) and keep on ice for 5 min.
15. Centrifuge the cells at 450-515 x g for 5 min.
16. Discard supernatant and observe for erythrocyte content (if there is visible amount of red cells in the pellet) repeat steps 15-16, until the red blood cells disappear.
17. Resuspend the cells in 10 ml of DMEM/RPMI supplemented with 10% FBS (this step eliminates the ELB activity).
18. Centrifuge the cells at 450-515 x g for 5 min.
19. Discard supernatant and resuspend cells in 1-2 ml of FACS buffer according to pellet size.
20. Examine the cells using FACS. Begin with the pre-injection sample, set the gates based on forward and side scatter (see figure below), and calibrate the laser intensity. Collect at least 2.5×10^5 cells. Draw a dot plot of FL-4 vs FL-2 (if using Dil) or FL-4 vs FL-1 (if using CFSE).
21. Read the lung samples. We recommend at collecting least 2.5×10^6 cells per sample
22. Note: cells can be resuspended in 2% formaldehyde and stored overnight at 4 °C in dark, prior to analysis by flow cytometry.
23. Flow cytometric analysis (Figure 1): Y axis FL-1 intensity X axis FL-4 intensity. The square frame marks the "experiment" cell population; the dashed square frame marks the "internal control" cell population. The lower left square are lung cells of the mice (FL-1, FL-4 negative).

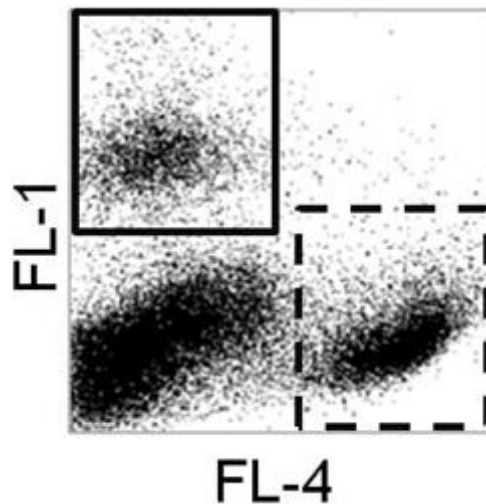


Figure 1. Flow cytometric analysis

24. To obtain the killing percentage, the amount of experimental cells (NK sensitive) divided by the amount of the control cells (NK insensitive) in the pre-injection group is calculated and this ratio is set up to be the reference control. Accordingly, similar calculation is performed regarding the cells recovered in the lungs 5 h post infection and tumor cell survival is calculated.

Recipes

1. Erythrocyte lysis buffer (ELB)
 - NH₄Cl 16.4 g
 - KHCO₃ 2 g
 - EDTA 0.5 M 400 µl
 - 2 L ddH₂O
 - Titrate with HCl to PH 7.2-7.4
2. FACS buffer
 - 1x PBS
 - 0.5% BSA
 - 0.05% NaN₃ (Sodium Azide)

Acknowledgments

The authors would like to acknowledge Noam Stern-Ginossar, Chamutal Gur, Ariella Glasner, and Mazal El-Nekaveh.

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