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Comparison of three monocyte depletion techniques for lymphocyte isolation from peripheral blood mononuclear cell

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ABSTRACT

Background: Lymphocytes are crucial cells in the immune system. Studying lymphocyte function can lead to better understanding of the immune system. Essentially, lymphocyte isolation technique is required for studying lymphocyte function. Several techniques were developed to prepare lymphocytes, including depletion of monocytes from peripheral blood mononuclear cells (PBMCs).

Objectives: To compare utilization of three different techniques for lymphocyte isolation by monocyte depletion from PBMCs.

Materials and methods: Lymphocytes were isolated from PBMCs by depletion of monocytes using (i) magnetic beads phagocytosis, (ii) Percoll density gradient centrifugation, and (iii) anti-FITC antibody conjugated micro-magnetic beads. The number of cells collected was counted using Turk's solution. The cellular profiles of PBMCs and monocyte-depleted PBMCs were determined by immunofluorescence and flow cytometry.

Results: The highest yield and purity of monocyte-depleted PBMCs were achieved using the anti-FITC antibody conjugated microbeads depletion method. However, this method consumed the longest time and had the highest cost. Magnetic beads phagocytosis depletion method required the shortest time; however, the wide range of collected yield was a concern. Percoll gradient centrifugation method was the cheapest, but the percentage yield was the lowest among the three methods.

Conclusion: The utilization of three different methods was able to deplete monocytes from PBMCs. However, each technique had some advantages and disadvantages. The information obtained from this study might give some guidance for selecting a suitable method for isolation of lymphocytes based on the monocyte-depleted PBMC strategy.

Introduction

The immune system is an important host defense system that relates to several types of cells and soluble molecules, which collaborate systematically to protect our body from infectious microorganisms and tumors.[1, 2] In the immune system, lymphocytes are one of the crucial cells that play several roles in both innate and adaptive immunity. Lymphocytes are divided into three major groups that play distinct roles, including B cells, T cells, and natural killer (NK) cells.[1-3] B cells, upon antigen stimulation, differentiate into plasma cells to secrete antibody and pro-inflammatory cytokines to fight pathogens. Moreover, B cells also act as antigen presenting cells (APCs), which recognize antigens and present peptide-major histocompatibility complex (MHC) class II complex to induce T cell activation.[4, 5] T cells

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play an important function in cell-mediated immunity, including directly killing infected cells, producing cytokines to activate and regulate other immune cells.[6] NK cells are innate lymphocyte subsets, which rapidly respond and kill virus-infected and tumor cells via direct killing or the ADCC mechanism.[7] Since lymphocytes play an important role in immune responses, studying lymphocyte function will build a path toward better understanding the immune system.

Venous blood contains several types of unstimulated leukocytes, including lymphocytes, monocytes, and granulocytes. These leukocytes are different in cell density and surface molecule markers.[8, 9] In order to study the function of lymphocytes, a method for lymphocyte preparation is necessary. The rapid and non-altering processing of cell function is an essential approach in clinical and basic research.[9, 10] Nowadays, several strategies are employed to prepare lymphocytes from peripheral blood, such as immune precipitation, flow cytometry sorting, magnetic particle sorting, and sorting by microfluidic chips blood.[9, 11, 12] However, lymphocyte isolation from whole blood using the available methods is difficult. Each technique has different advantages and disadvantages.[11, 13] In this study, we compare three different techniques for lymphocyte preparation by depletion of monocytes from peripheral blood mononuclear cells (PBMCs). The methods conducted in this study include Percoll density gradient centrifugation, anti-FITC mAb-conjugated microbeads, and magnetic beads-phagocytosis. The technique selected was based on physical properties and functions of cells, i.e. cell density, specific surface molecules, and phagocytosis ability.[11, 14] For Percoll density gradient centrifugation, monocytes were separated from lymphocytes by their density using isosmotic Percoll density gradient.[15, 16] In magnetic beads-phagocytosis method, monocytes were depleted by their phagocytosis function.[11] PBMCs were incubated with magnetic beads and then placed on a magnetic stand to remove beads-phagocytosed monocytes. For anti-FITC mAb-conjugated microbeads, monocyte-depleted PBMCs were prepared by depletion of monocytes (negative selection) from PBMCs using FITC-conjugated anti-CD14 mAb. Anti-FITC micro magnetic beads were added and monocyte-beads complexes were trapped in a magnetic column placed in a magnetic field. We demonstrated that each technique showed different advantage and limitations. Thus, the information obtained from this study could provide guidance for the selection of the appropriate method of lymphocyte preparation.

Materials and methods

Antibodies and reagents

PE-conjugated anti-CD3 mAb was purchased from ImmunoTools (Friesoythe, Germany). PE-conjugated anti-CD19 mAb was obtained from BD Bioscience (San Jose, CA, USA). FITC-conjugated anti-CD14 mAb, PerCP-conjugated anti-CD45 and Alexa Fluor647-conjugated anti-CD56 mAb were purchased from BioLegend (San Diego, CA, USA). Percoll reagent was ordered from Amersham Biosciences (Uppsala, Sweden). Magnetic beads sized 1.23 µm and anti-FITC microbeads were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany).

Peripheral blood mononuclear cells

Heparinized whole blood was obtained from healthy donors. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized whole blood using Ficoll-Hypaque gradient centrifugation. In brief, heparinized blood was diluted with phosphate buffer saline (PBS), pH 7.2, at a ratio of 1:1. Then, diluted blood was overlaid on Ficoll-hypaque solution and centrifuged at 25°C, 400 ×g for 30 minutes. After centrifugation, the mononuclear cells were collected and washed 3 times with PBS. The number of cells was counted using Turk's solution. Cells were resuspended in the appropriated buffer for further experimentation. It was noted that PBMCs used in this study were chosen in various ranges of monocytes to determine each technique's monocyte depletion ability.

Monocyte-depleted PBMC preparation using phagocytosis of magnetic beads

PBMCs ($1x10^7$ cells) and magnetic beads were resuspended in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS). Then, magnetic beads were added into cells to obtain cell-to-bead ratios of 1:1, 1:2 and 1:5 in total volume of 250 µL. The cell and beads mixtures were rotated at room temperature for 30 minutes. After incubation, cells were placed in a magnetic stand for 5 minutes to remove phagocytosed and free magnetic beads. The unbound solution containing monocyte-depleted PBMCs was collected and counted using Turk's solution. Cells were stained with either a cocktail of PerCP-conjugated anti-CD45 mAb and PE-conjugated anti-CD14 mAb or a cocktail of FITC-conjugated anti-CD3 mAb, Alexa Fluor 647-conjugated anti-CD56 mAb, and PE-conjugated anti-CD19 mAb at 4°C for 30 minutes. The percentages of monocyte contamination and remaining T cells, B cells, and NK cells were analyzed by flow cytometer.

Monocyte-depleted PBMC preparation using Percoll density gradient centrifugation

PBMCs ($1x10^7$ cells) were resuspended in PBS. PBMCs were then overlaid on Percoll solution (48.5% Percoll, 0.16 M sodium chloride in ddH_2O) and centrifugated at 865 ×g at room temperature for 40 minutes. After centrifugation, monocyte-depleted PBMCs were pelleted at the bottom of the tube. The enriched monocyte layer and Percoll solution was discarded. Monocyte-depleted PBMCs were washed three times with PBS by centrifugation at 1000 xg for 5 minutes. After depletion, cells were stained with either FITC-conjugated anti-CD14 mAb, FITC-conjugated anti-CD3 mAb, PE-conjugated anti-CD19 mAb, or Alexa Fluor 647-conjugated anti-CD56 mAb at 4°C for 30 minutes. The percentages of monocyte contamination and T cells, B cells, and NK cells remaining were analyzed by flow cytometer.

Monocyte-depleted PBMC preparation using anti-FITC mAb-conjugated microbeads

PBMCs ($1x10^7$ cells) were stained with 10 µg/mL of FITC-conjugated anti-CD14 mAb at 4°C for 30 minutes. After incubation, PBMCs were washed to remove unbound antibodies. Anti-FITC mAb microbeads were then added and incubated in a refrigerator for 15 minutes. The stained cells were washed with PBS containing 0.5% bovine serum

albumin (BSA) and 2 mM EDTA. Then, magnetic separation process was performed by placing an LD column in the magnetic field. The column was prepared by rinsing with 1 mL of PBS containing 0.5% BSA and 2 mM EDTA 3 times. After rinsing, cell suspension was applied into the column. The anti-CD14 antibody binding cells were trapped in the column by the magnetic force. The column was washed again with 1 mL of PBS containing 0.5% BSA and 2 mM EDTA 3 times. The unbound solution containing monocyte-depleted PBMCs was then collected. Percentages of monocyte contamination and T cells, B cells, and NK cells remaining were analyzed by flow cytometer.

Results

Monocyte-depleted PBMC preparation using phagocytosis of magnetic beads

Monocytes are phagocytic cells which could engulf the pathogens as well as particles.11 Therefore, lymphocytes could be prepared by depleted monocytes from PBMCs via the phagocytosis function of monocytes. For optimal cell-to-bead ratio titration, the magnetic beads were incubated with PBMCs at cell-to-bead ratios of 1:1, 1:2 and 1:5 for 30 minutes to allow phagocytosis of monocytes. By this method, after gating cell population by forward size scatter (FSC) and side scatter (SSC) plot, CD14 positive monocytes remaining in PBMCs were decreased from 18.5% to 9.42%, 6.99%, 1.65% at cell-to-bead ratios of 1:1, 1:2 and 1:5, respectively, (Figure 1A). Moreover, anti-CD45 mAb was used to gate white blood cells and analyze the percentage of monocytes. It was found that monocytes in PBMCs were decreased from 18.5% to 9.45%, 7.01% and 1.6% at cell-to-bead ratios of 1:1, 1:2 and 1:5, respectively (Figure 1A). FSC and SSC plot demonstrates that there were no beads remaining after depletion. According to the results, the cell-to-bead ratio of 1:5 was selected for further monocyte depletion experiments. As shown in Figure 1B, the percentages of each cell subpopulations in PBMCs compared with monocyte-depleted PBMCs were determined. After depletion, B cells were decreased from 10.9% to 4.20% but T cells and NK cells were not altered. Based on our findings, this method was able to deplete monocytes in PBMCs from 8.52-19.3% to 0.98-1.49%, as shown in Figure 1C, and the required time for the depletion step was around 60 minutes. The percentage yield of monocyte-depleted PBMCs was obtained in a range of 30.3-60% of PBMCs, which is unstable when compared with other methods, as shown in Table 1. The isolation cost to obtain $1x10^7$ cells of lymphocytes was approximately 1.56 USD (Table 1).

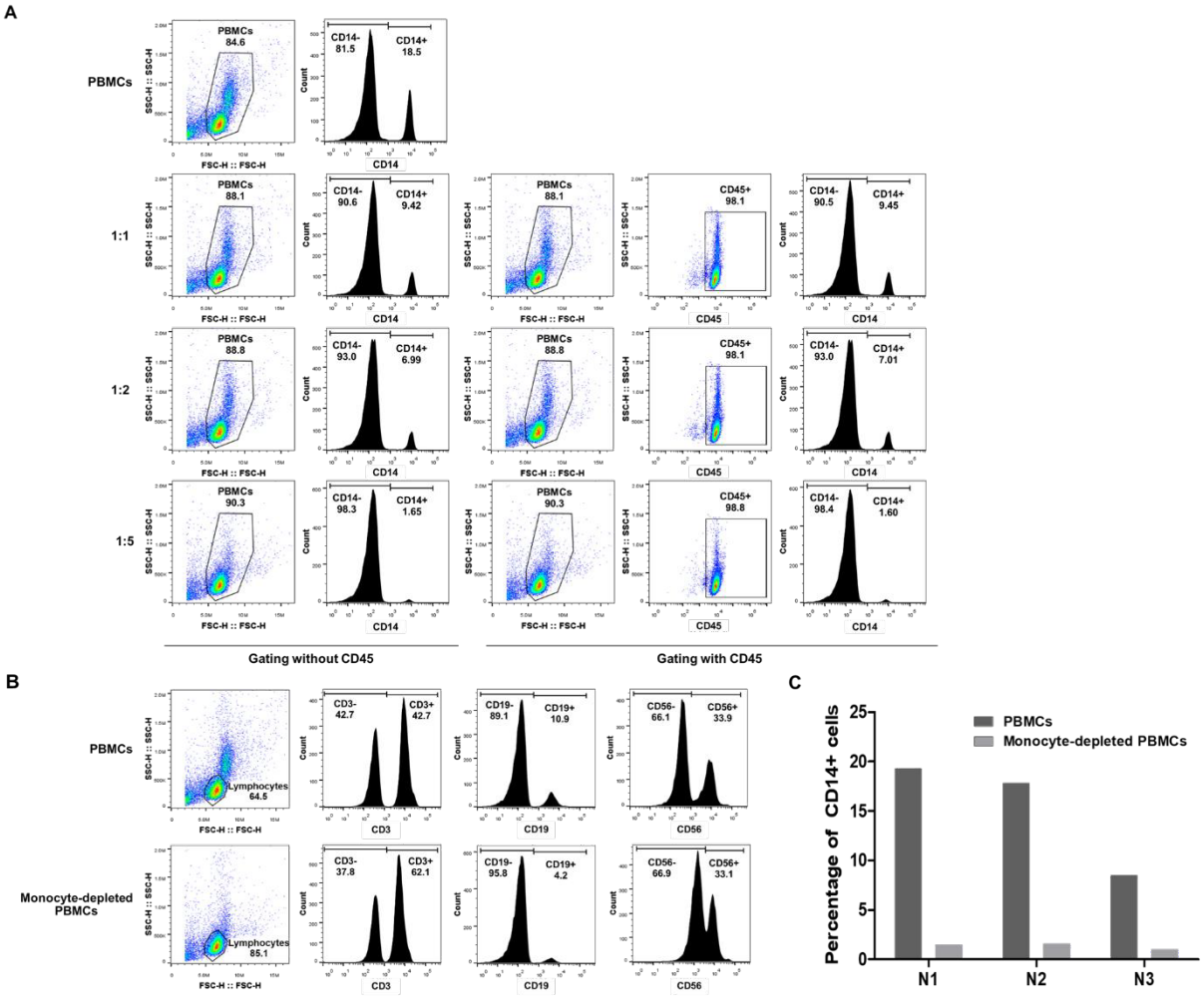

Figure 1. *Percentage of monocytes, T cells, B cells and NK cells after monocyte depletion by phagocytosis of magnetic beads.*

PBMCs were incubated with magnetic beads at cell-to-bead ratios of 1:1, 1:2 and 1:5. The beads-phagocytosed monocytes and free beads were removed. Percentages of each cell subpopulations in PBMCs and monocyte-depleted PBMCs were analyzed by flow cytometry. (A) Percentages of CD14+ cells were compared by gating with forward scatter and side scatter plot in combination with or without PerCP-conjugated anti-CD45. (B) Percentages of indicated cell subpopulations after monocyte depletion at cell-to-bead ratio of 1:5 is shown in histograms. CD3+, CD19+ and CD56+ cells were gated from CD45+ in lymphocyte gate. (C) Three individual representative data of percentage of CD14+ cells in PBMCs and monocyte-depleted PBMCs are shown (n=6).

Table 1 Summary comparison of the lymphocyte isolation using three different monocyte depletion methods.

Monocyte depletion methods	Depletable range of Monocyte (%)	Monocyte contamination (%)	Estimated time used for lymphocyte isolation (minutes)	Estimated cost for preparation of $1x10^7$ lymphocyte (USD)	Yield of isolated lymphocyte from PBMCs (%)*
Magnetic beads	8.52-19.3	0.98-1.49	60	5.13	30.3-60
Percoll solution	8.52-26.8	0.4-1.67	120	1.56	35.5-44.1
Anti-FITC microbeads	10.4-19.3	0.65-1.47	150	25.1	55-66.9

** % Yield of isolated lymphocyte from PBMCs = (total number of isolated lymphocyte – total number of monocyte in isolated lymphocyte) / (total number of PBMCs – total number of monocytes in PBMCs) x 100*

Monocyte-depleted PBMC preparation using Percoll solution

Lymphocytes can be separated from monocytes based on their different density using a low viscosity density gradient medium. In this experiment, we prepared lymphocytes from PBMCs by using Percoll gradient centrifugation method. As shown in PBMC gate, monocyte contamination in monocyte-depleted PBMCs was 1.67%, which was reduced from 20.4% of monocytes in PBMCs (Figure 2A). B cells were also reduced in monocyte-depleted PBMCs from 7.98% to 3.77%, while T cells and NK cells were changed only slightly. In Figure 2B, data shows that monocytes were reduced from a range of 8.52-26.8% to 0.4-1.67% using this method. This method can achieve a 35.5-44.1% yield of monocyte-depleted PBMCs in around 120 minutes (Table 1). The isolation cost to obtain 1x107 cell of monocyte-depleted PBMCs was approximately 5.13 USD (Table 1).

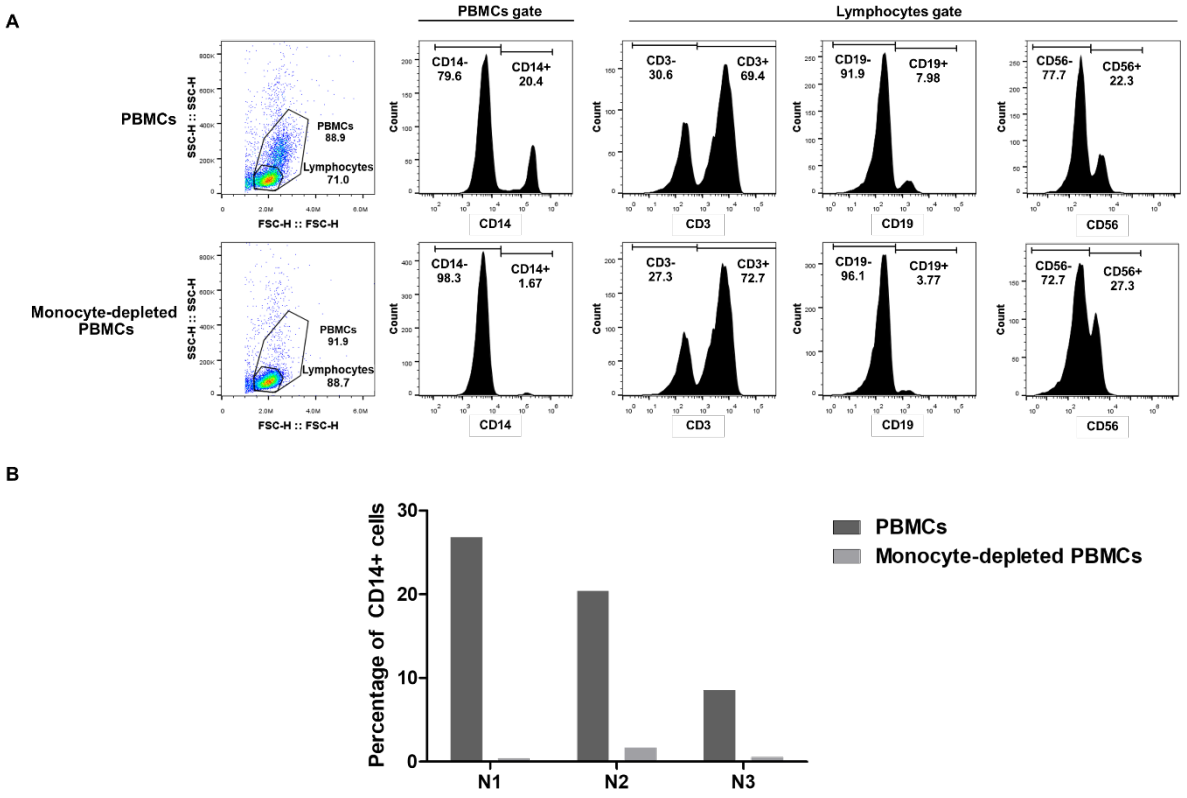

***Figure 2**. Comparison of the percentage of monocytes, T cells, B cells and NK cells after lymphocyte isolating using Percoll gradient centrifugation method.*

Monocytes were depleted from PBMCs using Percoll solution. (A) Percentage of each cell subpopulation in PBMCs compared with monocyte-depleted PBMCs from representative data are shown in histograms. (B) Three individual representative data of percentage of CD14+ cells in PBMCs and monocyte-depleted PBMCs are shown (n=6). CD14+ cells were gated from PBMCs while CD3+, CD19+ and CD56+ cells were gated from lymphocytes.

Monocyte-depleted PBMC preparation using anti-FITC antibody conjugated micro-magnetic beads

Immunomagnetic selection method is a technique used for the isolation of cells from the blood using high-affinity antibodies and magnetic bead technology.[9, 10,17] In this experiment, monocyte-depleted PBMCs were prepared using anti-FITC magnetic beads based on negative selection method. CD14+ cells were bound with anti-CD14 FITC mAb and trapped into a column with anti-FITC micro-magnetic beads under a magnetic field. As shown in Figure 3A, monocytes in PBMCs were depleted from 15.1% to 0.65%, whereas T cells and NK cells were altered only slightly. In addition, B cells were also depleted from 10.6% to 5.96%. After depletion by anti-FITC microbeads, the monocyte contamination was 1.47-0.65%, which was reduced from monocytes in PBMC ranging from 19.3-10.4%, as shown in Figure 3B. The percentage yield of monocyte-depleted PBMCs prepared by this method was 55-66.9%. Estimated time for depletion in this method was around 150 minutes (Table 1). The isolation cost to obtain $1x10^7$ cell of lymphocytes was approximately 25.1 USD, as shown in Table 1.

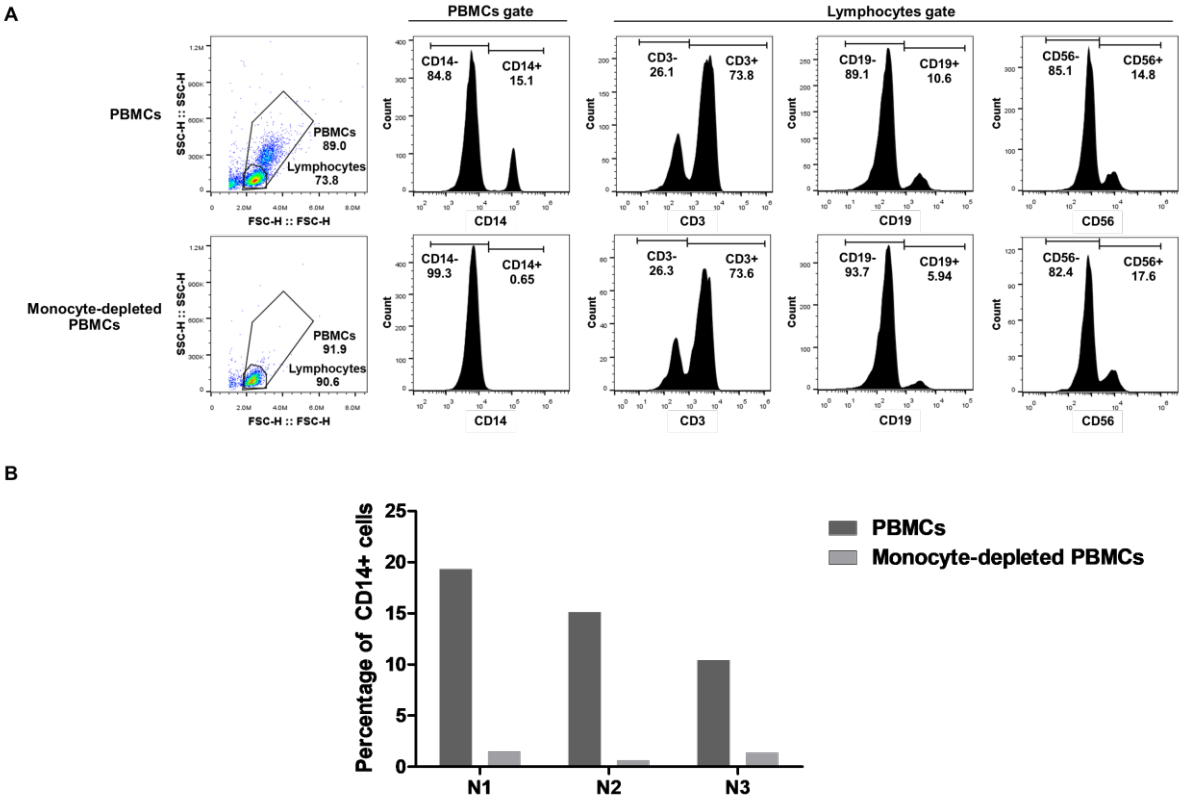

***Figure 3**. Comparison of the percentage of monocytes, T cells, B cells, and NK cells after monocyte depletion using anti-FITC microbeads.*

PBMCs were stained with FITC-conjugated anti-CD14 mAb. Anti-FITC microbeads were added to deplete monocytes from PBMCs using LD column. (A) Percentage of cell subpopulation in PBMCs compared with monocyte-depleted PBMCs is expressed from representative data in histograms. (B) Three individual representative data of percentage of CD14+ cells in PBMCs and monocyte-depleted PBMCs are shown (n=6). CD14+ cells were gated from PBMCs, whereas CD3+, CD19+ and CD56+ cells were gated from lymphocytes.

Discussion

Lymphocytes are crucial cells that play several roles in immune systems. In order to study the function of lymphocytes, a method for cell preparation from peripheral blood without altering cellular function is important.[10] Conventionally, a density gradient medium, such as Ficoll-Hypaque, is used to isolate PBMCs, in which the majority of cells are lymphocytes and monocytes, from granulocytes, erythrocytes and platelets. In this method, diluted blood with PBS is placed in Ficoll-Hypaque solution. After centrifugation, layers of cells are separated depending on the cell density. The layer in the middle between Ficoll-Hypaque solution and plasma contains PBMCs.[18,19] However, the densities of lymphocytes and monocytes overlap so that efficient separation proves difficult on the basis of density difference alone, since PBMCs consist of two major cell populations, including lymphocytes and monocytes.[20, 21] The responses of monocytes to stimuli can induce cytokine production, which affects the survival, proliferation, and immune deviation of other cell types.[22,23] Moreover, the contact between monocytes and lymphocytes stimulates the signaling triggering pro-inflammatory

mechanisms.[24, 25] The previous study reported that T cell stimulation with anti-CD3/CD28 antibody-coated beads is less effective due to non-specific binding of contaminated monocytes with beads.[26] Furthermore, the efficiency of gene transduction on lymphocytes can be decreased by monocytes that compete in the gene vector.[26] Therefore, the study of lymphocyte function can be interfered with by monocyte contamination.[27] The appropriate lymphocyte isolation techniques are required for studying roles of lymphocytes. In this study, we demonstrated 3 different methods for the isolation of lymphocytes by depletion of monocytes from PBMCs, using naked magnetic beads, Percoll density gradient centrifugation and anti-FITC mAb-conjugated microbeads.

Monocytes are classified as phagocytes, which engulf and eliminate invading pathogens, foreign particles, and cell debris.[1, 2] According to their function, PBMCs were incubated with magnetic beads and then monocytes were depleted by bead phagocytosis in a magnetic stand. The naked magnetic beads, sized 1.23 µm, were used to induce monocyte phagocytosis. Ben *et. al*. reported that beads sized 1.0–3.0 µm are taken up to monocytes via phagocytosis and 1.0 µm beads were optimal targets for monocytes.[28] In this study, after optimization, bead-to-cell ratio of 1:5 was optimal for monocyte depletion. Monocytes in the range of 19.3-8.52% in PBMC were almost completely depleted. This method takes the shortest times when compared with other methods. However, the percentage of lymphocyte yield obtained had a wide range of 30-60%. The difference in phagocytic activity of each individual was reported.[29] Monocytes from each individual might engulf different numbers of beads, resulting in different magnetic forces required to trap phagocytosed monocytes in the magnetic stand. The phagocytosed monocytes might bring non-phagocytosed cells trapped together with the magnetic stand in different levels for each individual. This might be one possible reason that a wide range of lymphocytes was obtained after monocyte depletion by magnetic beads phagocytosis method. B cells were also depleted because this cell can also phagocytose beads.[30] Study of B cell might not be appropriate, however, due to the limited number of B cells obtained after cell preparation using this method. The lymphocyte preparation using this method was able to study T cell proliferation induced by mIgG2b and anti-CD3 mAb.[31] For Percoll density gradient centrifugation, monocytes were depleted from lymphocytes using isosmotic Percoll density gradient. Percoll consists of various sizes of silica particles (15 to 30 nm diameter) coated with non-dialyzable PVP. These particles have a specific density to form density gradient in the range of 1.0-1.3 g/mL during centrifugation.[32] After centrifugation, monocytes in the middle layer were separated from lymphocytes, which were present in the bottom of the tube. Therefore, the step that discards monocytes and collects lymphocytes by pipette should consider monocyte contamination. By this method, monocytes in the range of 26.5.4-8.52% in PBMCs were almost depleted. This method takes moderate time and uses inexpensive reagents when compared with other methods. However, the yield of cells obtained by this method is lower than other methods. Besides monocytes, platelets and granulocytes that interfere with lymphocyte function can also be depleted by Percoll technique.[27] A previous study reported that the functional activity of cells is not harmed by polyvinyl-coated colloidal silica particles. The lymphocytes obtained from Percoll isolation responded to mitogens, including pokeweed mitogen and phytohemagglutinin in a close level with the lymphocytes obtained from a Ficoll-Hypaque gradient.[32, 33] Moreover, T cell proliferation assay with immobilized anti-CD3 mAb plus anti-CD28 mAb stimulation could be performed by monocyte-depleted PBMCs obtained from Percoll.[25] However, this method was not suitable for the study of B cell function due to the loss of cells during the lymphocyte isolation process.

In the last two decades, the isolation of cells from blood has been prepared by using high-affinity antibodies and magnetic beads technology.[10] Processes involved in cell separation including positive selection and negative selection have been developed. Positive selection enriches cells by antibodies, which directly bind to target cells and allow the labeled cells to be retained in the isolated fraction, whereas negative selection enriches cells by using antibodies specific to the non-target cells to deplete unwanted cells in the cell fraction.[9-11] Importantly, the study of cell function should use negative selection method because the binding of antibody to target cell could alter their features and functions. Antibody pull down is a well-known method for isolating lymphocytes using the specific antibody to bind to the cell surface.[9] For monocyte-depleted PBMCs preparation using anti-FITC mAb-conjugated microbeads, monocytes were stained with FITC-conjugated anti-CD14 mAb. Then, anti-FITC mAb-conjugated microbeads were added to bind with labeled monocytes. In order to deplete monocytes, monocyte-beads complexes were subjected to a magnetic LD column. Then, the unbound solution containing monocyte-depleted PBMCs was collected. This column is appropriate to deplete cells because the matrix of columns is composed of ferromagnetic spheres, which amplify the magnetic field by 10,000-fold when the column is placed in a magnetic stand. Moreover, the unique specifications of LD columns have a specific shape and matrix, resulting in a slow flow rate. This is crucial for the efficient isolation of the cells, which are minimally magnetically labeled. The space between the sphere matrix is larger than primary cells to allows the cells to freely flow through the column. Furthermore, the MACs magnetic beads do not activate or alter the status of target cells isolated.[34] In this study, when compared with other methods, the yield of cells obtained by this method is the highest and has the lowest monocyte contamination. Monocytes in the range of 19.3-10.4% in PBMC were depleted. However, this method takes a long time because several steps are required, including staining cells with FITC-conjugated anti-CD14 mAb, incubating cells with anti-FITC beads and subjecting cells in an LD column with a low flow rate. Moreover, this method is quite expensive because the reagent and column are a commercial kit. In our laboratory, lymphocyte isolation by anti-FITC beads was used for studying T cell proliferation assay using immobilized anti-CD3 mAb plus anti-CD28 mAb stimulation. Moreover, T cell proliferation

and cytokine production in Toll-like receptor 2 agonist were determined in monocyte-depleted PBMC preparation by anti-CD14-conjugated magnetic microbeads.[37] The interaction between monocytes and NK cells in IFN-γ and CD107a production during Zika virus infection was also studied by monocyte-depleted PBMCs. In addition, isolating lymphocytes by immunomagnetic separation column was previously reported to be used for studying quantitative and qualitative cell viability assay.[34]

In this study, we demonstrated the utilization of three different methods for lymphocyte isolation from PBMCs by monocyte depletion. All of them were able to remove the monocytes from PBMCs. The isolated lymphocytes can be used for studying their function. However, each technique has some advantages and disadvantages. Therefore, the lymphocyte isolation method should be considered and appropriately selected for each experiment.

Conflict of interest

The authors declare that they have no conflict of interests.

Human ethics approval

The human ethics of this study was approved by the Ethics Committee of the Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai, Thailand (AMSEC-61EX-080).

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