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Isolation of natural killer (NK) cells from human blood products V.3

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We use this protocol and it's working

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Abstract

Standard isolation procedure for peripheral blood mononuclear cells (PBMC) from human blood leukopheresis products.

Guidelines

One trimacone will generally provide 500 million - 2 billion PBMCs.
NK cells are generally 5% of PBMCs (ranging from about <1% to 30%), so 100 million PBMCs will routinely yield 5 million NK cells. Be aware that the lower the percentage of NK cells, the lower the post-enrichment purity with a negative selection kit. This may negatively impact later assays if the percentage of NK cells amongst PBMC was <1%. Routine post-enrichment purity checking by flow cytometry with a live/dead marker, anti-CD56 and anti-CD3 antibodies, is encouraged

Blood products carry the risk of blood borne pathogens. Follow your local safety guidance.

Post isolation culture
If needed, NK cells can be cultured and activated with cytokines for enhanced function. Increasing function can help differentiate between experimental conditions, but does change NK cell behavior compared to endogenous function.

1. Base media is **R10**: RPMI (Gibco Cat. No. 2240-089) + 10% fetal bovine serum (Gibco Cat. No. 26140079) + 100 U/mL Penicillin and Streptomycin (Gibco Cat. No. 15140122).
2. NK cells like to be at high density, we recommend 4×10^6/mL in flat bottomed TC-treated plastic
e.g. 4×10^6 cells in 1 mL in a 24 well plate well. Or 1.5×10^6/mL in 200 µL U-bottom 96-well plate wells.
3. 1 ng/mL IL-15 will enhance survival, but will not overly activate them or drive excessive proliferation (although we find batches from different manufacturers have differing potency, so check your batch with a **proliferation assay**).
4. 10 ng/mL IL-15 or 100 U/mL IL-2 will strongly activate the NK cells driving enhanced cytotoxicity after overnight treatment and enhanced proliferation of 7 days.

Materials

Isolation

Healthy donor blood product (Leukopak) referred to as 'trimacones'
Biological safety cabinet referred to as 'the hood'
50mL plastic syringe
T75 flask
1X sterile PBS
50mL conical tubes
Ficoll-Paque (GE Healthcare)
Scissors
Sterile transfer pipettes
Alcohol swap
70% ethanol for sterile technique.

Post isolation culture

RPMI (Gibco Cat. No. 2240-089)
fetal bovine serum (Gibco Cat. No. 26140079)
Penicillin and Streptomycin (Gibco Cat. No. 15140122)
IL-15 (e.g. from the national cancer institute or R&D systems cat. 247-ILB)
or IL-2 (e.g. Prometheus cat. NDC 65483-116-07 or R&D systems cat. BT-002-AFL)

Troubleshooting

Overview

1 Healthy donor blood products (Leukopaks) were obtained from Memorial Blood Bank (Minneapolis, MN). All samples were de-identified and their use was approved by the University of Minnesota and NMDP institutional review board in accordance with the Declaration of Helsinki.

2 Peripheral blood mononuclear cells (PBMCs) were separated through density gradient filtratation (Ficoll-Paque; GE Healthcare).

3 For experiments with enriched NK cells, PBMCs were processed fresh using negative magnetic bead separation (EasySep Human NK Cell Enrichment Kit; Cat. No:19055, STEMCELL Technologies).

4 Experiments with PBMCs or enriched NK cells were performed in RPMI 1640 supplemented with 10% Fetal Bovine Serum and 1% penicillin/streptomycin. Where specified, culture was supplemented with recombinant human IL-15.

Detailed instructions for density gradient separation of PBMCs

5 Drain the contents of a trimacone into a T75 flask:
- For each trimacone, you will need one T75 flask and two 50mL conical tubes.
- Add 40mL 1X sterile PBS to T75 flask.
- Open the bag containing the trimacone in the hood and wipe down the outside of the trimacone with 70% ethanol.
- Cut the bottom tube off at its base and place the trimacone in the mouth of the T75 flask.
- Upon cutting the top tube, the blood will begin dripping into the flask.
- *Prepare Ficoll while you wait (see step 6)*
- If the trimacone is taking too long to drain, you can use the 50mL syringe to force air into the top of the cone and push it through faster. Some blood will remain stuck to the sides; you do not need to wash it out.

6 Layer the blood product onto Ficoll:
- While waiting for the trimacone to empty, add 15mL Ficoll to each 50mL conical tube.
- Check the date the Ficoll was opened—if it is older than 3-4 months, toss it and open a new tube. **Be sure to write the date on the label!**
- Using a 25mL serological pipette, mix the blood and PBS in the flask by pipetting up and down a few times.
- Take 25mL of the blood/PBS mix and pipette it **VERY** slowly and gently down the side of the 50mL conical tube.
- It helps to hold the tube at an angle and drip the blood down the side. You can turn down the outflow rate of the pipette gun as well.

- The goal is to layer the blood on top of the Ficoll and not to mix them. If you accidentally pipette too quickly and there is some mixing, the PBMC extraction will still work, but you may have a lower yield.
- Rinse the flask with 5mL PBS to get any remaining blood.

7 Spin the layered blood product and remove the interface:
- Before spinning, **use a scale to balance your tubes**. If necessary, add some PBS (in the hood) gently to tube(s) to achieve equal weights. Even a slight imbalance can negatively impact the Ficoll layering and your final yield.
- Spin the tubes at room temperature at **2000rpm (approx. 940g)** for **20 minutes** with **acceleration 5 andno brake.** It usually takes a total of 30-35 minutes before the spin is complete and the centrifuge stops on its own.
- Carefully remove the tubes. You should see layers like in the description.
- The only layer you need to collect is the buffy coat. This layer moves almost as a single unit—when you extract it, it often will stick to itself and allow you to collect it all at once. Taking too much of the ficoll will prevent your cells pelleting later.
- If you prefer, pipette off some of the plasma. This can make the buffy coat easier to see from above.
- Using a sterile transfer pipette/dropper, draw up the buffy coat and put it into a new 50mL conical tube. You may need to go back and get some residual bits of the buffy coat.

8 Wash the cells in PBS:
- Add 20mL PBS to the new conical containing the buffy coat. Spin down at **2200rpm (small centrifuge = 750g) for 5 minutes**.
- You should get a large, distinct pellet after this spin. If the pellet seems “loose” or like there are cells in suspension, vortex the tube to mix and split the suspension between two new 50mL conicals. Add 20mL PBS to each and repeat the spin.
- Wash again with PBS.

9 Lyse red blood cells (optional):
- Resuspend pellet in 5-10mL ACK buffer for 3 minutes.
- Wash 2x with PBS. Resuspend in 10mL (expecting 200 million/ml max)

10 Count cells:
- Red blood cells can be difficult to tell apart from PBMC, depending on the counting method performed.
- We use a Cellaca, which only counts nucleated cells (AOPI= acridine orange / propidium iodide which are membrane permeable / non-permeable, respectively, nucleic acid dyes), not red blood cells.
- Dilute 25 µL cells into 475 µL PBS (1/20 dilution), then mix this 1:1 with AOPI to get cells into a measurable range (10million/mL-1million/mL). Final dilution = 1/40.

Detailed instructions for NK cell enrichment

11 Calculate the appropriate number of PBMCs to enrich from:
- NK cells are generally 5% of PBMCs (ranging from about <1% to 30%), so 100 million PBMCs will routinely yield 5 million NK cells.

12 Magnetically enrich NK cells according to the manufacturer's instructions:
- We prefer purity over yield (once in the magnet, not twice).
- Count cells and check purity by flow cytometry (stained with live/dead marker, CD56 and CD3 marker).
- Each lot of the kit should be checked for purity yield. These kits routinely yield 90%-95% CD56+ CD3- cells.

13 Store excess PBMCs:
- One trimacone will generally provide 500 million - 2 billion PBMCs, so there may well be excess PBMCs to your needs.
- However, if the proportion of NK cells is low, the purity of the NK cells following enrichment can also be low (<90%), so it is not worth storing donors with NK cell proportions <2%.
- For storage of excess cells, freezing 100 million PBMCs and enriching after thaw yields better results than freezing enriched NK cells.
- 100 million PBMC can be frozen in 1 mL of 90% heat inactivated fetal bovine serum, 10% DMSO.
- Freeze in styrofoam or controlled temperature cooler e.g. Mr Frosty in a -80 °C freezer overnight.
- Move to long-term storage in liquid nitrogen as soon as possible for best recovery.

14 Enriching from frozen PBMC:
- Upon thaw, wash the cells to remove DMSO.
- Culture the cells overnight at 4×10^6/mL in R10 at 37°C, 5% CO_2 to allow recovery of antigens.
- Proceed with magnetic negative isolation of NK cells the following day.
- Confirm purity of NK cells (stained with live/dead marker, CD56 and CD3 marker) by flow cytometry.