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Peripheral Blood Mononuclear Cells: Isolation, Freezing, Thawing, and Culture

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Abstract

The work with peripheral blood mononuclear cells (PBMCs), which comprise lymphocytes and monocytes, is indispensable in immunological diagnostics and research. The isolation of PBMCs takes advantage of differences in cell density of the different blood components. Density gradient centrifugation of diluted whole blood layered over a density gradient medium yields PBMCs; two subsequent washing steps remove remaining platelets. To store the cells for future assays, they can be frozen and thawed when required. Dimethyl sulfoxide (DMSO) serves as a cryoprotectant for freezing PBMCs, but must be removed by washing after thawing, as it can become toxic to the cells on longer exposure.

Keywords: PBMC, Density gradient, Freezing, Thawing, Cryopreservation

1 Introduction

The investigation of the human immune system is a broad field of research and the key to understanding many different diseases and their possible future cure or prevention. Immunological research contributes to deciphering the etiology and pathogenesis of autoimmune diseases like multiple sclerosis, to characterizing the role of immune cells during infections, to the development of vaccines and to a better understanding of the pathogenesis and possible future therapies of malignancies. For analysing adaptive immune responses, a starting point and fundamental method is the isolation of peripheral blood mononuclear cells (PBMCs) from whole blood. PBMCs comprise lymphocytes and monocytes. In subsequent experiments, they can be further separated, for example by magnetic cell separation or fluorescence-activated cell sorting. Lymphocytes and monocytes can also be employed together in assays like ELISPOTs or proliferation assays, where antigen-presenting cells are necessary to present antigenic peptides on MHC-I or MHC-II to the responding T cell populations.

Often it is not possible to work with freshly isolated PBMCs. Then the cells can be frozen and thawed at a later time point. This allows a batched thawing of different samples and a direct comparability in assays, reducing inter-assay variability, as well as a future analysis of later emerging issues. Many studies have been conducted exploring the viability and antigen-specific response of cryopreserved mononuclear cells, most of them in the context of HIV research. It has been shown that cryopreservation does not functionally impair cell cycle or cytokine-production of ($CD4^+$ and $CD8^+$) T cells upon stimulation (1, 2). Percentages of $CD4^+$ and $CD8^+$ cells could be shown to be equal in fresh and cryopreserved PBMC and were also stable in cryopreserved cells for more than a year (3, 4). However, it must be taken into consideration that cryopreservation can reduce the fraction of naïve and central memory T cells, can lead to a decrease of CCR-5 expression and to an increase of effector $CD8^+$ cells (4). All in all, a good quality of assays with cryopreserved cells can be maintained.

The isolation of PBMCs from whole blood is based on density differences between PBMCs and other components of the peripheral blood. The origins of this method go back to the 1960s when Arne Boyum first described the separation of white blood cells using Ficoll and other density gradient media (5). Since then, the technique has been refined, but the underlying principles have remained the same: By density gradient centrifugation, a separation of blood components according to their density is possible with the help of a density gradient medium (e.g. Lymphoprep or Ficoll) containing sodium diatrizoate, polysaccharides, and water, reaching a density of 1,077 g/ml. The density gradient medium leads to an aggregation of red blood cells. The medium is therefore denser than lymphocytes, monocytes, and platelets, but less dense than granulocytes and red blood cells. Accordingly, erythrocytes and most of the granulocytes will sediment and pellet at the bottom of the tube after centrifugation; over this phase, the density gradient medium is found. The top layer consists of plasma and platelets. Mononuclear cells band at the interface between the plasma and the density gradient medium. Two subsequent washing steps at a lower speed help to remove remaining platelets.

For cryopreservation, dimethyl sulfoxide is widely used as a cryoprotectant. It reduces the formation of ice crystals and resulting cell damage. Despite its benefits, it can be toxic to cells (6). When PBMCs are thawed the comprised DMSO should therefore be quickly diluted and removed by two subsequent washing steps. In the following, we describe how PBMCs can be isolated, cryopreserved, and thawed. In addition, we point out some basic principles for culturing PBMCs.

2 Materials

All materials and reagents coming into contact with cells must be sterile. Keep attention that your plastic material, reagents, and media are not only sterile, but also free of endotoxin. Store all reagents as indicated.

1. 1× Phosphate Buffered Saline (PBS).
2. 50 ml tubes.
3. Sterile plastic pipettes (*see* **Note 1**).
4. Transfer pipettes.
5. Lymphoprep: 9.1 % w/v Sodium Diatrizoate, 5.7 % w/v Polysaccharide, water. Density of 1.077 g/ml (Stemcell Technologies, Oslo, Norway).
6. 1× RPMI 1640 Medium (*see* **Note 2**).
7. Cell medium: 10 % Fetal Bovine Serum (FBS) (*see* **Note 3**), 1 % 200 mM L-Glutamine and 1 % Penicillin/Streptomycin in RPMI 1640 medium.
8. 0.4 % Trypan blue: Make a 0.4 % trypan blue solution by weighing the requested amount of trypan blue powder and diluting it with PBS (*see* **Note 4**).
9. Neubauer Counting Chamber.
10. DMSO (*see* **Note 5**).
11. Freezing medium: 80 % FBS and 20 % DMSO.
12. Cryogenic Vials: Vials must be sterile and resistant against liquid nitrogen.
13. 96-well or 24-well plastic plates.
14. Phytohemagglutinin.

3 Methods

Take care that everything coming into contact with cells remains absolutely sterile.

3.1 Isolation of PBMCs

1. Dilute the blood (*see* **Note 6**) with a double volume of PBS.
2. Prepare 50 ml tubes filled with 15 ml of Lymphoprep each. You need one tube per 10 ml whole blood or 30 ml blood dilution, respectively.
3. Carefully layer 30 ml of the blood dilution upon the Lymphoprep solution. Avoid an intermingling of the two phases (*see* **Note 7** *and* Fig. 2).

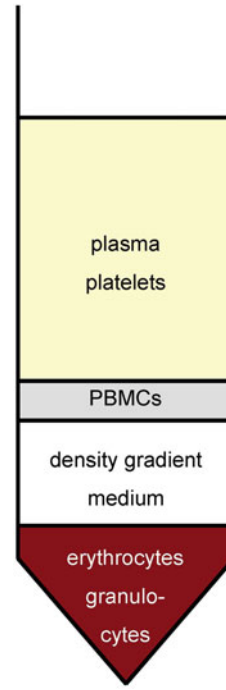

Fig. 1 After density gradient centrifugation, different blood cells are found in different layers according to their density. From bottom to top you find red blood cells and granulocytes, followed by the density gradient medium (e.g. Lymphoprep), the layer containing the PBMCs and on top the plasma phase containing platelets

4. Centrifuge for 25 min at 670–800 × *g* at 18 °C (*see* **Note 8**). Make sure the centrifuge brake is switched off to prevent a disruption of the gradient layers.
5. Collect the cloudy-looking phase containing the PBMCs, which you find above the Lymphoprep layer and beneath the phase containing plasma and platelets (*see* Fig. 1 *and* **Note 9**). Transfer PBMCs of three 50 ml tubes into one new 50 ml tube. Add PBS to make up to 50 ml.
6. Centrifuge for 10 min at 150 × *g* at 4 °C with the centrifuge brake switched off.
7. Decant the supernatant, resuspend the pellet in RPMI medium and centrifuge again for 10 min at 150 × *g* and 4 °C with the centrifuge brake off (*see* **Note 10**).
8. Decant the supernatant and resuspend the resulting pellet in cell medium (*see* **Note 11**).
9. Count the cells (*see* **Note 12**).
10. Freeze the cells or perform an assay with the freshly isolated cells.

3.2 Cryopreservation of PBMCs

1. Prepare cryovials and label them with cryotags or with a permanent marker (*see* **Note 13**).
2. Define the concentration at which you want to freeze the PBMCs and the quantity of cells contained in one aliquot. Bring the cell suspension to the double concentration of your end concentration by adding cell medium.
3. Add an equal volume of freezing medium in three subsequent steps in intervals of 3–4 min to give your end concentration (*see* **Note 14**).
4. Mix the cell suspension and transfer aliquots of the PBMCs to the cryovials.
5. Freeze the cells in a −80 °C freezer as fast as possible.
6. Transfer the cells to liquid nitrogen or to a freezer providing temperatures lower than −150 °C within 2–3 days. Store them at this low temperature until use.

3.3 Thawing PBMCs

1. Warm RPMI medium in a water bath at 37 °C for washing your thawed cells in (*see* **Note 15**).
2. Thaw the cells in the water bath (*see* **Note 16**).
3. Quickly resuspend the cells in the medium and centrifuge at 150 × *g* for 10 min at 4 °C with the centrifuge brake off.
4. Decant the supernatant, resuspend the cells in RPMI and wash again as in step 3 (*see* **Note 17**).
5. Resuspend the cells in cell medium and count them.

3.4 Culturing Cells

1. For culturing the PBMCs, bring them to the desired concentration by diluting with cell medium.
2. Add the cells to 96-well plates or 24-well plates (*see* **Notes 18–21**).
3. Incubate the cells at 37 °C and 5 % CO_2 in an incubator.

3.5 Stimulating Cells

1. When you perform an assay with PBMCs and stimulate them with different antigens, always introduce positive and negative control wells (*see* **Note 22**).
2. Pre-establish the optimal concentrations of your stimuli in titration experiments.
3. After the incubation time of your assay has ended, collect the supernatant and freeze it at −80 °C. You can later perform multiplex analyses to determine the prevalent cytokine milieu in different stimulatory situations.

4 Notes

1. It can be highly recommended to use one-way plastic pipettes in immunological settings. Lipopolysaccharide, when sticking to glass pipettes, can withstand autoclaving and stimulate cells coming into contact with it.
2. Cell media contain phenol red, a pH indicator. As the medium contains 5 % CO_2, the pH rises when the contact with ambient levels of CO_2 increases. In case the medium turns pink it should be discarded.
3. Do not use untested serum. Even small quantities of contaminants like stimulators (e.g. bacterial endotoxin) or suppressors can ruin the result of possible subsequent assays. Stick to the use of one serum and always use serum of one batch, if possible.
4. Trypan blue is cancerogenic. Avoid skin contact when weighing the trypan blue powder. Trypan blue does not need to be handled sterilly, but it is recommended to add sodium acid or to filter the solution from time to time to avoid a growth of fungi which can obscure your view when counting cells.
5. DMSO is combustible and irritating to eyes, respiratory system, and skin. It is absorbed through the skin. Avoid contact.
6. Try to draw the blood as carefully as possible. We can recommend the use of sodium heparin as an anticoagulant, but other anticoagulants like acid citrate dextrose (7) or lithium heparin can be used. Do not chill the blood during the transport to the laboratory. It was found that a cryopreservation of PBMCs within 8 h is significantly superior to a cryopreservation within 24 h, which can reduce the viability and functional activity of your cells (7). Our experience is that it is realistic to freeze the cells within 4 h after venipuncture, which we observe has positive effects on cell viability.
7. The blood can be layered over the Lymphoprep medium in two ways:
   (a) Hold the tube containing the Lymphoprep solution at a tilted angle. Use a pipette for taking up the blood and make sure the pipetting speed is set to a minimum. Place the tip of the pipette against the wall of the tube (*see* Fig. 2b).
   (b) Prepare the same number of tubes as the ones filled with Lymphoprep and pipette 30 ml of the blood dilution into each. If you are right-handed, take a tube containing Lymphoprep in your left hand and a tube containing blood dilution in your right hand. Hold both tubes nearly horizontally and make the winding of the blood tube meet the rim of the Lymphoprep tube. Layer the blood dilution over the Lymphoprep medium by pouring the blood dilution very carefully into the Lymphoprep tube (*see* Fig. 2c).

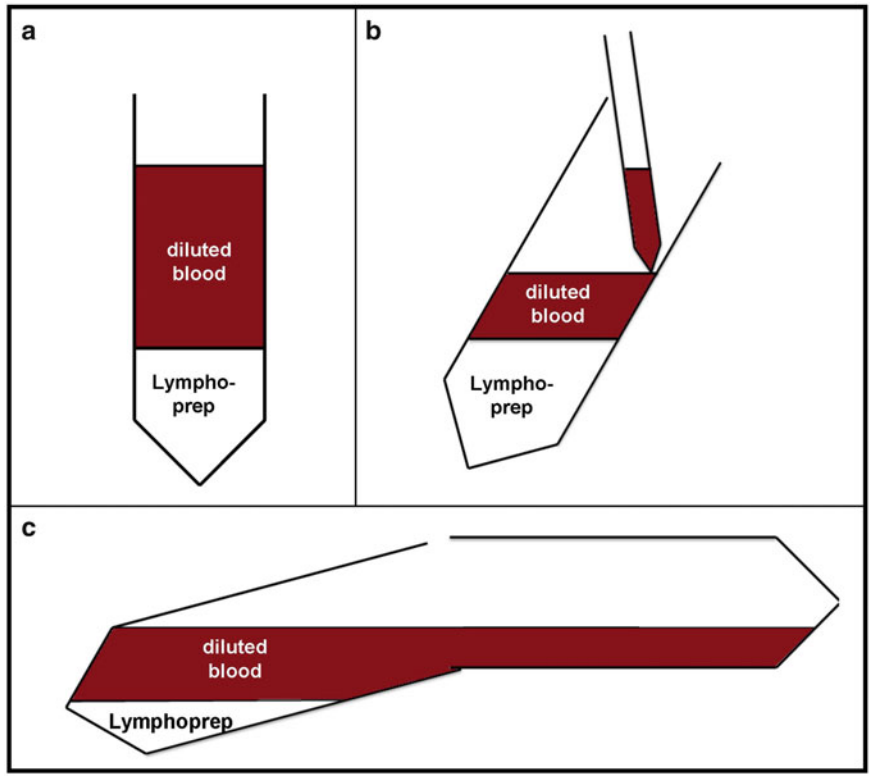

Fig. 2 This shows how the diluted blood can be layered over the Lymphoprep phase. (**a**) The two phases should not be intermingled. There should be a clear contour between the phases. (**b**) A pipette can be used for achieving this or (**c**) the blood dilution can be poured carefully from one tube into the other upon the Lymphoprep phase

8. Centrifugation at higher temperatures than 18–20 °C leads to lower cell yields, as more lymphocytes become trapped between aggregated erythrocytes. Lower temperatures increase the density of the density gradient medium and can cause a contamination of the PBMC layer with red blood cells and granulocytes.
9. It is best to use a transfer pipette for collecting the layer containing the PBMC. In contrast to bigger plastic pipettes, it is easier to avoid collecting either little volumes of the Lymphoprep phase or the plasma phase, leading to a contamination with granulocytes or platelets, respectively. Hold the tube so that the PBMCs are at eye level. So you can see best if the pipette remains in the right phase. Dispose of the tube containing the rest of the blood cells in a biohazard waste container.
10. If no pellet is visible after the centrifugation of the collected PBMC layer, it is recommendable not to discard the supernatant, but to decant it into new sterile falcon tubes, to add some

PBS and centrifuge it again in the next round. If there forms a pellet, you have saved the mononuclear cells you would have lost otherwise.

11. Normal PBMC yields range from 0.5 to 2.5 $\times$ 10^6 PBMCs per milliliter whole blood. If you freeze the cells afterwards, the quantity of cell medium you resuspend your cells in depends on the cell numbers frozen in one aliquot. For resuspending the cell pellet, we use about 5 ml cell medium per 100 ml starting blood volume and freeze aliquots of 5–10 $\times$ 10^6 PBMCs.
12. Mix the cell suspension thoroughly, then take about 10–20 µl of the cell suspension and transfer it to a cell culture plate. Mix it with an equal volume of trypan blue and dilute about 16–32-fold. Sometimes the gradient may be contaminated with some red blood cells. This can e.g. be due to problems at drawing the blood. When you count your cells with the trypan blue solution, you cannot discriminate between viable red blood cells and mononuclear cells, but the ratio of viable cells in all cells can be determined. Counting the cells again with Türk's solution reveals the number of mononuclear cells, as Türk's solution destroys red blood cells, but allows no discrimination between viable and apoptotic cells. Using both systems, the number of viable mononuclear cells can be estimated.
13. We prefer the use of printed cryotags, since their labeling withstands alcoholic disinfection.
14. A stepwise addition of DMSO helps the PBMCs to "get accustomed" to the cryoprotectant. However, the cells should best be frozen within 10 min. Make sure you have opened all the cryovials before you add the last portion of the freezing medium so that you can start aliquotting immediately.
15. It has been shown that adding cold medium rapidly to cold cells after thawing reduces the viability of the cells (8, 9). However, the volume of your thawing medium (e.g. 15 ml vs. 50 ml) does not influence cell viability (9).
16. Disinfect cryovials after taking them out of the water bath. Water baths are very susceptible to contamination.
17. Washing twice after thawing increases the viability of cells, since DMSO is diluted (8). The influence of DMSO on cell viability and functionality is dependent on the duration of exposure and concentration (10).
18. It is essential you have equal cell numbers in each well for good results of your assay. Make sure to mix your cell suspension thoroughly.
19. For transferring cells to a 96-well plate, you can use a multipette or a multichannel pipette. If you prefer a multichannel pipette, first transfer your cells into a sterile reagent reservoir.

20. There are different types of plates with U-shaped, V-shaped, and flat-bottom wells. Use plates with U-shaped or V-shaped wells for culturing cells when much cell-cell interaction is required. Use flat-bottom plates when you culture adherent cells.
21. Keep in mind that monocytes strongly adhere to plastic when you harvest your cells after culture.
22. As negative controls use wells containing PBMCs without stimuli. It is advisable to add the solvent of your stimuli (e.g. DMSO) to negative wells in the same concentration as in wells containing antigen. For positive control wells, you can add mitogens like phytohemagglutinin.

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