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Rapid Magnetic-microbead Method for Efficient Purification of Low-density Neutrophils

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Citation

Blanco-Camarillo, C., Alemán, O.R., Naranjo-Pinto, N., Mora, N., Rosales, C. Rapid Magnetic-microbead Method for Efficient Purification of Low-density Neutrophils. *J. Vis. Exp.* (), e69407, doi:10.3791/69407 (2025).

Date Published

November 3, 2025

DOI

10.3791/69407

URL

jove.com/t/69407

Abstract

Neutrophils, important leukocytes of innate immunity, have traditionally been considered a homogeneous cell population. Nevertheless, recent evidence has shown that neutrophils exist in several subpopulations. One such subpopulation is low-density neutrophils (LDN). LDN ~~is~~ are found in small numbers in the blood of healthy individuals, but ~~its~~ their numbers increase significantly in diseases such as systemic lupus erythematosus, autoimmune disorders, cancer, and infections. In these cases, LDN may participate in the pathogenesis of the disease. The only way to isolate LDN is through density-gradient centrifugation of peripheral blood. However, after centrifugation, LDN co-~~purifies~~ purify with mononuclear cells. Thus, studying this neutrophil subpopulation is challenging. There is no standard methodology to separate LDN from mononuclear cells. Typically, LDN~~s~~ are separated by cell sorting in a flow cytometer. However, this method requires long sorting times (hours) to obtain enough pure cells for further functional studies. This seriously affects the viability and function of cells. Here, we propose a practical method to obtain large numbers of pure and viable LDN in a short time. After density-gradient centrifugation, the mononuclear cell fraction is incubated with anti-CD66b magnetic microbeads, and then LDN are separated through magnetic columns in < 30 min. Purified LDN ($CD66b^+$ cells) are labelled with monoclonal antibodies against CD10, CD11b, CD14, CD15, CD16b, CD33, CD62L, CD66b, and CD98, and are analyzed by flow cytometry for confirmation. Purified LDN~~s~~ are completely functional as indicated by their capacity to produce reactive oxygen species, and to form neutrophil extracellular traps. This new purification method results in LDN with high purity (more than 90%) and viability (more than 96%) in a short time period. This method can easily be scaled up to obtain large numbers of pure LDN to evaluate LDN functions in different diseases through biochemical or other omics analysis.

Introduction

Neutrophils, the predominant leukocytes in human blood[1], are key participants of the innate immune system. They arrive in large numbers to tissues with inflammation or infection[2]. There, neutrophils activate several effector functions, such as phagocytosis[3,4], degranulation[5,6], and formation of neutrophil extracellular traps (NETs)[7]. Additionally, neutrophils also participate in the adaptive immune response[8]. The classical view of neutrophils considers them as homogeneous cells produced in the bone marrow with predetermined responses[9,10]. However, recent studies reveal that neutrophils are heterogeneous cells with multiple phenotypic and functional states under both healthy and disease states[11,12,13,14,15].

Among several neutrophil subpopulations, the low-density neutrophils (LDN) have attracted much interest because of their intrinsic properties and because they increase in numbers in several diseases[16,17,18]. LDN were found in the blood of systemic lupus erythematosus (SLE) patients in 1986[19]. They were detected following the process for separating leukocytes in a density gradient[20,21]. After centrifugation of blood or leukocyte-rich plasma on a density medium (e.g., Ficoll-Paque), monocytes and lymphocytes (known as peripheral blood mononuclear cells [PBMC]) form a band in the upper (low-density) part of the tube. Neutrophils sediment at the bottom of the tube. Among the PBMC, a few neutrophils were also found; these are LDN (**Figure 1**). Small numbers of LDN are in the blood of healthy individuals[22]. However, LDN numbers increase significantly in multiple immunosuppression and chronic inflammation conditions, including SLE[23,24], sepsis[25], psoriasis[26], asthma[27], juvenile idiopathic arthritis[28], anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis[29], HIV infection[30], malaria[31] and tuberculosis[32]. Although LDN numbers increase in all the mentioned pathologies, LDNs have been mostly studied in the context of SLE[17]. LDN from the blood of SLE patients seems to have a greater capacity to form NETs[33], to secrete large amounts of proinflammatory mediators[34], and to activate T cells[24]. However, in other conditions, such as cancer, LDNs are reported to consist of mature and immature neutrophils[35], with T-cell suppressive properties[36,37,38]. Similarly, in COVID-19 patients, LDNs are also reported to suppress T cell proliferation[39], but contrary to SLE, LDN seems to produce fewer NETs[39]. Therefore, the origin, composition, and functional properties of LDN are still controversial.

Because LDN are found together with PBMC, studying them is technically complicated. To fully explore LDN properties in different pathologies, it is necessary to purify them. A common procedure of LDN separation is fluorescent cell sorting[22,40,41,42,43,44,45]. However, cell sorting requires a long time for cell separation. This may lead to cell loss or low recovery if the sorting time is not sufficient. In addition, cells are subjected to excessive stress, causing variable results while studying the function of these cells. Long sorting time also increases the cost of experimental procedures and requires specialized personnel.

Here, we present a rapid and efficient method for obtaining large numbers of pure and viable human LDN in a very short time. After density-gradient centrifugation, LDNs are separated by magnetic cell sorting (MACS; **Figure 1**). The main advantage of this purification method is that LDNs are separated with high purity (more than 90%) and viability (more than 96%). Also, purified LDN is are completely functional as

indicated by ~~its~~ their capacity to generate reactive oxygen species (ROS) and to release NETs. This protocol can be easily implemented in any laboratory interested in neutrophil biology to quickly separate LDN from the blood of people with multiple diseases in order to further study these cells.

Protocol

All procedures in this protocol follow the guidelines of the Human Research Bioethics Committee at Instituto de Investigaciones Biomédicas - Universidad Nacional Autónoma de México (UNAM). All participants provided informed consent.

1. Isolation of peripheral blood mononuclear cells (PBMC) and neutrophils from human blood

1. Obtain about 10 mL of blood from a healthy adult volunteer by venipuncture. Add 10 U/mL of heparin as an anticoagulant.

   CAUTION: Dispose of the needle into a biohazard needle disposal container. Syringes and other materials in touch with blood should be placed in a bag and autoclaved before disposal.

2. In a 15 mL conical centrifuge tube, add 2 mL of 6% dextran T500 in PBS. Then add 10 mL of blood by draining it down the side of the tube. Invert the tube a couple of times to mix the blood and dextran.

3. Let the tube rest for 45 min, while erythrocytes sediment. The leukocyte-rich plasma appears above the erythrocyte layer.

4. In another 15 mL centrifuge tube, add 5 mL of density gradient medium. Carefully pipette the plasma, without touching the erythrocytes, and layer it on top of the medium. Two separate phases must be formed.

5. Centrifuge the tube at 516 x *g* for 20 min at 4 °C. Mononuclear cells (PBMC) form a band between plasma and the medium layers. Neutrophils form a pellet at the bottom (**Figure 1**).

6. Isolation of PBMC

   1. Eliminate by aspiration the plasma on top of the PBMC without touching the cells. Collect the cells from the band between the plasma and the medium. Make sure to aspirate as little medium as possible.

   2. Place cells into a 50 mL conical centrifuge tube. Add 20 mL of PBS. Centrifuge the tube at 400 x *g* for 5 min at 4 °C.

   3. Carefully aspirate the supernatant and scrape the tube to separate the cells. Add 10 mL of cold PBS to resuspend the cells. Count the PBMC using a Neubauer chamber.

      NOTE: Do not use a pipette to resuspend the cells, because this may damage the cells.

7. Isolation of neutrophils

   1. Remove the density gradient medium. Separate the cells by scraping the tube and add 10 mL of cold PBS.

   2. Put the cells into a fresh 50 mL centrifuge tube and centrifuge at 400 x *g* for 5 min at 4 °C. Aspirate the supernatant and separate the cells as described in step 1.6.3.

8. To eliminate residual erythrocytes, add 10 mL of cold hypotonic solution (0.2% NaCl, 1% BSA, 20 mM Hepes, pH = 7.4), and mix gently for exactly 1 min.

9. Quickly add 10 mL of cold hypertonic solution (1.6% NaCl, 1% BSA, 20 mM HEPES, pH = 7.4) to make the solution isotonic.

10. Count the neutrophils using a Neubauer chamber (purity > 95%). Pellet the cells by centrifuging as in step 1.6.2. and resuspend them in cold PBS. Maintain the tube on ice.

    NOTE: It is reported that the half-life of neutrophils *in vitro* is 8 - 12 h[46,47,48]. In our experience with this protocol, neutrophils maintain their functions for approximately 6 to 8 h.

2. Purification of low-density neutrophils (LDN)

1. Centrifuge PBMC at 400 x *g* for 5 min at 4 °C. Remove supernatant and resuspend cells in 120 µL of cold wash buffer (1% BSA in PBS).
2. Add 35 µL of CD66b magnetic microbeads. Incubate the mixture in the dark at 4 °C for 30 min. Mix gently every 10 min to prevent cell aggregation.
3. Add 1 mL of cold wash buffer. Place cells in a microcentrifuge and spin at 400 x *g* for 3 min.
4. Remove supernatant, separate the cell pellet by scraping the tube, and resuspend cells in 1 mL of wash buffer.
5. Place a magnetic separation column onto a magnet. Add 0.5 mL of wash buffer to the column and let it pass through the column.
6. Transfer cells (1 mL) onto the column. Let the buffer pass through the column drop by drop.
7. Add 0.5 mL of wash buffer into the column and let it pass through. Add another 0.5 mL of wash buffer into the column.
8. Remove the column from the magnet and put it in a microcentrifuge tube. Add 1 mL of wash buffer to the column.
9. Insert the plunger on top of the column and gently apply pressure to elute the cells. Remove the plunger and put the column on top of a new microcentrifuge tube.
10. Add another 1 mL of wash buffer. Insert the plunger on top of the column and gently apply pressure to elute the cells.
11. Place both tubes in a microcentrifuge and spin them at 800 x *g* for 3 min. Finally, resuspend the cells (LDN) from both tubes into 1 mL of cold PBS. Keep on ice.

    NOTE: The first 2 mL of flow-through contains the rest of the PBMC. These cells can be used for other studies.

3. Multicolor flow cytometry

1. Resuspend purified cells at 1 x 10^6 cell/mL in labeling buffer (1% FBS in PBS). Add 250 µL of cells into a 1.5 mL microcentrifuge tube.
2. Add the corresponding antibodies against neutrophil membrane molecules (for details, see **Table of Materials**). Incubate the cells for 30 min at 4 °C, protecting them from light.
3. Add 1 mL of PBS. Place tubes in a microcentrifuge and spin them at 800 x *g* for 3 min.
4. Aspirate the supernatant, break the cell pellet by tapping the tube, and resuspend cells in 0.5 mL of 1% paraformaldehyde.
5. Keep the cells at 4 °C protected from light, until analyzed by flow cytometry. Analyze cells by flow cytometry, capturing 10,000 events per sample. Perform cell sorting as previously described[22].

    CAUTION: Paraformaldehyde is an irritant to the skin and the respiratory tract. Make sure you do not breathe the vapors.

4. Detection of reactive oxygen species (ROS)

1. In a 1.5 mL microcentrifuge tube, add 2.5 x 10^5 cells. Place tubes in a microcentrifuge and spin at 800 x *g* for 3 min.
2. Aspirate the supernatant, scrape the tube to separate the cells, and resuspend the cells in 100 µL of 15 µM dihydrorhodamine 123 in PBS.
3. Incubate the cells protected from light at 37 °C for 20 min. Add 1 mL of PBS.
4. Place tubes in a microcentrifuge and spin at 800 x *g* for 3 min. Aspirate the supernatant, scrape the tube to separate the cells, and resuspend them in 100 µL of 50 nM phorbol 12-myristate 13-acetate (PMA) in PBS.
5. Incubate the cells protected from light at 37 °C for 45 min. Add 1 mL of PBS.
6. Place tubes in a microcentrifuge and spin them at 800 x *g* for 3 min. Aspirate the supernatant, scrape the tube to separate the cells, and resuspend them in 0.5 mL of 1% paraformaldehyde in PBS.
7. Keep the cells at 4 °C protected from light until analyzed by flow cytometry. Analyze cells by flow cytometry, capturing 10,000 events per sample.

5. Visualization of neutrophil extracellular traps (NETs)

1. Place coverslips in wells of a 12-well plate and cover them with 10 µg/mL poly-L-Lysine. Incubate the plate overnight at 4 °C with gentle agitation.
2. Remove the poly-L-Lysine, and wash the coverslips with PBS, incubating the plate for 5 min at room temperature with gentle agitation. Wash the coverslips with PBS two more times.
3. Remove the PBS and let the coverslips dry by placing the plate inside a laminar-flow hood for 2 h.
4. Resuspend LDN at 1 x 10^6 cell/mL in RPMI-1640 medium with 5% FBS. Take 350 µL (3.5 x 10^5 cells) of the cell suspension and put them into a well of the 12-well plate containing the coverslips.
5. Keep the plate for 30 min in a 5% CO_2 incubator at 37 °C. Add 3.5 µL of 5 µM PMA diluted in PBS to each well (final concentration of PMA is 50 nM).
6. Keep the plate for 4 h in a 5% CO_2 incubator at 37 °C. Add 350 µL of 8% paraformaldehyde in PBS, and keep the plate overnight in a 5% CO_2 incubator at 37 °C.
7. Place a transparent film over a test tube stand as previously described[49], so that wells are formed on the tube holes.
8. Fill each well with the various solutions to wash or stain the coverslips, forming a big drop. Remove the coverslips and put them upside down on a drop of water for 5 min. In the same manner, wash the coverslips three more times with water.
9. Place the coverslips upside down on a drop of blocking buffer (5% BSA in PBS) and incubate for 20 min at room temperature.
10. Transfer the coverslips to a drop of the corresponding primary antibody (e.g., anti-elastase or anti-citrulline) in blocking buffer and incubate for 60 min at room temperature.
11. Wash coverslips 2x in 0.01% Tween-20 in PBS. Transfer the coverslips to a drop of the corresponding secondary antibody in blocking buffer containing 150 nM DAPI, and

incubate for 60 min at room temperature, protected from light.

12. Wash coverslips 2x in 0.01% Tween-20 in PBS. Place a drop of antifade mounting medium onto a glass slide and put coverslips upside down.

13. Seal coverslips around the perimeter with nail polish. Store mounted slides at 4 °C, protected from light. To visualize NETs use a fluorescence microscope. ~~cells, see step 6.4. below~~.

6. Detection of neutrophil extracellular traps (NETs)

1. Resuspend cells at 5 x 10^5 cell/mL in 500 nM Sytox Green, diluted in RPMI-1640 medium with 5% FBS.

2. Take 100 µL (5 x 10^4 cells) of the cell suspension and put them into a well of a 96-well tissue culture plate.

3. Keep the plate for 20 min in a 5% CO_2 incubator at 37 °C. Add to each well 20 µL of 300 nM PMA diluted in RPMI-1640 medium (final concentration of PMA is 50 nM).

4. Transfer the plate to a pre-warmed microplate reader. Incubate the plate at 35 °C for 4 h, taking fluorescence measurements from the bottom of the plate every 5 min (using the 485 nm excitation and 528 nm emission filters).

Representative Results

Low-density neutrophils (LDN) in healthy individuals represent around 5% of the PBMC (**Figure 2A**). The protocol described here typically delivers pure (> 90%) LDN. Cells are also efficiently recovered (yield around 98%) with high viability (> 95%; **Figure 2B**). For comparison, LDN were also purified by fluorescent-activated cell sorting as previously described[22]. Briefly, PBMC were labelled with anti-CD16b antibody and were sorted in a fluorescent-activated cell sorter. LDN were sorted for 3 h and recovered in heat-inactivated FBS (**Figure 2**). Magnetically (MACS) purified LDN are mature cells displaying similar membrane molecules (markers) as neutrophils. Neutrophils express the membrane markers CD10, CD11b, CD15, CD62L, and CD66b (**Figure 3A**). Similarly, LDN purified by magnetic isolation are positive for expression of the same molecules (**Figure 3B**). Both neutrophils and LDN ($CD66b^+$ cells) are cells characterized by the phenotype $CD16b^{high}$, $CD15^{high,}$ and $CD10^{high}$ (**Figure 3**), indicating they are mature cells. Also, neither neutrophils nor LDN express CD33 or CD98 (not shown). Supporting the mature nature of LDN is the fact that purified LDN cells have a multilobulated nucleus and a similar size to neutrophils (**Figure 4**). However, very few cells with low or negative expression of CD16b were also present among purified neutrophils (2.8% ±0.523; n = 3) and among purified LDN (7.7% ±0.658; n = 3) (**Figure 3**, lower panels). These cells exhibited lower expression of CD15 and CD10 (**Figure 3**, lower panels), indicating that they are immature cells. These results suggest that among both normal neutrophils and LDN, there is a small proportion of immature cells[22,35,40,41,42,43,44,45].

Because purified LDN displays, right after isolation, an activated phenotype as previously reported[22], we explored the generation of ROS after stimulating the cells with PMA. Neutrophils or LDN were loaded with dihydrorhodamine 123, a well-known fluorescent indicator of ROS production[50,51]. Next, cells were treated with PMA and analyzed by flow cytometry. As anticipated, neutrophils stimulated with PMA produced large amounts of ROS (**Figure 5**). Likewise, purified LDN also generated ROS following PMA treatment (**Figure 5**). However, LDN produced more ROS than neutrophils (**Figure 5**). This result further supports that LDN are primed cells that, upon stimulation, produce higher levels of ROS.

As indicated, neutrophils also produce NETs as a key antimicrobial mechanism. Therefore, we assessed the capacity of LDN to produce NETs after PMA stimulation. Purified LDN released NETs in response to PMA treatment (**Figure 6**). NETs are DNA fibers decorated with proteins from neutrophil granules, such as elastase or myeloperoxidase (MPO)[7]. To visualize the NETs, they were stained with DAPI (to detect DNA) and with antibodies against neutrophil elastase. Elastase is colocalized with DNA fibers (**Figure 6A**). Similarly, citrullination of histones by the enzyme peptidylarginine deiminase 4 (PAD4) leads to chromatin decondensation. Thus, citrullinated histones are considered a marker of NETs[52,53]. DNA fibers also colocalized with citrulline (**Figure 6B**). Together, these results confirmed that LDN produces bona fide NETs. Next, NETs production was quantified using Sytox Green, a dye that fluoresces only when bound to DNA and cannot enter the cells. Thus, Sytox Green fluorescence indicates NETs formation, as DNA that has been released from cells[22,54,55,56]. Both purified neutrophils and purified LDN released NETs with the same kinetics and to the same amount (**Figure 7**).

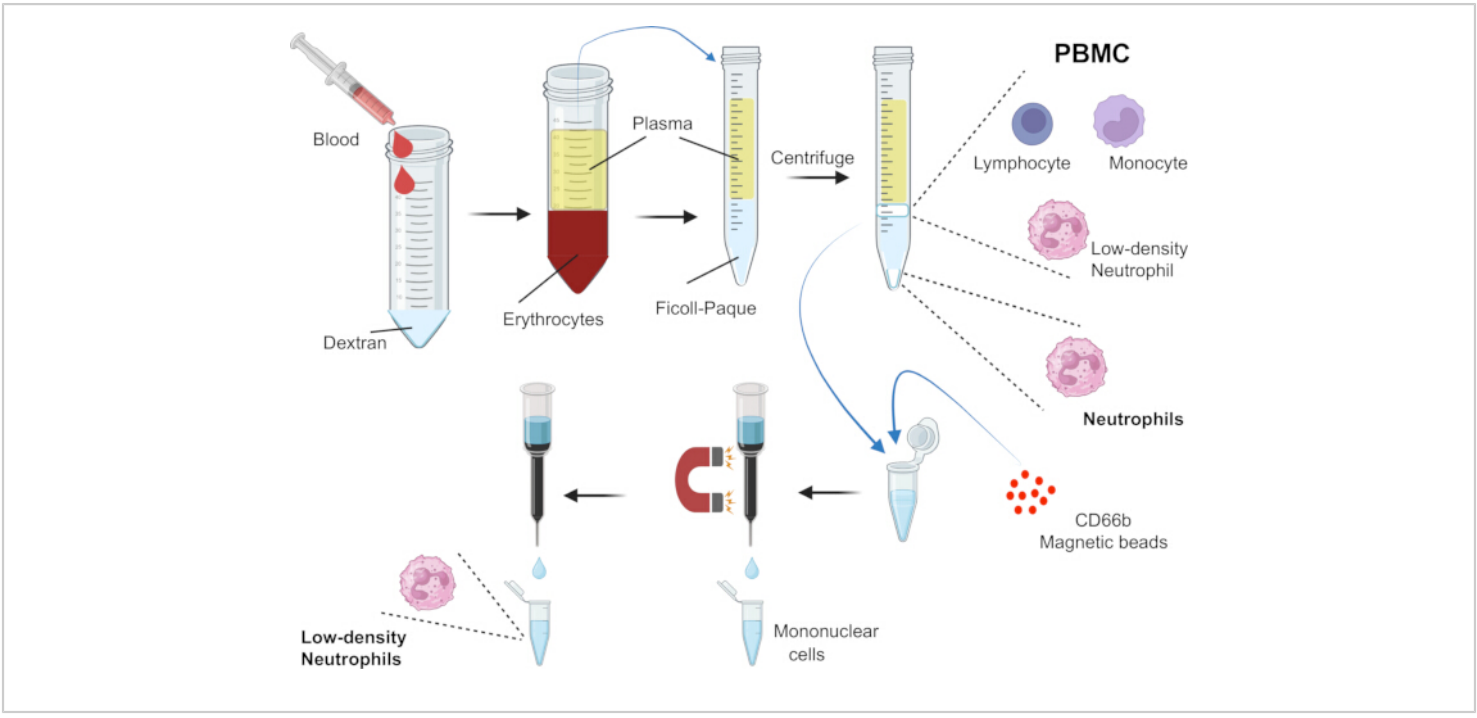

Figure 1: Purification of neutrophils and low-density neutrophils. Human blood from healthy donors was combined with heparin and dextran to allow erythrocyte sedimentation. Plasma was then layered above Ficoll-Paque and centrifuged. Neutrophils form a cell pellet at the bottom of the tube. Peripheral blood mononuclear cells (PBMC) form a band between the Ficoll-Paque and plasma layers. PBMC were mixed with CD66b magnetic beads and passed through a separation column with the magnet on. Mononuclear cells (monocytes and lymphocytes) pass through. Then, the magnet is removed and LDN are recovered in the eluate. **Figure created** with BioRender.com. Please click here to view a larger version of this figure.

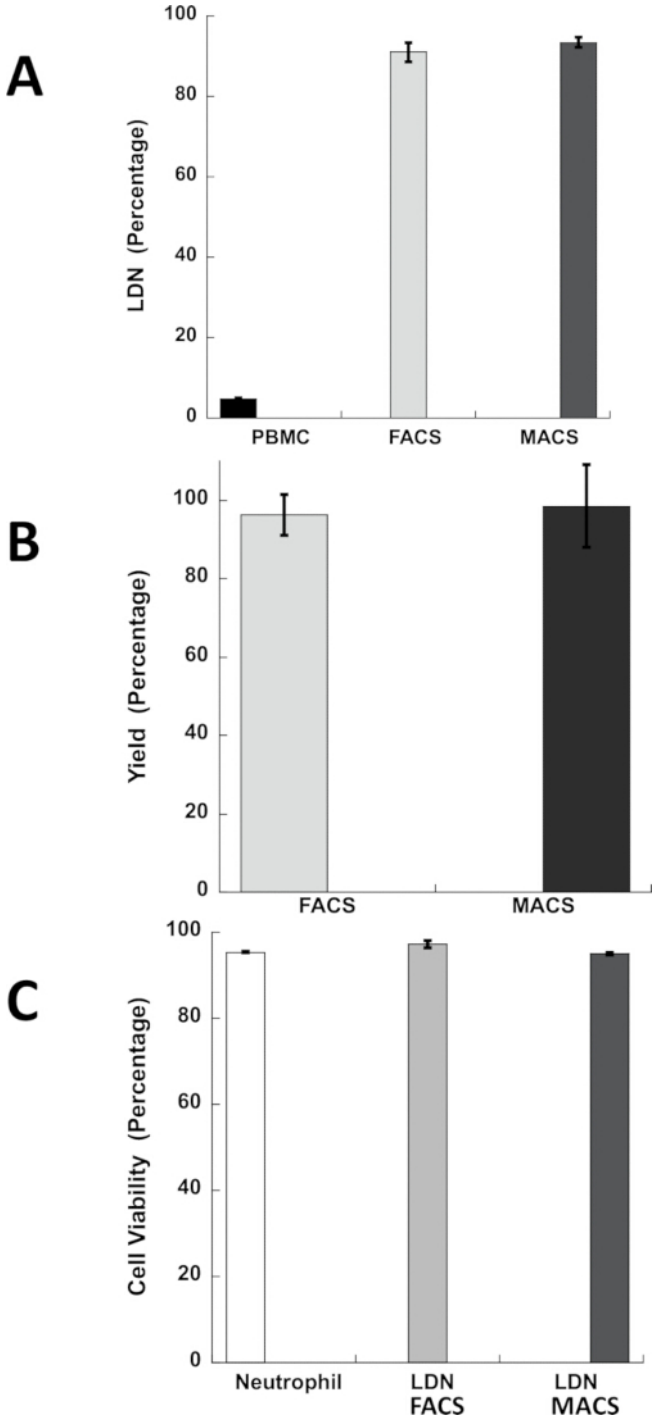

Figure 2: Enrichment and yield of LDN purification methods. Low-density neutrophils (LDN) were quantified within peripheral blood mononuclear cells (PBMC) before and after purification by fluorescent-activated cell sorting (FACS) or by the protocol presented here of magnetic isolation (MACS). (**A**) Percentage of LDN among PBMC and in the purified fractions after FACS or MACS. (**B**) Recovery (yield) of LDN after purification by FACS or MACS. (**C**) Cell viability of neutrophils or LDN after purification by FACS or MACS. Data are Mean ± SEM of three independent experiments. Please click here to view a larger version of this figure.

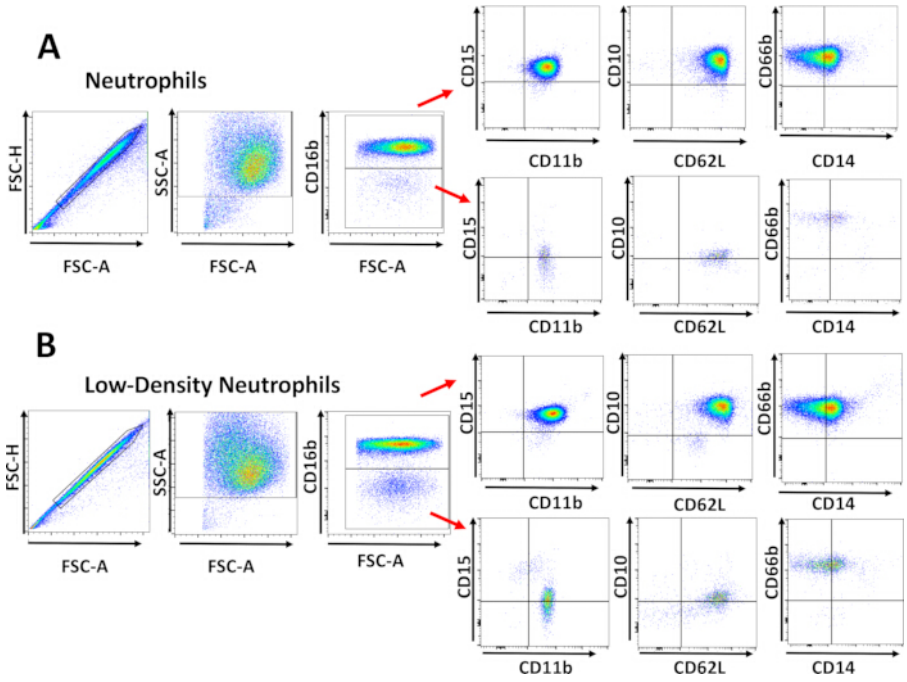

Figure 3: Low-density neutrophils have a mature phenotype. (**A**)*Purified neutrophils or* (**B**) purified low-density neutrophils (LDN) were examined for membrane expression of the molecules CD10, CD11b, CD14, CD15, CD16b, CD62L, and CD66b, by flow cytometry. Neutrophils and LDN with high expression of CD16b also expressed CD10, CD11b, CD15, CD62L, and CD66b (upper panels). Few cells with low or negative expressions of CD16b were also present (lower panels). Dot plots are representative of three independent experiments with similar results. Please click here to view a larger version of this figure.

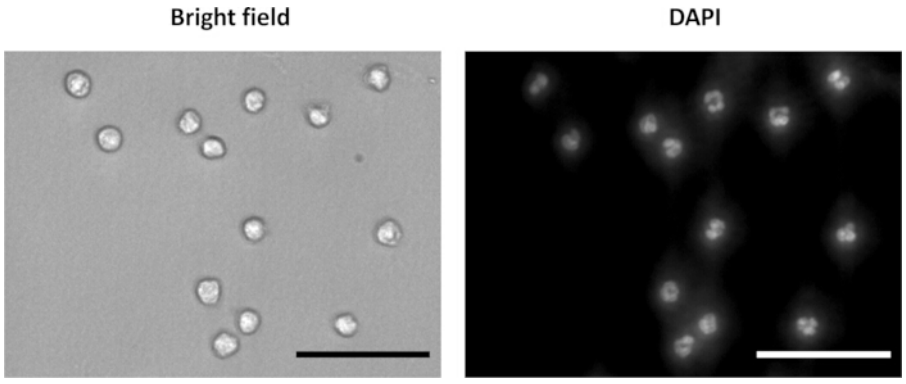

Figure 4: Low-density neutrophils are mature cells. Purified low-density neutrophils were treated with 150 nM DAPI and examined microscopically at a 40x magnification. Cells had a normal morphology (bright field) and showed multilobulated nuclei under fluorescent light (DAPI). Scale bar represents 50 µm. Images are representative of three independent experiments. Please click here to view a larger version of this figure.

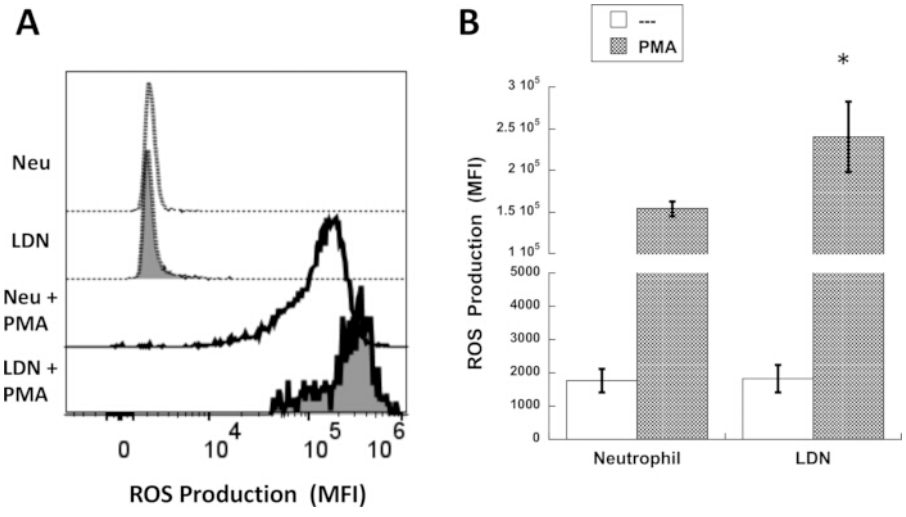

Figure 5: Low-density neutrophils generate more ROS. Purified neutrophils (Neu) or purified low-density neutrophils (LDN) were given dihydrorhodamine 123 and left alone (---) or treated with phorbol 12-myristate 13-acetate (PMA). (**A**) Histograms of cells alone or cells with PMA and their associated mean fluorescence intensity (MFI), indicating the generation of reactive oxygen species (ROS). (**B**) Quantification of ROS generation. Data are the Mean ± SEM of four independent experiments. Asterisk indicates statistical differences between PMA-treated neutrophils and PMA-treated LDN by t-test (*p = 0.029). Please click here to view a larger version of this figure.

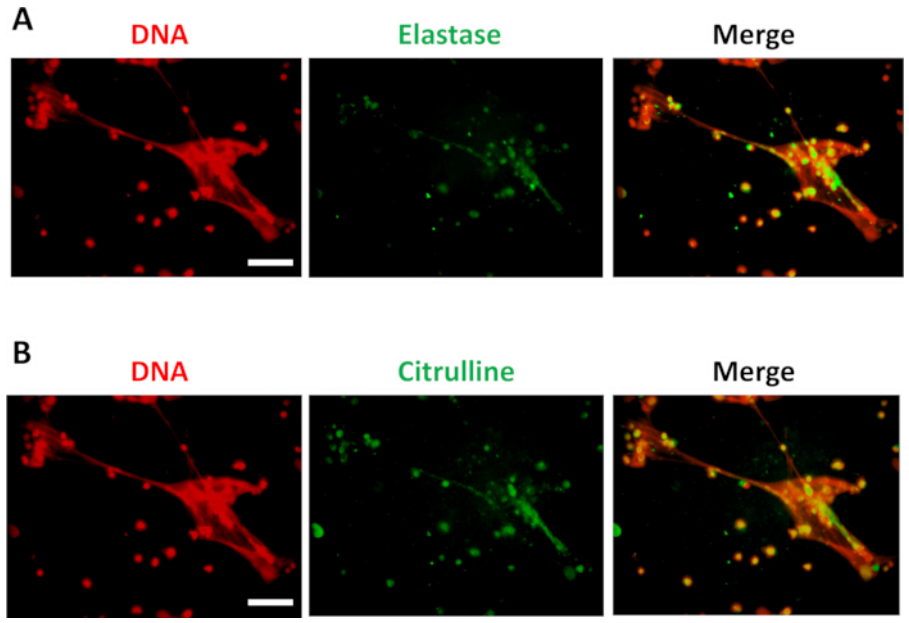

Figure 6: Low-density neutrophils form NETs. Purified low-density neutrophils were stimulated with 50 nM phorbol 12-myristate 13-acetate (PMA). After 4 h, cells were fixed and immunostained with (**A**) anti-elastase (green) or (**B**) anti-citrulline (green) antibodies, followed by the corresponding fluorescent-labelled secondary antibody. DNA was stained with DAPI (red). The right panels show co-localization (merge) of proteins with DNA. Scale bar represents 50 µm. Images are representative of two independent experiments. Please click here to view a larger version of this figure.

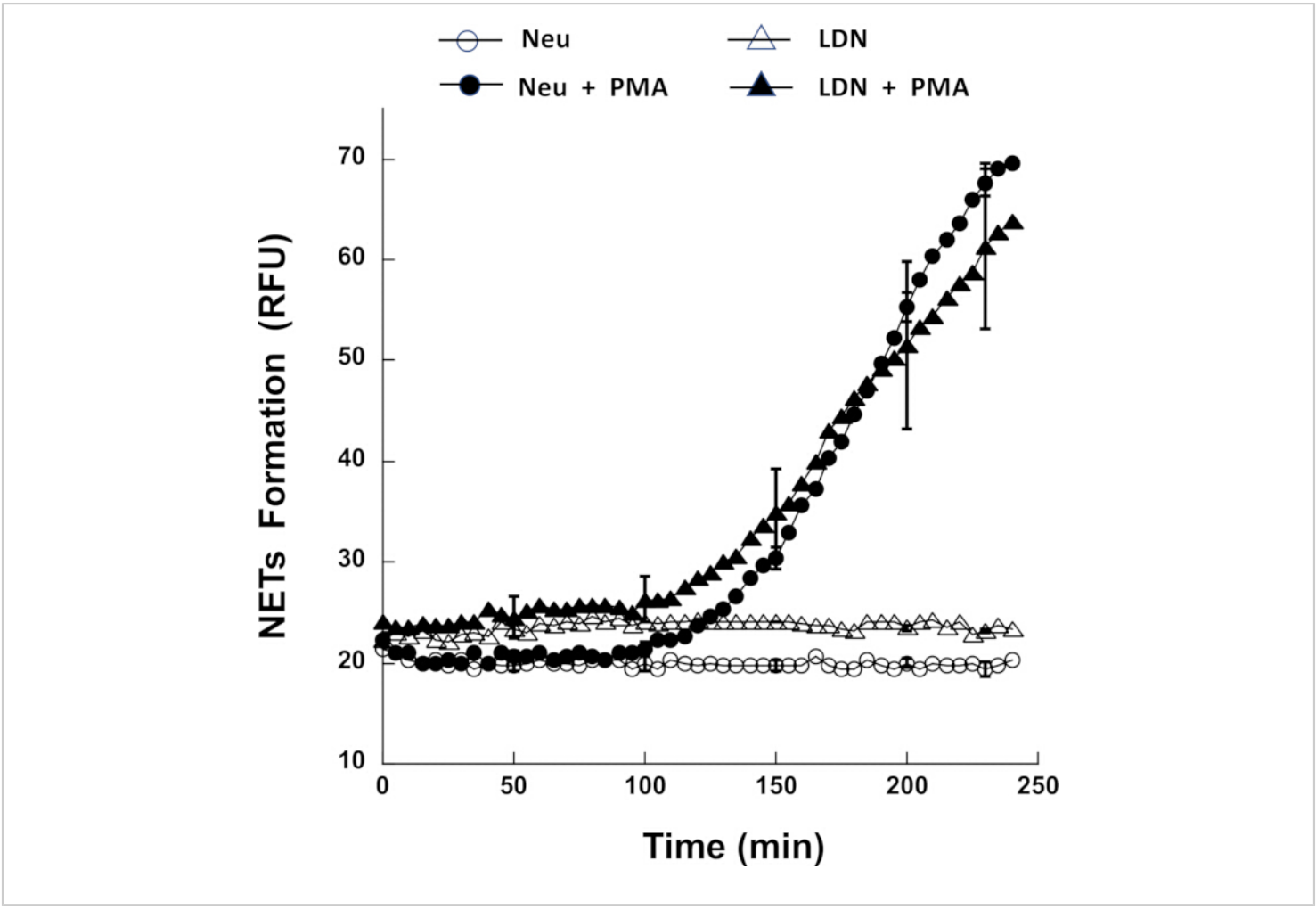

Figure 7: Neutrophils and low-density neutrophils release NETs with similar kinetics. Purified neutrophils (Neu) or purified low-density neutrophils (LDN) were placed in 500 nM Sytox Green diluted in RPMI-1640 medium and left untreated (open symbols) or treated with 50 nM phorbol 12-myristate 13-acetate (PMA) (closed symbols). Fluorescence intensity (RFU) of Sytox Green (external DNA), indicating NETs formation, was monitored for 4 h. Data are the Mean ± SEM (shown only for some time points) of three independent experiments. Please click here to view a larger version of this figure.

Discussion

Generally, neutrophils were thought of as homogeneous cells. However, recent evidence has shown that neutrophils could exist as cells with different activation states and/or multiple phenotypes. Thus, various neutrophil subpopulations may exist[13,14,15]. One neutrophil subpopulation, the low-density neutrophils (LDN), has acquired great interest due to their particular intrinsic properties and also because they increase in multiple diseases[16,17,18]. LDN are isolated from blood together with PBMC[22]. Therefore, studying them is technically complicated. In order to fully explore LDN properties in different pathologies, it is necessary to purify them. Now, we describe an easy, rapid, and efficient protocol for obtaining large numbers of pure and viable LDN.

After density-gradient centrifugation, LDN are separated by magnetic cell sorting.

The functions of LDN remain controversial. In some diseases, such as cancer, LDN have been reported to display immunosuppressive functions[37,38,57,58]; while in SLE, LDN produce large amounts of proinflammatory cytokines[34], and activate T cells[24]. Hence, LDN behavior may depend on the context of the disease involved. Consequently, it is important to purify LDN in order to better understand ~~its~~ their properties. Because LDN are found together with PBMC, separating them is technically complicated. A common procedure of LDN separation has been fluorescent cell sorting[22,40,41,42,43,44,45]. However, since there is no molecular marker that defines LDN[59], it is necessary to use a panel of several antibodies to clearly identify neutrophils among the PBMC[22]. Once LDNs are selected, cell sorting can be performed. This, in turn, requires at least 3 h of sorting time to obtain enough pure LDN for downstream analysis[22]. The long time involved in cell sorting could affect recovery, viability, and function of LDN, since neutrophils survive only a short time *in vitro* (half-time life is 8 - 12 h)[46,47,48]. In addition to the requirement of several distinct antibodies to identify neutrophils, fluorescent cell sorting is also expensive due to the long times involved and the need for specialized technical personnel to operate the cytometer. Therefore, alternative purification methods to isolate LDN are necessary.

Here, we described an efficient and rapid method to purify LDN from the PBMC fraction of cells after density gradient centrifugation. The method involves the separation of LDN from PBMC using magnetic beads. The molecule CD16b (Fcγ receptor IIIb) is exclusively expressed on human neutrophils[60,61,62]. Thus, CD16b would be an ideal marker for neutrophils. However, CD16b is very similar to its homolog receptor CD16a (Fcγ receptor IIIa), which is found abundantly expressed on natural killer cells[63,64]. In addition, CD16b is shed from the neutrophil cell membrane upon activation[65], making this receptor unsuitable to follow neutrophils. Other molecular markers, such as CD11b, CD15, and CD62L, also display differential expression depending on the activation state of the neutrophil[22,66,67]. Therefore, CD66b is a better choice for detecting neutrophils[68] and further isolating LDN. PBMC were mixed with magnetic beads that carry antibodies against CD66b. LDN ($CD66b^+$ cells) are recovered from the magnetic column as a homogeneous cell population with high viability (**Figure 1**). The method is also very efficient, since more than 95% of the LDN in the PBMC fraction are recovered, resulting in a highly pure cell preparation. This provides enough pure cells for more precise further downstream analyses. A potential concern related to this protocol is that the use of CD66b may bias the selection of LDN towards a more mature phenotype. This seems very unlikely, because characterization of neutrophil membrane markers using high-throughput flow cytometry has identified CD11b, CD16, and CD66b as molecules that are consistently expressed on neutrophils independently of cell location, activation level, or disease state[68]. Also, previous studies isolating LDN by cell sorting using CD66b as the selection molecule have identified both mature and immature LDN, all of them expressing CD66b[43,45]. Therefore, selecting neutrophils with CD66b magnetic beads will not prefer mature over immature cells.

Purified LDN are indeed neutrophils since they are positive for expression of the same membrane molecules as neutrophils (**Figure 3**). Although LDN has a slightly higher expression of CD15, suggesting an activated phenotype. Also, in a previous report, when analyzing the whole PBMC from healthy individuals by flow cytometry, we found that LDN

appeared to be a homogeneous cell population of mature neutrophils[22]. However, in other conditions such as cancer, LDN have been reported to be a heterogeneous population composed of mature and immature neutrophils[35]. Now, when analyzing purified LDN, we detected a small number of cells (about 7%) with low or negative expression of CD16b (**Figure 3**). Among purified neutrophils, these $CD16b^-$ cells (about 3%) were also present. Both $CD16b^-$ neutrophils and $CD16b^-$ LDN had a lower expression of CD15 and CD10 (**Figure 3**), suggesting that they are immature cells. This finding indicates that among normal neutrophils and also among LDN, there is a small proportion of immature cells. Whether this immature cell fraction increases in certain pathologies remains to be elucidated. The protocol presented here to isolate LDN will certainly help in further characterizing LDN in different diseases.

The protocol presented here is simple and straightforward. However, there are some steps that require particular attention. The amount of magnetic microbeads described works well with about 30 x 10^6 total cells. If the number of cells increases, the column begins to clog up, and the buffer takes a long time to pass through. If large numbers of cells need to be processed to purify large amounts of LDN, it is better to split the sample and use several magnetic columns. Also, in the elution step, the plunger should be pressed gently and only until all the liquid is out of the column. If the plunger is pressed all the way down to the end of the column, foam is formed. This seems to damage the cells, because fewer cells were recovered.

One possible disadvantage of the purification method presented here is that it involves positive selection of the cells. This means that antibodies binding to membrane molecules potentially could activate neutrophils and trigger cell responses. Crosslinking of CD66b molecules on the membrane of neutrophils could cause activation of cells[69,70]. However, this activation requires a high concentration of antibodies in order to achieve crosslinking of the receptors, and also extracellular calcium[70]. The magnetic-activated cell sorting (MACS) system used in the present protocol involves nano-sized immunomagnetic beads coupled to special columns. The magnetic beads are covered with a low concentration of antibodies, which does not allow crosslinking of the corresponding antigen (CD66b)[71]. In addition, there is no extracellular calcium in the buffer used to incubate the cells with the anti-CD66b antibody-coupled beads. Moreover, even the higher concentrations of anti-CD66b antibodies used in most flow cytometry studies of neutrophils cannot induce crosslinking and therefore are not capable of activating the cells[68,72]. The low concentration of antibodies on the beads is enough to capture the cells because the ferromagnetic spheres packed in the columns amplify 10,000-fold the magnetic field of the magnet used[71]. Together, these elements allow for cell separation with minimal cell labelling, resulting in no cell activation. We confirmed that total expression of CD66b molecules was not affected by the binding of the magnetic microbeads (**Figure 3**), which are therefore not necessary to be eliminated after the purification protocol.

The purification protocol presented here involves positive selection of cells. An alternative approach is the use of negative selection of cells. In some studies, this alternative approach to separate LDN has been used[24,34,73]. In this case, all cells except neutrophils would be labelled with antibodies to remove them using magnetic beads. This method requires many more antibodies against multiple cell types. This increases the cost of the process and does not completely guarantee that all other cell types have been

selected and eliminated because antibodies recognizing rare cell types may not be included in the antibody cocktail used. Still, the main reason to use negative selection is that the cells of interest (LDN in our case) would not be activated by the action of antibodies. However, as we discussed above, the use of positive selection with CD66+ microbeads does not result in cell activation.

LDN purified with the present method are viable and functional. After MACS isolation, purified LDN produce higher amounts of ROS than neutrophils upon stimulation with PMA, indicating a pre-activated phenotype (**Figure 5**). Neutrophils and LDN are both capable of producing NETs[7,22,74]. MACS-purified LDN, after PMA stimulation, produced similar amounts of NETs as neutrophils did (**Figure 7**). These results again indicate that purified LDN remains functional, and they are consistent with our previous finding that LDN from healthy individuals can produce higher amounts of ROS than neutrophils and similar NETs as neutrophils[22]. However, because PMA is not a physiologic stimulus, it is important to explore other stimuli for neutrophil activation, such as cytokines, immune complexes, and microorganisms. This protocol can be easily implemented in any laboratory interested in neutrophil biology, to quickly separate LDN from the blood of people with multiple diseases in order to further study these cells. The protocol can also be easily scaled up to obtain large numbers of pure LDN to evaluate LDN functions with multiple stimuli via biochemical or some other omics analysis.

In conclusion, we present a rapid and efficient protocol for obtaining large numbers of pure and viable LDN in a very short time. After density-gradient centrifugation, LDN are separated by magnetic cell sorting (MACS), resulting in LDN with high purity and viability. In addition, purified LDN are completely functional as indicated by their capacity to generate ROS and to release NETs.

Disclosures

The authors state that this study was carried out without any commercial or financial ties that might be perceived as a potential conflict of interest.

Acknowledgments

The authors thank César Díaz-Godínez (Instituto de Investigaciones Biomédicas-UNAM) for his help with fluorescent microscopy to visualize NETs. This research was supported by grant PAPIIT IN205523 from Dirección General de Asuntos del Personal Académico, Universidad Nacional Autónoma de México (UNAM), Mexico, and by grant CF-2023-I-610 from Secretaría de Ciencias, Humanidades, Tecnología e Inovación (Secihti), Mexico.

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