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Quantification of synapse polarization ("the conjugate assay")

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ABSTRACT

NK cells form structured interfaces with tumor cells, known as immune synapses. The forces and receptor-ligand interactions at the immune synapse determine whether the NK cell kills the tumor cell or not. Several mechanistic steps govern the efficiency of this process: adhesion molecules, such as LFA-1, and activating receptors, such as FcγRIIIA/CD16 or NKp30 gather at the point of contact; perforin-containing cytotoxic granules gather around the microtubule organizing center (MTOC); and the MTOC polarizes towards the synapse, where granules are released across the synapse onto the tumor cell.

If NK cells are allowed to form synapses with tumor cells in a short term assay (normally 5 min) they can then be fixed and stained for the cytotoxic machinery described above, then scored for individual cytolytic steps to determine the efficiency of the process in the experimental conditions at that early timepoint. This assay can also be used to identify if there is a specific deficit which is leading to inefficient cytotoxicity in the experimental setup.

GUIDELINES

There are two options for this protocol - stain in a 96 well plate then transfer conjugates onto coverglass or stain on coverglass. Suspension tumor cells (e.g. K562 or P815) can be easily stained in a 96 well plate and then mounted on coverglass. Adherent cell lines can be grown on coverglass and then conjugates are stained on the coverglass itself, either in chamber slides or on coverslips.

Considerations when selecting the staining method:

- Staining adherent cells in chamber slides with coverglass bottoms eases handling, but uses a comparatively large volume of staining solutions, which increases costs. Most chamber slides cannot be easily mounted, which decreases the quality of images obtained.
- Staining adherent cells on coverglass (inverted onto parafilm) uses less staining solution, but requires dexterous handling.
- Staining suspension cells in 96 well plates is simple to handle and minimizes reagent usage.

There are many stages where this protocol can be paused and stored in the fridge if necessary. Wrap the edge of the staining plate in parafilm to avoid the wells drying out

MATERIALS

Main protocol

poly-L-lysine (Sigma, Cat. P88920)
paraformaldehyde (Electron Microscopy Sciences, Cat. 15710)
0.1% Triton X in PBS (Sigma, Cat. 100261-4890)
anti-perforin-AlexaFluor488 (RRID:AB_493252, clone δG9, mouse IgG2b, Biolegend, Cat. 308108)
anti-LFA1 (RRID:AB_10662540, clone TS2/4, mouse IgG1, Biolegend Cat. 350602)
anti-pericentrin (RRID:AB_304461, rabbit IgG, Abcam, Cat. ab4448)
goat anti-rabbit AF647 plus (RRID:AB_2633282, ThermoFisher Scientific, Cat. A32733)
goat anti-mouse IgG1 AF568 (RRID:AB_2535766, ThermoFisher Scientific, Cat. A-21124)
DAPI e.g. NucBlue fixed cell stain ReadyProbes (ThermoFisher Scientific, Cat. R37606)
or Vectashield mounting media with DAPI (Vector Laboratories, Cat. H-1200)
1.5H Coverglass e.g. 22x22mm (Bioscience Tools, Cat. CSHP-No1.5-22x22)
Mounting media e.g. Dako fluorescent mounting medium (ThermoFisher Scientific, Cat. S3023)
Bovine serum albumin (BSA; Sigma Aldrich, Cat. A9430)
Human AB serum (Valley Biomedical, Cat. HP-1022HI)
Phosphate buffered saline (PBS; Corning, Cat. 21-040-CV)

Options for staining of adherent cells

Fibronectin bovine plasma (Sigma-Aldrich, Cat. F1141)
Chamber slides e.g. µ-Slide 8 well polymer bottom 1.5 (Ibidi, Cat. 80826)

Prepare coverglass for adherent cells

1 Optional: Coat coverglass with fibronectin (10µg/mL in PBS, Sigma-Aldrich, Cat. F1141) to allow tumor cells to spread across the coverglass and NK cells to migrate across the surface.

Options for coating
- 30 min at 37°C
- 45 min at room temperature
- overnight at 4°C

2 When ready to continue, rinse wells 3 times with PBS to remove excess fibronectin.

Staining

3 To prepare the cells, NK cells and K562 were co-mixed in V-bottom 96 well plate wells and incubated for 5 min at 37°C 5% CO_2, before being fixed with 4% paraformaldehyde/PBS (Electron Microscopy Sciences, Cat. 15710) for 30 min at room temperature.

4 Cells were permeabilized in 0.01% Triton X/PBS (Sigma Aldrich, Cat. 100261-4890) for 5 min at room temperature.

5 Cells were washed with PBS, then incubated for 1 h at room temperature in **Blocking Solution: 3% bovine serum albumin (cat. A9430, Sigma), 1% human AB serum (cat. HP-1022HI, Valley biomedical) in PBS.**

6 Primary antibodies against pericentrin (0.5 µg/ml; RRID:AB_304461, rabbit IgG, Abcam, Cat. ab4448), LFA-1 (1µg/ml; RRID:AB_10662540, clone TS2/4, mouse IgG1, Biolegend Cat. 350602) and AlexaFluor488-conjugated antibody against perforin (1.25 µg/ml; RRID:AB_493252, clone δG9, mouse IgG2b, Biolegend, Cat. 308108) were incubated in blocking solution for 1 h at room temperature.

7 Cells were washed again in PBS, then incubated with a blocking solution containing AlexaFluor647plus conjugated anti-rabbit antibody (1µg/ml; RRID:AB_2633282, ThermoFisher Scientific, Cat. A32733), AlexaFluor568 conjugated goat anti-mouse IgG1 (1µg/ml; RRID:AB_2535766, ThermoFisher Scientific, Cat. A-21124) and DAPI (NucBlue fixed cell stain ReadyProbes, ThermoFisher Scientific, Cat. R37606) for 1 h at room temperature.

8 Cells were washed and post-fixed with 4% paraformaldehyde/PBS for 5 min at room temperature. Cells were mounted onto coverslips with Dako fluorescent mounting media (ThermoFisher Scientific, Cat. S3023) and allowed to cure at room temperature overnight.

9 Slides were then stored at 4°C.

Microscopy

10 NK cell conjugates with tumor cells were visualized on a Nikon A1Rsi HD confocal microscope, using 60x 1.4 NA oil immersion objective with 405 nm, 488 nm, 561 nm, 640 nm laser excitation, detected using four PMT detectors, two of them GaAsP detectors, and a transmitted light

detector.

11 When imaging, conjugates were identified in the transmitted light channel and a z-slice through the contact point between the tumor cell and NK cell was obtained at the plane containing the microtubule organizing center (MTOC) of the NK cell.

Image analysis

12 Images were analyzed using ImageJ (RRID:SCR_003070). The mean intensity of LFA-1 was measured for the synapse and the back of the cell using the line tool in order to calculate a ratio for LFA-1 enrichment at the synapse.

13 The shortest distance between the MTOC and the synapse was measured and normalized to the distance between the synapse and the back of the cell for the z-slice where the MTOC appeared.

14 Perforin granules were qualitatively scored 1 (majority of granules are gathered at the MTOC) or 0 (granules are dispersed throughout the cell).

15 These values were then aggregated for all conjugates within a given condition.