Research Article

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A method for high-purity isolation of neutrophil granulocytes for functional cell migration assays

Nötrofil Granülositlerinin Yüksek Saflıkta İzolasyonu ve Fonksiyonel Hücre Göçü Analizi

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Abstract

Background: Neutrophil-mediated killing of pathogens is one of the most significant functions of the primary defense of the host. Neutrophil activity and migration play a key role in inflammatory conditions. To gain insights into the interactions between neutrophils and neutrophil migration-related disorders, a large number of sophisticated methods have been developed. The technical limitations of isolating highly purified neutrophil populations, minimizing both cell death and activation during the isolation process, and the short lifespan of neutrophils present challenges for studying specific functions of neutrophils in vitro. In this study, we aimed to evaluate a separation medium-based density gradient method to obtain highly purified neutrophil populations and combined this protocol with a model for studying neutrophil migration in-vitro.
Materials and methods: Human granulocytes were isolated using Lympholyte-poly solution. The purity and viability of isolated neutrophils were assessed by flow cytometry and morphological analysis. Neutrophil activation was confirmed by immunocytochemistry. Lastly, filter assay was performed to measure neutrophil chemotaxis.
Results and discussion: All validation experiments revealed that this method was capable of generating a highly purified neutrophil population for further functional in-vitro assays. Consequently, this study demonstrates a quick, cost effective, and easy-to-follow model, and may be a significant alternative to isolation methods that need extra subsequent steps such as flow cytometry-based cell sorting for reaching highly purified neutrophil population.
Conclusion: The suggested combination of methods for the isolation and cell migration analysis of human neutrophils is highly recommended to use for disease models involving neutrophil migration such as autoinflammatory disorders.

Keywords: Neutrophil isolation; Cell migration; Purity of neutrophils; In vitro cell migration assay; Autoinflammatory diseases.

Öz

Amaç: Doğal bağışıklık sisteminde, konağın birincil savunması ve patojenlerin öldürülmesi nötrofillerin en önemli işlevlerinden biridir. Nötrofil aktivitesi ve göç, inflamatuvar koşullarda önemli bir rol oynar. Nötrofiller ve nötrofil göçü ile ilişkili bozukluklar hakkında bilgi edinmek için çok sayıda yöntem geliştirilmiştir. Ancak yüksek saflıkta nötrofil popülasyonlarının izole edilmesinin teknik sınırlamaları, izolasyon sırasında hem hücre ölümünü hem de aktivasyonu en aza indirgeyen nötrofillerin kısa ömrü, nötrofil göçü ile ilişkili hastalıklarda yapılan araştırmalarda nötrofillerin fonksiyonlarını inceleme aşamasında zorluklara neden olmaktadır. Bu çalışmada, yüksek oranda

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saflaştırılmış nötrofil popülasyonları elde etmek için yoğunluk gradyanı temelli ayırma çözeltisi kullanılan bir metodu değerlendirdik ve bu protokolü, nötrofil göçünü in vitro koşullarda incelemek için bir deney sistemi ile birleştirdik.

Gereç ve Yöntemler: İnsan granülositleri, Lenfolit poli çözeltisi kullanılarak izole edildi. İzole edilmiş nötrofillerin saflığı ve canlılığı akım sitometrisi ve morfolojik analizler ile değerlendirildi. Nötrofillerin aktivasyonu immünositokimya ile doğrulandı. Son olarak, nötrofil göçünü-kemotaksisini ölçmek için filtre deneyi yapıldı.

Bulgular ve Tartışma: Tüm validasyon deneyleri, bu izolasyon yöntemi ile, fonksiyonel deneyler için saflaştırılmış bir nötrofil popülasyonu elde edilebildiğini gösterdi. Sonuç olarak, nötrofil izolasyonu ve devamında hücre göçü analizlerini içeren bu çalışma; hızlı, uygun maliyetli ve takip edilmesi kolay bir model ortaya koymaktadır ve nötrofil popülasyonunu elde etmek için akım sitometrisi temelli hücre sınıflaması gibi ilave adımlar gerektiren izolasyon yöntemlerine önemli bir alternatif olabilir.

Sonuç: İnsan nötrofillerinin izolasyonu ve fonksiyonel hücre göçü analizi için geliştirilen bu yöntemlerin, nötrofil göçünü içeren hastalık modellerinde kullanılması önerilir.

Anahtar kelimeler: Nötrofil izolasyonu; Hücre göçü; Nötrofillerin saflığı; In vitro hücre göçü deneyi; Otoinflamatuar hastalıklar.

Introduction

Neutrophils play important roles during the inflammation process in the innate immune system and represent the primary defense of the host against invading microorganisms [1]. The central functions of neutrophils include recognizing and killing pathogens. When encountering cytokine or chemokine signals caused by pathogens, neutrophils migrate rapidly from circulation to the infected sites and become activated, leading to the activation of a series of defense mechanisms such as phagocytosis, release of reactive oxygen species (ROS), formation of neutrophil extracellular traps (NETs), and production of antimicrobial peptides in order to protect the host, before finally undergoing apoptosis [2–8] Inappropriate neutrophil activation can contribute to the pathology of clinically significant inflammatory diseases. The elucidation of the key role of neutrophils in inflammation will lead to improvements of knowledge in numerous auto-inflammatory diseases including Familial Mediterranean Fever (FMF) [9–11], tumor necrosis factor receptor-associated periodic syndrome (TRAPS), hyper-IgD syndrome (HIDS), periodic fever, aphthous stomatosis, pharyngitis, and cervical adenitis (PFAPA) [11, 12], as well as conditions where inflammation is a component in the disease progression such as rheumatoid arthritis [13], acute respiratory distress syndrome [14], and even cancer [15].

Owing to their specialized structures and functions in several conditions, neutrophils are of major interest to scientists. Performing well-designed, advanced in vitro studies using live human neutrophils isolated from peripheral blood is an important strategy for further understanding the neutrophil migration mechanism. Nowadays, there are plenty of methods available to isolate neutrophils from peripheral blood samples. However, technical limitations in terms of viability, purification, and difficulties in obtaining a highly purified neutrophil population are frequently encountered. One of the major obstacles is the existence of mononuclear cell contamination, which cannot be excluded completely.

The most commonly used methods for neutrophil isolation include dextran sedimentation or Ficoll-Hypaque method in combination with a subsequent Percoll gradient. Even though these methods cause far less neutrophil activation, they are inadequate in providing highly purified neutrophil populations [16, 17]. Assessing neutrophil function and further studies require highly pure or enriched cell populations. Thus, subsequent to isolation, a great deal of strategies, such as specific inflammatory stimulus, magnetic beads, or cell-surface markers have been developed in human and murine settings in order to purify granulocytes [18, 19]. One of the most commonly used strategies involves enriching human neutrophils through fluorescence-activated cell sorting (FACS) following the isolation. However, FACS is known to be unsuitable for the purification of neutrophils. When neutrophils are exposed to antibody-labeled cell surface markers during FACS, neutrophil activation is triggered via crosslinking to surface receptors, especially binding with the Fc region of the antibody [20]. Neutrophils can be extremely sensitive to in vitro studies; therefore, the methods used for neutrophil isolation should be considered cautiously. In addition to these aspects, it is known that obtaining a highly purified neutrophil population is an essential step for functional studies. Monocyte contamination has been shown to have effects on neutrophil survival as well as on cytokine production [21, 22].

Investigations of cell migration can be performed by several methods including filter assays, two-dimensional (2D) migration assays, three-dimensional (3D) migration

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assays, wound healing model, and directional migration assays [23].

In this article, we report a highly purified neutrophil population-based functional model for studying cell migration. The present protocol consisted of a combination of methods for the isolation and functional cell migration analysis, representing a quick, cost effective, and easy-to-follow tool for the analysis of inflammatory neutrophil migration. We believe that elucidating primordial functional and biochemical properties and homeostasis of neutrophils are the key points in understanding the pathological basis of neutrophil-dominant inflammatory diseases.

Materials and methods

All blood samples from healthy donors who participated in this project were obtained after informed consent had been given according to guidelines by the local ethics committee (Ethical Approval: GO15/90-20 Date: 04.02.2015). All experiments were performed in triplicates.

Human neutrophil isolation

Peripheral blood samples (~10 mL) were taken from 11 healthy donors into tubes coated with EDTA (final concentration 4.5 mmol/L). Female/male ratio was 1.75 with a median age of 10 years. Blood samples were transferred to the laboratory at room temperature for analysis and processed within 30 min after the blood draw. Next, 5 mL of the anticoagulant blood was layered onto 5 mL of Lympholyte-poly solution (Cederlane Laboratories, Cat. No: CL5071, Burlington, Southern Ontario, Canada) at the dilution of 1:1. Specific attention was paid to avoid mixing the blood with Lympholyte-poly solution, which consists of dextran 500 and sodium diatrizoate. Following the preparation of the tubes, samples were centrifuged at $500 \times g$ for 35 min in a swing-out rotor. After this first centrifugation, two distinct bands as mononuclear cells (upper band) and polymorphonuclear cells (lower band) were observed. The high-density neutrophil layer was collected from the lower pellet fraction and centrifuged at $350 \times g$ for 10 min. Following the centrifugation, neutrophils were suspended in 6–7 mL of 1X erythrocyte lysis buffer (Biolegend, Cat. No: 420301, San Diego, CA, USA) to remove contaminating erythrocytes. After mixing gently the lysis buffer with the pellet, the mixture was centrifuged at $250 \times g$ for 5 min. The addition of lysis buffer and centrifugation were repeated until erythrocytes were completely removed. The final neutrophil pellet was suspended in 100 µL of PBS for flow cytometry analysis and cytospin, or in RPMI-1640 media, containing 2 mM L-glutamine, 100 U/mL penicillin (1%), 100 µg/mL streptomycin (1%) and FBS (10%) (Sigma Aldrich) was used for immunocytochemistry and chemotaxis assays. The concentration of neutrophils from 10 mL of blood samples from healthy individuals was generally between 0.7×10^6/100 µL and 2×10^6/100 µL. From the initial blood draw to obtaining highly purified neutrophils, the total process took approximately 2 h. In a successful isolation at least 1×10^6 cells from 10 mL blood sample could be isolated.

Neutrophil purity

Isolated neutrophils suspended in PBS were distributed into flow cytometry tubes. Next, the cells were labeled with monoclonal antibodies for 40 min at 4°C against CD45 (2D1), CD11b (D12), CD14 (MφP9), CD66b (G10F5), CD125 (A14), and CD33 (P67.6) (Becton Dickinson, San Jose, CA, USA). The flurochroms for the selected antibodies were CD11b- APC-Cy-7, CD45- PercP, CD66b-FITC, CD33-Pe-Cy7, CD125-PE. The percentage of positive cells was calculated by comparison with the appropriate isotype-matched antibody controls. The detection was performed on a FACSAria II flow cytometer (Becton Dickinson Biosciences, Franklin Lakes, NJ, USA) and FlowJo Software was used for the analysis. Alternatively, neutrophil purity was measured by May Grünwald-Giemsa staining (Sigma Aldrich, Taufkirchen, Germany) based on their characteristic morphological appearance [24]. The suspended neutrophil pellet in 100 µL PBS was cytocentrifuged at $300 \times g$ for 3 min and then fixed. The cells were spun onto the slide, stained by May Grünwald-Giemsa staining, and examined by light microscopy (×1000 magnification; Carl-Zeiss Axioplan 2 equipped with an ICAM photosystem, Oberkirchen, Germany).

Neutrophil viability and ROS production capacity

Cell viability was assessed by propidium iodide (PI) (Sigma Aldrich, Germany) staining and analyzed by flow cytometry. PI (1 mg/mL) was immediately added in each sample just before the flow cytometric analyses. For the analysis of ROS production, the granulocytes collected were incubated with 5-(and-6)-Carboxy-2′,7′-Dichlorofluorescein Diacetate (H_2DCFDA; Anaspec, San

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Jose, CA, USA) (10 μM, 106 cells/mL), a cell permeant tracer which is a chemically reduced form of fluorescein used as an indicator for ROS in cells. H_2DCFDA, which penetrates the cellular membrane, enzymatically deacetylated by esterases. The conversion of H_2DCFDA into the non-fluorescent compound H_2DCF which is then oxidized to highly fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of ROS. The fluorescence signals from H_2DCFDA probe gives information for the quantification of ROS at cellular level [25]. In order to assess the ROS production capacity, 0.5×10^6 cells/mL incubated at 37°C for 24 h were stimulated with phorbol-12-myristate-13-acetate (PMA) (800 nM) for 25 min. The median fluorescence intensity is measured by flow cytometer (FACS Aria II, Becton Dickinson, Franklin Lakes, NJ, USA).

Neutrophil longevity and storage

For neutrophil longevity and storage we aimed to test if neutrophils can be used for further analysis after 24 h and if these cells are resistant to freezing and thawing. For this purpose, isolated neutrophils (10^6 cells in 500 μL RPMI-1640 media supplemented with 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin; Sigma Aldrich) were placed into glass-bottom chamber slides (CC2, 4-welled Nunc Lab-Tek® II Chamber Slide™ system, Thermo Scientific, Waltham, MA, USA) and incubated at 37°C with 5% CO_2. Some of the glass-bottom slides were coated with a thin diluted collagen layer (Sigma Aldrich) in order to mimic the in vivo extracellular matrix environment. Next, 250 μL of fresh culture medium was added to the cell suspension every other day, but the medium was not completely replaced. Following the replacement of medium for each day, the cells were stimulated by N-formylmethionyl-leucyl-phenylalanine (fMLP) and stained by Alexa Fluor AF488 Phalloidin as described below. Morphological changes were visualized by light microscopy (400×; Leica DMIL equipped with Leica Application Suit 3.1 software, Germany), and the response of cells to the fMLP stimuli was examined by fluorescence microscopy (630×; Carl-Zeiss Axioplan 2 equipped with an ICAM photosystem). The time course assay continued for a period of 4 days. In addition, cryopreservation of isolated neutrophil in liquid nitrogen was performed. The neutrophils (1×10^6 cells/mL) were stored in 2 mL-cryotubes (Greiner) using 10% of DMSO as a cryoprotectant. A gradual cooling process was used, and cells were cryopreserved for up to 9 weeks. Following the post-thaw handling of neutrophils carefully, the cells were placed into glass-bottom chamber slides, incubated at 37°C with 5% CO_2 for 2 h, activated by fMLP for 1 h, stained with Alexa Fluor AF488 Phalloidin, and visualized by both light microscopy (200× and 400×; Leica DMIL equipped with Leica Application Suit 3.1 software) and fluorescence microscopy (630×; Carl-Zeiss Axioplan 2 equipped with an ICAM photosystem) as described below.

Neutrophil polarization and immunocytochemistry

The protocols used for neutrophil polarization and immunocytochemistry were optimized in our previous report [26]. Neutrophils (4×10^5/500 μL in RMPI-1640) were incubated for 2 h at 37°C for them to adhere to the glass-bottom slide. Following the incubation, neutrophils were treated with 50 μM fMLP (Sigma Aldrich) for 1 h at 37°C in order to trigger polarization, which is an initial step for cell migration. Cells were then fixed with 4% paraformaldehyde at room temperature for 30 min, washed three times with PBS, and permeabilized in PBS with 1% Triton-X-100 (Sigma Aldrich) at room temperature for 15 min. Following rinsing with PBS containing Tween 20 (Sigma Aldrich), nonspecific staining was blocked by incubating with Fc Receptor blocker (Innovex Biosciences, USA) for 30 min and PBS containing 1% BSA and 3% goat serum at room temperature for 1 h. Cells were stained by Alexa Fluor AF488 Phalloidin (Thermo Fisher Scientific) at room temperature for 40 min and rinsed with PBS w/Tween 20 for 15 min. After rinsing at room temperature, cells were treated with DAPI (1 mg/mL) for 1 min, washed three times with PBS containing Tween 20 and examined by fluorescence microscopy (630×; Carl-Zeiss Axioplan 2 equipped with an ICAM photosystem).

Neutrophil chemotaxis assay

A 24-well plate fitted with 3-μm filters (Greiner, Germany), a suitable pore size for neutrophil, was used for the chemotaxis assay [23, 27]. Neutrophils (15×10^3/400 μL in RPMI-1640) were loaded onto the upper compartment of the filter and triggered to migrate towards the fMLP gradient (100 nM) in the lower compartment. An equal number of neutrophils was loaded onto the upper compartment as a control condition. However, the lower compartment contained fMLP-free medium. Cells were incubated at 37°C for 24 h. Following incubation, the filter was removed and cells were stained with 1 mM Calcein-AM (Sigma Aldrich) at 37°C for 15 min. Cells stained with Calcein-AM were visualized by fluorescence microscopy

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(200×; Leica DMIL microscope equipped with analysis software Leica Application Suite 3.1). The cells migrating toward the lower compartment were counted by the 'analyze particles' function in ImageJ 1.46 software.

Statistical analysis

All the graphs were created using GraphPad Prism 6.1 Software. A non-parametric Mann-Whitney U test was used for studies comparing differences between two groups. Differences were considered significant when $p < 0.05$.

Results

Human neutrophil isolation and the analysis of neutrophil purity

The combination of Lympholyte-poly solution and density gradient, as a tool to isolate neutrophils from peripheral blood samples, yielded between 0.7×10^6 and 2×10^6 cells/100 µL. These amounts were concordant to whole blood results representing a recovery of 70% of the neutrophils in the circulation. Flow cytometric immunophenotyping was performed to assess the proportion of neutrophil granulocytes after dextran sedimentation-based isolation. Since hypotonic lysis was applied to remove erythrocytes, the leukocytes obtained were highly pure ($CD45^+$, 99.06 ± 0.63%, n = 6). These cells were positively stained with CD11b (93.55 ± 3.43%, n = 6) and CD66b (93.61 ± 4.54%, n = 6), both of which are granulocyte-associated markers. In addition, a small portion was identified with CD125 (2.26 ± 1.07%, n = 5) surface expression, which corresponds to a basophil or eosinophil granulocyte sub-population. There were only minimal amounts of monocytes ($CD14^+$, 1.94 ± 1.38%; $CD33^+$, 1.26 ± 0.58%; n = 6) contaminating the peripheral blood granulocytes isolated by the Lympholyte-poly solution and density gradient method (Figure 1A, B). In addition to flow cytometry, the purity of neutrophil granulocytes was assessed based on morphological analysis (93.50%, n = 2) (Figure 2 and Table 1).

Neutrophil viability, ROS production capacity, longevity, and storage

The viability of the granulocytes isolated was not hampered (PI^+ dead cells, 4.8 ± 0.78%, n = 3) upon the use of Lympholyte-poly solution (Figure 1C). To maintain the viability of a high-purity neutrophil population, cells were processed and placed into glass-bottom chamber slides. Actin immunofluorescence staining was successfully performed as the glass surface notably triggered neutrophil adherence. No significant differences between collagen-coated and non-coated slides were observed in terms of neutrophil longevity. Neutrophils were cultured for 4 days by adding fresh media onto the cell suspension every other day after first seeding. However, 75–80% (n = 3) of neutrophils cultured for up to 24 h displayed gradually increasing apoptotic nuclear morphology, which was confirmed by DAPI staining. However, the cell membranes were intact when observed via light microscopy (Data is not shown). Although a small subset of cells were alive after first 24 h, non-specific phalloidin staining was observed in these cultured cells. In addition, the polarization capability of cryopreserved neutrophils was compared with the ones that were freshly isolated. The chemoattractant response of these cells (11.3%, SEM = 0.8, n = 3) was significantly lower from freshly isolated neutrophils (56%, SEM = 1.9, n = 6) ($p < 0.05$) (Figure 3). These experiments testing longevity showed that although a certain percentage of neutrophils can still be alive for 4 days, functional assays using these cells should be performed in the first 24 h after isolation.

Moreover, these cells retained full capacity to produce ROS. Under steady state conditions, the mean fluorescence intensity (MFI) of the ROS tracer was measured as 7430 ± 1940 (n = 3), whereas when these cells were stimulated with a protein kinase C (PKC) agonist such as PMA, ROS production was almost tripled (MFI, 20191 ± 7167, n = 3) (Figure 4A, B).

Neutrophil polarization and immunocytochemistry

Freshly isolated cells were stimulated with 50 µM fMLP, a classic chemoattractant for neutrophil migration for 1 h. The polarization state of cells was detected by Alexa Fluor AF488 Phalloidin (Thermo Fisher Scientific), which contains a fluorescent tag and selectively binds to F-actin responsible for the forward movement of plasma membrane at the cell's front. For each individual, 100 cells were counted, and the percentage of polarized cells was also detected. The actin staining showed that the polarization percentage of neutrophils for the fMLP-mediated condition was 56% (SEM = 1.9, n = 6), whereas it was 23.2% (SEM = 1.07, n = 6) for the non-fMLP condition (Figure 5A–F). A statistical analysis revealed that the fMLP-mediated polarization rate was significantly increased compared to

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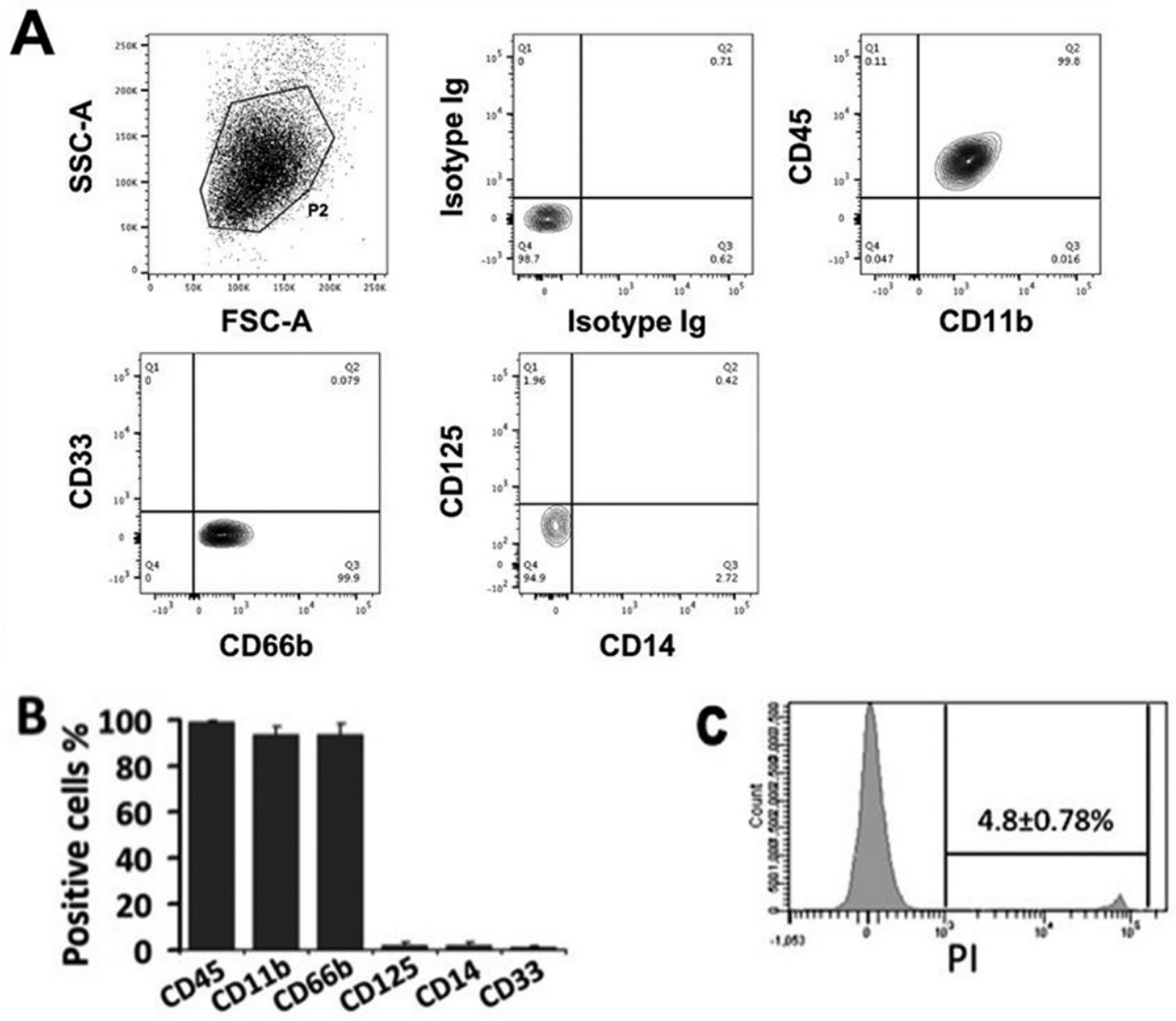


Figure 1: Lympholyte-poly solution and density gradient method can be efficiently used for high-purity isolation of peripheral blood neutrophils. Human granulocytes were isolated via a combination of Lympholyte-poly solution and density gradient from whole blood, and then analyzed by flow cytometry.
(A) According to the forward scatter (FSC) and side scatter (SSC) profiles, leukocytes were separated from contaminating erythrocytes. Granulocytes were confirmed by CD11b (93.55 ± 3.43%, n = 6) and CD66b (93.61 ± 4.54%, n = 6) positive staining with a small portion of basophils or eosinophil identified with CD125 (2.26 ± 1.07%, n = 5). In addition, a negligible contamination of monocytes was shown with CD14 (1.94 ± 1.38%) and CD33 (1.26 ± 0.58%, n = 6) positive staining. (B) The bar graphic of percentage of positive cells for the given markers. (C) The viability of isolated granulocytes was detected by propidium iodide staining (dead cells, 4.8 ± 0.78%, n = 3). Representative histograms from healthy donors are shown.

the non-fMLP state ($p < 0.05$) (Figure 5G). Actin was localized at the leading edge of neutrophils treated with 50 µM fMLP for 1 h, whereas it was observed under the plasma membrane in non-stimulated cells.

Neutrophil chemotaxis assay

Quantification of migrating cells was performed by a modified Boyden chamber (filter) assay. Exposure of freshly isolated neutrophils to fMLP resulted in a high chemotactic migratory response. The optimal stimuli dose was 100 nM of fMLP for 24 h. The cells that migrated into the lower compartment of the filter were stained by Calcein-AM, which is a cell-permeant dye that releases green fluorescence after hydrolysis and converts into Calcein by intracellular esterases in living cells. Following the Calcein-AM staining, 12 random areas were visualized under the microscope, and cells were counted for each individual. The average number of the migrated cells in fMLP-treated and

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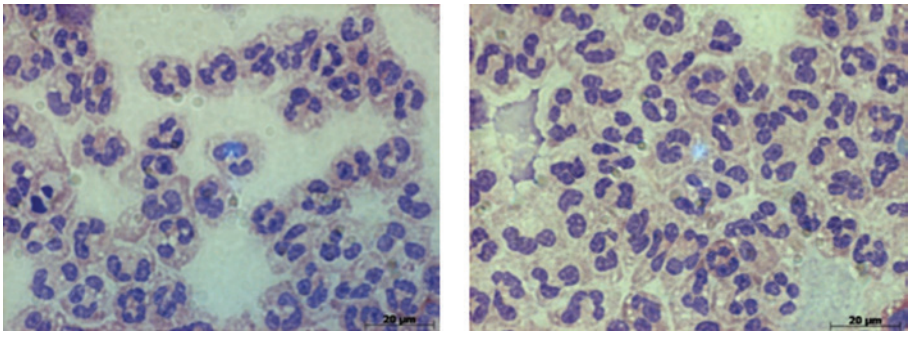


Figure 2: Morphology of a freshly isolated neutrophil population.
Human neutrophils, isolated by dextran sedimentation from whole blood, were stained with May Grünwald-Giemsa and examined by light microscopy (1000×). The morphological analysis demonstrated that the suggested isolation method was capable of yielding highly pure neutrophil populations with negligible amounts of contaminating cells (93.50%, n = 2).

Table 1: Evaluation of isolated neutrophil purity by morphological analysis and flow cytometry.

Measure	% Cells	SEM	Events counted	n
Morphological analysis	93.50	–	1000	2
CD45	99.06	0.63	1000	6
CD11b	93.55	3.43	1000	6
CD66b	93.61	4.54	1000	6
CD125	2.26	1.07	1000	5
CD14	1.94	1.38	1000	6
CD33	1.26	0.58	1000	6

SEM, Standard error of the mean.

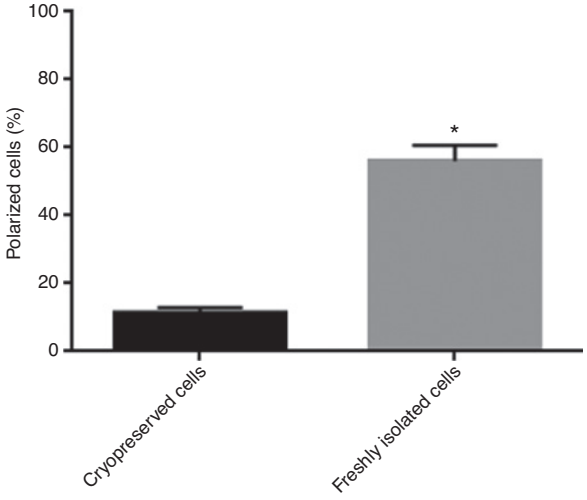


Figure 3: Chemoattractant response and polarization capability of cryopreserved neutrophils.
Freshly isolated neutrophils (1×10^6 cells/mL) were stored in 2 mL-cryotubes with 10% DMSO. The cells were cryopreserved for up to 9 weeks. Following the post-thaw handling process, the cells were activated by fMLP (50 μM) for 1 h, and the polarization capability was assessed by actin staining. According to the actin staining, the chemoattractant response and the polarization capability of cryopreserved neutrophils (11.3%, SEM = 0.8; n = 3) were significantly lower than freshly isolated neutrophils (56%, SEM = 1.9; n = 6) ($p < 0.05$). Error bars indicate standard deviations. All data are presented as the mean ± SD.

non-treated conditions was 84 and 6, respectively. Migration towards the fMLP gradient (86.5%, SEM = 3.2, n = 6) was higher in the lower compartment of the filter when comparing to basal levels of chemotaxis in the non-fMLP condition (17.5%, SEM = 4.1, n = 6) ($p < 0.05$) (Figure 6A–C). The neutrophil chemotaxis assay demonstrated that neutrophils isolated via Lympholyte-poly solution can be successfully used to determine the migration potential in different conditions.

Discussion

Neutrophil migration represents a highly coordinated and crucial process, which is characterized by changes in expression of integrins, E and P selectin receptors on the cell surface, morphological changes, and up-regulation of inflammatory mediators against invading pathogens [2–8, 28]. Therefore, inappropriate neutrophil activation and motility are key players of inflammatory migration in terms of pathology of inflammatory diseases. Many in vitro models are currently used to analyze the possible contribution of neutrophils in inflammatory diseases. This study reports a protocol for the isolation of human neutrophils and an inflammatory cell migration model using these neutrophils, which can be performed to study the role of neutrophils in several inflammatory conditions.

The combination of commercially available separation medium, Lympholyte-poly solution, and a density gradient was capable of acquiring high-purity neutrophil population. Flow cytometry-based immunophenotyping analysis of dextran sedimentation revealed high leukocyte

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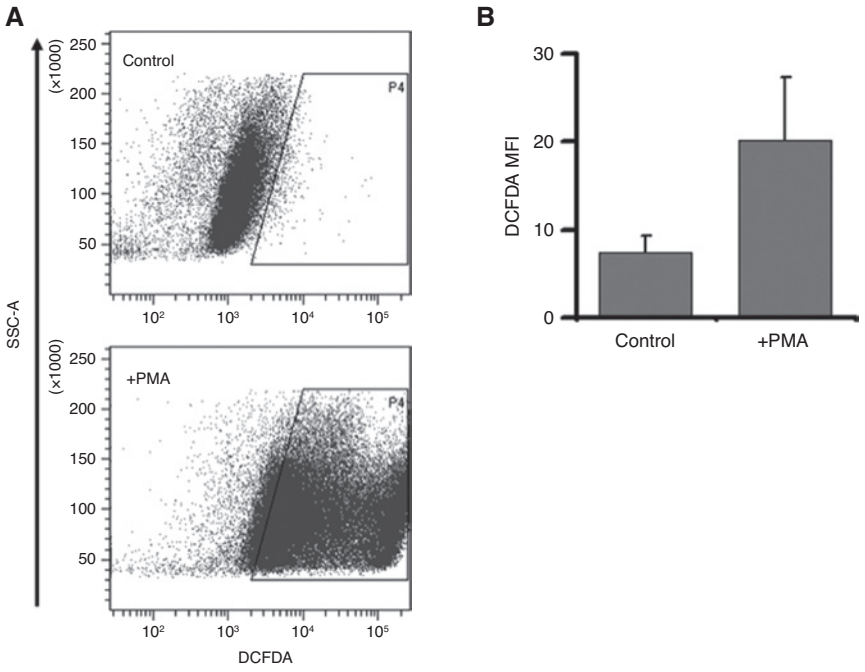


Figure 4: Functional capacities of neutrophils are retained with Lympholyte-poly solution and density gradient isolation.
(A) The capacity of neutrophil granulocytes to produce reactive oxygen species was evaluated by phorbol-12-myristate-13-acetate (PMA) stimulation. The mean fluorescence intensity (MFI) of the ROS tracer was measured as 7430 ± 1940 (n = 3). When compared to cells stimulated with PMA, ROS production were almost tripled (MFI, 20,191 ± 7167, n = 3). (B) The comparison of DCFDA levels in control cells and PMA stimulated cells was given as bar graphic. Error bars indicate standard deviations. All data are presented as the mean ± SD.

($CD45^+$, 99.06 ± 0.63%, n = 6) and neutrophil granulocyte purity (CD11b, 93.55 ± 3.43%, n = 6; CD66b, 93.61 ± 4.54%, n = 6), with a small portion of eosinophil or basophil granulocyte sub-population (CD125, 2.26 ± 1.07%, n = 5) and negligible contamination of monocytes ($CD14^+$, 1.94 ± 1.38%; $CD33^+$, 1.26 ± 0.58%, n = 6) (Figure 1A, B). In addition, the average purity of neutrophils was reconfirmed by morphological analysis (93.50%, n = 2) (Figure 2 and Table 1). This isolation method does not allow distinct separation between neutrophil and eosinophil populations. However, 1–6% of the granulocyte population represents eosinophils from healthy individuals with asymptomatic conditions; however, the reference ranges may vary depending on laboratory techniques. This eosinophil percentage was consistent with our flow cytometry-based eosinophil ratio and does not present an obstacle for the study of cell migration. As for the mononuclear cell contamination, that cannot be eliminated completely by the density gradient method [21, 29]. However, the viability of the isolated granulocytes was high (PI^+ dead cells, 4.8 ± 0.78%, n = 3) (Figure 1C).

Neutrophils are extremely delicate and become easily activated when exposed to nearly every physicochemical stimulus. Many other isolation protocols, including the ones suggested in this study, involve hypotonic lysis solution in order to remove erythrocyte contamination. Hypotonic lysis of erythrocytes has been reported to result in high levels of cell activation [30, 31]. The activated cells have been demonstrated to be less reactive in response to chemoattractants. Given this concern of activation, analysis of ROS generation capacity via flow cytometry was performed, and basal neutrophil ROS release and response to PMA were compared. While the MFI of the ROS tracer was measured as 7430 ± 1940 (n = 3), the production of ROS in these cells stimulated with PMA were almost tripled (MFI, 20191 ± 7167, n = 3) (Figure 4A, B). The observations of a low basal ROS release capacity suggest that our isolation protocol successfully minimized activation. In addition, the use of culture medium containing Ca^{2+} and Mg^{2+}, which have been demonstrated to activate PMNs, was particularly avoided, and tubes with EDTA helped prevent the activation and aggregation of cells [32].

In this protocol, a maximum of 2×10^6 neutrophils from 10 mL whole blood of a healthy donor were obtained. Based on clinical biochemical analysis of blood, neutrophils represent ~70–75% of leukocytes in circulation. Consistent with this percentage, the isolation method described above, which is elegantly simple and easy-to-follow, generates high-yield and pure population of neutrophils.

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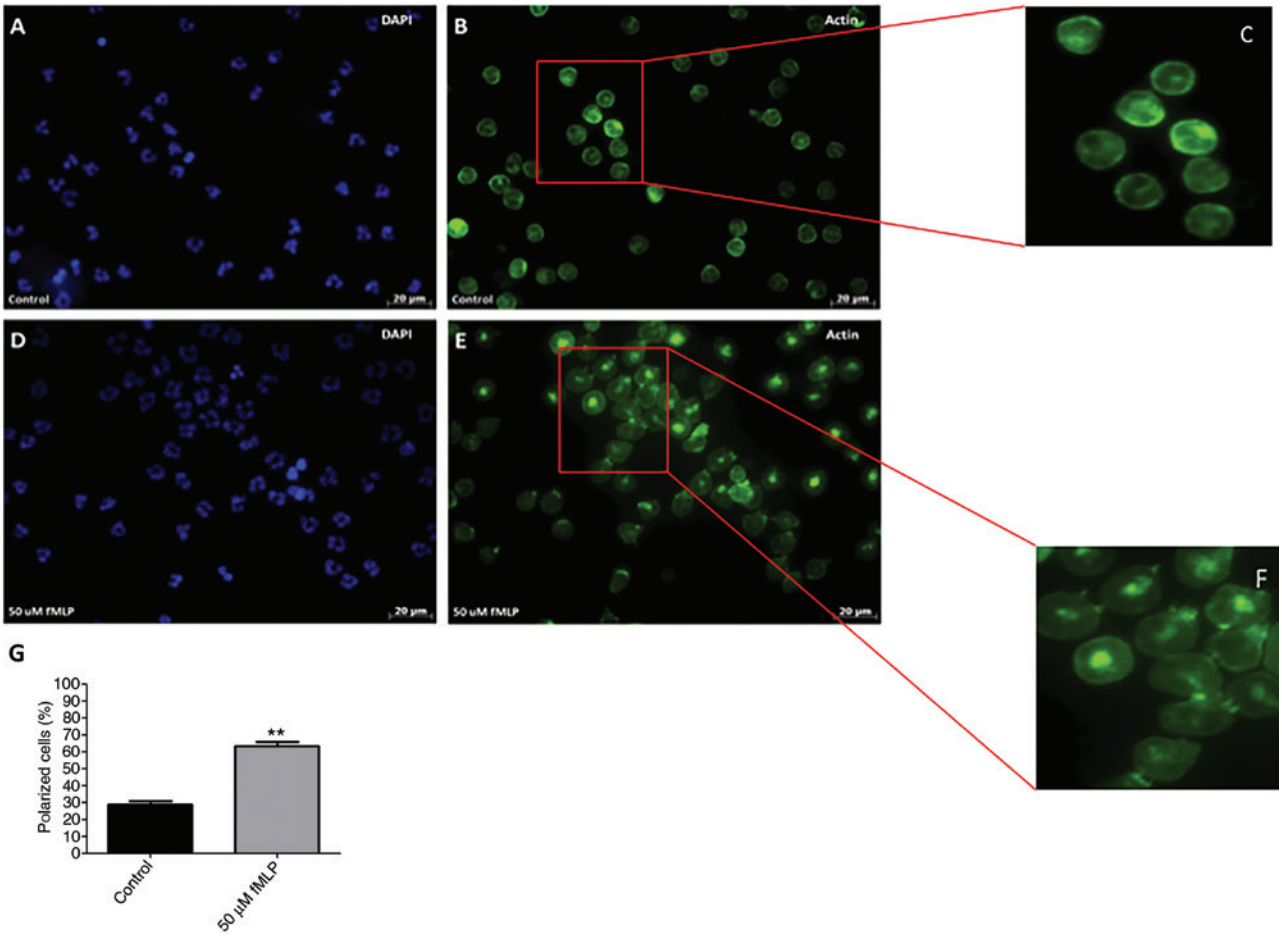


Figure 5: The polarization state of neutrophils by actin staining.
Immunofluorescence analysis of the control and chemoattractant-treated groups were performed in parallel. Non-stimulated and N-formylmethionyl-leucyl-phenylalanine (fMLP)-stimulated (50 µM) neutrophils were allowed to migrate for 1 h. Cell nuclei were stained by DAPI (blue), and the polarization percentage based on shape change characteristics was assessed by actin staining (green). (A–C) Immunofluorescence analysis of untreated control cells is shown. F-actin was observed near the plasma membrane in non-stimulated cells. (D–F) Immunofluorescence analysis of fMLP-treated cells is shown. F-actin cytoskeleton was reorganized and localized to the leading edge of neutrophils treated with 50 µM fMLP for 1 h. Neutrophil populations were examined by microscopy at 630×. However, the cells are magnified for clarity (C, F). (G) Actin staining showed that the polarization percentage of neutrophils for the fMLP-mediated condition was 56% (SEM = 1.9; n = 6), whereas it was 23.2% (SEM = 1.07; n = 6) in the non-fMLP condition. Statistical analysis revealed that the fMLP-mediated polarization rate was significantly increased compared to the non-fMLP state ($p < 0.05$).

The suggested method here has several apparent advantageous over a variety of other neutrophil isolation methods. The most significant advantage of the technique was that neutrophils were not labeled by antibodies targeting cell surface markers. The use of FACS to generate highly pure neutrophil populations and immunomagnetic separation following density gradient isolation have been reported to cause a high activation state of human neutrophils. Antibody-based selection strategies have been shown to be unsuitable for the purification of neutrophil populations and use of these cells for further functional assays as a result of antibody-induced surface receptor activation [29]. Our total process can be completed in approximately 2 h from the initial blood draw to obtaining highly purified neutrophils, which provides rapid availability of neutrophils for further functional assays. In addition, this cost-effective system promotes neutrophil longevity for up to 24 h via glass-bottom chambers without the need to treat cells with additional biological survival factors such as G-CSF and GM-CSF. Previous studies have reported that neutrophils are degranulated during freeze-thaws cycles. Once they are degranulated, their functions and response to inflammatory stimuli are altered [33–35]. In our experiments, consistent with reported data, the

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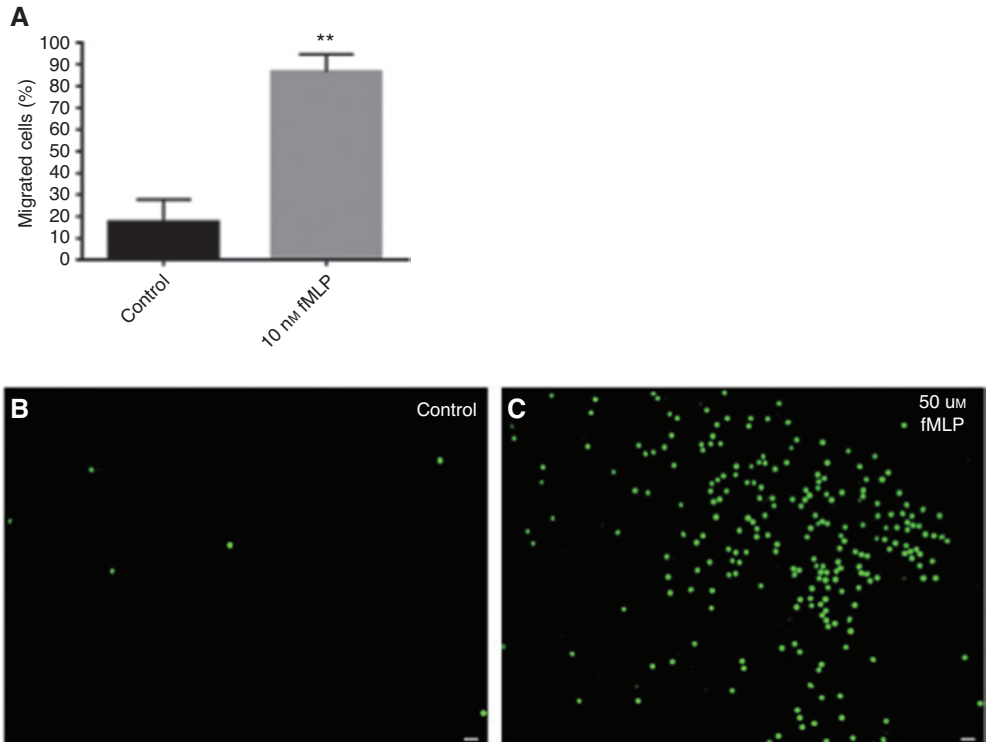


Figure 6: Migration potential of human neutrophils towards the fMLP gradient via chemotaxis assay.
Untreated and N-formylmethionyl-leucyl-phenylalanine (fMLP)-treated (100 nM) neutrophils were allowed to migrate through a porous membrane filter for 24 h. The filter assays for the control and chemoattractant-treated groups were performed in parallel, and the percentage of migrated cells was evaluated by Calcein-AM (1 mM) after 24 h. (A) The average number of migrated cells in fMLP-treated and non-treated conditions was 84 and 6, respectively. Migration in the presence of a 100-nM fMLP gradient (86.5%, SEM = 3.2; n = 6) was higher when compared to basal levels of chemotaxis in the non-fMLP condition (17.5%, SEM = 4.1; n = 6) ($p < 0.05$). Error bars indicate standard deviations. All data are presented as the mean ±SD. (B, C) Representative immunofluorescence microscopy of control and fMLP-treated neutrophils stained by Calcein-AM is shown (200× magnification, scale bar is 20 μm).

chemoattractant response and polarization capability of cryopreserved neutrophils (11.3%, SEM = 0.8, n = 3) were significantly lower than freshly isolated neutrophils (56%, SEM = 1.9, n = 6) ($p < 0.05$) (Figure 3).

Our data also demonstrated that isolated neutrophils cultured for 24 h are highly functional and of good quality. The quality and capability of isolated neutrophils were determined by actin immunofluorescence staining and filter assay. The optimal fMLP dose for neutrophil polarization was 50 μM fMLP for 1 h. Characteristic changes in morphology were investigated by phalloidin staining followed by statistical analysis, which revealed that the fMLP-mediated polarization rate was significantly increased (56%, SEM = 1.9, n = 6) compared to the non-fMLP state (23.2%, SEM = 1.07, n = 6) ($p < 0.05$) (Figure 5A–G). According to our results, F-actin aggregated at the leading edge of neutrophils treated with 50 μM fMLP for 1 h, whereas it was observed near the plasma membrane in non-stimulated cells. The optimal concentration of fMLP that triggered neutrophil chemotaxis in the filter assay was 100 nM for 24 h. The obtained data indicated that migration towards the fMLP gradient (86.5%, SEM = 3.2, n = 6) was significantly higher when compared to basal levels of chemotaxis in the non-fMLP condition (17.5%, SEM = 4.1, n = 6) ($p < 0.05$) (Figure 6A–C). These results are concordant with the accepted hypothesis that high chemoattractant doses result in neutrophil polarization and chemotaxis. In light of these results, we have demonstrated the chemotactic ability of isolated neutrophils to respond to fMLP by this approach.

The percentage of cell contamination is approximately between 2 and 5% in other isolation protocols in the literature. In our method, the percentage of other cells, mostly eosinophils or monocytes, that contaminated the neutrophils are <5%. In one comprehensive study, it was shown that this contamination ratio did not affect the overall gene expression profile of cytokine-stimulated neutrophils in high-sensitivity assays like RNA-Seq or mass spectrometry. Also the morphology of the cells were remain unchanged [36].

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In summary, herein we report a quick and cost-effective neutrophil purification method as well as a cell migration assay using these neutrophils. This purification method yields highly pure neutrophil populations, which is a fundamental step in functional assays. The in vitro models used in our system aiming to understand the initial step of inflammation successfully demonstrated the polarization and migration capability of the cells. The two different methods, polarization and filter assays carried out in parallel, provide a basis to determine the migration status of the cells both qualitatively and quantitatively. Therefore, the developed protocol may be a good alternative to models that are most commonly used in the research field, and can be applied to both healthy individuals and patient neutrophils. We believe that the recommended neutrophil isolation method and subsequent cell migration assays will be instrumental to understanding the relationship between cell migration and several inflammatory conditions as well as the molecular pathology underlying disorders related with inflammatory cell migration.

Ethical considerations: All blood samples from healthy donors who participated in this project were obtained after informed consent had been given according to guidelines by the Hacettepe University Local Ethics Committee (Ethical Approval: GO15/90-20 Date: 04.02.2015).

Conflict of interest disclosure: The authors declare that there is no conflict of interest regarding the publication of this article.

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