3-19-2021

Flow cytometry-based ex vivo murine NK cell cytotoxicity assay

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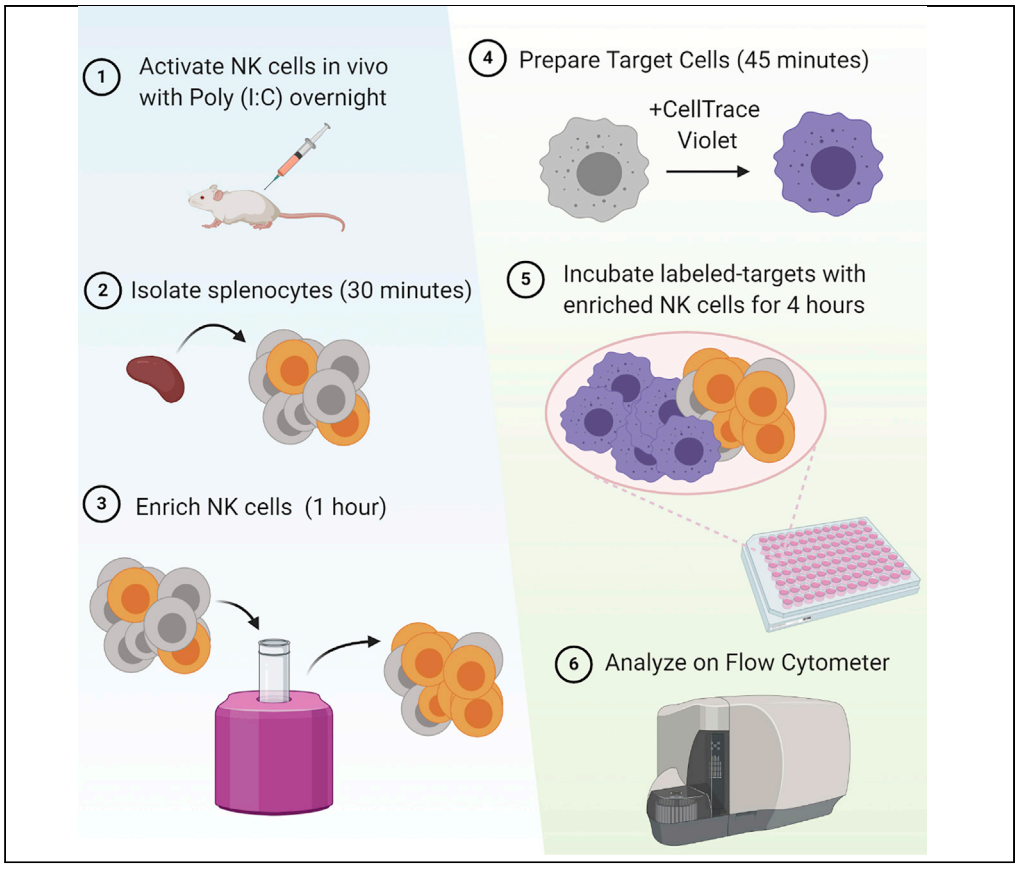

Direct killing of diseased cells is a hallmark function of NK cells. This protocol describes a flow-based assay to measure *in vivo* activated murine NK cells' ability to kill target cells *ex vivo*. Existing published protocols for assaying *ex vivo* NK cell killing utilized the radioactive chromium release assay or were designed for human NK cells. This protocol details specifically an *ex vivo* cytotoxicity assay using primary murine NK cells enriched from splenocytes that were activated *in vivo* with poly(I:C).

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HIGHLIGHTS

This protocol describes a flow-based assay to assess mouse NK cell killing *ex vivo*

Protocol details the procedure for enriching NK cells from mouse splenocytes

In vivo poly(I:C) activated NK cells are used as effectors to kill labeled targets

Wong et al., STAR Protocols 2, 100262
March 19, 2021
https://doi.org/10.1016/j.xpro.2020.100262

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Flow cytometry-based *ex vivo* murine NK cell cytotoxicity assay

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https://doi.org/10.1016/j.xpro.2020.100262

SUMMARY

Direct killing of diseased cells is a hallmark function of NK cells. This protocol describes a flow-based assay to measure *in vivo* activated murine NK cells' ability to kill target cells *ex vivo*. Existing published protocols for assaying *ex vivo* NK cell killing utilized the radioactive chromium release assay or were designed for human NK cells. This protocol details specifically an *ex vivo* cytotoxicity assay using primary murine NK cells enriched from splenocytes that were activated *in vivo* with poly(I:C).

For complete details on the use and execution of this protocol, please refer to Wagner et al. (2020).

BEFORE YOU BEGIN

Prepare murine IL-15 working stock from lyophilized powder

Timing: 10 min

This step describes the preparation of IL-15 stock from lyophilized powder. IL-15 will be used during the killing assay to maintain NK cell viability.

1. Reconstitute 10 μg recombinant murine IL-15:
   a. Add 100 μL sterile H_2O to lyophilized powder and mix by pipetting
   b. Add 900 μL sterile PBS + 0.1% w/v BSA(A7906) and mix by pipetting, so that the final concentration is 10 ng/μL
2. Divide into aliquots and store at −20°C to −80°C for up to 3 months. Avoid repeated freeze-thaw cycles.

Culture RMA-S cell line

Timing: >2 days before killing assay

The target cell line to be used in this protocol should be thawed and cultured >2 days before the killing assay.

Alternatives: RMA-S is the MHC-I-deficient target cell line used in this protocol, but any alternative NK-sensitive target cell line of choice can be used.

Note: The RMA cell line can be used as a negative control for NK cell killing. Because RMA-S is a well-established MHC-I-deficient target cell line for NK cell killing, killing assays against RMA-S are commonly performed without comparison to RMA cells.

3. Thaw RMA-S cells
   a. Warm 10 mL of cRPMI media in a 15-mL conical
   b. Remove RMA-S frozen stock from liquid nitrogen tank
   c. Place RMA-S cryovial in the 37°C water bath, and as soon as it is thawed, slowly transfer the cells into the pre-warmed 10 mL cRPMI media
   d. Centrifuge tube at 400 × *g*, 4 min at 20°C–25°C
   e. Resuspend in appropriate amount of media so that the concentration is between 2–5 × 10^5 cells/mL, transfer to a tissue culture flask
4. Maintain RMA-S in culture for up to 1 month; perform media change regularly to prevent over-growth of the cells

Recommended: assess baseline Granzyme B expression

Timing: day before killing assay; 2 h plus flow analysis time

This step describes the assessment of baseline Granzyme B expression of NK cells harvested from peripheral blood of the mice before activation.

5. Collect samples, prior to poly(I:C) administration
   a. Perform cheek bleed on mice to collect blood into EDTA tubes
   b. Transfer all blood volume into 15-mL conicals containing 4 mL 1× ACK lysis buffer
   c. Lyse for 1 min, stop lysis by quantum statis (q.s.) to 15 mL, using DPBS
   d. Centrifuge the tubes at 400 × *g*, 4 min at 20°C–25°C, aspirate supernatant
   e. Lyse cells again with 4 mL 1× ACK lysis buffer for 1 min, q.s. to 15 mL with DPBS
   f. Centrifuge tubes at 400 × *g* , 4 min at 20°C–25°C, aspirate supernatant
   g. Resuspend cells in 250 μL FACS buffer and transfer through a 70 μm filter into a 96-well for staining

6. Stain cells for baseline Granzyme B expression before activation
   a. Prepare surface stain master mix:

Surface stain master mix

	Amount per well
anti-NKp46 PE	2 μL
anti-NK1.1 PerCPCy5.5	4 μL
anti-CD3 PB	1 μL
anti-CD45 BV605	1 μL
Fc Block	1 μL
FACS buffer	91 μL
Total:	100 μL

   b. Add 100 μL surface stain master mix to each well
   c. Incubate for 15 min at 4°C in the dark
   d. After the incubation, centrifuge at 400 × *g*, 4 min at 20°C–25°C, tap out supernatant
   e. Wash the plate twice with 200 μL FACS buffer
      i. Add FACS buffer, centrifuge at 400 × *g*, 4 min at 20°C–25°C, tap out supernatant. Repeat once more.

f. Make up 200 μL of eBioSciences Fixation/Permeabilization(Fix/Perm) solution per well following manufacturer's instructions (1 part Concentrate : 3 part Diluent)
g. Resuspend each well in 200 μL eBioSciences Fix/Perm Solution
h. Incubate at 4°C for 30 min up to 18 h
i. Centrifuge plate at **750 × *g***, 4 min at 4°C, tap out supernatant
j. Prepare 1× Permeabilization Buffer by diluting 10× Permeabilization buffer in water, according to manufacturer's instructions
k. Add 200 μL 1× Permeabilization Buffer to each well, mix
l. Centrifuge plate at 750 × *g*, 4 min at 4°C, tap out supernatant
m. Make up Granzyme B master mix:

Granzyme B master mix	
	Amount per well
anti-Granzyme B APC	2 μL
Goat serum	1 μL
1× Permeabilization Buffer	97 μL
Total:	100 μL

n. Add 100 μL of Granzyme B master mix to each well, mix
o. Incubate for 30 min at 4°C in the dark
p. Wash plate twice with 1× Permeabilization Buffer
   i. Add 1× Permeabilization Buffer, centrifuge at 750 × *g*, 4 min at 4°C. Tap out supernatant. Repeat once more.
q. Resuspend in 200 μL FACS buffer and analyze on the flow cytometer

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
APC Granzyme B anti-human mAb (GB12)	Thermo Fisher	Cat# MHGB05
PE-Cyanine7 NKp46 anti-mouse mAb (29A1.4)	Thermo Fisher	Cat# 25-3351-82
PerCP-Cy5.5 anti-mouse NK-1.1 mAb (PK136)	BD Biosciences	Cat# 561111
Brilliant Violet 605 anti-mouse CD45 mAb (30-F11)	BioLegend	Cat# 103139
Pacific Blue anti-mouse CD3e(500A2)	BD Pharminogen	Cat#558214
Chemicals, peptides, and recombinant proteins		
Mouse BD Fc Block	BD Bioscience	Cat# 553142
Goat serum (heat-inactivated)	Sigma-Aldrich	Cat#9023
HyClone classical liquid media: RPMI 1640	Thermo Fisher	Cat# SH30027LS
Fetal bovine serum	Sigma-Aldrich	Cat# F0926
Cytofix/Cytoperm fixation and permeabilization solution	BD Bioscience	Cat# BDB554722
Murine IL-15	Peprotech	Cat#210-15
Poly(I:C) LMW	Invivogen	Cat#tlrl-picw
7-AAD	Sigma-Aldrich	Cat#A9400
Hyclone L-glutamine	Thermo Fisher	Cat# SH3003402
Hyclone HEPES solution	Thermo Fisher	Cat# SH3023701
HyClone non-essential amino acids	Thermo Fisher	Cat# SH3023801
HyClone sodium pyruvate solution	Thermo Fisher	Cat# SH3023901
HyClone penicillin streptomycin 100× solution	Thermo Fisher	Cat# SV30010
Potassium bicarbonate	Fisher Scientific	Cat#235-500
0.5 M EDTA solution	Corning	Cat#46-034-CI

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Ammonium chloride	Sigma	Cat# A9434
HyClone DPBS (calcium, magnesium)	GE	Cat# SH09928.03
2-Mercaptoethanol (2-ME)	Life Technologies	Cat# 21985-023
Critical commercial assays		
CellTrace Violet Cell Proliferation Kit	Thermo Fisher	Cat# C34557
EasySep Mouse NK Cell Isolation Kit	STEMCELL Technologies	Catalog # 19855
eBioscience fixation/permeabilization Concentrate	Invitrogen	Cat# 00-5123-43
eBioscience Fixation/Permeabilization diluent	Invitrogen	Cat# 00-5223-56
Permeabilization buffer (10×)	Invitrogen	Cat#00-8333-56
Experimental models: cell lines		
Mouse: RMA-S	ATCC	RRID:CVCL_2180
Experimental models: organisms/strains		
Mice (8–12 weeks old) to serve as your source of NK cells (2–3 mice per group will be pooled together)	N/A	N/A
Other		
Biosafety cabinet	N/A	N/A
Cell culture incubator	N/A	N/A
Dissection tools	N/A	N/A
Beckman Coulter Allegra X-15R centrifuge (or similar)	N/A	N/A
Beckman Coulter Gallios flow cytometer (or similar)	N/A	N/A
Nexcelom cellometer (or similar)	N/A	N/A
EasySep magnet	STEMCELL Technologies	Cat#18000
1 L/500 mL 0.22 μm filter	Fisher	REF# 09-761-107
70 μm cell filter	Fisher	REF#352350
100 mm × 15 mm Petri dish or similar	N/A	N/A
Insulin syringe or similar	N/A	N/A
15-mL conical tubes	N/A	N/A
T-75 cell culture flasks	N/A	N/A
96-well staining plate	Fisher	REF#3365
3 mL syringe	BD	REF#309657
5 mL polypropylene round-bottom tube	Fisher	REF#352063
96-well cell culture plate, V-bottom with lid	Fisher	REF#3894

MATERIALS AND EQUIPMENT

Media and buffer		
Complete RPMI (cRPMI) media		
Reagent	Amount	Final concentration
RPMI 1640 medium	430 mL	–
Heat-inactivated FBS	50 mL	10%
100× Penicillin/streptomycin	5 mL	1%
Non-essential amino acids	5 mL	1%
Sodium pyruvate	5 mL	1%
L-Glutamine	5 mL	1%
Total volume	**500 mL**	–

Set up 0.22 μm filter, pour ~200 mL of RPMI into filter, then add in the rest of the reagents, finish by adding the remaining RPMI medium. Filter after all reagents are added. Store at 4°C for up to 1 month.

FACS buffer		
Reagent	Amount	Final concentration
DPBS	489 mL	–
Heat-inactivated FBS	10 mL	2%
0.5 M EDTA	1 mL	1 mM
Total volume	500 mL	–

Set up 0.22 μm filter, pour ~300 mL of DPBS into filter, then add in the rest of the reagents, finish by adding the remaining DPBS. Filter after all reagents are added. Store at 4°C for up to 3 months.

10× ACK Lysis buffer		
Reagent	Amount	Final concentration
Ammonium chloride	80.24 g	1.5 M
Potassium bicarbonate	10.01 g	0.1 M
0.5 M EDTA solution	2 mL	1 mM
DPBS	to 1 L	–
Total volume	1 L	1 L

Filter through 0.22 μm filter after all reagents are added. Store at 4°C. To prepare **1× ACK Lysis buffer**, dilute 10× ACK Lysis buffer in sterile H_2O. Store 1× ACK Lysis buffer at 20°C–25°C for up to 6 months.

STEP-BY-STEP METHOD DETAILS

Activate NK cells

Timing: 18–24 h before killing assay, after obtaining pre-poly(I:C) blood samples (optional); 30 min

This step uses poly(I:C) to activate NK cells *in vivo*.

1. For each mouse prepare 300 μg poly(I:C) in DPBS, 200 μL total volume
2. Inject poly(I:C) intraperitoneally, using an insulin syringe, into each mouse

Enrich NK cells from splenocytes

Timing: 1.5 h

The enrichment step not only serves to increase the percentage of NK cells in the resulting enriched sample but also normalize the NK cell percentages in experimental groups that could have started with different NK cell numbers. In our experience, the purity after enrichment is similar between groups in each experiment even if they had different NK cell percentages to start as we did in Wagner et al. (2020).

Note: All of the following steps should be performed in a tissue culture hood to ensure sterility

3. Sacrifice mice and isolate splenocytes
   a. Isolate spleen from mice, pooling spleens from every 2–3 mice to ensure sufficient NK cell numbers for the killing assay

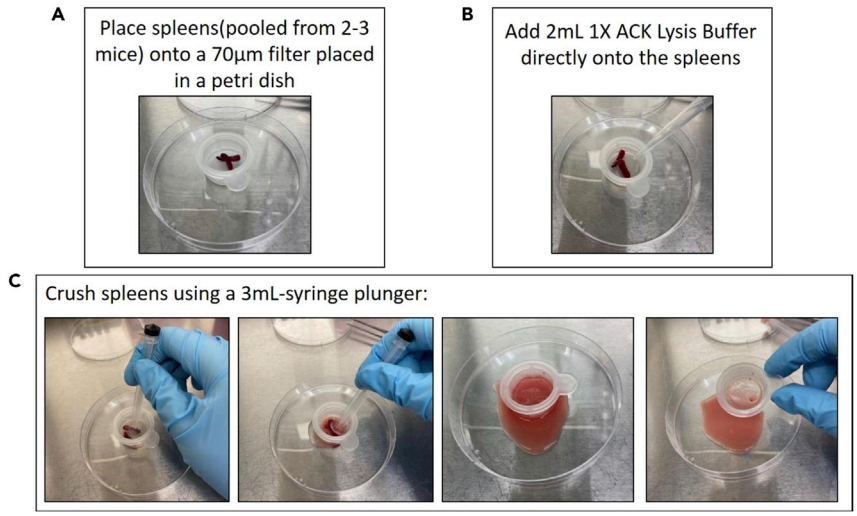

Figure 1. Dissociation of spleens through filter using a 3-mL syringe plunger

Note: We recommend placing the isolated spleens in 1.5 mL cRPMI media and on ice while waiting to be processed.

Note: We recommend performing steps (b)–(f) one group of pooled spleen at a time. Then proceed to step (g) once all the spleens are ready.

b. Place spleens onto a 70 µm cell filter placed into a petri dish (Figure 1)
c. Add 2 mL of 1X ACK lysis buffer directly onto the spleens on the filter (Figure 1)
d. Using a 3-mL syringe plunger, crush spleens through the filter (Figure 1)
e. Transfer all volume into a 15-mL conical
f. Rinse petri dish with 13 mL DPBS and transfer into the same 15-mL conical
g. Centrifuge cells at 400 × *g*, 4 min, 20°C–25°C, aspirate supernatant
h. Resuspend cells in 500 µL sterile FACS buffer

Note: To make sterile FACS buffer, filter FACS buffer through a 0.22 µm filter. Keep at 4°C and open only in sterile tissue culture hoods.

i. Count live cells with AOPI on the cellometer

4. Enrich for NK cells using the STEM CELL TECHNOLOGIES NK EasySep purification kit

Note: this is a slightly modified version of the official manufacturer's protocol (https://cdn.stemcell.com/media/files/pis/28043-PIS_1_0_4.pdf?_ga=2.67415057.1840857299.1607959444-1584491980.1604954550). Here we perform the isolation cocktail and RapidSpheres incubation steps in the same 15-mL conicals that the cells were resuspended in step 3h to minimize cell loss and to incubate all cells from the same sample in the same tube. We transfer the sample to 5-mL tubes after these incubation steps while the manufacturer's protocol recommends this to be done in 5-mL tubes from the start.

a. Using cell counts from 3i, bring cells to the concentration of 1×10^8 cells/mL in the 15-mL conical in sterile FACS buffer

Note: If the cell count indicates the concentration is less than 1×10^8 cells/mL, proceed with the 500 µL of samples as that is the recommended minimum volume for the kit.

b. Add 50 µL of Isolation Cocktail (from kit) per mL of sample, pipette up and down to mix
c. Incubate at 20°C–25°C for 10 min

d. Vortex RapidSpheres for 30 s to disperse the particles thoroughly
e. Add 100 µL of RapidSpheres per mL of sample. Pipette up and down to mix
f. Incubate at 20°C–25°C for 5 min
g. If the sample volume is greater than 2 mL at this point, divide into 2 tubes, so that each tube has not more than 2 mL of samples
h. Q.S. to 2.5 mL with sterile FACS buffer
i. Mix by gently pipetting then carefully transfer the whole 2.5 mL into a 5-mL polypropylene tube
j. Plate the tube without lid into the magnet, and incubate at 20°C–25°C for 5 min
k. Carefully pick up the magnet with the tube inside it, then invert the magnet, and tube to decant the enriched cells into a new 15-mL conical tube
l. Remove the 5-mL polypropylene tube from the magnet, q.s. to 2.5 mL with sterile FACS buffer and mix
m. Plate the tube back into the magnet again, and incubate at 20°C–25°C for 5 min
n. Carefully pick up the magnet with the tube sitting inside it, then invert the magnet and tube to decant the enriched cells into the same 15-mL conical as step (k). Immediately proceed to "Set Up Killing Assay" step.

Note: NK cell purity post-enrichment ranges from ~50%–80% and will be assessed in the Granzyme B activation panel ("Assess NK cell Activation" step).

Alternatives: NK enrichment can be omitted so that the killing assay is performed with unenriched splenocytes as effectors, though the effector:target ratios for the killing assay would have to be adjusted as noted in step 6d (Berrien-Elliott et al. 2019). If using unenriched splenocytes for effectors, after step 3g, proceed to step 6b.

Set up killing assay

Timing: 1 h setup; 4–6 h incubation; variable flow time

This step describes the preparation and plating of effector and target cells for the killing assay and the assessment of killing on the flow cytometer after a 4–6 h incubation.

5. Prepare complete NK media:

Complete NK media

Reagent	Amount	Final concentration
cRPMI media	To 40 mL	–
1,000× 2-ME	40 µL	55 µM
10 ng/µL mIL-15	40 µL	10 ng/mL

⚠ CRITICAL: This media should be prepared fresh every time.

6. Prepare enriched NK cells
   a. Centrifuge enriched cells at 400 × *g*, 4 min at 20°C–25°C
   b. Resuspend in 300–500 µL **complete NK media**
   c. Count live cells and bring concentration to 2 × 10^6 cells/mL

Note: This assay as written would require around 4 × 10^5 cells total, which includes some extra to assess purity and activation in "Assess NK Cell Activation" step. If not enough cells, this

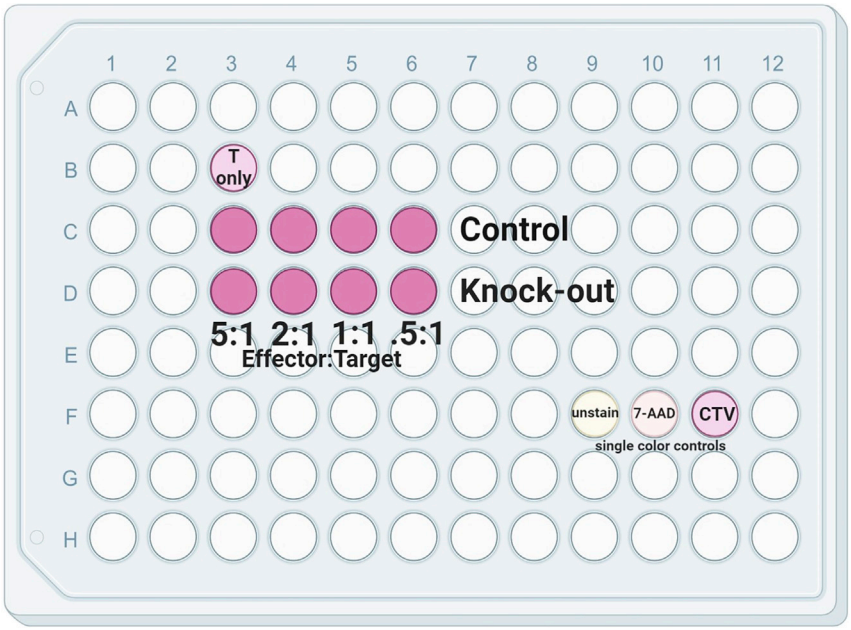

Figure 2. Example killing assay plating scheme
T only, target cells only.

assay can be scaled down accordingly. We have had great success with half the number of effectors and target cells scaled down accordingly. In such cases where we scaled down the numbers of cell, we kept the volume of each well the same as described here.

d. Label a **V-bottom 96-well plate** for assay (Figure 2 for example)

e. Plate appropriate number of enriched NK cells for each effector to target ratio and bring volume of each well up to 100 μL:

For 5:1 wells (5 enriched NK: 1 Target) → 100 μL of enriched NK cells (2×10^5 enriched NK cells)
For 2:1 wells → 40 μL enriched NK cells (8×10^4 enriched NK cells) + 60 μL complete NK media
For 1:1 wells → 20 μL enriched NKcells (4×10^4 enriched NK cells) + 80 μL complete NK media
For 0.5:1 wells → 10 μL enriched NK cells (2×10^4 enriched NK cells) + 90 μL complete NK media

Note: Make sure to include a well with Target Cells only and single-color control wells.

Alternatives: If using unenriched splenocytes as effectors, the effector to target ratios commonly used are 100:1, 50:1, 25:1, and 12.5:1 (Berrien-Elliott et al. 2019). The concentration to resuspend the effector cells prior to plating (step 6c) would have to be adjusted accordingly.

f. Place plate into 37°C incubator until target cells are prepared

g. Reserve and keep left over NK cells on ice for staining in "Assess NK Cell Activation" step to assess NK cell activation and purity

7. Prepare Target Cells

a. Remove some RMA-S from culture flask and count live cells

b. Transfer 5×10^6 cells into a 15-mL conical and q.s. to 15 mL with DPBS

c. Centrifuge cells at 400 × *g*, 4 min at 20°C–25°C, and aspirate supernatant

d. Wash cells once more with 5 mL DPBS; centrifuge cells at 400 × *g*, 4 min at 20°C–25°C, and aspirate supernatant

e. Make up 10 μM CellTrace Violet(CTV) working solution:

i. Reconstitute stock CTV in 20 μL DMSO per manufacturer instructions (5 mM after reconstitution)
ii. 10 μM CellTrace Violet: 2 μL of 5 mM CTV stock in 1 mL DPBS total

f. Resuspend target cell pellet in 1 mL DPBS and then add 1 mL of 10 μM CTV, pipet up and down to mix
g. Incubate for 15 min in 37°C incubator
h. After incubation add 10 mL warm cRPMI media into the tube then incubate 5 more minutes in 37°C incubator
i. Centrifuge at 400 × *g*, 4 min at 20°C–25°C
j. Resuspend cells in 1 mL **complete NK media**
k. Count live cells and dilute cells to 4 × 10^5 cells /mL
l. Plate 100 μL target cells into each well (40,000 target cells per well)
i. At this point, each well should have 200 μL total volume
m. Gather cells at the bottom of the well by letting centrifuge to get up to ~200 × *g*, then stopping the centrifuge
n. Incubate for 4 h (up to 6 h) in 37°C incubator
o. At the end of the incubation, make up 7-AAD master mix:

7-AAD master mix

	Amount per well
1 mg/mL 7-AAD	0.4 μL
FACS Buffer	9.6 μL
Total:	10 μL

p. Add 10 μL of 7-AAD master mix into each well, mix by pipetting, and transfer into flow tubes
q. Incubate at 20°C–25°C for 5 min, and analyze on flow cytometer immediately

Note: It is best to flow 7-AAD stained samples within an hour. If working with large number of wells that will take longer than an hour to finish analyzing the whole plate on the flow cytometer, divide samples into groups, and add 7-AAD to each group immediately before analyzing on the flow cytometry; keep the other groups at 4°C until ready for 7-AAD to be added.

Note: When setting up voltages on the flow cytometer, use the CTV single color control to make sure the voltage for the CTV channel is set appropriately so that the CTV+ cells are clearly visible.

Note: The flow cytometry approach of this protocol allows for the inclusion of markers like Annexin V to further identify early apoptotic cells from dead cells. If that is of interest, we suggest spinning down the assay plate after the incubation and perform 7-AAD/Annexin V staining in a buffer suitable for Annexin V staining instead of the FACS buffer.

Assess NK cell activation

Timing: 2 h plus flow time; this staining can be performed during the 4 h killing assay incubation

This step assesses whether the NK cells have been activated and NK-enriched samples' purity. This will provide a context in which the data from the killing assay will be interpreted.

To ensure NK cells are incubated with target cells as soon as possible after they have been harvested from the mice, we perform this step after the killing assay is set up.

8. Stain enriched NK cells for activation and purity
   a. Plate left over enriched NK cells in to a 96-well staining plate, no more than 1–2 × 10^6 cells per well; 30,000 cells minimum if possible
   b. Centrifuge plate at 400 × *g*, 4 min at 20°C–25°C, tap out supernatant
   c. Prepare surface stain master mix:

Surface stain master mix	
Reagent	Amount per well (μL)
anti-NKp46 PE	2
anti-NK1.1 PerCPCy5.5	4
anti-CD3 PB	1
anti-CD45 BV605	1
Fc Block	1
FACS buffer	91
Total:	100

   d. Add 100 μL surface stain master mix to each well, mix
   e. Incubate for 15 min at 4 degrees in the dark
   f. After the incubation, centrifuge at 400 × *g*, 4 min at 20°C–25°C, tap out supernatant
   g. Wash the plate twice with 200 μL FACS buffer
      i. Add FACS buffer, mix, and centrifuge at 400 × *g*, 4 min at 20–25°C. Tap out supernatant, repeat once more.
   h. Resuspend each well in 200 μL eBioScience Fix/Perm Solution, mix

Alternatives: We have also performed fix/perm and Granzyme B staining using the BD fix/perm kit (Cat#554714) with success. Please follow the BD kit instructions from step 8h onwards if using it instead of the eBioScience kit described here.

   i. Incubate at 4°C for 30 min up to 18 h
   j. Centrifuge plate at 750 × *g*, 4 min at 4°C, tap out supernatant
   k. Prepare 1× Permeabilization Buffer by diluting 10× Permeabilization buffer in water
   l. Add 200 μL 1× Permeabilization to each well, mix
   m. Centrifuge plate at 750 × *g*, 4 min at 4°C, tap out supernatant
   n. Make up Granzyme B master mix:

Granzyme B master mix	
	Amount per well (μL)
anti-Granzyme B APC	2
Goat Serum	1
1× Permeabilization Buffer	97
Total:	100

   o. Add 100 μL of Granzyme B master mix to each well, mix
   p. Incubate for 30 min at 4°C in the dark
   q. Wash plate twice with 1× Permeabilization Buffer
      i. Add 1× Permeabilization Buffer, centrifuge at 750 × *g*, 4 min at 4°C. Tap out supernatant. Repeat once more.
   r. Resuspend in 200 μL FACS buffer and analyze on the flow cytometer

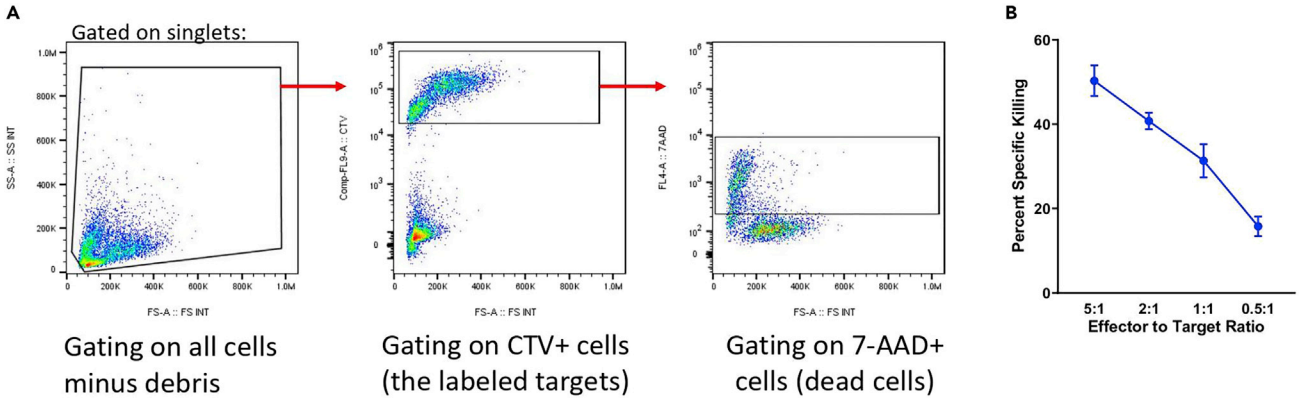

Figure 3. Example killing assay data

(A) Example gating strategy for assessment of target cell killed by effectors. Data acquired on the Beckman Coulter Gallios flow cytometer and analyzed on Flowjo 10.

(B) Example data of killing assay performed with wild-type enriched NK cells against RMA-S targets at indicated effector:target ratio (n = 2–3). Data are presented as means ± SEM.

EXPECTED OUTCOMES

At the end of the 4-h incubation, when analyzed on flow cytometer, cells that are double positive for CTV and 7-AAD are the labeled-target cells that have been killed (Figure 3A). For each E:T ratio, Percent Specific Killing is calculated as [% 7-AAD+ of CTV+ cells] $_{(Effector+Target)\ well}$ – [%7-AAD+ of CTV+ cells] $_{Target\ only\ well}$. In a killing assay performed C57BL/6 mice with wild-type NK cells against RMA-S targets, the %specific killing should be around 50% at 5:1 enriched NK : RMA-S as reported in Wagner et al. (2020) and also Figure 3B here. *In vivo* poly(I:C) activation should result ≥ 90% of NK cells expressing Granzyme B (Figure 4).

LIMITATIONS

This protocol is optimized to examine short-term killing within a 4-h time frame, when the granyzme B/perforin mediated killing pathway dominates (Prager et al. 2019). If studying cytotoxicity mediated by death-ligand interaction is of interest, an alternative strategy is to perform prolonged killing assays and/or use alternative methods, instead of the flow cytometry, such as the Incucyte to monitor killing overtime.

Because this protocol requires pooling spleens from multiple mice, individual mouse to mouse differences are not captured. Therefore, if performing studies where collecting data from each individual mouse is critical, we suggest scaling down the number of cells used in the killing assay or only perform one E:T ratio to accommodate for lower cell numbers. Alternatively, NK enrichment can be omitted, and this protocol can be performed with unenriched splenocytes instead.

NK cell percentage post-enrichment using the STEMCELL TECHNOLOGIES NK isolation kit as described in this protocol ranges from 50%–80% in our experience, and we have been able to consistently achieve killing of RMA-S targets with this range of NK percentage in the samples. To our knowledge, there are no commercially available beads-based selection product that can achieve better purity. If >80% NK purity is desired, sorting by FACS, a much less accessible approach, will be required.

If working with precious mouse strains with limited numbers where the number of different analyses performed on each mouse needs to be maximized, systemic *in vivo* activation with poly(I:C) may not be ideal, as this precludes opportunities to study the mice at homeostasis. In such cases, one may choose to activate NK cells in vitro by incubating them with IL-15 for 18–24 h in complete NK media

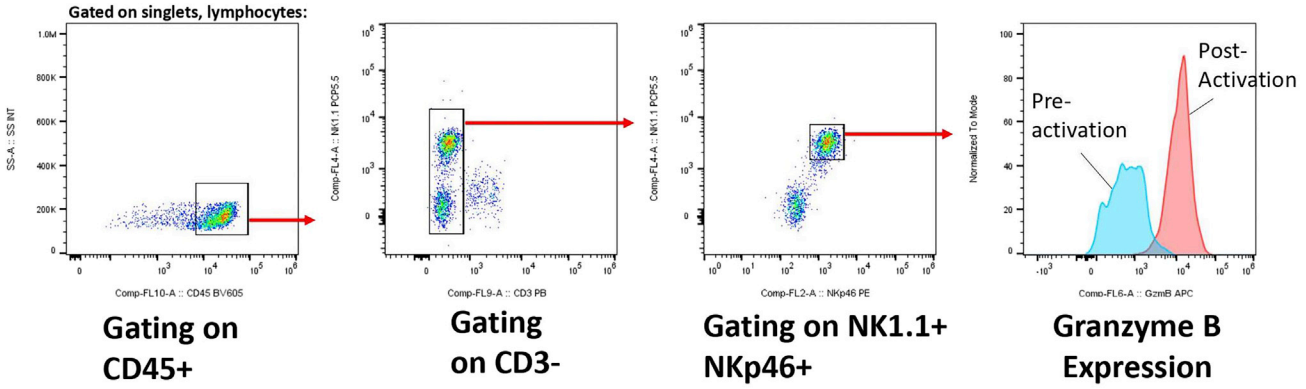

Figure 4. Example gating strategy for Granzyme B activation assessment on post-poly(I:C) enriched NK cell compared to pre-poly(I:C) baseline sample from cheek bleed

Data acquired on the Beckman Coulter Gallios flow cytometer and analyzed on Flowjo 10.

instead and then performing the killing assay described. However, such method may not reflect the *in vivo* NK biology as closely as activating *in vivo* and performing the killing assay directly *ex vivo* as described here.

TROUBLESHOOTING

Problem 1

Insufficient numbers of enriched NK cells (step 4)

Potential solution

Solution 1: Pool spleens from more mice

Solution 2: Scale down the assay to use fewer cells. We have had great success with using half the number of enriched NK cells (with the target cell numbers scaled down accordingly for each effector:target ratio).

Solution 3: Perform assay with fewer E:T ratios. Instead of doing all 4 ratios listed above, pick only 1 or 2 that you have sufficient amount of cells to perform.

Problem 2

CellTrace reagent signal too high on flow cytometer (step 8r)

Potential solution

Solution 1: Use CellTrace labeled single color control cells to set the voltage appropriately before running samples

Solution 2: Dilute CellTrace reagent to be less concentrated when labeling cells

Problem 3

Florescent reporter protein expressed by the cells cannot be detected when staining with the eBioscience Fix/Perm kit (step 8h)

Potential solution

Solution 1: Use BD Cytofix/Cytoperm Fixation/Permeablization Kit (BD Biosciences Cat# 554714) instead, following the manufacturer's instructions for intracellular staining

Solution 2: After surface staining, wash once with FACS buffer, then pre-fix cells with 1% PFA for 2 min at 20°C–25°C, immediately centrifuge cells and tap out PFA, then proceed with eBio Fix/Perm protocol.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Todd A. Fehniger (tfehnige@wustl.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate datasets or codes.

ACKNOWLEDGMENTS

This work was supported by grants from the NIH: T32 HL007088 (P.W. and J.A.W.), the Howard Hughes Medical Institute (Medical Fellow Award, J.A.W.), K12 5K12CA167540 (M.M.B.-E.), R01 AI102924 (T.A.F.), R01 CA205239 (T.A.F.), and P50 CA171963. We acknowledge the use of the Siteman Flow Core for this study. Schema figures were created with Biorender.com.

AUTHOR CONTRIBUTIONS

P.W., J.A.W., and T.S. performed the experiments. P.W., J.A.W., and M.M.B.-E. designed the study and wrote the protocol. T.A.F. conceived and designed the study. All authors reviewed the data, edited, and approved the final version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests

REFERENCES

Berrien-Elliott, M.M., Sun, Y., Neal, C., Ireland, A., Trissal, M.C., Sullivan, R.P., Wagner, J.A., Leong, J.W., Wong, P., Mah-Som, A.Y., et al. (2019). MicroRNA-142 is critical for the homeostasis and function of type 1 innate lymphoid cells. Immunity *51*, 479–490.e6.

Prager, I., Liesche, C., van Ooijen, H., Urlaub, D., Verron, Q., Sandström, N., Fasbender, F., Claus, M., Eils, R., Beaudouin, J., et al. (2019). NK cells switch from granzyme B to death receptor-mediated cytotoxicity during serial killing. J. Exp. Med. *216*, 2113–2127.

Wagner, J.A., Wong, P., Schappe, T., Berrien-Elliott, M.M., Cubitt, C., Jaeger, N., Lee, M., Keppel, C.R., Marin, N.D., Foltz, J.A., et al. (2020). Stage-specific requirement for Eomes in mature NK cell homeostasis and cytotoxicity. Cell Rep. *31*, 107720.