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# Degranulation and cytokine production (functional assay)

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ABSTRACT

Two primary functions of NK cells - their cytotoxcity and ability to produce cytokines, are measured in a flow-based assay that assesses these responses at the single cell level when NK cells are challenged with tumor cells. Cytotoxicity is not directly measured, instead it is inferred from cytotoxic granlue release. For this reason, this assay should be accompanied by a direct measure of cytotoxicity, such as a *Time - lapse killing assay*. Cytotoxic granlue release is measured by the accumulation of the cytotoxic granule membrane protein, CD107a (LAMP1), at the surface of the NK cell, when fusion of recycling endosomes with the lysosome is blocked by monensin. Cytokines accumulate in NK cells when Golgi transport is blocked by brefeldin A. At the end of an effector:target co-culture, these proteins and other lineage surface markers are detected by flow cytometry.

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1 Effector cells (PBMCs or enriched NK cells) are resuspended in **R10 (RPMI + 10% fetal calf serum + 1% penicillin/streptomycin)**.

2 5 x $10^5$ cells/well are added to a U-bottomed 96 well plate.

These numbers can be decreased to 1 x $10^5$ effector cells/well if the number of effector cells is limited, so long as target numbers are decreased accordingly.

Effector cells can be plated one day in advance in 200 µL R10 and cultured at 37°C 5% $CO_2$ overnight, if required. In this case, plates are spun at 300*g* for 5 min the following day in order to remove excess media.

3 Target cells are added to each well at an effector:target ratio of 2:1. At the same time, FITC conjugated anti-CD107a (5 µL/well; clone H4A3, BioLegend) is added to each well. The cells are then incubated at 37°C 5% $CO_2$. **00:00:00**

4 One hour after the addition of anti-CD107a, cells are given monensin (GolgiStop Cat. No. 554724, BD Biosciences) and brefeldin A (GolgiPlug Cat. No. 555029, BD Biosciences). GolgiStop (1/150) and GolgiPlug (1/100) are diluted in R10 and 20 µL is added to each well. Cells are incubated for a further 4 h at 37°C 5% $CO_2$.

5 Cells are washed twice in PBS, as defined below. This definition also applies to subsequent washes.

5.1 The plate is spun in a centrifuge at 300*g* for 5 min. The supernatant is removed and replaced with 200 µL PBS/well (first wash).

5.2 The plate is spun again in a centrifuge at 300*g* for 5 min. The supernatant is removed and replaced with 200 µL PBS/well (second wash).

5.3 The plate is spun for a final time at 300*g* for 5 min and the supernatant is



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removed.

6 Cells are resuspended in 200 µL PBS cells contained a dead cell marker (1/1000 dilution; Live/Dead Fixable Aqua Staining Kit, Cat. No: L-34966, Thermo Fisher) and incubated for 30 min at 4°C in the dark.

7 Cells are washed twice in **flow buffer (1% AB serum, 0.5mM EDTA in PBS).**

8 Cells are resuspended in 50 µL flow buffer containing PE-CY7 conjugated anti-CD56 and PE-CF594 conjugated anti-CD3 and incubated for 15 min at 4 °C. After the incubation, wells are topped up to 200 µL with flow buffer and washed once with flow buffer.

9 Cells are resuspended in 100 µL 2% paraformaldehyde/PBS and incubated for at room temperature in the dark for 10 min to fix them. Afterwards, wells are topped up to 200 µL with flow buffer and washed once with flow buffer.

10 Cells are resuspended in 100 µL 0.1% Triton X/PBS and incubated at room temperature in the dark for 5 min to permeabilize them. Afterwards, wells are topped up to 200 µL with flow buffer and washed once with flow buffer.

11 Permeabilized cells are resuspended in flow buffer containing BV650 conjugated IFNγ (4S.B3, BioLegend) and BV421 conjugated TNFa (Mab11, Biolegend). They are incubated for 30 min at 4 °C in the dark.

12 After staining, wells are topped up to 200 µL with flow buffer and washed once with flow buffer. Cells are resuspended in 200 µL of flow buffer and transferred to bullet tubes.

13 The tubes are covered and stored in the dark at 4 °C until they are ready to be run on a flow cytometer (LSR II, BD Biosciences).

14 Data is analyzed using FlowJo software (Tree Star Inc., RRID:SCR_008520)

Degranulation (CD107a+) and cytokine production (IFNγ+ or TNFα+) are assessed for the live (dead cell marker-) NK cell (CD56+ CD3-) population.



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