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Protocol status: Working
We use this protocol and it's working

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Expansion of NK cells on feeder cells

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ABSTRACT

This protocol contains the details of how we implement the protocol devised by Somanchi et al. (https://www.jove.com/v/2540/expansion-purification-functional-assessment-human-peripheral-blood).

5 million enriched NK cells should give approx. 100 million cells by day 7 and >2 billion by day 14. This assay can be scaled down or up depending on how many cells are needed.

Expanded NK cells are typically frozen and stored until required.

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GUIDELINES

For preparing NKEM media:
IL-2 can be added to the NKEM stock at the start of the experiment or added fresh as needed. We see no difference in expansion rate or viability.

Wilson Wolf offer instructions for appropriate use of G-rex plasticware:
https://www.wilsonwolf.com/literature/?lit=g-rex-ifu

General advice for refreshing media in G-Rex wells:
Carefully remove media from the top of the wells. Do not disturb the layer of cells at the bottom of the wells. If cell layer disturbance occurs, rest G-Rex in the incubator again for a couple hours before attempting to remove media.

General advice for harvesting cells from G-Rex:
Carefully remove 60mL media from the top of the wells, then resuspend the cell layer with leftover 40mL media in the well. Transfer the resuspended cells into 50-mL conical tubes. This allows you to fit one well of cells into one 50-mL conical tube.

For thawing frozen eNKs:
Spot check each batch of frozen cells by thawing at least one vial and recording (1) the yield, (2) the viability straight after thaw and (3) the viability the day after thaw. Keeping an eye on these values ensures reproducibility of the cell product.

General advice for preparing and irradiating feeder cells:
For convenience and reproducibility, we irradiate feeder cells in large batches then store them as frozen aliquots until needed. We generally use freshly processed trimacones to start these expansions, but the assay should work with thawed cells. If working with thawed PBMC, rest the cells overnight ($4x10^6$/mL in R10; 5% CO_2, 37°C) then proceed with NK cell enrichment.

Let feeders grow for at least one week post thaw before irradiating. Grow feeders in R10, splitting down to $1x10^5$/mL on Mondays and Wednesdays, and down to $5x10^4$/mL on Fridays. Our irradiator (BioRad) can work with tubes or flasks, but maximally fits 7x 50mL tubes at $1x10^7$/mL. Seal the tube caps with parafilm. After irradiating, place cells on ice, return to the lab and freeze down the cells (using the cell counts obtained prior to irradiation). We recommend aliquots of 10-$50x10^6$cells/mL in 90% FBS, 10% DMSO. There is some loss upon thaw - be sure to wash cells and recount before adding them to NK cells.

MATERIALS

Catalog numbers are provided here to indicate what we use. Generic replacements would also be suitable.

EasySep Human NK Cell Enrichment Kit, Cat. 19055, STEMCELL Technologies
K562 mIL21 41BBL cells kindly provided by Fate Therapeutics (Zhu et al., 2020).
6M well plate, Cat. 80660M, Wilson Wolf.
RPMI-1640, Cat. 2240-089, Gibco
heat inactivated fetal bovine serum, Cat. 26140079, Gibco.
Penicillin and Streptomycin, Cat. 15140122, Gibco.
IL-2, Cat. NDC 65483-116-07, Prometheus.
DMSO, Cat. BP231-100, Fisher Bioreagents.

Prepare irradiated feeder cells

1 K562 overexpressing mIL-21 and 41BBL (Zhu et al. https://doi.org/10.1182/blood.2019000621) are cultured in R10 at 1x10^5/mL split 2-3 times to week before they reach 1x10^6/mL.

1.1 **R10**: RPMI (Gibco Cat. No. 2240-089) + 10% heat inactivated fetal bovine serum (Gibco Cat. No. 26140079) + 100 U/mL Penicillin and Streptomycin (Gibco Cat. No. 15140122)

Enrich NK cells

2 Start with 5x10^6 NK cells enriched from PBMC (EasySep Human NK Cell Enrichment Kit, Cat. 19055, STEMCELL Technologies)

3 For each 5x10^6 NK, count and irradiate 1x10^7 K562 mIL-21 41BBL using a gamma irradiator at 100 Gy

4 Post irradiation, wash the cells with PBS and resuspend in NK cell expansion media (**NKEM**):

4.1 **NKEM:**
RPMI 1640 medium (Cat. 2240-089, Gibco)
10% heat inactivated fetal bovine serum (Cat. 26140079, Gibco)
100 U/mL Penicillin and Streptomycin (Cat. 15140122, Gibco)
50 U/mL IL-2 (Cat. NDC 65483-116-07, Prometheus)

5 Seed $5x10^6$ NK cells with $10x10^6$ irradiated K562 mIL21 41BBL (1:2 ratio) in 40 mL of NKEM in a T75 flask and place it upright in an incubator at 37°C and 5% CO_2.

Day 3 and Day 5 - media refresh

6 Recover cells by centrifugation at 400g for 5 min and replace half of the media with fresh NKEM (adding fresh IL-2 for the entire media volume) and continue culture.

Day 7 - re-stimulation

7 Count the number of cells in culture at the end of one week

8 For each $5x10^6$ cells to be re-stimulated, count and irradiate $5x10^6$ K562-mIL21-41BBL using a gamma irradiator at 100 Gy.

9 Add an equal number of irradiated K562 mIL21 41BBL (1:1 ratio) and resuspend in NKEM at $2.5x10^5$ total cells/mL

10 Seed cells in G-Rex 6M flask (Cat. 80660M, Wilson Wolf) at 10 million total cells/well in 40mL ($2.5x10^5$ total cells/mL; cell area is $10cm^2$; $1x10^6$ total cells/cm^2)

Day 10 - refresh media

11 Remove 20ml and top up with fresh media (80mL) containing IL-2 for full culture*. Count the cells and check viability.
**G-rex 6M wells can each hold 100mL.*

Day 12 - refresh media

12 Remove 80ml of the media and replace with fresh media containing IL-2 for full culture. Count the cells and check viability.

Day 14 - freezing

13 Freeze $1x10^8$/aliquot and $5x10^7$/aliquot in 90% heat inactivated fetal bovine serum (Cat. 26140079, Gibco) 10% DMSO (Cat. BP231-100, Fisher Bioreagents).