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Isolation of human neutrophils from whole blood and buffy coats.

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TITLE:

Isolation of Human Neutrophils from Whole Blood and Buffy Coats

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KEYWORDS:

Neutrophil, gradient, dextran, RBC lysis, whole blood, buffy coat

SUMMARY:

This protocol details a method for the isolation of neutrophils from whole blood, buffy coats, or leukapheresis membranes, achieving good yield, high purity, and minimal cell activation. We utilize gradient purification, red blood cell (RBC) sedimentation, and RBC lysis to obtain a high-quality/purity neutrophil preparation.

ABSTRACT:

Neutrophils (PMNs) are the most abundant leukocytes in human circulation, ranging from 40 to 70% of total blood leukocytes. They are the first cells recruited at the site of inflammation via rapid extravasation through vessels. There, neutrophils perform an array of functions to kill invading pathogens and mediate immune signaling. Freshly purified neutrophils from human blood are the model of choice for their study, as no cell line fully replicates PMN functions and biology. However, neutrophils are short-lived, terminally differentiated cells and are highly susceptible to activation in response to physical (temperature, centrifugation speed) and biological (endotoxin, chemo- and cytokines) stimuli. Therefore, it is crucial to follow a standardized, reliable, and fast method to obtain pure and non-activated cells. This paper presents an updated protocol combining density gradient centrifugation, red blood cell (RBC) sedimentation, and RBC lysis to obtain high purity and minimize cell activation. Furthermore, methods to assess neutrophil isolation quality, viability, and purity are also discussed.

INTRODUCTION:

The innate immune system is comprised of many cell types that maintain immune homeostasis and pathogen clearance along with many other physiological functions. Neutrophils comprise the

largest pool of white blood cells in human circulation[1]. Most mature neutrophils are stored in the bone marrow, which is also the site of the generation of new neutrophils, also called granulopoiesis. In the bone marrow, granulocyte progenitors exit the cell cycle and terminally differentiate, acquiring their characteristic segmented nuclei and granules[2]. Under inflammatory conditions, in response to chemokines, cytokines, and damage-associated and pathogen-associated molecular patterns, neutrophils are mobilized from the bloodstream and out of the bone marrow to perform a wide array of functions. These include cytokine secretion, direct phagocytosis of the pathogen, the release of reactive oxygen species, degranulation of antimicrobial proteins, and formation of neutrophil extracellular traps.

The molecules used by neutrophils to fight infection are toxic to the microbes and the host. Thus, the life span and the proper removal of aging/dying neutrophils are highly regulated, and they have a limited life span in circulation (<48 h)[3]. Due to this short survival, the human body produces 100 billion new neutrophils on average every day to maintain population homeostasis[4]. Emergency granulopoiesis can further increase the release of neutrophils, both mature and immature, in the blood during inflammation and infection[5]. The importance of neutrophils in the innate immune response is highlighted by patients with acquired or congenital neutropenia, who are susceptible to bacterial and fungal infections[6].

Many challenges arise when studying neutrophil biology and their roles in the immune response due to their nature, including their short survival and cytotoxic content. Neutrophil-like cell lines have been commonly differentiated from human promyelocytic leukemia HL-60 cells and PLB-985 cells[7,8]. Although they can display neutrophil-like morphology and function such as migration, these cell lines cannot fully recapitulate the biology of neutrophils. *In vitro* assays using these cell lines are also unable to recapitulate *in vivo* experiments. Furthermore, the differentiation of these cells needs to be induced and could be adversely affected by gene manipulation before differentiation.

Recently, methods have been developed to circumvent these issues by using inducible promoters to modulate gene expression post differentiation in HL-60 cells[9]. Even with such tools, primary human PMNs are required to validate targets using pharmacological approaches. Thus, it is imperative to obtain pure and inactivated neutrophils isolated from blood to validate the findings of cell line and animal models. This paper presents a revised PMN isolation protocol in which the pros and cons of current methods were evaluated[10]. A combination was devised consisting of gradient centrifugation to separate PMNs from other immune cells, short dextran-based sedimentation to remove the bulk of RBCs, rapid residual RBC lysis via osmotic pressure, and low-speed centrifugation to remove platelet contamination.

PROTOCOL:

NOTE: Human neutrophils were isolated from venous blood from discarded white blood cell filters obtained from the Blood Bank Lab at the Boston Children's Hospital. Blood donors were unidentifiable, and there was no interaction with living individuals or knowledge of identifiable personal information. Therefore, this work is not classified as human subjects research under the

HHS human subjects regulations (45 CFR Part 46). The Boston Children's Hospital Institutional Review Board (IRB) approved the protocol.

1. Layering the gradient

1.1. After sterilizing the buffy coat packaging and the laminar hood, divide the blood into 50 mL tubes with 10 mL of blood in each tube.

1.2. Bring the volume up to 35 mL with 5% fetal bovine serum (FBS)/Hank's Balanced Salt Solution (HBSS) to dilute the blood for a cleaner gradient.

NOTE: When using a leukapheresis membrane, cells can be flushed out using a 60 mL syringe and 30 mL of 5% FBS/HBSS per filter. Dilution is necessary when working with freshly drawn whole blood.

1.3. Close the 50 mL tube lid, mix it several times by inversion, and keep it upside down to have the bottom devoid of RBCs.

1.4. Add 10 mL of density gradient medium (see the **Table of Materials**) directly beneath the blood. Ensure that the medium and the blood do not mix and that the interface is sharp (**Figure 1A**).

NOTE: This step is crucial. Ensure that the density gradient solution is at room temperature (RT) and well-mixed before each gradient. The first milliliter of the density gradient medium needs to be carefully and steadily layered as slowly as possible. Having the electronic pipette speed set to low is recommended. Similar results can be achieved using Histopaque-1077.

1.5. Gently place the tube in a centrifuge without disturbing the gradient and spin at 400 × *g* for 30 min at RT, making sure to disable the brake. Observe how the spun gradient separates into a top serum/plasma layer, a middle white ring of peripheral blood mononuclear cells (PBMCs), a cloudy density gradient medium layer, and a bottom pellet consisting of a white, thin neutrophil band on top of the RBCs (**Figure 1B**).

NOTE: A cloudy or opaque side of the tube after centrifugation can suggest that the cells (neutrophils) are activated and may not be suitable to use.

1.6. Remove the PBMCs first by emerging the suction pipette directly into the PBMC layer. Be sure to aspirate it completely while the serum/plasma layer decreases as the ring is removed. Scrape the side of the tube where cells pelleted with the suction pipette to maximize the removal of the PMBC. Carefully remove the cloudy density gradient medium layer between the PBMC ring and the neutrophil/RBC pellet.

NOTE: Scraping the pelleted cells on the side of the tube increases the purity of the isolation considerably. Be careful not to aspirate the pellet, as most neutrophils are sitting directly on top of the RBCs.

2. Sedimentation of erythrocytes (RBCs)

2.1. Using a 10 mL pipette, transfer the neutrophil/RBC pellet into a clean tube. Do not pipette up and down. Add 5% FBS/HBSS to a final volume of 25 mL. Gently mix by inversion.

NOTE: Performing the RBC sedimentation after the PBMC removal improves yield and decreases activation[10].

2.2. Directly add 25 mL of prewarmed (37 °C) 3% Dextran/0.9% NaCl/H_2O into the tube containing the diluted neutrophil/RBC pellet and mix gently by inversion. Place the tube on a level, non-vibrating surface for 15 min (**Figure 1C**).

NOTE: Longer sedimentation reduces RBC contamination but also decreases the yield. Furthermore, prolonged dextran exposure may lead to neutrophil activation or cell death[11].

2.3. Gently bring the tube back into the hood (for sterile isolation). By only slightly immersing the pipette in the liquid, collect the top layer (~30 mL) following the liquid surface downwards.

NOTE: If the sedimentation produces a sharp interface between the medium and the RBCs, the RBC pellet is smaller, or if a higher yield is desired, collect up to 35 mL.

2.4. Spin the tube (400 × *g*, 10 min, RT, using low brake (3)), resulting in a red pellet with no particles floating in the media.

3. Lysis of the residual RBCs

3.1. Gently aspirate the supernatant without disrupting the pellet.

3.2. Add 25 mL of sterile ultrapure water directly into the tube and gently mix by inverting for 28 s to lyse the RBCs. Do not use a pipet to resuspend the pellet.

NOTE: Do not exceed 30 s as prolonged hypotonic conditions can activate and lead to neutrophil death[12].

3.3. Immediately add 25 mL of sterile 1.8% NaCl/H_2O into the tube and gently mix by inverting to bring the solution back to isotonic conditions.

NOTE: The solution should be red but without turbidity.

3.4. Spin down at 200 × *g* for 3–5 min with low brake (level 3) to minimize RBCs and platelet sedimenting together with the neutrophils (**Figure 1D** and **Figure 2**)[13,14].

NOTE: The pellet should be white with a minimal RBC layer on top, which can be gently removed while the supernatant is aspirated.

3.5. Resuspend neutrophils by pipetting the culture medium (10% FBS/RPMI1640) directly on the pellet, but do not pipette up and down. Rock the tube horizontally from side to side to minimize cell activation.

NOTE: Cells should be kept at a concentration of ~2 × 10^6 cells/mL, as higher density will lead to increased cell activation/death[15]. For the same reason, the cell pellet should be resuspended as soon as possible.

3.6. If cell aggregation or clumping is observed, filter the cell suspension using a 70 µm mesh to discard clumped neutrophils.

4. Determining neutrophil isolation quality

4.1. Stain the cells with markers specific for neutrophils (CD66b, CD11b), eosinophils (CD193) (**Figure 3**), and an activation marker such as CD62L (**Figure 4**). Acquire 20,000 cells by flow cytometry. Analyze cell purity and activation using the gating strategies proposed in **Figure 3** and **Figure 4**.

NOTE: The quality of the neutrophil preparation can be assessed using a 3% acetic acid–methylene blue solution to visualize the unique lobed nucleus of neutrophils.

4.2. Determine cell viability using Annexin V/propidium iodide (PI) (**Figure 5**).

NOTE: Trypan blue staining can be used to evaluate cell viability.

REPRESENTATIVE RESULTS:

When using density gradient to purify neutrophils, it is critical for the interface between the blood and the density gradient medium to be as sharp as possible, and that a distinct layer separation remains after centrifugation (step 1.4). Following RBC lysis, the buffer should be a clear red and not turbid (step 3.3). If the preparation is cloudy, a second round of lysis (step 3) may be required, although this may affect neutrophil survival (**Figure 1**). Following lysis, low-speed centrifugation (200 × *g*) is recommended when purity is prioritized, as it reduces platelet contamination considerably. However, high-speed centrifugation (400 × *g*) increases the yield at the expense of purity (step 3.4, **Figure 2**). After neutrophil isolation, fluorescence-activated cell sorting can be used to assess isolation purity (step 4.1) and should be chosen over microscopy. Although the FSC/SSC distribution of cells alone provides an estimate of the cell isolation quality (**Figure 3A**), the use of specific cell markers should be preferred. In this case, the most common contaminating cell populations are stained with specific antibodies together with CD66b, specifically expressed

on granulocytes. CD45 staining is used to distinguish leukocytes (CD45+) and red blood cells and platelets (CD45-).

Other contaminants include lymphocytes (CD3+ or CD19+, **Figure 3F**), monocytes (CD14+, **Figure 3D**), and eosinophils (CD193+, **Figure 3G**). CD11b is an integrin expressed on the myeloid lineage; neutrophils and monocytes are CD11b+, whereas lymphocytes are CD11b- (**Figure 3C**). As neutrophil activation can affect downstream experiments, the expression of CD62L should be assessed; neutrophils become CD62L- once activated (step 4.1). The peptide fMLP can be used as a positive control for CD62L shedding (**Figure 4**). It is also important to evaluate the health of neutrophils before performing assays; neutrophils have a relatively short half-life, and activation can further shorten it (step 4.2). A standard Annexin V and PI staining can give information on the live/dead status of the neutrophil culture at designated times (**Figure 5**).

FIGURE AND TABLE LEGENDS:

Figure 1: Density gradient medium-based separation of granulocytes. (**A**) Before and (**B**) after centrifugation. Note the sharp interface between the blood and the density gradient medium layers. (**C**) Sedimentation of the pellet in **B** resuspended (1:1) in 5% FBS/HBSS and 3% Dextran-0.9% NaCl. (**D**) PMN pellet after lysis of the residual RBCs in the supernatant of (**C**) with H_2O. Abbreviations: PBMC = peripheral blood mononuclear cell; PMN = neutrophil; RBC = red blood cell; FBS = fetal bovine serum; HBSS = Hank's Balanced Salt Solution.

Figure 2: Spinning at lower speeds after RBC lysis decreased platelet contamination. After lysis of RBCs with H_2O, cells were spun down at 200 × *g* (**A**) or 400 × *g* (**B**). Neutrophils were stained, and flow cytometry analysis was performed, as described in **Figure 3**, with the addition of anti-CD41 to label the platelets. Abbreviations: RBC = red blood cell; CD41 = cluster of differentiation 41; SSC-A = side scatter-Area.

Figure 3: Assessment of neutrophils isolated from buffy coat. Isolated neutrophils were stained with anti-CD66b, anti-CD11b, anti-CD14, and anti-CD193 following a standard protocol. Single cells (**B**) were gated from total cells (**A**). (**C**) $CD11b^+$ cells were gated from single cells. (**D**) Dot plot showing neutrophils ($CD66b^+$, CD14 low/-) and a very low monocyte contamination ($CD66b^-$, $CD14^+$, Q1). (**E**) $CD66b^-$ and $CD66b^+$ cells were gated. (**F**) $CD66b^-$ cells were positive for lymphocyte markers (CD3, CD19). (**G**) Expression of CD11b and CD193 in $CD66b^+$ cells, showing distinct neutrophil ($CD66b^+$, $CD11b^+$, $CD193^-$) and eosinophil ($CD66b^+$, $CD11b^-$, $CD193^+$) populations. In the representative result shown here, neutrophil purity is ~93% with ~3.7% lymphocyte and ~3.7% eosinophil contamination. (**H**) Quantification of neutrophil purity after purification. Data are compiled from 5 individual trials and presented as mean ± SD. Abbreviations: CD = cluster of differentiation; SSC-A = SSC-A = side scatter-Area; FSC-A = forward scatter-Area.

Figure 4: Gradient purification did not cause CD62L shedding. (**A–B**) Control (**A**) or fMLP-stimulated neutrophils (**B**) (1 mM fMLP for 15 min at 37 °C) were stained with neutrophil markers and CD62L. (**C**) Fluorescent mean intensity for CD62L is decreased in fMLP-treated cells, indicating CD62L shedding and neutrophil activation. Abbreviations: CD26L = L-selectin; fMLP = N-Formylmethionyl-leucyl-phenylalanine; SSC-A = SSC-A = side scatter-Area; FSC-A = forward

scatter-Area; PE = phycoerythrin.

Figure 5: Spontaneous death of neutrophils purified by density gradient or neutrophil isolation kit. Neutrophils were purified with density gradient (**A** and **B**) or commercial microbeads (**C** and **D**) and cultured for 0 h (**A** and **C**) or 24 h (**B** and **D**) in RPMI-10% FCS. Cells were stained using Annexin V and PI at respective time points following a standard protocol. (**E**) Quantification of purified neutrophil spontaneous death. n=5, mean ± SD. Abbreviations: SSC-A = SSC-A = side scatter-Area; FSC-A = forward scatter-Area; PI = propidium iodide; FCS = fetal calf serum; FITC = fluorescein isothiocyanate; AV5 = Annexin V.

DISCUSSION:

Due to the short life span, terminal differentiation status, and lytic content of neutrophils, studying these cells has always been a challenge. Apart from utilizing mouse models or cells from patient cohorts, cell lines are useful tools to help study neutrophil biology[16]. However, neutrophil-like cell lines cannot completely reiterate all aspects of neutrophil biology, adding an extra layer of difficulty in studying these cells. The most commonly used *in vitro* model is the HL-60 cell line, which can be differentiated into neutrophil-like cells by treatment with dimethylsulfoxide or retinoic acid[17,18]. Although these cells are useful in the study of migration and respiratory burst, they are not suitable for studying the microbiocidal activity of neutrophils. Other cell lines exist (PLB-98, NB4), and they are also associated with their set of limitations[19].

It is pivotal to validate with primary human neutrophils observations made with mouse disease models and cell lines. Neutrophils cannot be cryopreserved efficiently and thus are often freshly isolated from whole blood or buffy coats obtained from donors and immediately processed. Once isolated, the cells start to undergo a complex form of spontaneous death, regulated by oxidation, cytoplasmic caspases, and proteases found in neutrophil granules[20,21]. Improper isolation methods or techniques can lead to the activation of neutrophils, only accelerating cell demise. It is imperative to have a reliable and consistent method to obtain pure and high-quality neutrophils from donors.

There have been many methods published on human neutrophil isolation[10,22]. They mainly fall into two categories, with some shared strategies. The first category is antibody-based, being either through positive or negative selection. Positive selection would label neutrophils directly, therefore providing a highly pure cell population, although it also leads to rapid cell activation, cell death, as well as unwanted tagging of neutrophils[23]. Negative selection, though leaving the cells unlabeled and giving a very pure population, accelerated neutrophil death, although the precise mechanism is unknown (**Figure 5**). Whether gene or protein expression is also altered after positive or negative selection needs to be further investigated. Moreover, due to the amount of antibody needed to deplete the other types of cells, these methods cannot output large amounts of neutrophils. However, antibody-based assays can still be used for short-term culture and experiments on a smaller scale and are methods of choice for experiments requiring very high cell purity, such as gene and protein expression studies.

The second type of isolation method is gradient- and density-based. It usually involves Percoll,

Ficoll-Paque, or other polysaccharide/polyvinylpyrrolidone components and utilizes centrifugation force to separate different types of blood cells based on cell density. These methods often are complemented with the sedimentation of red blood cells by dextran. These methods can handle larger scales of starting material and can achieve high purity as well. One caveat of density-based isolation is the inefficient separation of other far less abundant granulocytes (mostly eosinophils) from neutrophils, and it is, therefore, the major limitation of the protocol presented, as even the presence of small cell contamination can affect neutrophil response[24].

Presented here is a summarized method based on gradient isolation, refining previous methods[10,22]. We utilize the current understating of neutrophil specificities to reliably isolate pure human neutrophils with limited residual platelet and RBCs, preventing neutrophil activation and accelerated death. The most crucial step is the layering of the gradient, which is much more effectively obtained by adding the density gradient medium underneath the blood to obtain a sharp interface. A quick examination of the PBMC ring and the gradient after centrifugation can reveal possible contamination, activation, and low yields. When working with a leukapheresis membrane or buffy coat, diluting the blood is important as excessive cell density would lead to cell aggregation, leading to impurities and cell activation.

This protocol should be completed within 2 h to ensure cell freshness, and steps involving the density gradient medium, dextran, and lysis be done immediately, as exposure to these solutions can alter neutrophils. With this protocol, the expected yield of neutrophils is at least ~10 million/10 mL of whole blood and at least ~60 million/10 mL of buffy coat. Evaluation of isolation quality should be done as follows: activated neutrophils should be less than 10%, lymphocyte contamination lower than 5%, minimal eosinophil (same side scatter but lower forward scatter population), and cell viability should be over 90%. Lower purity can result from improper layering or storage of the density gradient medium or due to the quality and freshness of the starting blood product.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors declare that the research was conducted in the absence of any conflict of interest.

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Figure1

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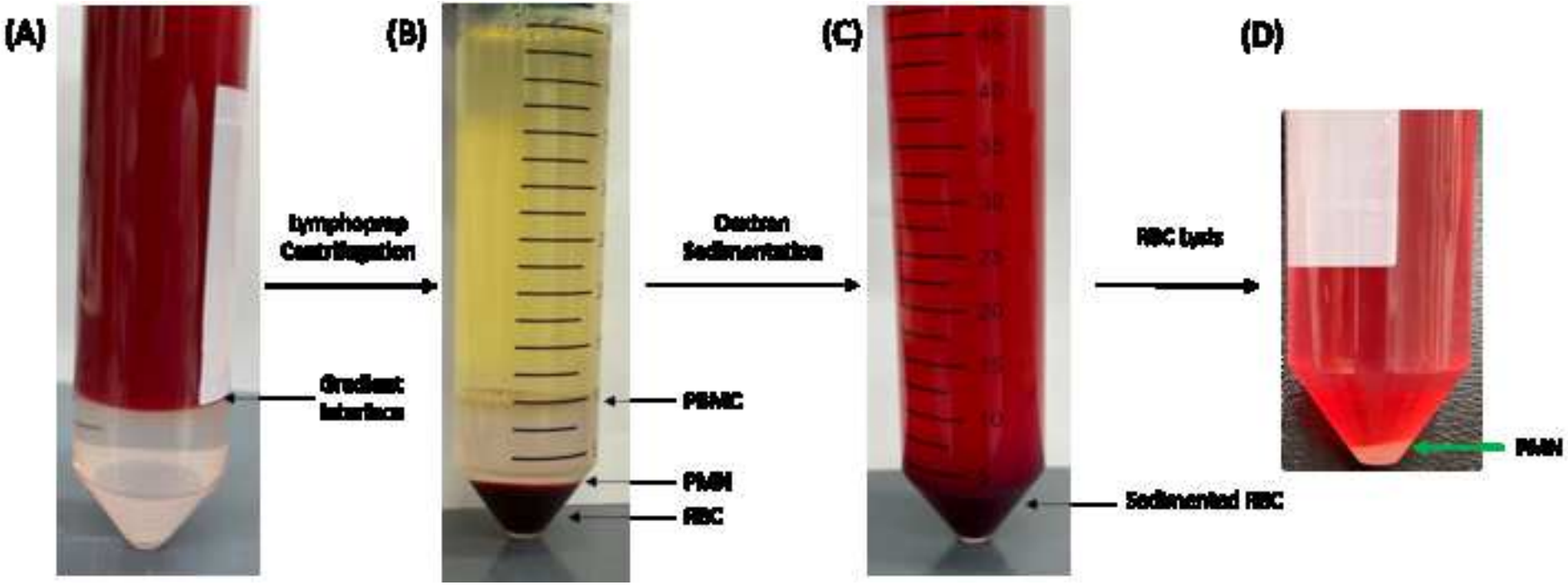
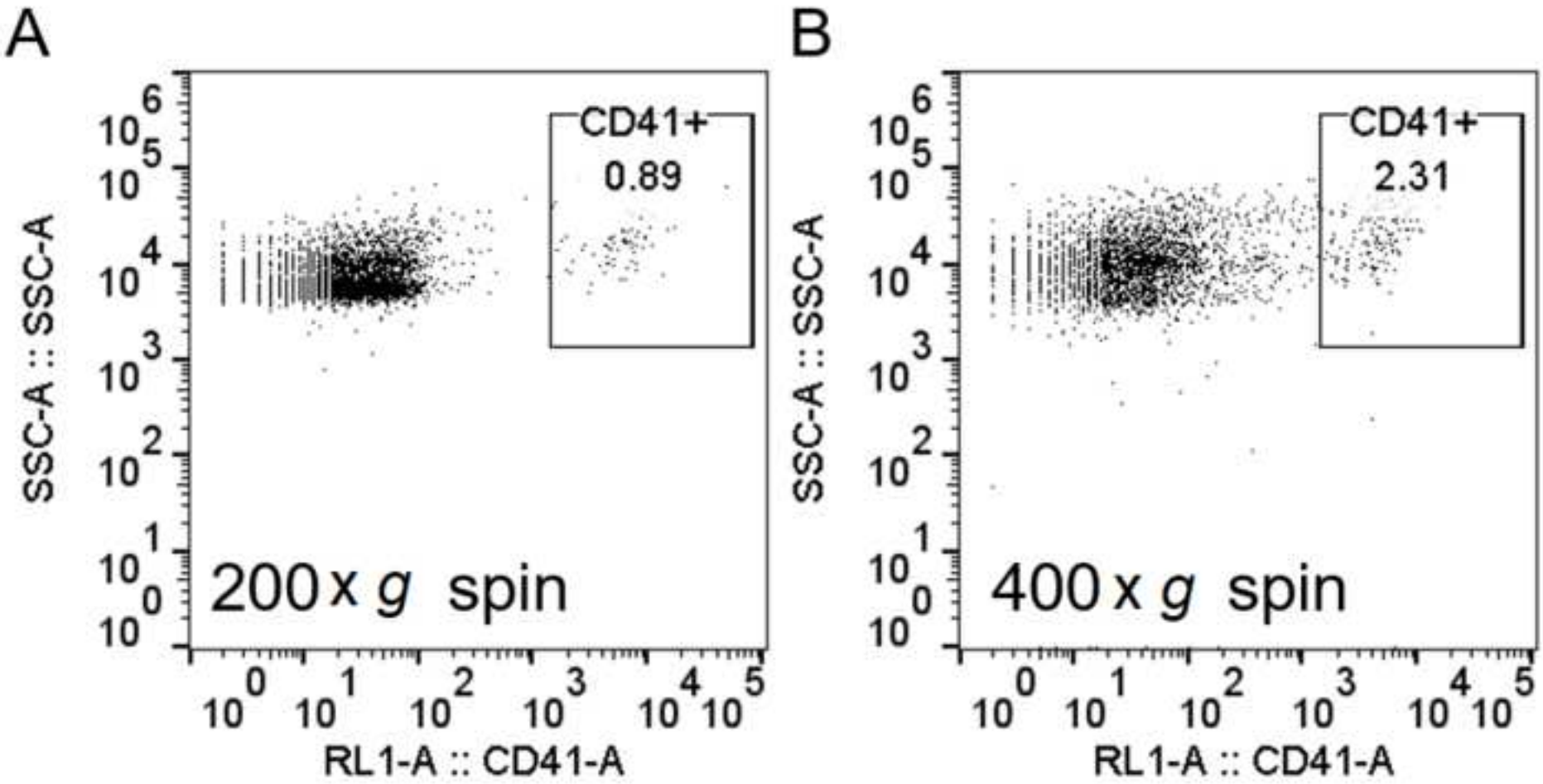


Figure 2



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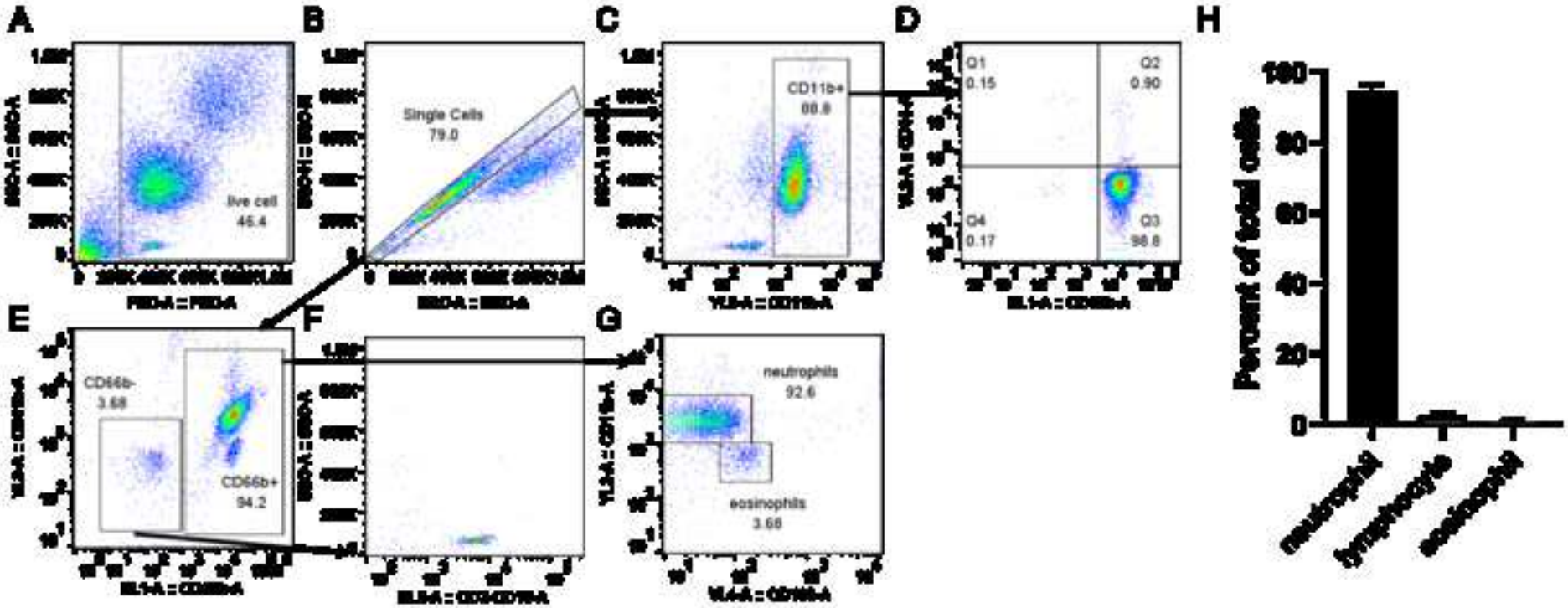

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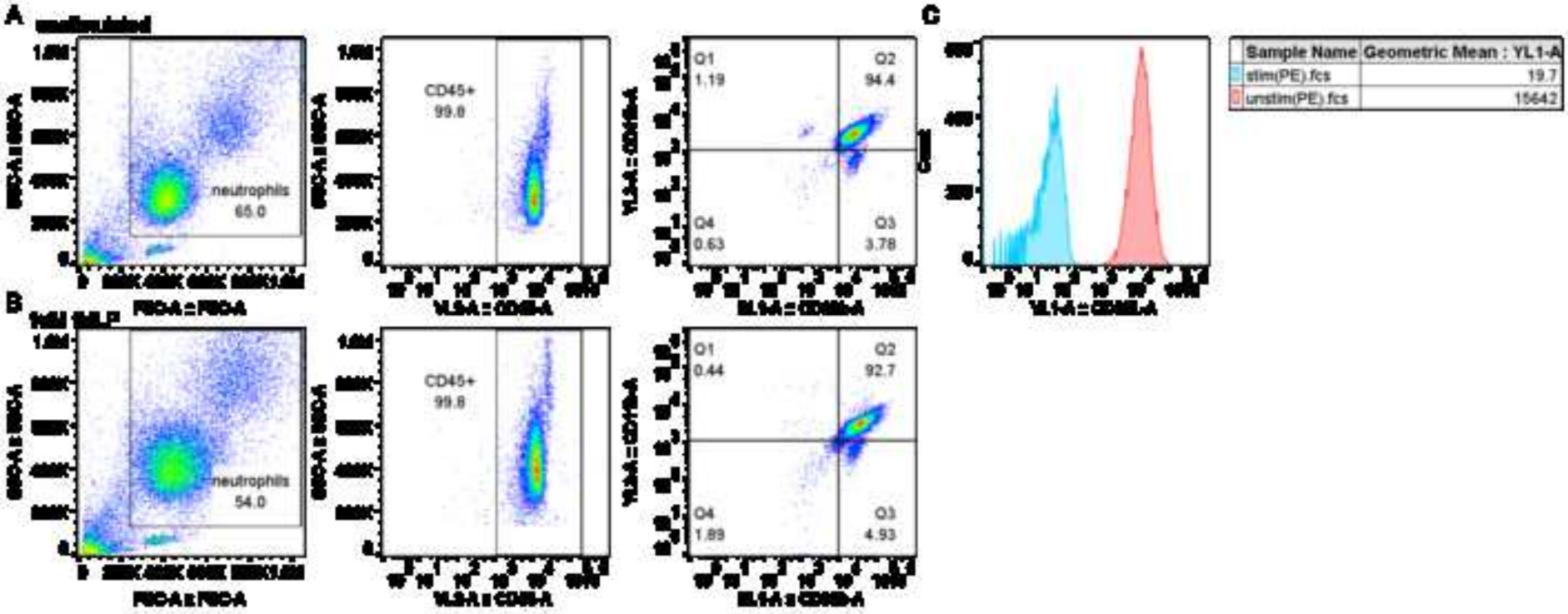
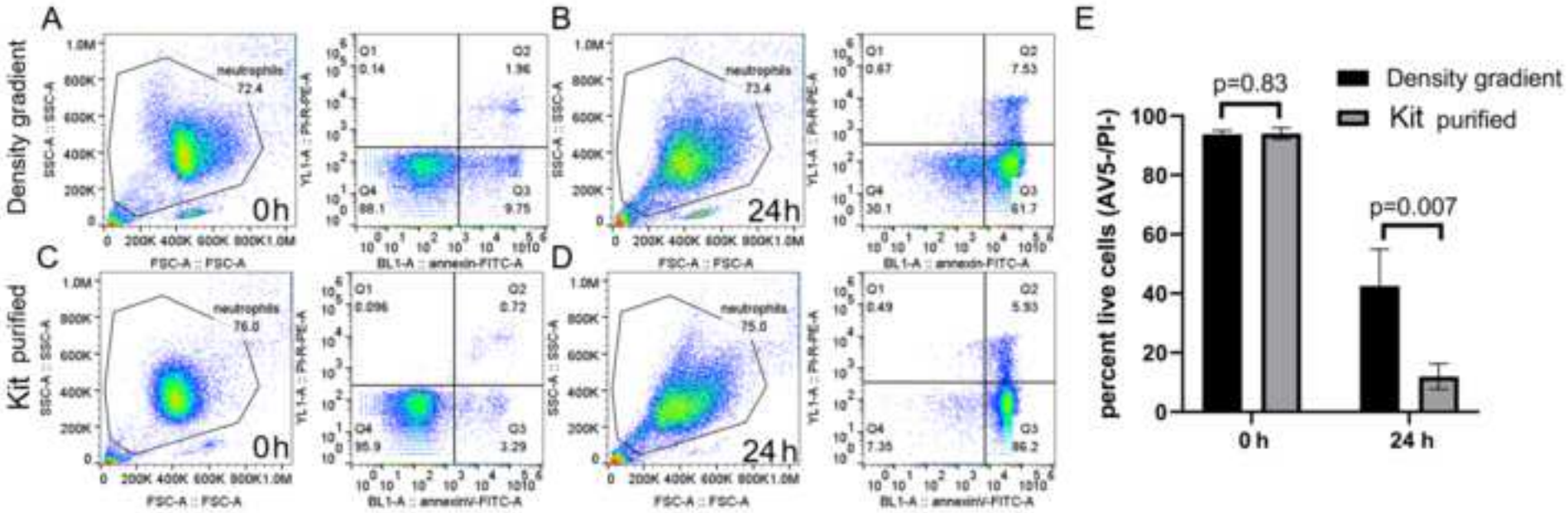


Figure 5



Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.
2. Please provide an email address for each author.
3. Please remove the citation from the abstract. Citation of references should start only from the introduction onwards.
4. Whose blood are you using here? Was approval required to conduct these experiments? Please provide details about the human volunteers or source of human blood (if from a blood drive or bank) before the numbered steps. Please include an ethics statement before all of the numbered protocol steps indicating that the protocol follows the relevant guidelines of your institution.

5. Please revise the text, especially in the protocol, to avoid the use of **any personal pronouns (e.g., "we", "you", "our" etc.).**

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., **"Do this," "Ensure that**," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "**could be," "should be," and "would be" throughout the Protocol**. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

7. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

8. After including a one line space between each protocol step, highlight up to 3 pages of protocol text for inclusion in the protocol section of the video. This will clarify what needs to be filmed.

9. Please add limitations of your technique to your discussion.

We have added discussion regarding the importance to use highly purify cells for gene and protein expression experiment.

10. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source (ITALICS). Volume (BOLD) (Issue), FirstPage–LastPage (YEAR).] For 6 and more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references, and do not abbreviate the journal names.

We have used the endnote style "JOVE".

Reviewers' comments:

Reviewer #1:

Dr. Luo and co-authors presented a standard protocol of isolating neutrophils from fresh blood using density gradient centrifugation, RBC sedimentation, and RBC lysis. The protocol is straightforward. Here is some minor points below:

1. Figure 2, are eosinophils considered? I think they also express both CD66b and CD11b.

Thanks for the useful suggestion. We have added the staining (CD193) we routinely use to determine the percentage of eosinophil contamination in a revised version of figure 3.

2. Figure 2A and 3A, the gating is not live cells, but leukocytes, I believe. You don't know whether they are live or dead showing viability staining.

Thanks for highlighting the mislabeled graphics. Mention of "live cells" has been removed from figure 3. In the revised version of the manuscript, we have added the assay we routinely perform to measure cell survival immediately after cell isolation. These results are shown in figure 5.

3. Figure 3B, the upon-right corner is doublets I believe, not activation. Activated neutrophils will degranulate, which do not have a big change in ssc but a decrease in fsc. So that will be a left shift. You can confirm this by stimulation.

Thanks for highlighting the misleading labeling. Figure 3 has been modified and the mention of cell activation has been removed.

4. A positive control is needed for the AnnexinV/PI staining, such as cells 1 day after isolation, or treated with some toxin. Also a negative control is missing, such as stained whole blood with RBC lysis after staining.

We have added the assay we perform to measure cell survival immediately after cell isolation. These results are shown in figure 5. Of note, the cell survival measured by flow cytometry immediately after isolation is similar using the protocol described here or using a commercially available negative selection kit. In both cases, cell survival is >= at 90%.

5. At least 3 repeats are needed for each experiments and show a statistics, such as purity mean±SD, live/dead mean±SD.

We thank the reviewer for this important remark. We have added quantification and statistical analysis in figure 3 and 5.

Reviewer #2:

Manuscript Summary:

The authors describe studies for isolating neutrophils from human blood or buffy coats.

Major Concerns:

There are a number of methods publications about how to isolate neutrophils. None of these are referenced even though the methods are essentially the same. For example, see Nauseef, W.M. (2014) Methods in Molecular Biology 1124: 13-18 or Siemsen, D.W., et al. Methods in Molecular Biology 1124: 19-37. How do yields and purity compare with these methods or others already published?

Our method is a revised version of previously published methods, based on the references the reviewer kindly highlighted. These references will be cited, as they should have been in the initial version of this manuscript. Thanks for pointing this important omission.

Nauseef, W.M., 2014. Isolation of Human Neutrophils from Venous Blood. , in: Quinn, M.T., DeLeo, F.R. (Eds.), Neutrophil Methods and Protocols, Methods in Molecular Biology. Humana Press, Totowa, NJ, pp. 13–18. https://doi.org/10.1007/978-1-62703-845-4_2

Siemsen, D.W., Malachowa, N., Schepetkin, I.A., Whitney, A.R., Kirpotina, L.N., Lei, B., Deleo, F.R., Quinn, M.T., 2014. Neutrophil isolation from nonhuman species. Methods Mol Biol 1124, 19–37. https://doi.org/10.1007/978-1-62703-845-4_3

We feel that our protocol brings important updates to previously published methods.

One is that yield is better when RBC sedimentation was performed after centrifugation. We believe that it is due to the activation of monocytes by dextran, as it has been reported by

others. However, we believe exploring the mechanisms are beyond the scope of this revise method.

The cited references do not provide a detail gating strategy and adequate markers for the precise identification of neutrophils and contaminating cells. The yield and purity reported in these studies are based on cell counting using an hemacytometer, which is not has accurate as flow cytometry. These works used veinous whole blood. Our results are comparable to the one reported in the works in term of purity, but yields cannot be compared as buffy coats were used for the present work.

Minor Concerns:

1) There are a number of typographical errors and grammatical errors.

2) Figure 1 is very poor quality and the text is not readable and the images don't show the layers very well. Better photos of the gradients are needed. **Use of a backlight and white background would help.**

Thanks for the useful comment. We have improved our photos by using a better camera and a dark background (Fig 1B and 1D).

Reviewer #3:
This is a very interesting and important manuscript about neutrophil isolation with few activated cells. However, some aspects need to be taken into consideration.

Manuscript Summary:
The abstract must be rewritten in a better way to avoid redundancy. There is a number 1 with no connection. Please, it is not elegantly written in the abstract to the readers to perform the assay in a correct form.

Line 55-56: please rewritten this sentence.

We have rewritten the sentence.

Major Concerns:
The authors mentioned that the used protocol and with "**low-speed centrifugation" is able "to remove platelet contamination"**. However, this approach was not confirmed like using a platelet cell marker to show this aspect and to confirm the affirmative sentence. The

same observation can be conducted when the authors mentioned a few numbers of other polymorphonuclear cells like eosinophils. It is important to use an eosinophil cell marker to show this aspect.

We thank the reviewer for the useful suggestions and comments.

Low speed centrifugation is commonly used to prepare platelet rich plasma and as been previously described, as for example in the following publications:

Dhurat, R., Sukesh, M., 2014. Principles and Methods of Preparation of Platelet-Rich Plasma: A Review and Author's Perspective. J Cutan Aesthet Surg 7, 189–197. https://doi.org/10.4103/0974-2077.150734
Etulain, J., Mena, H.A., Meiss, R.P., Frechtel, G., Gutt, S., Negrotto, S., Schattner, M., 2018. An optimised protocol for platelet-rich plasma preparation to improve its angiogenic and regenerative properties. Sci Rep 8, 1513. https://doi.org/10.1038/s41598-018-19419-6

These references are now included in the protocol. We have also included figure 2, showing the difference of platelet contamination between low speed and normal speed centrifugation.

We have also included the staining of eosinophils we commonly use to assess neutrophil purity in our flow cytometry antibody panel in figure 3.

Both are important observations when we are trying to show the methodology used and standardized are good enough as mentioned: "a neutrophil preparation of high purity with minimal activation when performed correctly".

Another point observed this protocol was conducted first of all with Lymphoprep. Did the authors try to used Hystopaque?

Histopaque-1077 and Lymphoprep are both commercial solutions of polysaccharides and sodium diatrizoate with a density of 1.077 g/ml. Similar results can be obtained using either solutions. This is now mentioned in the main protocol (line 95).

Where is the panel with the activation marker (CD62L) (figure 2)? The plots need to be better described in the results section.

We apologize for the omission. Figure 4 shows the level of CD62L in neutrophils freshly isolated before or after stimulation with the positive control fMLP.

What was the flow cytometer used? What was the gate strategy? This can be a problem for the reproduction of the protocol "in a correct form" as mentioned in summary.

We have improved the description of the gating strategies for all flow cytometry figures (Figure 2, 3,4 and 5), as well as have indicated the equipment used for the flow cytometry sample acquisition.

Line 228-229: The sentence should be moved to the beginning of the Discussion. Here is a disconnected sentence.

Minor Concerns:
The photographs are of bad quality and with different backgrounds. Please, try to use white paper behind the tube for a better photograph.

Thanks for the suggestion. We have improved our photos by using a better camera and a dark background (Fig 1B and 1D).

Reviewer #4:
The manuscript by Hsu and colleagues describes a classical method to isolate human granulocytes from buffy coats or blood, i.e., based on density gradient centrifugation followed by red blood cells (RBC) sedimentation and RBC osmotic lysis. The various steps listed for the procedure are clearly explained, with crucial issues well highlighted along the protocol. The major concern about this manuscript is that, by quickly searching for methods for neutrophil isolation already published in JOVE, I found at least three studies describing similar protocols (doi: 10.3791/745; doi: 10.3791/53846; doi: 10.3791/52687) which were not even discussed by the authors.

Our method is a revised version of previously published methods, based on the references the reviewer kindly highlighted. These references will be cited, as they should have been in the initial version of this manuscript. Thanks for pointing this important omission.

Nauseef, W.M., 2014. Isolation of Human Neutrophils from Venous Blood. , in: Quinn, M.T., DeLeo, F.R. (Eds.), Neutrophil Methods and Protocols, Methods in Molecular Biology. Humana Press, Totowa, NJ, pp. 13–18. https://doi.org/10.1007/978-1-62703-845-4_2

Siemsen, D.W., Malachowa, N., Schepetkin, I.A., Whitney, A.R., Kirpotina, L.N., Lei, B., Deleo, F.R., Quinn, M.T., 2014. Neutrophil isolation from nonhuman species. Methods Mol Biol 1124, 19–37. https://doi.org/10.1007/978-1-62703-845-4_3

We feel that our protocol brings important updates to previously published methods.

One is that yield is better when RBC sedimentation was performed after centrifugation. We believe that it is due to the activation of monocytes by dextran, as it has been reported by others. However, we believe exploring the mechanisms are beyond the scope of this revise method.

The cited references do not provide a detail gating strategy and adequate markers for the precise identification of neutrophils and contaminating cells by flow cytometry. The yield and purity reported in these studies are based on cell counting using an hemacytometer, which is not has accurate as flow cytometry. These works used veinous whole blood. Our results are comparable to the one reported in the works in term of purity, but yields cannot be compared as buffy coats were used for the present work.

In addition, there are some issues:
- The title is misleading. The current protocol allows the isolation of total granulocytes (neutrophils and eosinophils) and is not specific for neutrophils. The title and the text should be changed accordingly.

We believe that the title is appropriate as this type of approach has been described widely in the literature as a proper neutrophil isolation methods, and as contamination by eosinophils is low, as illustrated in figure 3.

- Authors should specify that, for gene expression and/or protein production studies, their method does not guarantee a sufficient grade of neutrophil purity.

Discussion has been modified to highlight the necessity to use ultra-pure neutrophils in gene and protein expression studies. We also discussed to effect of antibody-based isolation methods on neutrophil survival, and possibly on other cell functions (line 243-246 and 249-250).

- The assessment of neutrophil purity is not very accurate and must be implemented. It is essential to extend the antibody panel for flow cytometry analysis proposed by the authors, to also include recognition of monocytes and various DCs, which, together with lymphocytes and eosinophils, could contaminate granulocyte isolation.

We appreciate the suggestions of the reviewer. We have included the panel of antibody we use to assess cell purity after each isolation. As show in figure 3, most contaminating cells are eosinophils and lymphocytes. Monocyte contamination is usually very low. We do not

believe it is necessary to include markers for the detection of dendritic cells, as they are even scarcer than the other cell populations in the peripheral blood of healthy donors.

Hasskamp, J.H., Zapas, J.L., Elias, E.G., 2005. Dendritic cell counts in the peripheral blood of healthy adults. American Journal of Hematology 78, 314–315. https://doi.org/10.1002/ajh.20296

Orsini, G., Legitimo, A., Failli, A., Massei, F., Biver, P., Consolini, R., 2012. Enumeration of human peripheral blood dendritic cells throughout the life. International Immunology 24, 347–356. https://doi.org/10.1093/intimm/dxs006

- On line 147, authors correctly suggest to use CD62L to measure neutrophil activation. A flow cytometry plot of CD62L expression in resting and activated neutrophils should be shown.

Thanks for the recommendation. We have included figure 4, that shows the expression level of CD62L on freshly isolated neutrophils or after activation using fMLP.

- On lines 204-206, authors state that, in their hands, neutrophil purification by negative selection has been shown to accelerate neutrophil death and activation. Authors should support this statement adding a reference or showing related data.

This is correct, a quantification of cell survival following isolation by both methods should have been included. The results of this experiments are now presented in figure 5.

- Similarly, on lines 136-137, authors state that cells should be kept at a concentration of ~2million cells/ml, as higher density leads to increased cell activation/death. Also in this case, authors should support their statements by adding a reference or showing related results.

A reference reporting the effect of cell density on neutrophil survival has been added (line 168).