EXPERIMENTAL PROTOCOL: ISOLATION, PURIFICATION AND EXPANSION OF HUMAN NK CELLS FROM HUMAN PBMCs

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Introduction

This protocol is used when we want to isolated untouched NK cells from human PBMCs by depleting the other cells types, i.e., T and B cells or monocytes, which are magnetically labeled thanks to a cocktail of biotin-conjugated antibodies and microbeads cocktail.

Reagents

- Lymphoprep™ (Stem Cell, Catalog #07851)
- PE Buffer (*see below*)
- PBE Buffer (*see below*)
- NK Cell Isolation Kit (Miltenyi Biotec, Order no. 130-092-657)
- NK MACS Basal Medium and NK MACS Supplement (*see below*)
- Human IL-2 IS premium grade (*see below*)
- Human IL-15 premium grade (*see below*)

Instruments and tools

- OctoMACS™ Starting Kit (Miltenyi Biotec, Order no. 130-042-108)
- Sterile falcon tubes, glass pipettes and micropipettes
- Neubauer chamber
- Culture plates and suspension T25 and T75 flasks
- Laminar flow cabinet
- Centrifuge with swinging-bucket

Before start

Preparation of Reagents:

PE Buffer

Component	Company/Cat#	Stock Conc.	Volume	Final Conc.
PBS (tablets)	Applichem/A9202	100 tablets	1 L	1X
Ethylenediaminetetraacetic acid disodium salt	Sigma-Merck/E5134	100 g	For 1 L	2 mM

dihydrate (EDTA)				

PBE Buffer

Component	Company/Cat#	Stock Conc.	Volume	Final Conc.
PBS (tablets)	Applichem/A9202	100 tablets	1 L	1X
Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA)	Sigma-Merck/E5134	100 g	For 1 L	2 mM
A1310 Albumin, Bovine Serum, Cohn Fraction V, pH7 (BSA)	US Biological/ 9048-46-8	100 g	For 1 L	0.5%

Complete NK MACS Medium

Component	Company/Cat#	Stock Conc.	Volume	Final Conc.
NK MACS Basal Medium	Miltenyi Biotec/ 130-114-429	500 ml	100 ml	100 ml
NK MACS Supplement	Miltenyi Biotec/ 130-114-429	5 ml	1 ml	1%
Fetal Bovine Serum (FBS)	Biowest/S1810	500 ml	5 ml	5%
ZellShield®	Minerva Biolabs/ 13-0050	50 ml	1 ml	1%

Expansion NK MACS Medium

Component	Company/Cat#	Stock Conc.	Volume	Final Conc.
Complete NK MACS Medium	*See above*	100 ml	100 ml	100 ml
Human IL2S premium grade	Miltenyi Biotec/ 130-097-746 (200 µg)	200µg	2 ml	0.1 mg/ml
Human IL15 premium grade	Miltenyi Biotec/ 130-095-764 (25 µg)	25 µg	250 µl	0.1 mg/ml

PROCEDURE

Priorities:

1) Isolation of PBMCs
2) Isolation of NK cells
    - Manual magnetic labelling

- Manuel cell separation

3) Culture and expansion of NK cells

Isolation of PBMCs

1. Dilute blood with 2-4X the volume of PE buffer, i.e., 20 ml of blood and PE buffer up to 40-50 ml.
2. Add 15 ml of Lymphoprep in a sterile 50 ml falcon tube and, carefully, layer 20-35 ml of diluted blood suspension.
3. Centrifugate at 2500 rpm for 20 minutes in a swinging-bucket with acceleration 1 and without brake.
4. Carefully transfer the mononuclear cell layer using a 10 ml glass pipette to a new 50 ml falcon tube. Do not aspire the Ficoll-phase.
5. Centrifugate at 300 g for 10 minutes in a swinging-bucket.
6. Discard the supernatant, add 50 ml of PE buffer and centrifugate at 200 g for 10 minutes for removal the platelets. Repeat this step if high purity is desired.
7. Count the number cells using a Neubauer chamber and *Trypan Blue stain*.
8. Centrifugate the cell suspension at 300 g for 10 minutes and resuspend the cell pellet in an appropriate volume of PBE buffer. Proceed to the first step of "Isolation of NK cells".

Isolation of NK cells

IMPORTANT NOTE: work fast, keep cells cold and use pre-cooled solutions (4ºC). The volumes indicate below are for **up to 10^7 cells**. When working with fewer number of cells, use the same volumes indicated; on the contrary, scale up all reagent volumes.

Manual magnetic labelling

1. Resuspend cell pellet in 40 µl of PBE buffer.
2. Add 10 µl of NK Cell Biotin-Antibody Cocktail, mix well with a pipette and incubate for 5 minutes in refrigerator (4ºC). Do not use vortex, it can damage the cells.
3. Add 30 µl of PBE buffer and 20 µl of NK Cell MicroBeads Cocktail.
4. Mix well with a pipette and incubate for 10 minutes in refrigerator (4ºC).
5. Add up to 500 µl per column of PBE buffer and proceed to the magnetic cell separation.

Manual cell separation

1. Place the column (i.e., MS column) in the magnetic field of a OctoMACS Separator.
2. Rinse the MS column with 500 µl of PBE buffer. Wait until the column is empty.

3. Add 500 µl of cell suspension onto the MS column and collect the flow-through which contain unlabelled cells, representing the NK cells.
4. Wash the MS column with 500 µl of PBE buffer and combine with the effluent from step 3.

Culture and expansion of NK cells

1. Determine the total cell number by counting using a Neubauer chamber without stain.
2. Centrifugate at 300 g for 10 minutes. Discard supernatant.
3. Resuspend cell pellet at a concentration of 1 million per ml in expansion NK MACS Medium (complete NK MACS Medium supplemented with 500 UI/ml of IL2 and 140 UI/ml of IL15).
4. Pipet 700 µl of cell suspension into each well of a 24 well non adherent-plate (approximately $7x10^5$ cells/well).

Expansion process

- Upon reaching a well volume of 1 ml, switch to a 12-well plate (around day 5-7).
- Upon reaching a well volume of 2.5 ml, switch to a 6-well plate.
- Upon reaching a well volume of 5-6 ml, switch to a T25 suspension flask (around day 10-12).
- Upon reaching a flask volume of 7-10, switch to a T75 suspension flask (around day 12).
- Upon reaching a flask volume of 30 ml, switch to a T175 suspension flask (around day 14-17).

1. **Day 1-5**: do not disturb the cells.
2. **Day 5**: add 300 µl Expansion NK MACS Medium.
3. **Day 7**:
   a. Take 15 µl sample to determine cell count (without stain).
   b. If the cell density is less than 1 million per ml, double the culture volume with fresh expansion NK MACS Medium.
   c. If the cell density is higher than 1 million per ml, add enough fresh expansion NK MACS medium to dilute cells to a final concentration of $5x10^5$ cells per ml.
4. **Day 10 and 12**:
   a. Take 15 µl sample to determine cell count (without stain).
   b. Add enough fresh expansion NK MACS medium to dilute cells to a final concentration of $5x10^5$ cells per ml.
5. **Day 14**: take 15 µl sample to determine cell count (without stain) and perform cell analysis (i.e., *Cytotoxicity assay*).