Journal of Visualized Experiments

Development and identification of a novel subpopulation of human neutrophil-derived giant phagocytes in-vitro

--Manuscript Draft--

Manuscript Number:	JoVE54826R3
Full Title:	Development and identification of a novel subpopulation of human neutrophil-derived giant phagocytes in-vitro
Article Type:	Invited Methods Article - JoVE Produced Video
Keywords:	Neutrophil-derived giant phagocytes; human neutrophils; cluster of differentiation (CD)66b; microtubule-associated protein-1 light chain 3B (LC3B); autophagocytosis; oxidized low density lipoprotein (oxLDL); scavenger receptors; confocal microscopy
Manuscript Classifications:	1.11.118: Blood Cells; 1.11.118.637.415.583: Neutrophils; 1.11.733: Phagocytes; 1.11.733.689: Neutrophils; 1.15.382: Immune System; 1.15.382.490.315.583: Neutrophils; 1.15.382.680.689: Neutrophils
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Abstract:	Neutrophils (PMN) are best known for their phagocytic functions against invading pathogens and microorganisms. They have the shortest half-life amongst leukocytes and in their non-activated state are constitutively committed to apoptosis. When recruited to inflammatory sites to resolve inflammation, they produce an array of cytotoxic molecules with potent microbial killing. Yet, when these powerful cytotoxic molecules are released in an uncontrolled manner they can damage surrounding tissues. In recent years however, neutrophil versatility is increasingly evidenced, by demonstrating plasticity and immunoregulatory functions. We have recently identified a new neutrophil-derived subpopulation which develops spontaneously in standard culture conditions without the addition of cytokines/growth factors such as granulocyte colony-stimulating factor (GM-CSF)/interleukin (IL)-4. Their phagocytic abilities of neutrophil remnants largely contribute to increase their size immensely; therefore they were termed giant phagocytes (GΦ). Unlike neutrophils, GΦ are long lived in culture. They express the cluster of differentiation (CD) neutrophil markers CD66b/CD63/CD15/CD11b/myeloperoxidase (MPO)/neutrophil elastase (NE), and are devoid of the monocytic lineage markers CD14/CD16/CD163 and the dendritic CD1c/CD141 markers. They also take-up latex and zymosan, and respond by oxidative burst to stimulation with opsonized-zymosan and PMA. GΦ also express the scavenger receptors CD68/CD36, and unlike neutrophils, internalize oxidized-low

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Author Comments:	no comments
Additional Information:	
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Dear Editor,

We have corrected the manuscript according to the Editor's comments as marked in RED and add a clean manuscript as well. We hope that you will find this revised version suitable for publication in the JoVE.

Sincerely

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TITLE:
Development and identification of a novel subpopulation of human neutrophil-derived giant phagocytes *in-vitro*

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KEY WORDS:
Neutrophil-derived giant phagocytes; human neutrophils; cluster of differentiation (CD)66b; microtubule-associated protein-1 light chain 3B (LC3B); autophagocytosis; oxidized low density lipoprotein (oxLDL); scavenger receptors; confocal microscopy

SHORT ABSTRACT:
We describe here a method for obtaining and identifying a newly characterized subpopulation of neutrophil-derived giant phagocytes. These cells develop in culture from fresh human blood neutrophils, and are characterized by phagocytosis, autophagy, immensely large size, and extended lifespan. This method is essential to further investigate this unique neutrophil-derived subpopulation.

LONG ABSTRACT:
Neutrophils (PMN) are best known for their phagocytic functions against invading pathogens and microorganisms. They have the shortest half-life amongst leukocytes and in their non-activated state are constitutively committed to apoptosis. When recruited to inflammatory sites to resolve inflammation, they produce an array of cytotoxic molecules with potent microbial killing. Yet, when these powerful cytotoxic molecules are released in an uncontrolled manner they can damage surrounding tissues. In recent years however, neutrophil versatility is increasingly evidenced, by demonstrating plasticity and immunoregulatory functions. We have recently identified a new neutrophil-derived subpopulation, which develops spontaneously in standard culture conditions without the addition of cytokines/growth factors such as granulocyte colony-stimulating factor (GM-CSF)/interleukin (IL)-4. Their phagocytic abilities of neutrophil remnants largely contribute to increase their size immensely; therefore they were termed giant phagocytes (Gϕ). Unlike neutrophils, Gϕ are long lived in culture. They express the cluster of differentiation (CD) neutrophil markers CD66b/CD63/CD15/CD11b/myeloperoxidase (MPO)/neutrophil elastase (NE), and are devoid of the monocytic lineage markers CD14/CD16/CD163 and the dendritic CD1c/CD141 markers. They also take-up latex and zymosan, and respond by oxidative burst to stimulation with opsonized-zymosan and PMA. Gϕ also express the scavenger receptors CD68/CD36, and unlike neutrophils, internalize oxidized-low density lipoprotein (oxLDL). Moreover, unlike fresh neutrophils, or cultured monocytes, they respond to oxLDL uptake by increased reactive oxygen species (ROS) production. Additionally, these phagocytes contain microtubule-associated protein-1 light chain 3B (LC3B) coated vacuoles, indicating the activation of autophagy. Using specific inhibitors it is evident that both phagocytosis and autophagy are prerequisites for their development and likely NADPH oxidase dependent ROS. We describe here a method for the preparation of this new subpopulation of long-lived, neutrophil-derived phagocytic cells in culture, their identification and their currently known characteristics. This protocol is essential for obtaining and characterizing Gϕ in order to further investigate their significance and functions.

INTRODUCTION:
Polymorphonuclear neutrophils (PMN) constitute the largest population of leukocytes in the blood, serving as the first line of defense against invading pathogens by producing a wide range of cytotoxic molecules. The traditional view has long been that of blood circulating, short lived,

professional phagocytes, which are the first to arrive to acute inflammatory sites to combat infections and aid in the clearance of pathogens and harmful particles.[1] In their non-activated state, neutrophils are constitutively committed to apoptosis. When migrating from the blood to inflammatory sites, neutrophils undergo activation to resolve inflammation. They phagocytose and kill invading microorganisms, by producing an array of cytotoxic molecules as reactive oxygen species (ROS), lytic enzymes such as neutrophil elastase (NE) and cathepsins with potent microbial activity. In order to trap pathogens, neutrophils also release extracellular traps (NETs) which consist of nuclear chromatin threads containing antibacterial peptides and various lytic enzymes. However, uncontrolled release of these cytotoxic molecules from neutrophils may also perpetuate inflammatory responses and induce damage to surrounding tissues.[2] Therefore, an effective clearance of apoptotic neutrophils by macrophages (Mϕ) and dendritic cells (DC) is crucial to resolve inflammation.[3-6]

In recent years however, it has become increasingly evident that neutrophils are highly versatile cells, whose functions go far beyond phagocytosis and pathogen killing.[6,7] By undergoing priming or activation, neutrophil plasticity is gradually gaining attention. For instance, bacteria and mycobacteria challenged neutrophils were shown to secrete interleukin (IL)-10 and control the inflammatory response, suggesting the presence of immuno-regulatory responses.[8] Post-mitotic neutrophils were shown to trans-differentiate into Mϕ-like cells, or DC-like cells by digesting and presenting antigen fragments when treated with cytokines and growth factors, [9,10] thus, serving a critical role in integrating innate and adaptive responses.[3,6] Activation by growth factors promoted engulfment of apoptotic neutrophils or cell debris, thereby, facilitating clearance of debris at inflammatory sites and the resolution of inflammation, [3,9] particularly when the Mϕ/DC clearance system is insufficient or overwhelmed, [11,12] suggesting potential 'self-regulation' to help resolve the inflammatory response. This, since apoptosis is a form of regulated self-death which can inhibit the extracellular release of cytotoxic compounds and thus prevent injury to surrounding tissues.[6]

Prolonged survival is another feature of neutrophil activation and was demonstrated by treatment with various host derived factors such as granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), inflammatory cytokines such as interferon (IFN)-γ, tumor necrosis factor (TNF)-α and/or pathogen derived products, thus, allowing neutrophils to modulate their survival response.[6] In fact, neutrophil survival is a prerequisite for its plasticity and was associated with its ability to perform phagocytosis.[6,13] Accordingly, it was also shown to associate with phenotypic and functional changes which depended on upregulated gene expression by inducing the synthesis of new proteins involved in neutrophil lifespan extension, and diminished apoptosis.[10]

Unlike neutrophils which are short lived and constitutively undergo apoptosis in culture, or the cytokines/growth factors-activated neutrophils, described above, which have extended life span, we have recently identified a new, small subpopulation of neutrophils that develops spontaneously in prolonged standard culture conditions from freshly isolated human blood neutrophils without externally adding cytokines or growth factors.[14] These neutrophil-derived cells, which were not described before in the literature were termed giant phagocytes (Gϕ). The Gϕ have extended lifespan in culture, they are fully developed within 5-7 days, and are characterized by unique morphological features, phenotypic expression and functions. They are

vastly enlarged due to autophagocytosis of dead neutrophil remnants, vacuolated, and contain phagolysosomes. The Gϕ express the specific neutrophil granules marker - cluster of differentiation (CD)66b, the azurophilic granules markers - CD63 and myeloperoxidase (MPO) and additional neutrophils markers such as CD11b, NE, CD15, the NADPH oxidase subunits gp91-*phox* and p22-*phox,* and the autophagy marker -LC3BII.[14,15] Functionally, they actively take-up latex beads and zymosan particles, and generate ROS in response to zymosan and phorbol 12-myristate 13-acetate (PMA) stimulation. Interestingly, unlike fresh neutrophils, Gϕ also intensively express the scavenger receptors CD68 and CD36, take-up oxidized low density lipoprotein (oxLDL), and generate ROS in response to stimulation with oxLDL. Additionally, Gϕ are devoid of the monocytic lineage markers CD14, CD16 and CD163 or the dendritic markers CD1c and CD141. Moreover, phagocytosis and autophagy and likely functional NADPH oxidase are prerequisites for their development. This since, the phagocytosis-inhibitor cytochalsin B, the autophagy inhibitors 3-methyladenine (3-MA) and bafilomycin (BafA1) and the NADPH oxidase inhibitor – diphenylene iodonium (DPI) - prevented their development. Additionally, monocytes/neutrophils co-cultures as well as exposure to intermittent hypoxia hampered their development, whereas neutrophil adaptation to sustained hypoxia was evident.[14,15] Their suggested development in culture is illustrated in Figure 1.The protocol in the present paper describes step by step the preparation of Gϕ from freshly isolated circulating human blood neutrophils, their development, identification and some basic characteristics. This protocol can be used to further investigate and reveal the broad spectrum and the roles of these newly described and intriguing neutrophil-derived Gϕ in order to characterize their significance and their potential functions.

[Place figure 1 here]

PROTOCOL:

The protocol was approved by the local Human Rights Committee according to the declaration of Helsinki, and all participants signed an informed consent form.

1. Neutrophil isolation and development of Gϕ in culture

Note: All steps should be performed using sterile tissue grade lipopolysaccaride (LPS)-free solutions in a Bio-Safety Laminar flow hood. Do not add antibiotics cytokines or growth factors to the Roswell park memorial institute (RPMI)-1640 medium.

1.1. Obtain at least 40 ml venous blood from young healthy adults using a sterile scalp vein set. Draw blood into vacutainer tubes containing ethylenediamine tetra acetic acid K_3 salt (K3EDTA) and mix gently. Keep the blood at room temperature.

1.2. Isolate the neutrophils by two step discontinuous density gradient using polysucrose at 1.119 and 1.077 g/ml. Bring solutions to room temperature before using.

Note: During centrifugation, red blood cells (RBCs) are aggregated by the polysucrose and sediment rapidly. The mononuclear cells (monocytes/lymphocytes) are found between the upper plasma/polysucrose -1077 interface, whereas the neutrophils are found just above the RBCs, at

the polysucrose -1077/1119 interface (see Figure 2). This method allows simultaneous separation of mononuclear cells and neutrophils from the same individual.

[Place figure 2 here].

1.2.1. Add 12 ml polysucrose-1119 to the bottom of a 50 ml sterile polypropylene conical centrifuge tube.

1.2.2. Carefully layer 12 ml of polysucrose-1077 onto the polysucrose -1119.

1.2.3. Dilute 10-12 ml whole blood to a final volume of 24 ml blood with ion free phosphate buffered saline (PBS) containing 2 % heat inactivated fetal calf serum (HI-FCS). Carefully layer 24 ml of the diluted whole blood onto the upper gradient of the tube.

1.2.4. Centrifuge at 700 x g for 30 min at room temperature (20-24 °C) without brake.

Note: Centrifugation at lower temperatures may result in cell clumping and poor recovery.

1.2.5. Carefully remove the tubes from the centrifuge without disturbing the gradient. Two opaque layers should be observed (A - Mononuclear cells and B – PMN, depicted in figure 2).

1.2.6. Aspirate and discard the fluid up to 0.5 cm above layer A. Transfer (or discard) the cells from this layer to a tube marked “Mononuclear”.

1.2.7. Aspirate and discard the remaining fluid up to 0.5 cm above layer B. Transfer the cells from this layer to a tube labeled “PMN”.

1.2.8. Pool PMN from each two gradient tubes and wash with PBS containing 2 % HI-FCS to a final volume of 30 ml. Centrifuge for 12 min at 200 x g, remove the supernatant and discard.

1.2.9. To get rid of contaminating red blood cells (RBC), add 3 ml of hypotonic 0.2 % ice cold sterile NaCl while resuspending the pellet by gently drawing in and out with a 1 ml sterile pipet tip. Keep on ice for 30 s.

1.2.10. After 30 s, restore isotonicity by adding 3 ml of sterile 1.6 % ice cold NaCl to the tube.

1.2.11. To the 6 ml of isotonic saline, add 6 ml of pre-warmed (37 °C) RPMI-1640 medium supplemented with 2 % HI-FCS and centrifuge at 250 X g for 12 min. Discard the supernatant. The PMN pellet should be clean of RBC contamination.

Note: If contaminated by RBC, the PMN pellet appears reddish.

1.2.11.1. If some contaminating RBC remain, repeat steps 1.2.9 and 1.2.10 once more.

1.2.12. Resuspend the cell pellet in 4 ml RPMI-1640 supplemented with 10 % HI-FCS and count the cells to determine their concentration and viability by trypan blue exclusion.

1.2.13. Adjust the concentration to 1.25 – 1.5 X 10^6 PMN/ml (depending on the experimental needs), and plate 1.0 ml/well in a 24 well plate.

Note: The purity of neutrophils in the granulocyte population always exceeded 95 %, as assessed by May Grunewald-Giemsa staining and light microscopy.

1.2.14. After seeding, place the cells in a humidified 5 % CO_2 incubator at 37 °C.

1.2.15. Replace medium every 3 days by gently aspirating half of the medium and adding the same volume of fresh RPMI-1640 medium supplemented with 10 % HI-FCS. Use LPS free solutions and compounds and low LPS levels in HI-FCS (0.05 ng/ml or less).

Note: A gentle medium change is imperative since the Gϕ, which develop in culture do not firmly attach to the culture dish and vigorous washing may also wash out the developing cells. The appearance of Gϕ is noticeable at 3-4 days after PMN culturing, depending on the blood donor. Most of the analyses and assays described here are performed between 6-7 days in culture, when Gϕ are very large in size. It should be noted that addition of 1-10 ng/ml LPS to the RPMI-1640 medium did not affect Gϕ development in culture.[14]

2. Confocal Laser Scanning Microscopy

2.1. Prepare cytospins[16] from freshly isolated neutrophils, and from the 7 day developed Gϕ cultures (prepared in section 1).

Note: To increase the concentration of Gϕ in the dish for various analyses, gently remove half of the medium. Make sure that Gϕ are not detected in the removed medium by examining the medium under a light microscope. Then, intensively pipet the remaining medium to remove lightly adhered Gϕ. Centrifuge the medium for 10 min at 200 x g, and resuspend the pellet in 100-120 µl medium.

2.1.1 Use 100-120 µl of the medium containing cells for each slide. Prepare duplicate or triplicate slides from each treatment. Spin for 7 min at 84 x g.

2.2. Fix the cells with 4 % paraformaldehyde at room temperature for 10 min under a chemical hood. Wash 3X with PBS (~100 µl for a few seconds per wash). For intracellular staining, permeabilize cells with 0.5 % Triton X-100 in PBS at room temperature for 10 min and wash 5 X with PBS.

Note: At all stages, use appropriate buffer/solution volume to cover the perimeter of the cells on the slide.

Caution: Paraformaldehyde is toxic. Avoid contact with skin and eyes. Wear appropriate personal protective equipment.

2.3. Block cells with 10 % normal goat serum in RPMI-1640 medium and incubate overnight at 4 ºC or at room temperature for 40 min. Wash with PBS.

2.4. Incubate with single antibody (Ab) or a combination of mouse and rabbit primary antibodies (Abs) at a 1:100 dilution (~100 µl). Incubate overnight (18-20 h) at 4 °C.

Note: Here, mouse monoclonal Abs included: anti-CD14, anti-CD63, anti-CD66b, anti-CD1c, anti- CD15, and anti-Cytochrome b-245 light chain (p22-*phox* identification). Rabbit polyclonal Abs included: anti-CD68, anti-CD36, anti-LC3B, anti-Myeloperoxidase, anti-neutrophil elastase (NE), and anti-Nox2/gp91-*phox* Abs. Isotype controls included purified mouse IgG1 and IgG2, and rabbit IgG. Prepare the Abs according to manufacturer's instructions and use appropriate volume (about 100 µl) to cover the perimeter of the cells.

2.5. Wash the cells and incubate with 1/400 secondary antibodies Cy2-CF (488A)-conjugated goat anti-rabbit IgG (green) and/or Cy5 (CF 647)-conjugated goat anti-mouse IgG (red) at room temperature for 40 min.

Note: Dilute and prepare Abs according to manufacturer's instructions.

2.6. After washing, mount slides with one drop of mounting medium, containing 4',6-diamidino-2-phenylindole (DAPI) for nuclear staining, then immediately place the cover slip.

2.7. Analyze the slides by a confocal laser scanning system using fluorescence microscope and Plan Apo x 40 immersion oil objective. Perform the analysis within 30 min to 2 h after preparation of the slides or keep at 4 °C overnight.

2.7.1. Calculate the cell area and fluorescence intensity using an imaging software (e.g. Image J). For co-localization, quantify by software using Manders Overlap Coefficient (MOC)[17].

Note: Only cells with MOC > 0.6 can be considered as cells with significant co-localization.

3. Transmigration of PMN across endothelial cells: effects of IL-8 on giant phagocyte (Gϕ) formation

Note: Use 24-well permeable cell culture inserts for the cell transmigration assay.

3.1 Coat the upper chamber of the insert with 150 µl fibronectin at a concentration of 50 µg/ml, and keep at room temperature for 30 min.

3.2 Add to the upper chamber 5×10^4 EA.hy926 endothelial cells/well, resuspended in 150 µl of formulated Dulbecco's Modified Eagle's Medium (complete growth medium).

Note: Ensure that the endothelial monolayer is confluent before use.

3.3 To the lower chamber, add 700 µl of the complete growth medium.

3.4 Place the permeable cell culture inserts in cluster trays and culture the EA.hy926 endothelial cells for 2 days at 37° C in 5% CO_2.

Note: In parallel, on the second day prepare fresh PMN (as described in section 1).

3.5 After 2 days, replace the medium in the lower and upper chambers of the inserts.

3.5.1 To the lower chamber, add RPMI-1640 medium supplemented with 10% IH-FCS and interleukin (IL)-8 at a final concentration of 50 nM/ml. Do not add IL-8 to control lower chambers.

3.5.2 To each upper chamber, add 10^6 fresh PMN in 100 µl of RPMI-1640 medium supplemented with 10% IH-FCS.

3.6 Incubate the cluster trays at 37° C in 5% CO_2 for 90 min.

3.7 After 90 min incubation, remove the cells from the upper and the lower chambers separately and count each subpopulation. Express cells in each chamber as a percentage of the total cells added.

Note: Carefully remove cells from the upper chamber by pipetting gently in order to avoid removing endothelial cells and transfer to a sterile tube. Remove the cells from the lower chamber by pipetting and washing the lower chamber with 500 µl and transfer to a second sterile tube.

3.8 Pool 10^6 cells from several wells of transmigrating (lower chamber) and non-migrating (upper chamber) PMN fractions and culture each for 7 days without growth factors as specified in steps 1.2.13-1.2.15.

3.9 Spin cells onto slides[16] and analyze the developed cells in each culture condition by confocal microscopy as described in section 2.

REPRESENTATIVE RESULTS:

Neutrophil autophagocytosis and development in culture:

Neutrophil autophagocytosis and their development into Gϕ within 7 days in culture is shown in Figures 3 and 4. By days 4-7, their size was vastly enlarged,[15] and autophagosytosis was evident as early as 90 min after co-culturing the neutrophils with fluorescent membrane stains (PKH-26, red; PKH-67, green).[14] As a control to this neutrophil subpopulation, some neutrophil cultures were also treated with GM-CSF/IL-4. The cytokine-treated cells increased in size within 7-14 days in culture as previously described.[18,19] But, were smaller than Gϕ and had cytoplasmic projections resembling DC-like cells (Figure 5), as reported previously by Oehler et al.[19] Also, the GM-CSF/IL-4 treated cells were negative or had a low CD66b expression,[15] clearly demonstrating morphological and potentially functional differences as well.

[Place figure 3 - 5 here]

To further investigate the course of Gϕ development, their morphologic changes were also followed by time-lapse microscopy. Video-1 (day 3 to day 4) and video-2 (day 4 to day 5) demonstrate their development in purified neutrophil cultures. These Gϕ are non-adherent or lightly adherent with limited movement capacity and actively ingest surrounding neutrophil remnants and debris. In video-3, the movement of monocyte-derived Mϕ and Gϕ is compared in a mixed monocyte/neutrophil culture. The Mϕ actively crawls (left, unlabeled cell). The Gϕ (right,) is bright PKH-26 labeled cell.

[Place video 1-3 here]

Expression of markers in giant phagocytes:
The neutrophilic origin of Gϕ was verified by positive expression of the following neutrophil markers CD66b/CD63/MPO/NE/CD15 (Figure 6). The Gϕ also expressed NADPH oxidase, the oxLDL scavenger receptors - CD68 and CD36, and contained LC3B-coated vacuoles and aggregates (identified by Western blotting as LC3BII[15]), demonstrating the presence of an autophagy marker. However they were negative for monocytic lineage (CD14, CD16 and CD163) and dendritic cells (CD1c and CD141) markers, suggesting that Gϕ did not arise from contaminating monocytes.

[Place figure 6 here]

Functions of Gϕ - NADPH oxidase activation, ROS production and phagocytosis:
Phagocytosis of latex beads and opsonized zymosan was evident in Gϕ. Gϕ also generated basal ROS (Figure 7A), and responded to zymosan and PMA stimulation by oxidative burst (Figure 7B-D). However, unlike monocytes or neutrophils, Gϕ generated ROS also in response to oxLDL stimulation and were stained by Oil Red O (Figure 7B, F). Of note, treatment of fresh neutrophils with the NADPH oxidase inhibitor - DPI, not only inhibited ROS production, but also prevented Gϕ formation in culture, suggesting that ROS signaling is essential for Gϕ formation.[14,15]

[Place figure 7 here]

Transmigration of PMN across endothelial cells:
In order to identify potential neutrophils sub-populations that might develop into Gϕ, the migration of neutrophils through endothelial cell monolayers was determined (Figure 8A). After 90 min, 62.3 ± 12.2 % of the neutrophils transmigrated through endothelial cells towards IL-8 in the lower compartment. Of note, Gϕ positive for CD66b/CD15/LC3B developed only from the transmigrated population of neutrophils whereas the cells which developed from the non-migrating neutrophils fraction were smaller in size and negative for the neutrophilic markers CD66b/CD15 (Figure 8B, C).

[Insert figure 8 here]

Figure 1: Schematic representation of giant cells development in 7 day neutrophil cultures. It is suggested that at inflammatory sites (1) neutrophils undergo apoptotic cell death, and (2)

release membrane-encircled fragments containing nuclear debris, granules (green and red dots), and other subcellular constituents which trigger autophagy mechanisms. (3) Giant phagocytes (Gϕ) develop in long-term neutrophil cultures devoid of cytokines or growth factors by internalizing apoptotic bodies and neutrophil debris, while maintaining functional NADPH oxidase. They are characterized by various neutrophilic CD66b+/CD63+/MPO+/ CD15+/CD11b+/NE markers, large phagosomes enclosing granules and cell debris, and scavenger receptors CD36 and CD68. Gϕ are mostly mononucleated cells, capable of internalizing also various particles and oxidized LDL and generate ROS. The membranes of the vacuoles filling Gϕ contain LC3B (marked in dark blue), a marker of autophagosomal membrane, suggesting a strict association between autophagy and giant phagocyte formation. Gϕ do not develop in medium containing GM-CSF/IL-4. Also, inhibitors such as the NADPH oxidase inhibitor – diphenylene iodonium (DPI), the autophagy inhibitors 3-methyladenine (3-MA) and bafilomycin (BafA1) and the phagocytosis inhibitor cytochalasin B (Cyto. B) abolish their formation. (4) Potential Gϕ functions *in vivo* may include anti- or pro-inflammatory properties and participation in atherosclerotic processes (this figure is based on our findings[14,15] and was modified from the accompanying Editorial by Berton[20]).

Figure 2: Neutrophil isolation from human whole blood. Polysucrose at a 1.077 g/ml is carefully layered on top of polysucrose-1.119 g/ml to form a discontinuous gradient. The diluted whole blood is then layered on top of the polysucrose-1.077. The tubes are immediately subjected to centrifugation at 700 X g for 30 min, at room temperature without brake. Three distinct bands are noted. (A) Mononuclear cells, (B) polymorphonuclear cells (PMN), and (C) red blood cells (RBC) at the bottom of the tube.

Figure 3: Autophagocytosis in the developing giant phagocytes (Gϕ) in culture. Freshly isolated purified neutrophils were labeled with PKH-67 (green) or PKH-26 (red) membrane fluorescent dyes at zero time, and then co-cultured and followed up to seven days. Cells were spun onto glass slides, nuclei were stained with DAPI and samples were analyzed by confocal microscopy. Autophagocytosis is already noticeable after 90 min of co-culture. Merging of red and green into yellow and orange is clearly evident in the developing Gϕ.

Figure 4: Development of giant phagocytes (Gϕ) in culture. Freshly isolated purified neutrophils were followed up to 7 days in culture. Cells were spun onto glass slides at the indicated time intervals, stained with May Grunewald-Giemsa, and analyzed with a bright field microscopy. An individual with few eosinophils is presented for comparison. Note that the size of eosinophils remains unchanged in culture. Magnification oil X100.

Figure 5: Comparison between the development of giant phagocytes (Gϕ) and GM-CSF/IL treated neutrophils in culture. (A) May Grunwald-Giemsa stained neutrophils cultured without (Gϕ) and with GM-CSF/IL-4 for 7 days. Samples were analyzed with a bright-field microscopy. Magnification, X40. Cells developed in cultures with medium supplemented with GM-CSF/IL-4 show widespread cytoplasmic projections but are smaller than Gϕ. (B) Freshly isolated neutrophils were labeled with PKH-26 (red) dye and cultured in cytokine-free medium for 7 days or labeled with PKH-67 (green) dye and cultured in medium supplemented with GM-CSF/IL-4 for 7 days. Then, the developed cells were mixed in a 1:1 ratio and co-cultured for 2 h. Cells were fixed and analyzed by confocal microscopy. This figure has been modified from [15].

Figure 6: Expression of various markers for neutrophils, monocytes and dendritic cells in giant phagocytes (Gϕ) after 7 days in culture. Positive expression of the neutrophil specific granule marker CD66b, the azurophil granules markers CD63 and MPO, neutrophil elastase and CD15. Negative expression for the dendritic CD1c and CD141 markers and monocytic lineage markers CD14, CD16 and CD163. Additionally, Gϕ expressed the autophagy marker LC3B, the scavenger receptors CD68 and CD36 and the NADPH oxidase subunits gp91-phox/p22-phox subunits. Nuclei were stained with DAPI, and samples were analyzed by confocal microscopy. This figure has been modified from [14,15].

Figure 7: Oxidative burst, phagocytosis, and oxLDL uptake by giant phagocytes (Gϕ). (A) Basal ROS production is evident in lysosomes of Gϕ**.** (B**)** ROS production in response to oxidized LDL (oxLDL), PMA and zymosan (zymosan particles are clearly noted). (C) Nitroblue tetrazolium (NBT) test in Gϕ showing respiratory burst activity without and with PMA (slides are unstained, but the inserts are stained with May Grunwald-Giemsa). (D) NBT test and May Grunewald-Giemsa stained Gϕ with PMA and PMA/DPI which inhibited NADPH oxidase and ROS. (E) Phagocytosis of Latex and IgG-opsonized zymosan in PKH-26 (red) stained cells. (F) Oil Red O staining in untreated and oxLDL treated Gϕ. This figure has been modified from [14,15].

Figure 8: Effects of IL-8-dependent PMN transmigration through endothelial cells on giant phagocyte (Gϕ) formation. (A) A scheme illustrating neutrophil transmigration assay across endothelial cell monolayers (ECs) towards IL-8. This assay can be considered as a model for neutrophils recruitment to acute inflammatory sites. (B-C) In the cell migration assay (specified in protocol 3), transmigrating (B) and non-migrating (C) neutrophils fractions were cultured for seven days without growth factors (as in protocol 1). Then, cells were spun onto glass slides and analyzed by confocal microscopy. Fixed cells were stained for CD66b (red), LC3B (green) and CD15 (red). Nuclei were stained with DAPI (blue).

Video-1: Demonstrates the development of giant phagocytes in purified PMN cultures on days 3-4 by time-lapse microscopy. Neutrophils were followed-up in culture from day 3 to day 4 by time-lapse microscopy. The time-lapse microscopy system is composed of inverted motorized fluorescent microscope, and a high resolution B/W CCD camera, with an on stage incubator. Image capture acquisition of time-lapse was taken every 10 min. Originally published in [14].

Video-2: Demonstrates the development of giant phagocytes in purified PMN culture on days 4-5 by time-lapse microscopy. Neutrophils were followed-up in culture from day 4 to day 5 by time-lapse microscopy. The time-lapse microscopy system is composed of inverted motorized fluorescent microscope, and a high resolution B/W CCD camera, with an on stage incubator. Image capture acquisition of time-lapse was taken every 10 min.

Video-3: A giant phagocyte and a macrophage developed in co-culture. Monocytes/neutrophils co-culture was followed-up from day 4 to day 5 by time-lapse microscopy. Monocyte-derived macrophage (left); bright (PKH-26 stained cell) neutrophil-derived giant phagocyte (right). The time-lapse microscopy system for video is composed of inverted motorized fluorescent microscope, and a high resolution B/W CCD camera, with an on

stage incubator. Image capture acquisition of time-lapse was taken every 10 min. Originally published in [14].

DISCUSSION:

Giant phagocytes (Gϕ) are a newly defined subpopulation of neutrophil-derived cells expressing fundamental and specific neutrophilic markers such as CD66b/CD15/CD63/MPO/NE. This type of neutrophil-derived phagocyte was not described in the literature before. Unlike neutrophils that are short-lived and undergo apoptosis, Gϕ are Annexin-V-negative and display extended lifespan. Yet, like neutrophils, Gϕ also internalize particles and produce NADPH oxidase-dependent ROS in response to those particles and to PMA. However, their abilities to internalize OxLDL and consequently to produce ROS are unique features of Gϕ.[14]

A number of factors were shown to influence their development in culture. The lack of external cytokines or growth factors in the growth medium is essential (specifically GM-CSF/IL-4). However, neutrophils migration towards IL-8 proved a discriminating factor between those that developed into Gϕ and those that did not. Also, internalization of debris arising from apoptotic neutrophils, the expression of autophagy proteins (LC3B) and functional NADPH oxidase, were all shown to be imperative for their development, since their inhibition prevented Gϕ formation (Figure 1). Apparently, the development of these giant cells arising from neutrophils differs from that characterizing giant cell formation in the monocyte/macrophage lineage. The latter form multi-nucleated giant cells associated with diverse chronic inflammatory diseases,[20,21] whereas the neutrophilic Gϕ described here develop via autophagocytosis, by engulfing cell remnants and remain mostly mono-nucleated throughout their development,[14] (rarely however, sometimes a second nucleus can be observed). Moreover, a number of controls established their neutrophilic origin: (1) expression of the specific neutropilic markers and absence of dendritic and monocytic lineage markers, (2) their hampered development in monocytes/PMN co-cultures, (3) their different patterns of movement in culture from macrophages (as evidenced by live cell imaging and time-lapse microscopy),[14] (4) their light adherence to plastic dishes and (5) their development from pure $CD15^{+}/CD14^{-}$ PMN acquired by flow cytometry.

Some of the functions identified *in-vitro* may give us clues as to their potential functions *in vivo*. For instance, the abilities of Gϕ to consume large amounts of neutrophil granules and debris, the presence of large vacuoles, and the expression LC3B - an autophagy protein which contributes to diminishing inflammation through regulatory interactions with innate immune signaling pathways,[22] - all of which support scavenging abilities. As such, these findings also indicate that Gϕ might be functioning at inflammatory sites where the Mϕ/DC system is insufficient or overwhelmed, and thus contribute to the resolution of inflammation. This notion might be supported by the fact that in mixed monocyte/neutrophil cultures Gϕ development is hampered.[14] Also, given that Gϕ express oxLDL scavenger receptors (CD36, CD68), internalize oxLDL, and produce ROS in response to it, may indicate that they are involved in atherosclerotic processes to resolve inflammation. Since Gϕ developed only from neutrophils which migrated towards IL-8, and neutrophils' transmigration across endothelial monolayers towards IL-8 represents neutrophil recruitment to acute inflammatory sites, this finding also may support anti-inflammatory functions. Conversely, the performance of Gϕ in certain inflammatory conditions might enable them to discharge granule constituents and ROS, thus, contributing to persistent

inflammation and tissue damage.[20] However, overall, their autophagic abilities indicate that Gϕ are likely involved in diminishing the inflammatory response rather than perpetuating it.

Interestingly we have recently identified the presence of Gϕ in human atherosclerotic plaques. (in preparation). Yet, a great number of questions remain to be unraveled. For instance, are Gϕ pro- or anti-inflammatory? What are the factors which determine their formation and function *in vitro* or *in vivo*? Which specific neutrophil subpopulation is their precursor cell that facilitates their development into Gϕ? Are they associated with certain pathologies and which? Collectively, posing interesting questions as to their origin and potential functions.

However critical steps and pitfalls within the protocol should be kept in mind. A critical step in, the development of Gϕ is culturing the pure neutrophils in medium devoid of cytokines, growth factors or antibiotics. Another critical step is to rule out that Gϕ develop from contaminating monocytes and to ascertain the neutrophilic origin of Gϕ. Thus, after blood separation by discontinuous gradient, the neutrophils were further subjected to an additional step of purification by flow cytometry using granulocyte gating and $CD15^{+}/CD14^{-}$ markers. The developed Gϕ obtained from neutrophils that were further purified by flow cytometry separation did not differ from those that were not subjected to this step of purification. Therefore, most of the experiments were conducted without the flow cytometry step of purification due to additional cell loss. Of note, in some rare instances some eosinophils were noted in culture. Their size remained unchanged throughout the culture period. We should also note that although there are a number of methods for neutrophil separation from human blood, the method described here is the only method we employed and therefore we cannot compare Gϕ development by other available methods for neutrophil separation.

A major pitfall in investigating Gϕ results from the inability to obtain sufficient numbers of pure Gϕ population suitable for various biochemical assays. It is basically impossible in the conditions our experiments were conducted. First, the yield of Gϕ is low. From $1.0X10^{6}$ PMN seeded about 100-200 Gϕ develop after seven days in culture, depending on the blood donor. Second, it is basically difficult at the moment to separate the developed Gϕ in culture from the remaining neutrophil debris in the dish. These limitations made it practically impossible to analyze the cells by biochemical or molecular biology methods. Therefore, this protocol is focused at describing Gϕ identification and function by using light and confocal microscopy. Their morphological transformation from neutrophils into Gϕ in culture was also followed by live cell imaging and time lapse microscopy.[14] Apparently, much larger blood volumes may be needed in order to implement biochemical or molecular biology methods and overcome the low yield obtained and separating the viable Gϕ from neutrophils' debris in the dish.

In summary, we have recently described for the first time the development of Gϕ in culture, a subpopulation of long-lived phagocytes of neutrophilic origin. Therefore, this is the only method currently available to obtain Gϕ in culture, although the two major limitations mentioned above should be overcome (the low yield of the Gϕ obtained in culture and the inability to separate the developed Gϕ from the neutrophil debris in the culture dish). Still, their preparation and identification, presented in this protocol, is essential for scientists interested in inflammatory

responses and neutrophil biology and plasticity, in order to further investigate the potential significance and functions of Gϕ.

ACKNOWLEDGMENTS:
The authors thank Dr. Edith Suss-Toby for her invaluable help with the confocal microscopy studies. This study was supported by the Ministry of Immigration Absorption and the Committee for Planning and Budgeting of the Council for Higher Education under the framework of the KAMEA program (LD and AP). We also gratefully acknowledge the support of the Research Fellow from the Lady Davis Foundation Post-Doctoral Research Fellowship (OR).

DISCLOSURES:
The authors have nothing to disclose.

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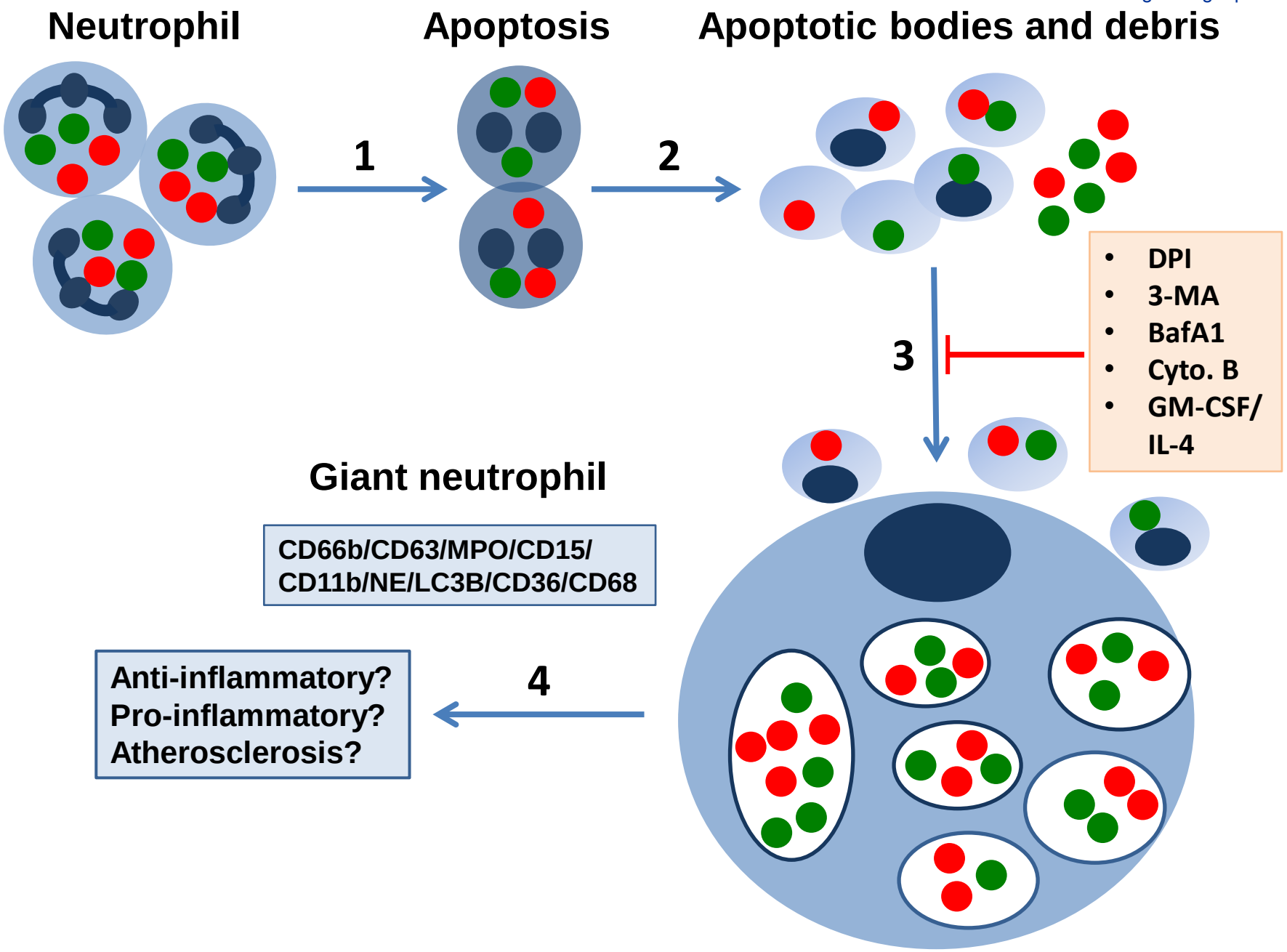
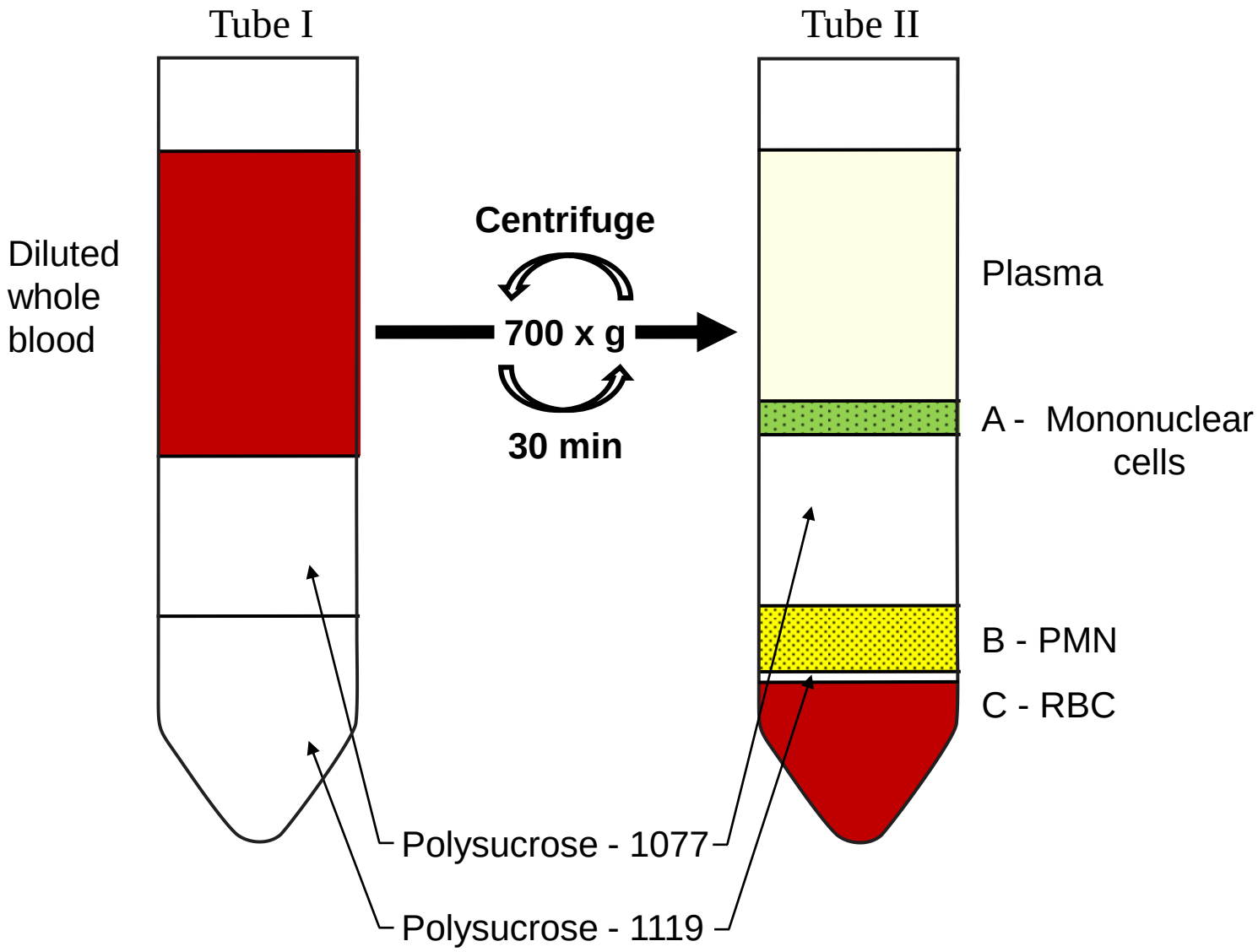


Figure 1



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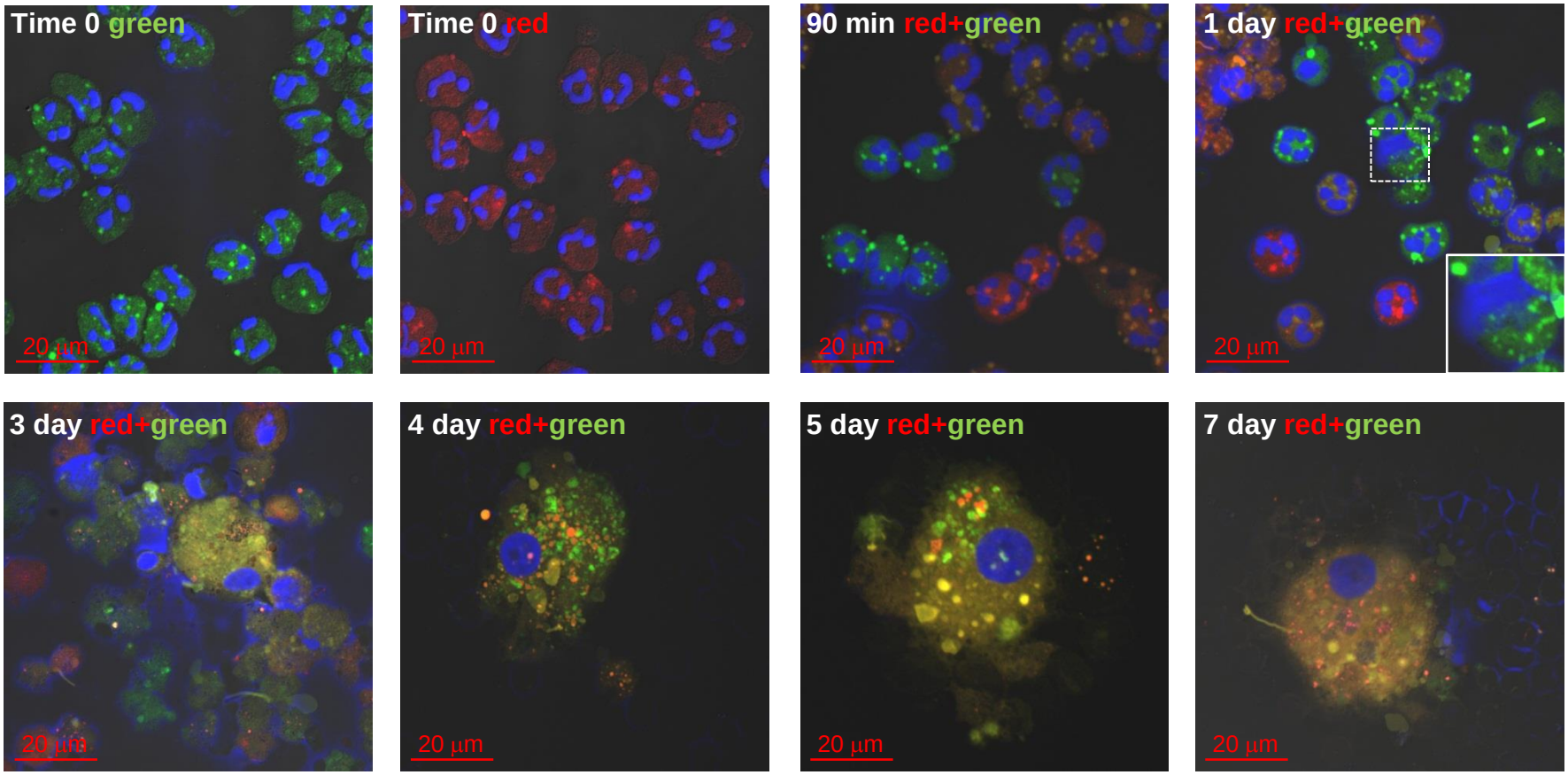

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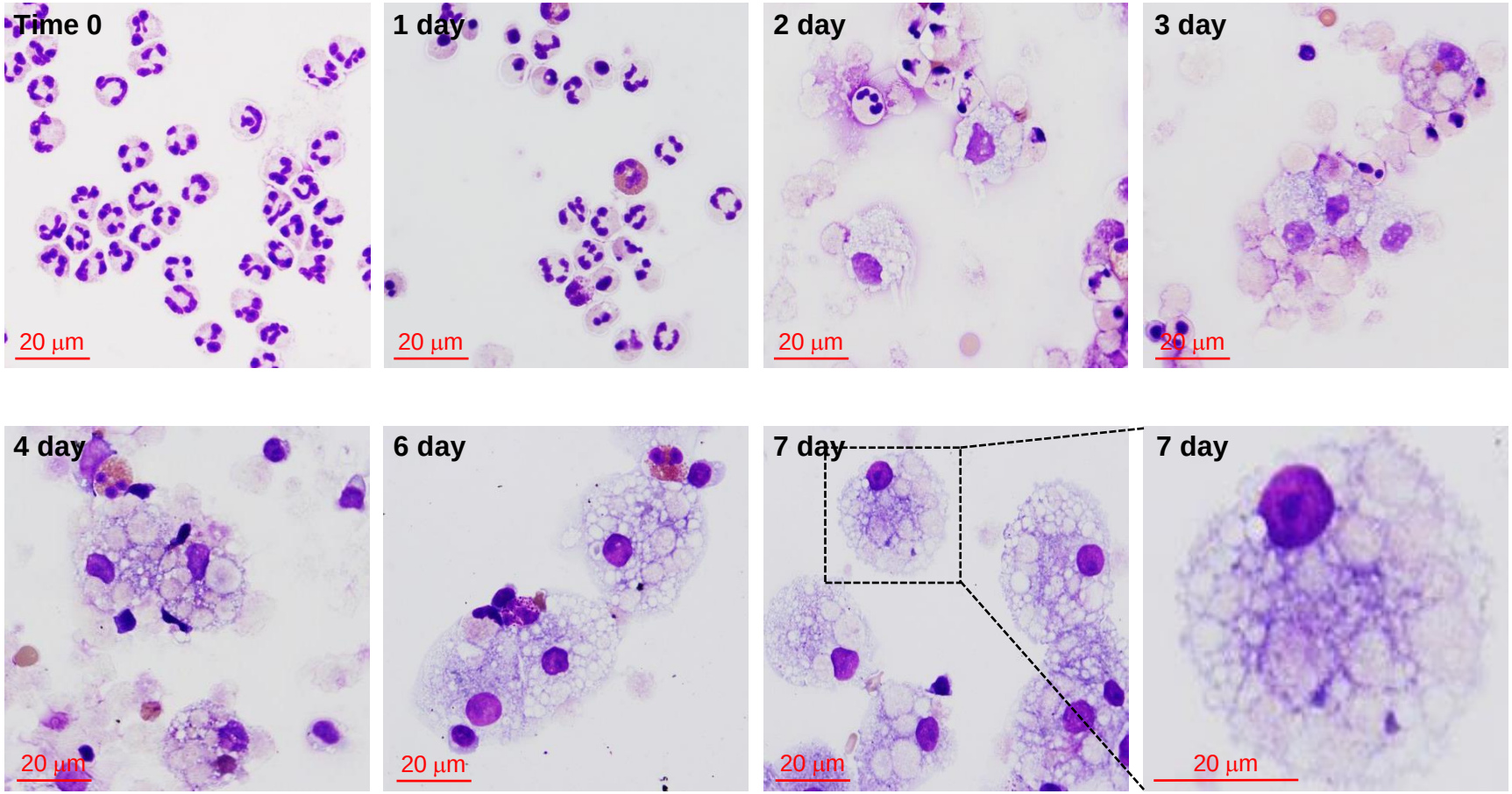

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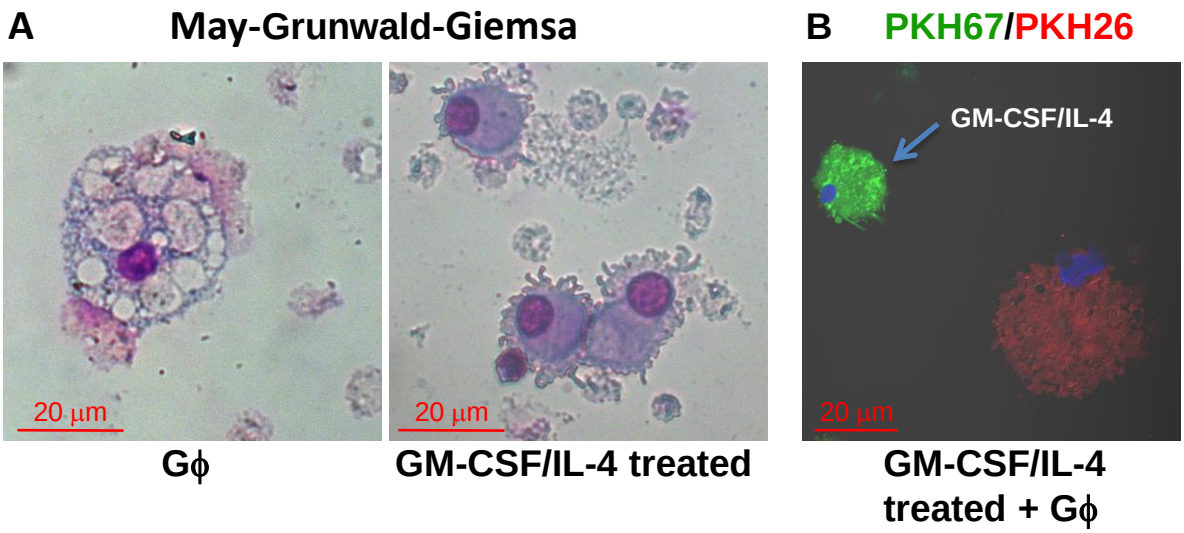

Figure 5

Figure

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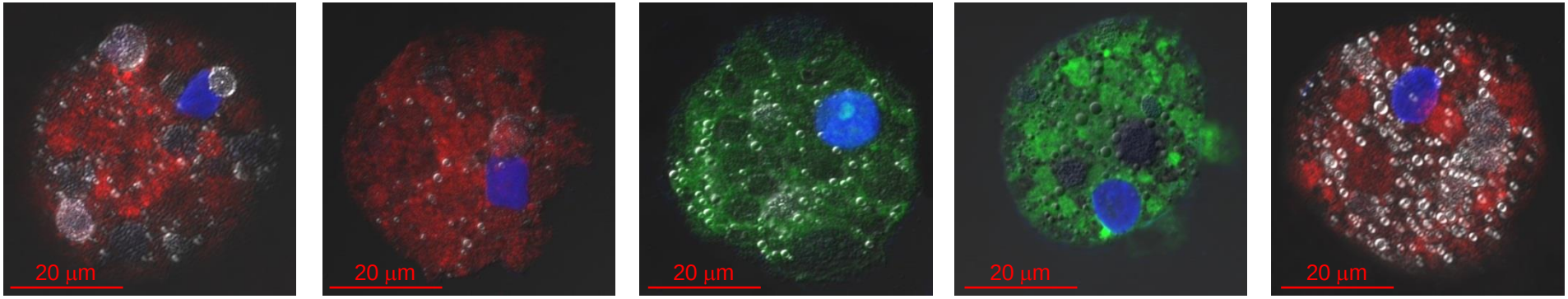
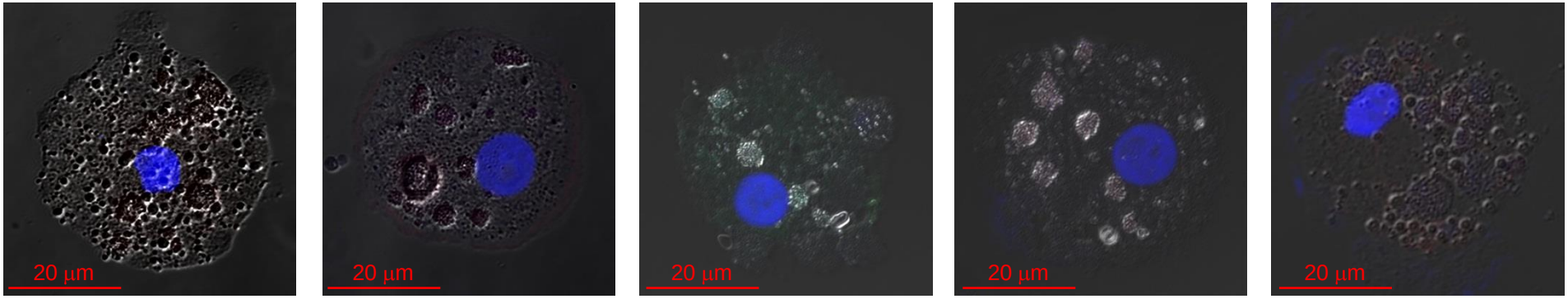
Markers of Neutrophil granules:

CD66b (specific granules) | CD63 (azurophil granules) | MPO (azurophil granules) | Neutrophil Elastase | CD15

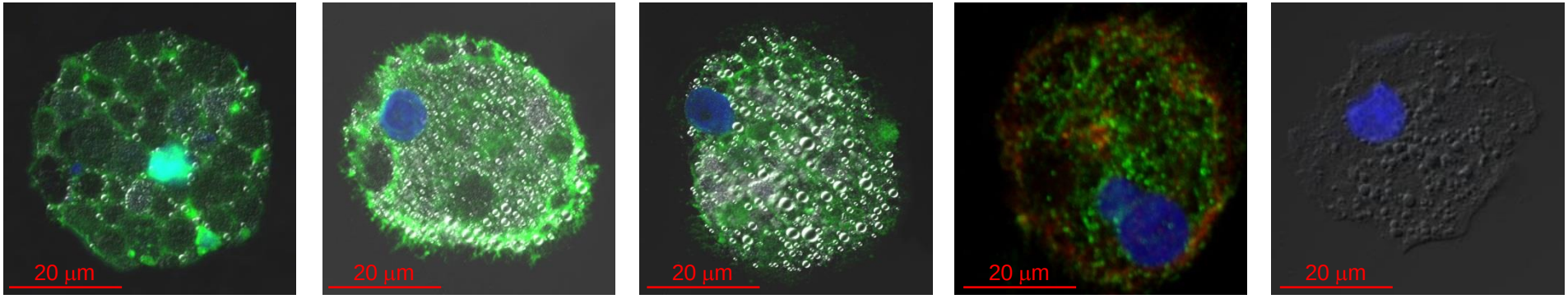


CD1c | CD141 | CD16 | CD14 | CD163



Scavenger receptors:

LC3B | CD68 | CD36 | Gp91-*phox*/p22-*phox* | Isotype control



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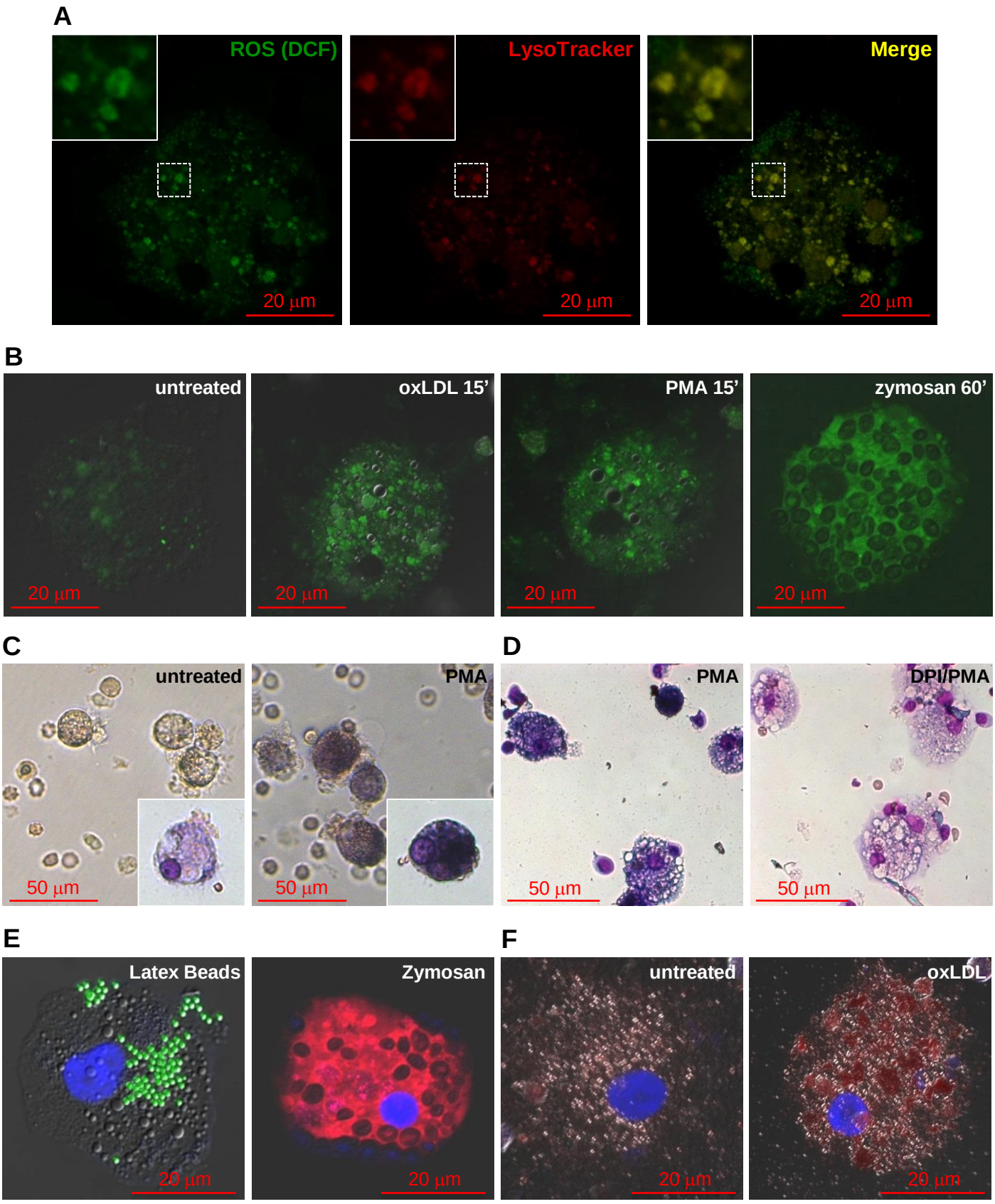

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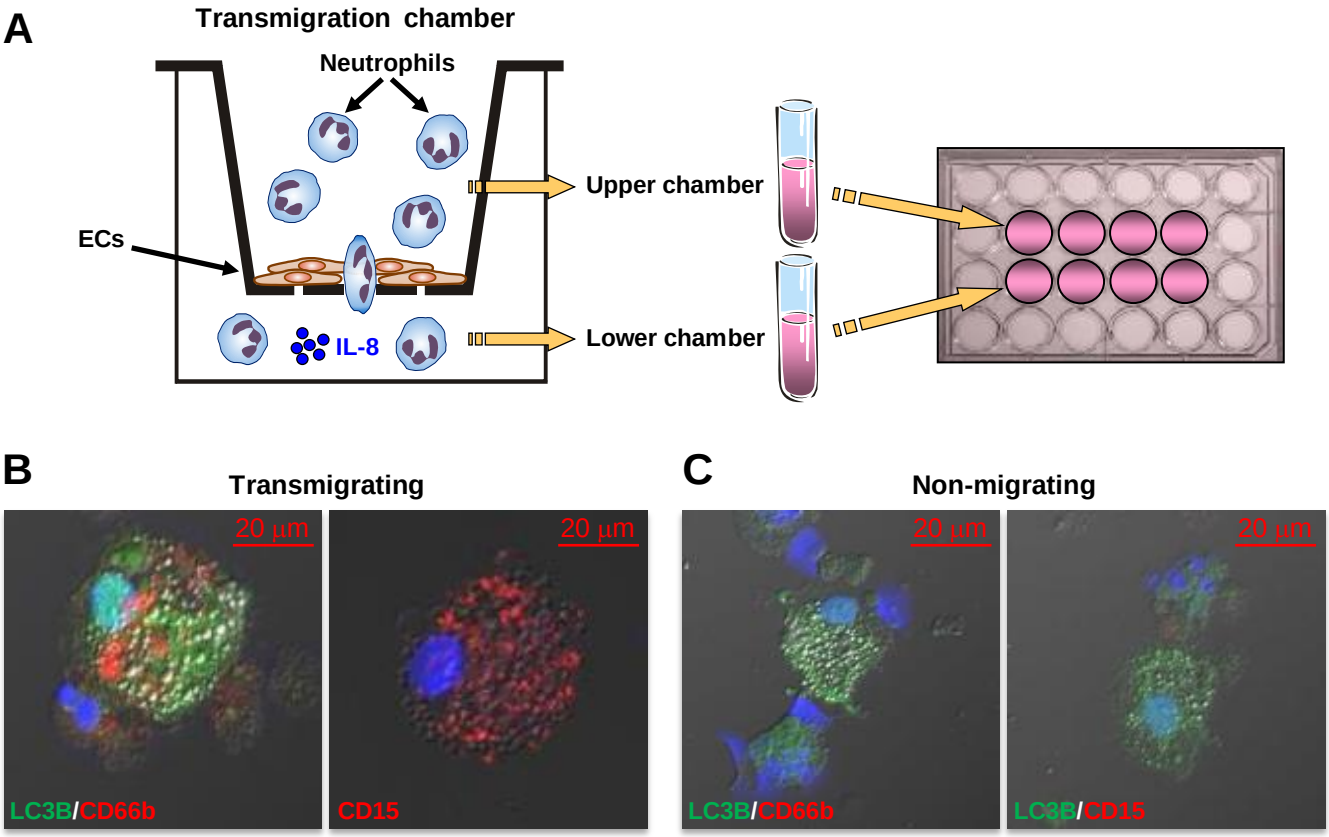

Figure 8

Excel Spreadsheet- Table of Materials/Equipment

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Name of Reagent/ Equipment	Company	Catalog Number
Sterile scalp vein set (21GX3/4)	Bio Diagnostics Ltd.	№ 20080312
VACUETTE HOLDEX Single-Use Holder PP	Greiner Bio-One	№ 450263
VACUETTE Tube K3E K3EDTA (16x100/9 ml)	Greiner Bio-One	№ 455036
Nunclon MultiDish (24 wellx1ml)	Thermo Scientific	№ 142475
Polypropylene conical centrifuge tube (50 ml)	Greiner Bio-One	№ E14103PJ
Transwell-24 well (transmigration assay)	Corning	№ CA-3415
RPMI-1640 medium	BioIndustries	№ 01-100-1A
EA.hy926 (ATCC CRL2922)	BioIndustries	№ CRL-2922
ATCC-formulated Dulbecco's Modified Eagle's Medium	BioIndustries	№ 302002
Polysucrose - Histopaque1119	Sigma-Aldrich	№ 1119-1
Polysucrose - Histopaque1077	Sigma-Aldrich	№ 1077-1
Phosphate buffered saline (PBS) - ion free	BioIndustries	№ 02-023-1A
Heat inactivated Fetal calf serum (HI-FCS)	BioIndustries	№ 04-121-1B
NaCl	Sigma	№ S3014
Paraformaldehyde, 16%	Electron Microscopy Sciences	№ 15710
Triton X-100	Sigma-Aldrich	№ 9002-93-1
Normal Goat Serum	BioIndustries	№ 04-009-1
Trypan blue	BioIndustries	№ 031021B
May-Grünwald	Sigma-Aldrich	№ MG500
Giemsa stain Kit	Sigma	№ 48900
Fibronectin	BioIndustries	№ 03090105
Human Interleukin-8 (CXCL8)	PeproTech	№ 200-08-5
Anti-CD14 (clone 5A3B11B5)	Santa Cruz Biotechnologies	№ sc-58951
Anti-CD63 (clone MX-49.129.5)	Santa Cruz Biotechnologies	№ sc-5275
Anti-CD66b (clone 80H3)	AbD Serotec	№ MCA216
Anti-CD1c (BDCA-1) (clone AD5-8E7)	MACS Miltenyi Biotec	№ 130-090-695
Anti-CD15 (clone MY-1)	Abcam	№ ab754
Anti-Cytochrome b-245 Light Chain (p22-phox) (clone 44.1)	BioLegend	№ 650001
Anti-CD68	Protein Tech	№ 16192-1-AP
Anti-LC3B	Sigma	№ L7543
Anti-Myeloperoxidase	Abcam	№ ab45977
Anti-Neutrophil elastase	Calbiochem	№ 481001
Anti-NOX2 (gp91-phox)	Abcam	№ ab131083

Name of Reagent/ Equipment	Company	Catalog Number
Anti-CD36 (SR-B3)	Novus Biologicals	№ NB400-144
Purified Mouse IgG1, κ Isotype Control (clone MG1-45)	BioLegend	№ 401401
Purified Mouse IgG2a, κ Isotype Control (clone MOPC-173)	BioLegend	№ 400263
Normal rabbit IgG	Santa Cruz Biotechnologies	№ sc-2027
CF488A Goat Anti-Rabbit IgG (H+L)	Biotium	№ 20012
CF647 Goat Anti-Rabbit IgG (H+L)	Biotium	№ 20043
CF488A Goat Anti-Mouse IgG (H+L)	Biotium	№ 20010
CF647 Goat Anti-Mouse IgG (H+L)	Biotium	№ 20040
Fluorescent Mounting Medium with DAPI	Vectashield H-1000; Vector Lab Inc.	№ E19-18
Confocal laser scanning microscope (LSM 700)	Carl Zeiss	Ser.№ 3523000380
Zeiss CLSM software (ZEN 2010)	Carl Zeiss MicroImaging GmbH	version 6.0
ImageJ software	Wayne Rasband, NIH, USA	version 1.49k
Light microscope (Axiovert 25)	Carl Zeiss	Ser.№ 201060153
Centrifuge (Megafuge 1.0 R)	Heraeus Instruments	№ D-37520
Inverted fluorescent microscope (Zeiss Axio Observer Z.1)	Carl Zeiss	Ser.№ 3834001470
Temperature-controlled incubation system (Cube&Box)	Life Imaging Services	
High resolution digital CCD camera (AxioCam HRm)	Carl Zeiss	Ser.№ 117090279

Comments/Description
A strile needle for venipancture
For securing during venipuncture
Sterile tube for blood collection
Polycarbonate membrane, 6.5 mm diameter, 3 µm pores)
Do not add antibiotics
complete growth medium
Tissue culture grade
Tissue culture grade
Cell biology and molecular biology grade
Cell biology grade or tissue culture grade, low LPS
Molecular biology grade, suitable for cell culture
Cell bology grade or tissue culture grade (only 16% PFA)
Molecular biology grade
Cell biology and molecular biology grade
Tissue culture grade
Cell biology grade-(procedure No GS-10)
Cell biololgy grade-(procedure No GS-10)
Mouse IgG2b; expressed by monocytes
Mouse IgG1; expressed by neutrophils
Mouse IgG1; expressed by neutrophils
Mouse IgG2a; expressed by dendritic cells
Mouse IgM; expressed by neutrophils
Mouse IgG2a; to recognize neutrophil NADPH oxidase complex
Rabbit IgG; to recognize oxLDL scavenger receptor
Rabbit IgG
Rabbit IgG
Rabbit IgG
Rabbit IgG

Comments/Description
Rabbit IgG
Antibody used as isotype control
Antibody used as isotype control
Antibody used as isotype control
Anti-Rabbit IgG with the green fluorescent dye CF488A
Anti-Rabbit IgG with the red fluorescent dye CF647
Anti-Mouse IgG with the green fluorescent dye CF488A
Anti-Mouse IgG with the red fluorescent dye CF647
Nuclear staining
Plan Apo x40 immersion oil objective
For colocalization analysis
For determination of cell areas and fluorescence intensity
Examination of cells in culture
Cells separation from blood; cytospins preparation
Demonstration of giant phagocytes development
Temperature control system for microscopes
For capturing high-contrast image data from an examined cell objects

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Signature:	Lena Lavie
Date:	august-18-2016

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RESPONSE TO EDITORIAL COMMENTS

Dear Prof. Lavie,

Your manuscript JoVE54826R2 "Development and identification of a novel subpopulation of human neutrophil-derived giant phagocytes in-vitro" has been peer-reviewed and the following comments need to be addressed. Please keep our formatting requirements in mind while revising the manuscript to address peer review comments. Please maintain the overall manuscript changes, e.g., if formatting changes were made, commercial language was removed, etc. **DONE**

Please track the changes to identify all of the manuscript edits. After revising the submission, please also upload a separate document that addresses each of the editorial and peer review comments individually with the revised manuscript. For each comment, please provide either (1) a description of how the comment was addressed within the manuscript or (2) a rebuttal describing why the comment was not addressed or out of the scope of this work for publication in JoVE. **DONE**

Your revision is due by **Aug 18, 2016.**

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Mala Mani,
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DONE – we used some pictures from our previous publication (references 14 (JLB) and 15(Hindawi Publishers). From both papers we have publishers permission to reuse data since both paper are free access and we attach the forms in supplemental files. We also cite the figures appropriately in the figure legend.

• Please remove Transwells (trademarked term) from Figure 8. **Removed**

• 2.7: Please provide a reference for analyzing the slides by fluorescence microscopy. Mentioning the microscope details in the Table of Materials is not sufficient. **Corrected, we analyzed the data by software (ImageJ 1.49) which is specified in the table.**

• Please update the access type to Open in the attached Author License Agreement (ALA). Scan the updated ALA and upload it with your manuscript files.

•Formatting:
-Unless some of the identification is to be shown in the video, please remove reference to it from the manuscript title. **N/A**

-Paraformaldehyde is toxic and requires a caution statement. **Corrected to** "…**with 100 µl 4% paraformaldehyde under a chemical hood….."** Using such a low concentration and a small volume under hood is safe. All other instructions for handling paraformaldehyde are specified in manufacturer's instructions.

•Grammar:
-Line 66 – Do not begin sentences with conjunctives like "but" or "and". **Corrected**
-1.1 – "from informed consent young healthy adults"; "vain" **Corrected**
-Line 185 – "allows to harvest at the same time mononuclear cells and neutrophils" – awkward phrasing - **Corrected**
-1.2.15 – "IH-FCS" or "HI-FCS"? **It is correct as is "HI-FCS"**

•Additional detail is required:
-1.2.7 – About what volume of PMN layer is transferred? – The volume of the band containing the PMN varies between individuals and therefore we cannot add a specific value. As mentioned the bands described fig 2 are opaque and can be identified easily.

-1.2.10.1 – How does one check for contaminating RBC? If judging by the pellet, this step should appear after 1.2.11.

While the PMN are white blood cells and appear white, the red blood cells are RED. Therefore, are clearly noted in the pellet. We added to 1.2.10.1 "note: If contaminated by RBC, the PMN pellet appears reddish."

-2.1 note – How would Gφ be detected in the removed medium? **Corrected in the note to 2.1**

-2.2 – How are washes performed? (ie volume, length of time, etc.). **Added a note.** The washing volume can vary depending on the perimeter of the cells that were cytospun on the slide and that depends on the specific centrifuge and rotor used. We used 100 µl, but in each lab this can vary according to the equipment used. The term "wash" it commonly used to be an immediate procedure of few seconds depending on the person capabilities for doing the washing. Again here we used 100 µl per wash but other labs can use more or less. The volume is not critical, only the perimeter of the cells is and that depends on the cytospin used.

-2.4, 2.5 – What are antibodies diluted in? **Added**

•Branding: 2.1, 2.1.1, 2.2, 3.9 plus 7 occurrences in figure legends – while cytospin is a method, it is also a trademarked term. We can allow the use in 2.1, but all other instances should use a generic term/phrase.

Unfortunately, I don't know any other term to replace "cytospin"- unless I write a whole sentence to describe the procedure over and over, and that will be confusing. Although this term was originally used by "Shandon" centrifuges over 40 years ago, currently it is used as a generic term to describe centrifugation of cells onto slides for microscopy analysis and many companies hold centrifuge rotors for cytospin preparation.
Definition of cytospin from the internet "The **cytospin process** is a simple **procedure**. Cells are washed in a serum and/or albumin based PBS or culture media solution. The cells are resuspended in up to 500 µl of this solution. A cytofunnel is attached to a glass slide and slide carrier." Thus, currently it is a generic term and should remain as such in the paper.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
In their manuscript „ Development and identification of a novel subpopulation of human neutrophil-derived giant phagocytes in-vitro „ Lavie and colleagues describe preparation of a neutrophil-derived phagocytic cell population.

Major Concerns:

The MS is fine with me. I have no criticism (a seldom case).

Minor Concerns:
The MS is fine with me. I have no criticism (a seldom case).

Additional Comments to Authors:
N/A

Reviewer #2:

Manuscript Summary:
This article describes an interesting new subpopulation of human neutrophil-derived giant phagocytes. The microscopy data is of an extremely high standard. The manuscript is enhanced by demonstrating this new cell population can undergo phagocytosis, autophagy and have an extended lifespan. The method is clear and well written.

Major Concerns:
No major concerns were raised in my review of this article.

Minor Concerns:
Please correct "vain" on in section 1.1. **Corrected**

Additional Comments to Authors:
N/A