

The development of giant phagocytes in long-term neutrophil cultures

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ABSTRACT

We tested the hypothesis that in long-term culture conditions, some neutrophils remain viable and participate in debris clearance, and autophagy is involved in their prolonged survival. Neutrophils, classified as professional phagocytes, have the shortest half-life among leukocytes and are constitutively committed to apoptosis. Apoptotic neutrophils are actively removed by M ϕ /DCs. However, early and acute inflammatory infiltrates primarily consist of neutrophils. Recently, neutrophils were suggested to facilitate debris clearance at inflammatory sites when the M ϕ /DC system is insufficient. Here, purified CD15⁺/CD66b⁺/CD63⁺ neutrophils were followed up to 7 days in culture using light, time-lapse, and confocal microscopy. After 3 days in culture, Annexin-V⁻/LC3B⁺ large vacuolated cells, engulfing cellular residues, were noted among apoptotic neutrophils and cell debris. Thereafter, these cells were vastly enlarged and exhibited a neutrophilic phenotype (CD15⁺/CD63⁺/MPO⁺/CD66b⁺), phagocytosis, and oxidative burst activity. They also expressed CD68 scavenger receptors and internalized oxLDL. But, unlike in fresh neutrophils or cultured monocytes, oxLDL treatment increased their ROS production. Additionally, these phagocytes contained LC3B-coated vacuoles and LC3B aggregates, indicating the activation of autophagy. An intensive LC3B accumulation was also noted during oxLDL internalization. Importantly, the inhibition of autophagy by 3-MA or BafA1 prevented their development. In conclusion, the internalization of neutrophil remnants may induce activation of autophagic mechanisms in some neutrophil subsets or precursors. This may lead to cell adaptation and survival, resulting in their transformation into long-lived G ϕ and potentially suggesting their involvement in inflammatory/anti-inflammatory processes. *J. Leukoc. Biol.* 96: 511–521; 2014.

Introduction

Neutrophils are the first line of defense against infection, playing a central role in the innate immune system [1]. They are classified as professional phagocytes and are important in the clearance of pathogens and harmful particles. Yet, they are also capable of inducing and perpetuating inflammatory processes and tissue damage [2]. Therefore, effective clearance of apoptotic neutrophils by M ϕ or DCs is a crucial component in the resolution of inflammation [3–5]. However, at the early phase of an acute inflammation, the inflammatory infiltrate primarily consists of neutrophils, whereas the number of M ϕ /DCs is low. In synovial fluid of rheumatoid joints, neutrophils comprise as much as 80% of all infiltrating cells [6]. Neutrophils are also the most abundant inflammatory cells in acute peritonitis and in the initial steps of pleural inflammation [6, 7]. Additionally, ingesting bacteria may delay neutrophil apoptosis, thereby facilitating neutrophil accumulation. In turn, extremely high numbers of apoptotic neutrophils within inflamed tissues may lead to failure of the M ϕ /DC clearance system. As a result, neutrophils undergo secondary necrosis, leading to the development of acute inflammatory diseases [8].

A number of studies have recently reported that the neutrophils themselves may participate in the clearance of dead neutrophils and/or debris in a form of cellular “cannibalism”, particularly when the M ϕ /DC clearance system is insufficient or overwhelmed [8–10]. For instance, a mouse model of LPS-induced lung inflammation revealed that at 24 h after LPS administration, almost 50% of the neutrophils contained phagosomes, which engulfed apoptotic nuclei and other neutrophil remnants and possibly whole apoptotic neutrophils [8]. A study conducted on human neutrophils *in vitro* has shown that LPS- or cytokine-activated neutrophils mostly ingested small-sized apoptotic material rather than whole apoptotic cells. Moreover, naive, unlike activated neutrophils, had a limited capacity to phagocytose apoptotic cells [9]. In addition, neutrophils phagocytosing apoptotic cells and nuclei were described in blood smears of patients with lupus erythematosus

Abbreviations: 3D=three-dimensional, 3-MA=3-methyladenine, BafA1=bafilomycin A1, DCF=2',7'-dichlorodihydrofluorescein diacetate, G ϕ =giant phagocyte, M ϕ =macrophage, Nox2=NADPH oxidase 2, oxLDL=oxidized LDL

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

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[11]. Moreover, by transforming or reprogramming into other cell types, such as M ϕ or DCs, neutrophils were suggested to facilitate debris clearance at inflammatory sites [3]. Such neutrophil transdifferentiation was shown previously by treatment with cytokines and growth factors [12].

Recently, the existence of long-living neutrophil subsets was suggested through their ability to modulate their survival response by intrinsic and extrinsic factors [10]. Importantly, the fate of neutrophils is intimately linked with their ability to perform phagocytosis [13]. This was also linked with phenotypic and functional changes that depended on de novo synthesis of proteins involved in neutrophil lifespan extension [14].

One of the mechanisms promoting cell survival is autophagy [15], which as apoptosis, is a process that regulates cellular stress responses, yet there is a complex and intricate functional cross-talk between the two [16]. Possibly, similar stressors can induce either apoptosis or autophagy. Interestingly, in some conditions, a single cell can express a mixed apoptotic and autophagic phenotype [17]. However, the exact factors that specifically determine the development of the autophagic process remain to be unraveled.

In the current study, we show, for the first time, that under long-term culture conditions, without adding external cytokines or growth factors, a small percentage of neutrophils remains viable and develops into G ϕ . Thus, we tested the hypothesis that autophagy, followed up by LC3B accumulation, might be involved in extending neutrophil survival under these long-term culture conditions. We suggest that these G ϕ may contribute to the resolution of inflammation by phagocytosis of apoptotic bodies and neutrophil-derived cell debris, possibly when the M ϕ /DC system is insufficient or overwhelmed. On the other hand, G ϕ may also exacerbate pathological processes by immediate and intensive ROS production in response to various stimuli, including oxLDL uptake.

MATERIALS AND METHODS

Isolation and culture of PMNs (neutrophils) and monocytes

Blood samples were obtained from 14 healthy, nonsmoker volunteers (12 males/two females) with a mean age of 27.6 ± 4.1 years and a body mass index of 24.9 ± 4.3 Kg/m², who were not taking medications for at least 2 weeks. The protocol was approved by the local Human Rights Committee, according to the declaration of Helsinki, and all participants signed an informed consent form. Each subject was tested between two and five times. Neutrophils were isolated using Ficoll gradient 1.119 (Sigma-Aldrich, Israel), according to the manufacturer's instructions, followed by lysis of red blood cells with 0.2% sodium chloride for 30 s on ice. Neutrophils were cultured in RPMI-1640 medium, supplemented with 10% heat-inactivated FCS (Biological Industries, Beit Haemek, Israel). LPS content in FCS was as follows: Lot 715149, 6.9 EU/ml (0.69 ng/ml); Lot 615305, 0.5 EU/ml (0.05 ng/ml); Lot 515296, 0.6 EU/ml (0.06 ng/ml); and Lot 715274, 0.8 EU/ml (0.08 ng/ml). The addition of 1–10 ng/ml LPS to the culture medium did not affect G ϕ formation. One-half of the growth medium was replaced every 3 days. To exclude possible monocyte contamination, neutrophils were purified further by flow cytometry (FACSAria), using granulocyte gating and CD15⁺/CD14⁻ markers. Annexin-V (Annexin V-Cy5 Apoptosis Detection Kit; BioVision, Milpitas, CA, USA) was used to assess apoptosis and PI for necrosis. The specific autophagy inhibitors 3-MA and BafA1 (Sigma-Aldrich) were added at Time 0 and after 48 h in culture (5 mM and 30

nM, respectively, dissolved in DMSO). Equal volumes of the vehicle (DMSO) were added under the same experimental conditions.

For coculture studies, mononuclear cells were isolated over Ficoll gradient (Sigma-Aldrich), according to the manufacturer's instructions. Monocytes were prepared from mononuclear cells by a 1-h adherent step, followed by extensive washing. Then, pure autologous neutrophils were added, and monocyte/neutrophil cocultures were followed up to 7 days in culture.

Flow cytometry

Flow cytometric analyses were performed using the CyAn ADP flow cytometer (Beckman Coulter, Brea, CA, USA) in fixed and saponin-permeabilized, purified neutrophils. Data were analyzed with Summit software 4.3 (Beckman Coulter).

Fluorescent labeling of cells

The fluorescent membrane stains PKH-26 (red) and PKH-67 (green; Sigma-Aldrich) were used to label freshly isolated neutrophils. Cells were washed in serum-free medium, and the pellet was resuspended in 0.5 ml PKH-26 or PKH-67 staining solution (1:500 in Diluent C; Sigma-Aldrich) and incubated for 5 min at room temperature. The labeling was stopped by adding 0.5 ml FCS. Then, cells were washed three times with complete medium.

Confocal laser-scanning microscopy

Cytospins were prepared from freshly isolated neutrophils [18] or from G ϕ , fixed with 4% PFA and washed with PBS. For intracellular staining, cells were permeabilized with 0.5% Triton X-100 (Sigma-Aldrich) in PBS at room temperature for 10 min. After blocking with 10% normal goat serum in RPMI 1640, cells were incubated overnight at 4°C using the primary mAb directed against: CD14 (5A3B11B5) and CD63 (MX-49.129.5; Santa Cruz Biotechnology, Santa Cruz, CA, USA); CD66b (80H3, AbD Serotec, Oxford, UK); CD1c (blood DC antigen 1; MACS; Miltenyi Biotec, Bergisch Gladbach, Germany); and CD15 (MY-1; Abcam, Cambridge, UK). Rabbit polyclonal antibodies included: anti-CD68 (ProteinTech, Chicago, IL, USA), anti-LC3B (L7543; Sigma-Aldrich), and anti-MPO (ab45977; Abcam). Then, the cells were washed and incubated with 1/400 secondary antibodies Cy2-conjugated goat anti-rabbit IgG and/or Cy5-conjugated goat anti-mouse IgG (Biotium, Hayward, CA, USA). After washing, slides were mounted with mounting medium containing DAPI for nuclear staining (Vectashield H-1000; Vector Laboratories, Burlingame, CA, USA). Slides were analyzed by a confocal laser-scanning system (LSM 700) using the Nikon E600 fluorescence microscope (Japan) and Plan Apo $\times 40$ immersion oil objective.

Phagocytotic activity of neutrophils

Freshly isolated neutrophils or 5- to 7-day G ϕ were incubated for 2 h with carboxylate-modified fluorescent yellow-green latex beads of 1 μ m diameter (Polyscience, Warrington, PA, USA), at a bead:cell ratio of 750:1. Zymosan was opsonized with IgG opsonizing reagents (Sigma-Aldrich) for 1 h at 37°C and then was added to the cells at a final concentration of 10 μ g/ml. Cytospins were prepared and fixed as described above and analyzed by confocal microscopy. A cytolocalization analysis was performed using 3D IMARIS software (Bitplane AG, Switzerland) to validate intracellular localization.

Oil Red O staining

Cytospins prepared from untreated or oxLDL-treated (2 or 20 h) cultures were fixed (as above) and stained for 2 min at room temperature in freshly diluted Oil Red O solution (six parts of 0.5% Oil Red O stock in isopropanol and four parts double distilled water). Fluorescence imaging was assessed by confocal microscopy.

ROS production

The intracellular ROS production was assessed by DCF (Molecular Probes, Invitrogen, Carlsbad, CA, USA). ROS production was stimulated by incubating the cells at 37°C with the following stimulants: 10 μ g/ml opsonized

zymosan for 60 min; 5 $\mu\text{g}/\text{ml}$ oxLDL for 15 min, 90 min, or 20 h; or 100 nM PMA for 15 min (Sigma-Aldrich). DCF was added at the last 15 min of each treatment. The fluorescence intensity of nonfixed cells was analyzed immediately by confocal microscopy.

Intracellular ROS was also determined by a NBT (Sigma-Aldrich) test. NBT was dissolved in RPMI 1640 (0.2%) without phenol red. Cells were incubated without or with 100 nM PMA at 37°C for 15 min, then kept at room temperature for 10 min, and assessed by light microscopy. G ϕ with cytoplasmic clumps of formazan deposits were considered positive.

Statistical analysis

Data are expressed as mean $\pm$ SD for each experimental group. A two-tailed Student's *t*-test was used for single comparison of parametric data. Values of $P < 0.05$ were considered significant. A paired two-tailed Student's *t*-test with Bonferroni correction was used for multiple comparisons. Therefore, only values of $P < 0.017$ were considered significant. The NCSS 2004 statistical package (NCSS Statistical Software, Kaysville, UT, USA) was used.

Online supplemental material

Supplemental Fig. 1 represents TEM photomicrographs of G ϕ with large phagosomes containing neutrophil granules. Samples for TEM were fixed with 3.5% glutaraldehyde; rinsed with cacodylate buffer, followed by secondary fixation in 2% osmium tetroxide for 1 h; and washed again in cacodylate buffer to remove excess osmium. Then, samples were immersed in saturated, aqueous uranyl acetate, dehydrated in graded alcohol solutions, immersed in propylene oxide, and embedded in Epon resin. Thin sections (80 nm) were cut and placed on a 300-mesh, thin-bar copper grid and stained with uranyl acetate and lead citrate. The samples were examined by a JEOL transmission electron microscope (JEOL JEM-1011). Supplemental Fig. 2A–D represents negative expression of CD1c, a DC marker, on freshly isolated neutrophils and in developing G ϕ compared with DCs (a positive control). Supplemental Fig. 2E and F represents Nox2 expression in untreated (Supplemental Fig. 2E) and oxLDL-treated (Supplemental Fig. 2F) G ϕ . For detection of acidified endosomes, viable neutrophils were incubated with 50 nM LysoTracker (Molecular Probes, Eugene, OR, USA) for 90 min at 37°C in the dark (red staining). Then, the prepared, fixed cytoplasts were stained with anti-Nox2/gp91phox antibodies (ab131083; green; Abcam). Arrows indicate Nox2 expression around phagolysosomes. Supplemental Fig. 3 represents a set of control experiments conducted to analyze in monocytes the G ϕ phenotypic markers (CD63, MPO, CD14, and CD68 expression), as well as to show ingestion of PMN by monocytes in monocyte/PMN cocultures. Supplemental Video 1 demonstrates the development of G ϕ in purified PMN culture by time-lapse microscopy. Supplemental Video 2 demonstrates the differences between monocyte-derived M ϕ , characterized by active movement (left, unlabeled cell), and G ϕ (right, bright PKH-26-labeled cell) in monocyte/neutrophil cocultures. The cells were followed by time-lapse microscopy. Briefly, neutrophils (Days 2–5) in culture or monocyte/neutrophil cocultures (Days 4–5) were subjected to live imaging. The time-lapse microscopy system is composed of the Zeiss Axio observer inverted, motorized fluorescent microscope and a high-resolution black/white charge-coupled device camera (Zeiss HRm) with an on-stage incubator (Live Imaging Services, Basel, Switzerland). Image captures were taken every 10 min. Time-lapse acquisition was made with AxioVision 4.7 software.

RESULTS

Neutrophil autophagocytosis

Freshly isolated, purified PMNs were equally divided into two parts and labeled with PKH-26 (red) or PKH-67 (green) dyes. Then, cells were cocultured and followed up to 7 days by confocal microscopy. Annexin-V staining was used to assess apo-

ptosis in PKH-67-dyed neutrophils. Concurrently, neutrophils were stained by Giemsa and analyzed with bright-field microscopy. As shown in Fig. 1A, at zero time of coculture, green- and red-labeled neutrophils could be easily distinguished by confocal microscopy. However, after 90 min, green-labeled neutrophils contained bright-red particles, whereas red-labeled neutrophils contained green particles, and ~20% of the neutrophils were orange and contained yellow particles (Fig. 1B, arrow). As expected, after 24 h, most of the neutrophils had typical apoptotic morphology, characterized by nuclear condensation and cell shrinkage (Fig. 1C), and were Annexin-V-positive (data not shown). During the following next 2 days in culture, cellular residues (debris), such as apoptotic bodies and nuclear materials, including chromatin fibers, were observed (Fig. 1D–F). Of note, during this period of time, a small number of large cells with aberrant morphology were noticed by confocal (Fig. 1D and E, arrow) and light (Fig. 1F, arrow) microscopy. As shown in Fig. 1E, Annexin-V-positive (dotted circle) and Annexin-V-negative (arrow) cells were seen. At a later point, giant cells resembling vacuolated M ϕ , with a nuclear incline toward the end of the cells, were noted. These large cells engulfed cellular residues at various stages of disintegration (Fig. 1E–H, J). TEM of G ϕ confirmed the presence of large phagosomes enclosing neutrophil granules (Supplemental Fig. 1).

These long-lived G ϕ were Annexin-V-negative (Fig. 1H) and could be followed during 2–3 weeks in culture. G ϕ (100–200) developed from 1×10^6 neutrophil cultures, derived from 14 healthy adult subjects (four of whom were used for time-lapse microscopy). All G ϕ were PI-negative (Fig. 1I), indicating that these cells were not necrotic.

To confirm the viability of G ϕ and to investigate the dynamics of neutrophil transformation into G ϕ , the morphologic changes were also followed by live-cell imaging and time-lapse microscopy. As can be seen in Supplemental Video 1, these G ϕ were nonadherent phagocytes with limited movement capacity and actively ingested surrounding debris. Their development occurred between 2 and 3 days in culture.

Origin and phenotype of G ϕ

To determine whether these G ϕ have a neutrophilic origin and to prevent a potential contamination by monocytes, Ficoll-isolated PMNs were subjected to a subsequent purification step by FACS using CD15 and CD14 antibodies and granulocyte gating, as described by Araki et al. [12]. The cell population obtained was morphologically identified as neutrophils and also expressed CD15⁺/CD11b⁺/CD63⁺/CD66b⁺ neutrophil markers (Fig. 2A and B). Also, this purified cell population did not express a bright CD14 marker, characteristic of monocytes (Fig. 2A and B). The development of G ϕ from this population was similar to the Ficoll-isolated neutrophils.

In addition, the phenotypic characteristics of G ϕ were determined by confocal microscopy, as depicted in Fig. 3 and in online Supplemental Fig. 2B. Specifically, all G ϕ intensively expressed the neutrophil receptor CD15 (Fig. 3A), a marker of specific neutrophil granules CD66b (Fig. 3B), as well as markers of azurophilic granules CD63 (granulophysin; Fig. 3C) and MPO (Fig. 3D), but did not express CD14 (Fig. 3E).

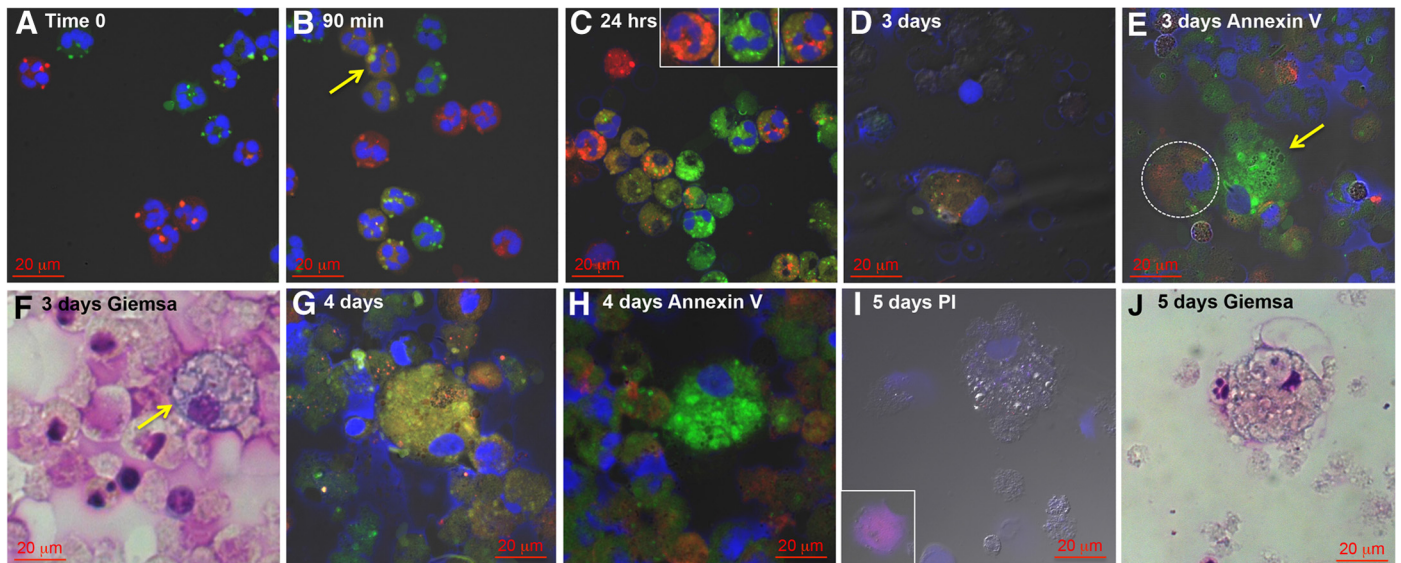
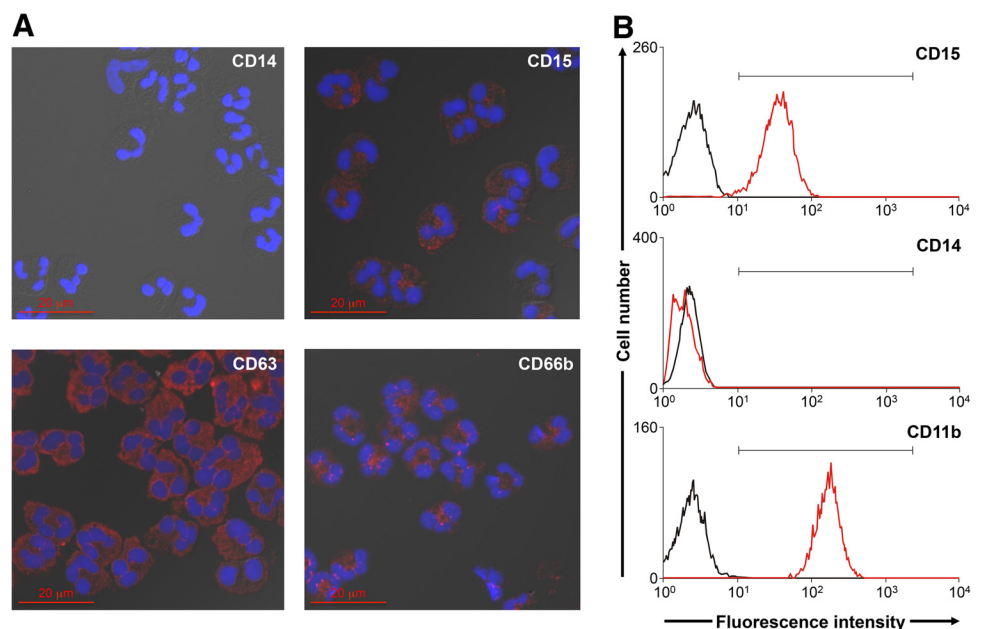


Figure 1. Development of G ϕ , up to 5 days in culture. (A–D) Freshly isolated, purified neutrophils were labeled with PKH-26 (red) or PKH-67 (green) dyes and cocultured for various times. (B) Arrow indicates orange neutrophil containing yellow particles. (C) Insets represent enlargements of single cells. (E) PKH-67 (green)-labeled neutrophils stained with Annexin-V-Cy-5 (red). The circled cell is Annexin-V-positive, and the arrow depicts an Annexin-V-negative, large neutrophil. (F) Giemsa-stained cytopins of cultured neutrophils. Arrow depicts G ϕ . (G) Four-day G ϕ developed in cocultures of PKH-26 (red) and PKH-67 (green). (H) PKH-67 (green)-labeled neutrophils were stained with Annexin-V-Cy-5 (red). (I) PI-negative G ϕ . Inset represents a PI-positive cell from the same culture. (J) Five day Giemsa-stained cytopsin of a G ϕ . (A–E and G–I) Nuclei were stained with DAPI, and samples were analyzed by confocal microscopy. (F, J) Samples were analyzed with a bright-field microscopy. Magnification oil, $\times 40$. Representative data out of 10 independent experiments.

Importantly, CD15 and CD66b were specifically expressed only by G ϕ and freshly isolated neutrophils, whereas cultured monocytes were negative for these markers (Fig. 3A and B). Moreover, also, monocyte-derived M ϕ , which developed in co-culture with autologous PMN, were devoid of CD15 and CD66b, thus ruling out the possibility that these markers in G ϕ resulted from phagocytosis of neutrophil remnants (Fig.

3A and B). Of note, a smaller number of G ϕ developed in these cocultures. Additionally, similar to neutrophils, G ϕ were negative for the dendritic marker CD1c (Supplemental Fig. 2A and B). Importantly, G ϕ intensively expressed the scavenger receptor CD68 (Fig. 3F). Intracellular CD68 expression was also noted in fresh neutrophils by confocal microscopy (Fig. 3F), as well as by flow cytometric analysis (Fig. 3G). Despite

Figure 2. Phenotype of freshly isolated neutrophils after FACS separation. (A) Cytospins were prepared and analyzed by confocal microscopy for CD14, CD15, CD63, and CD66b (see Materials and Methods). Nuclei were stained with DAPI. (B) Representative histograms depicting the expression of CD15, CD14, and CD11b, analyzed by flow cytometry. The red and black lines are indicative of the molecules expressed and isotype controls, respectively. Representative data out of three independent experiments.



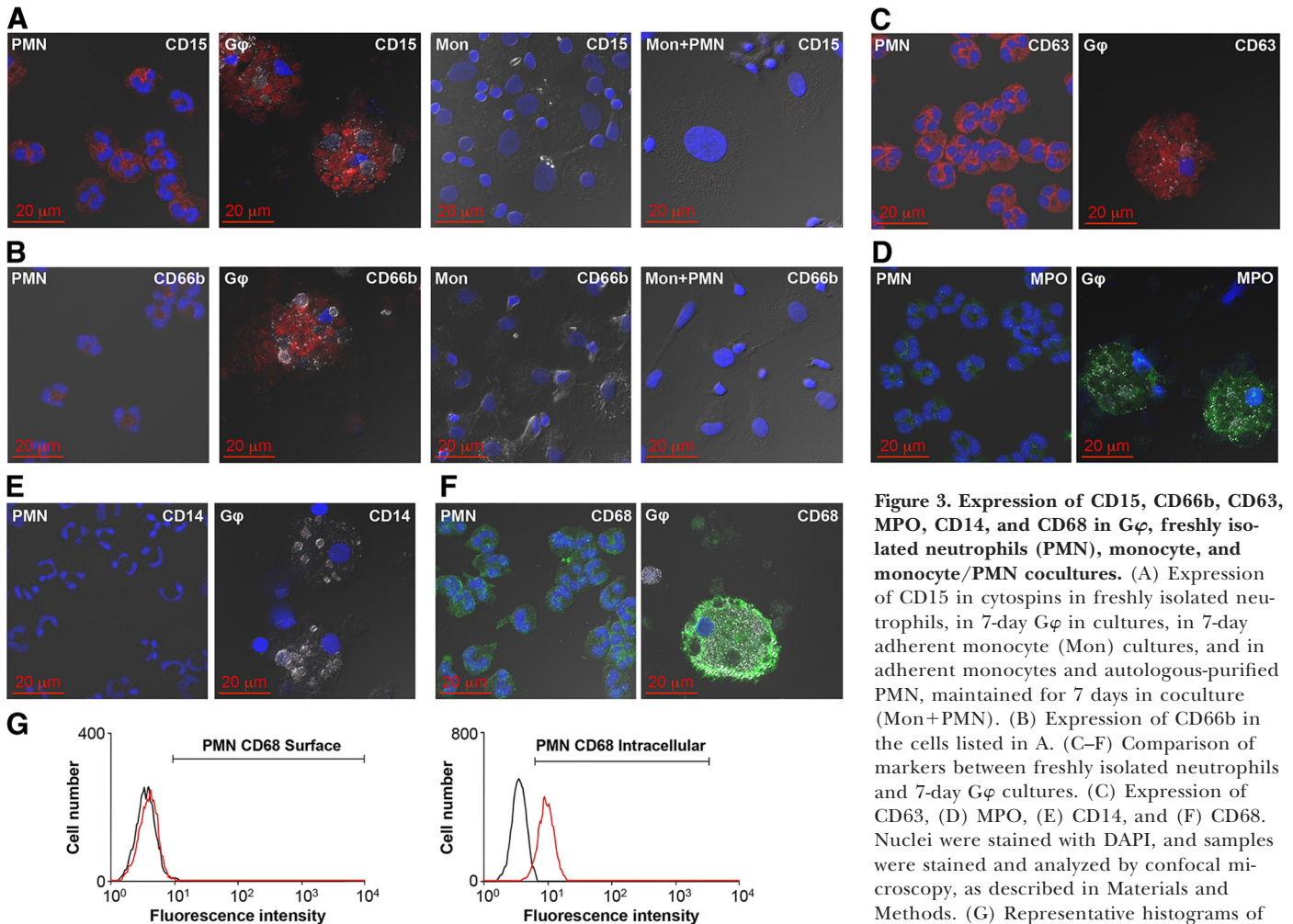


Figure 3. Expression of CD15, CD66b, CD63, MPO, CD14, and CD68 in Gφ, freshly isolated neutrophils (PMN), monocyte, and monocyte/PMN cocultures. (A) Expression of CD15 in cytoplasm in freshly isolated neutrophils, in 7-day Gφ cultures, in 7-day adherent monocyte (Mon) cultures, and in adherent monocytes and autologous-purified PMN, maintained for 7 days in coculture (Mon+PMN). (B) Expression of CD66b in the cells listed in A. (C–F) Comparison of markers between freshly isolated neutrophils and 7-day Gφ cultures. (C) Expression of CD63, (D) MPO, (E) CD14, and (F) CD68. Nuclei were stained with DAPI, and samples were stained and analyzed by confocal microscopy, as described in Materials and Methods. (G) Representative histograms of CD68 surface and intracellular (saponin-per-

meabilized) expression in freshly isolated neutrophils demonstrating high intracellular CD68 content. Representative data out of at least three independent experiments.

the low number of Gφ developed in PMN/monocyte cocultures, the intensity of CD68 expression in these Gφ was similar to Gφ developed in purified PMN cultures. The integrated density of green fluorescence in Gφ, developed in monocyte/PMN cocultures, was $845,788 \pm 228,307$ versus $843,614 \pm 374,423$ in Gφ, developed in purified PMN cultures ($P=0.7$).

When neutrophils were cocultured with autologous monocytes, two different types of large phagocytes were noted: slow rotating cells, identified as Gφ (as in Supplemental Video 1), and large Mφ with amoeboid-like movement (Supplemental Video 2). Moreover, in contrast to pure neutrophil cultures, after 2 days in monocytes/neutrophil cocultures, monocytes ingesting whole neutrophils (red) were detected (Supplemental Fig. 3F, inset). Monocytes ingesting neutrophils would give rise to, what is termed in pathology as, a Reiter cell.

Phagocytic activity and oxLDL uptake by Gφ

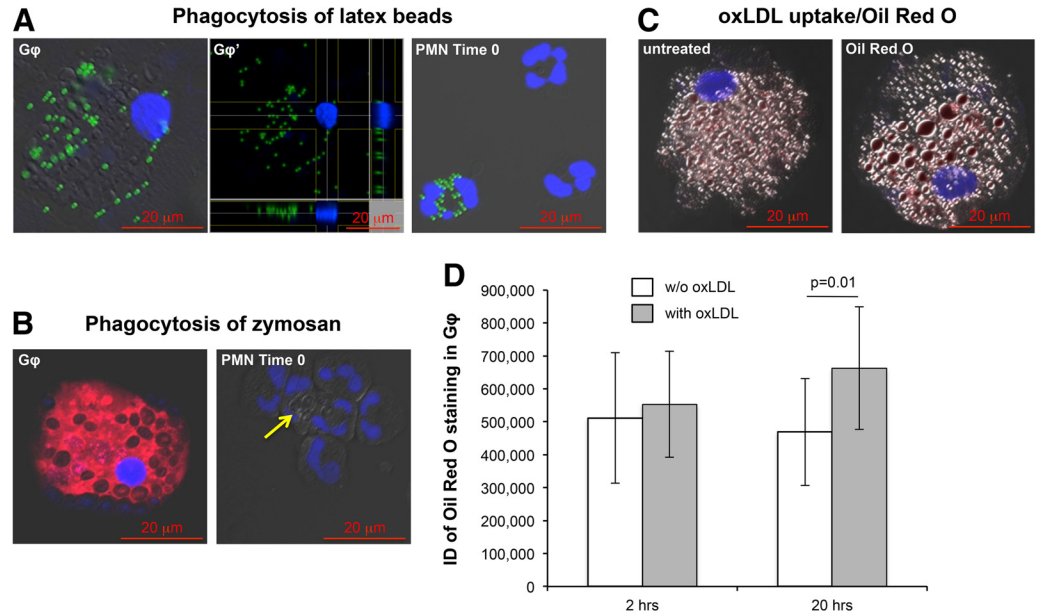
The phagocytic activity of Gφ was evaluated using carboxylate-modified fluorescent yellow-green latex beads and opsonized zymosan by confocal microscopy. All Gφ internalized latex

beads (Fig. 4A), as well as opsonized zymosan (Fig. 4B). By contrast, only 32% and 25% of the freshly isolated neutrophils internalized latex beads and opsonized zymosan, respectively (Fig. 4A and B). The intracellular localization of latex beads was confirmed further by 3D z-stack images (Gφ'; Fig. 4A).

Intensive expression of CD68 scavenger receptor by Gφ suggests that they may also participate in oxLDL uptake. To test this, Gφ were incubated with oxLDL for 2 and 20 h and stained with Oil Red O. Of note, although Oil Red O staining is commonly used to identify mainly exogenous or endogenous lipid deposits in Mφ, it also stains the granules in neutrophils [19]. Thus, it was not surprising that Oil Red O-positive staining was detected in all Gφ (Fig. 4C). Treatment of Gφ for 2 h with oxLDL had no effect on lipid droplet formation. However, after a long, 20-h exposure to oxLDL, large Oil Red O-positive lipid droplets were seen, indicating that oxLDL was taken up by Gφ (Fig. 4C). The integrated density of fluorescence analysis of Oil Red O revealed a 41% increase in oxLDL-treated cells over the controls (Fig. 4D; $P=0.01$).

Figure 4. Phagocytic activity of G ϕ compared with freshly isolated neutrophils. Cytospins were prepared and analyzed by confocal microscopy (see Materials and Methods).

(A) G ϕ (left and middle) and freshly isolated neutrophils (right) were incubated for 2 h with carboxylate-modified fluorescent yellow-green latex beads. G ϕ ', representative image (xy, xz, and yz cross-sections) of intracellular latex beads, localized by 3D reconstructing software IMARIS z-stack analysis. (B) Opsonized zymosan was added for 1 h to PKH-26 labeled (red) G ϕ (left) and freshly isolated neutrophils (right; arrow). (C) Untreated (left) and 20-h oxLDL-treated G ϕ (right), stained with Oil Red O (see Materials and Methods). (D) Integrated density (ID) of Oil Red O staining in G ϕ , treated with oxLDL for 2 h and 20 h compared with untreated cells [without (w/o)]. Significance ($P=0.01$) compared with G ϕ without oxLDL treatment. (A–C) Nuclei were stained with DAPI. Representative data out of at least three independent experiments.



ROS production by G ϕ and the effects of oxLDL

Production of ROS is the hallmark of neutrophil phagocytosis. It was determined with confocal microscopy in PMA and zymosan-stimulated G ϕ using DCF as the ROS fluorescent-sensitive probe and verified with NBT test (Fig. 5A, B, and E). As G ϕ also internalized oxLDL, ROS production was determined in response to oxLDL treatment for 15 min, 90 min, and 20 h. ROS production by G ϕ was compared with PMA stimulation at the indicated times after adding PMA for 15 min. As shown in Fig. 5A, oxLDL treatment for 15 and 90 min significantly increased ROS production by G ϕ compared with untreated G ϕ . However, after 20 h, there was no difference in ROS production between untreated and oxLDL-treated G ϕ . Importantly, 15-min PMA treatment induced significant ROS production at all of the indicated time intervals. Production of ROS was also noted in response to 1-h opsonized zymosan (integrated density of green fluorescence, $83,226 \pm 21,022$ with vs. $28,539 \pm 11,144$ without zymosan; $P=0.026$). Representative photomicrographs of ROS production in unstimulated and oxLDL PMA- and zymosan-stimulated G ϕ are depicted in Fig. 5B and compared with freshly prepared neutrophils in Fig. 5C. As expected, fresh neutrophils produced ROS in response to stimulation by PMA and to a lesser extent, by zymosan. However, they did not produce ROS under oxLDL treatment. Also, ROS production was detected in 7-day monocyte cultures, only in response to PMA but not to oxLDL stimulation (Fig. 5D). The ability of G ϕ to produce ROS was also verified using the NBT test. In response to stimulation by PMA, almost all G ϕ reduced NBT dye into blue formazan (Fig. 5E).

G ϕ are labeled with LC3B

The autophagic machinery plays a crucial role in neutrophil survival and death, and LC3B is a key and specific component

of the autophagosomal membrane [20]. Therefore, the induction of autophagy was determined in G ϕ by the autophagy-specific, endogenous LC3B marker using immunofluorescence microscopy. As shown in Fig. 6A (and inset), fresh neutrophils showed a weak LC3B expression at Time zero. After 2 days in culture, LC3B-coated vacuoles and redistribution of LC3B into punctuated structures were noted in Annexin-V-negative G ϕ (Fig. 6B, arrows). But, also, single LC3B⁺/Annexin-V⁺ neutrophils were detected (Fig. 6B, inset). After 3 days, punctuated LC3B distribution was noted in all G ϕ (Fig. 6C). Importantly, after 6 days, LC3B-coated vacuoles were clearly noted in untreated G ϕ , as shown in Fig. 6D, indicating that autophagy is a constitutive trait of G ϕ . However, as shown in Fig. 6E, oxLDL treatment induced LC3B accumulation. The integrated density of green fluorescence units after 90 min of oxLDL treatment was $803,576 \pm 149,616$ versus $647,832 \pm 81,754$ U in untreated G ϕ ($P<0.01$). An intensive LC3B accumulation was also noted during zymosan phagocytosis (Fig. 6F). Additionally, nuclear LC3B staining was noted in G ϕ , as reported previously for other cell types [21, 22]. However, as indicated in the legend, the integrated density of LC3B nuclear expression was similar in untreated and oxLDL-treated G ϕ .

To ascertain further the involvement of LC3B and autophagy in the development of G ϕ , two specific autophagy inhibitors—3-MA and BafA1—with different modes of action were used [23, 24]. These inhibitors were used at concentrations that have been used previously for autophagy blockage in neutrophil studies (3-MA, 5 mM; BafA1, 30 nM) [25, 26]. The effects of these inhibitors in all culture conditions were verified by following the cells daily up to 7 days in culture. Treatment with the inhibitors did not cause toxic effects on PMN. The percentage of dead neutrophils detected by trypan blue exclusion at 24 h, after adding the inhibitors to freshly iso-

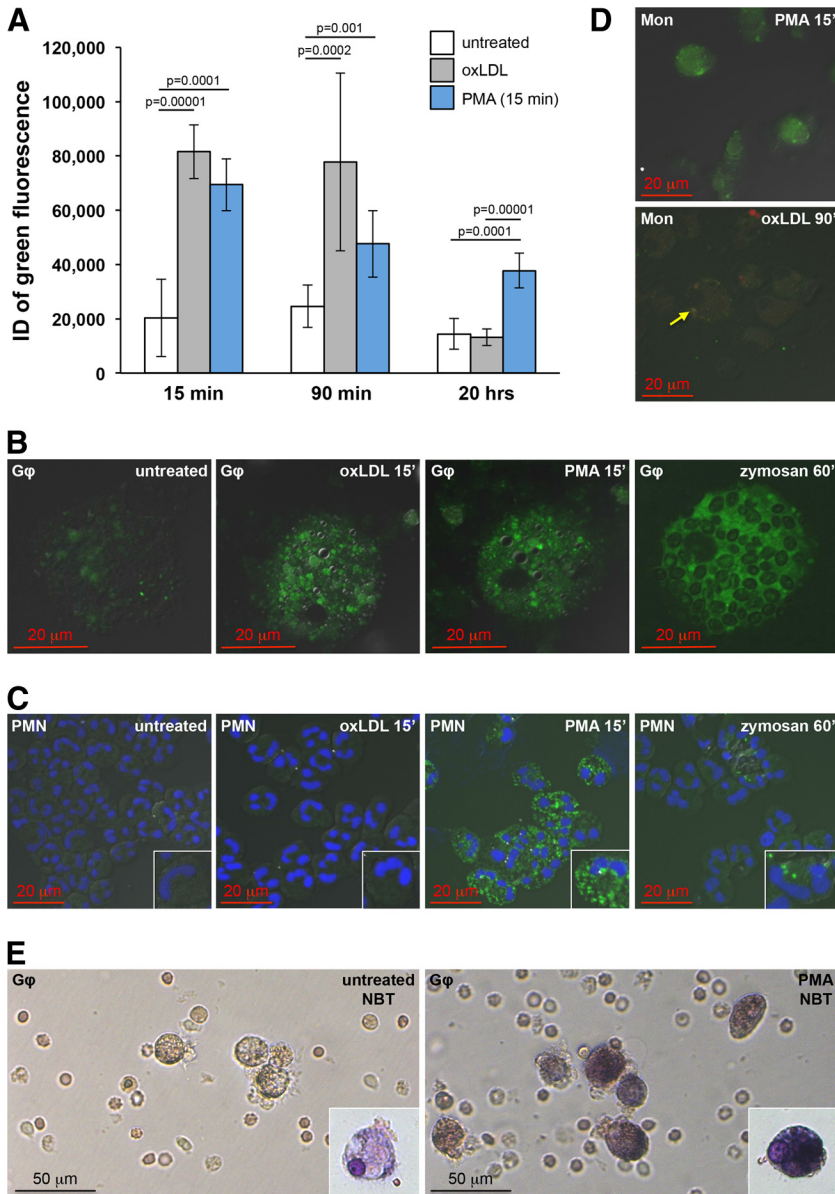


Figure 5. ROS production by Gφ, freshly isolated neutrophils, and cultured monocytes. (A–D) Intracellular ROS production was assayed by DCF (described in Materials and Methods). The fluorescence intensity of nonfixed cells was analyzed immediately by confocal microscopy. (A) DCF values at various time-points after oxLDL and PMA stimulation. Two-tailed *t*-test with Bonferroni correction was used for the analysis. (B) Representative photomicrographs of untreated, oxLDL-treated, PMA, and opsonized zymosan-stimulated Gφ. (C) Fresh neutrophils, stimulated with oxLDL, PMA, and opsonized zymosan as in B. Insets represent enlargements of a single PMN. (D) Intracellular ROS production in 7-day cultured monocytes stimulated with PMA or oxLDL. Arrow represents negative ROS production in monocyte. (E) Intracellular ROS production by NBT test in control untreated and PMA-stimulated Gφ. Insets represent examples of untreated and PMA-stimulated Gφ from the same culture in fixed and Giemsa-stained cytopins. Representative data out of at least three independent experiments.

lated PMN, was similar to untreated PMN (3-MA- and BafA1-treated cells: 9.0% and 7.5%, respectively, vs. 11.0% in untreated). The addition of 3-MA to PMN cultures at Time zero had no effect on Gφ development (Fig. 7B), further indicating the absence of toxic effects. However, 3-MA abolished the formation of Gφ when it was added to 48-h cultures (Fig. 7E), which was the time frame at which LC3B-coated vacuoles and redistribution of LC3B into punctuated structures were noted (Fig. 6B and C). Treatment with BafA1 at Time zero decreased and after 48 h in culture, abolished (Fig. 7C and F) the formation of Gφ.

DISCUSSION

In the present study, we provide new findings on phagocytic cells that arise from pure CD15⁺/CD63⁺/CD66b⁺ human

neutrophil populations maintained in prolonged culture conditions. These cells displayed an extended lifespan and a vastly enlarged size, were able to internalize neutrophil remnants, and demonstrated the following characteristics: (1) Gφ were positive for CD15/CD66b/CD63/MPO markers; (2) they produced ROS in response to PMA and opsonized zymosan; (3) Gφ expressed CD68 scavenger receptors and internalized oxLDL, subsequently producing ROS; (4) Gφ also contained LC3B-coated vacuoles and LC3B aggregates, indicating that autophagy is a constitutive trait, and the addition of oxLDL or zymosan induced further accumulation of LC3B around phagosomes; and (5) treatment with 3-MA or BafA1, two well-established autophagy inhibitors, abolished the development of Gφ.

Neutrophils, like Mφ, are considered the main arm of the myeloid phagocyte system to rid of pathogens and harmful

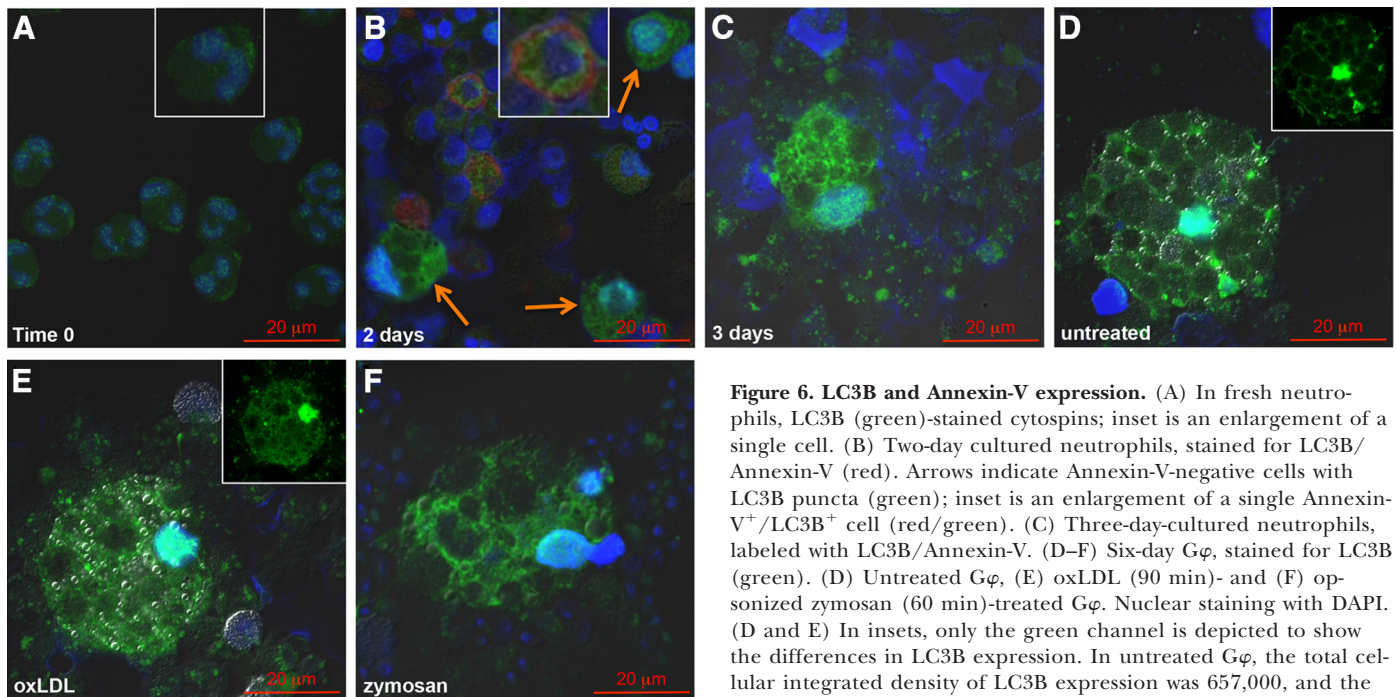


Figure 6. LC3B and Annexin-V expression. (A) In fresh neutrophils, LC3B (green)-stained cytopins; inset is an enlargement of a single cell. (B) Two-day cultured neutrophils, stained for LC3B/Annexin-V (red). Arrows indicate Annexin-V-negative cells with LC3B puncta (green); inset is an enlargement of a single Annexin-V⁺/LC3B⁺ cell (red/green). (C) Three-day-cultured neutrophils, labeled with LC3B/Annexin-V. (D–F) Six-day G ϕ , stained for LC3B (green). (D) Untreated G ϕ , (E) oxLDL (90 min)- and (F) opsonized zymosan (60 min)-treated G ϕ . Nuclear staining with DAPI. (D and E) In insets, only the green channel is depicted to show the differences in LC3B expression. In untreated G ϕ , the total cellular integrated density of LC3B expression was 657,000, and the nuclear was 180,000. In oxLDL-treated G ϕ , the total cellular integrated

density for LC3B expression was 785,000, and the nuclear was 178,000. Representative data out of at least three independent experiments.

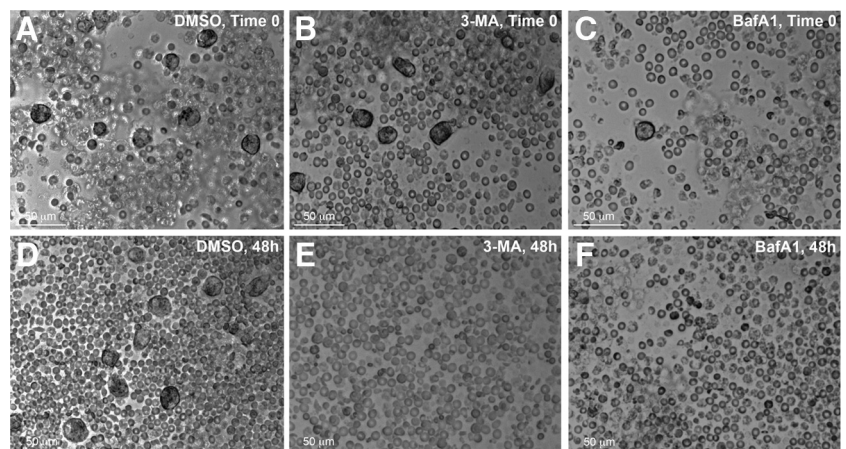
particles [2]. However, their clearance by M ϕ /DCs is a crucial step in the resolution of inflammation [27]. This process is essential for maintaining tissue homeostasis and if hampered, may lead to “nonresolved inflammation”, a condition that contributes to many diseases [28]. Thus, our experimental model in vitro simulates an important element in certain in vivo inflammatory conditions, namely, the presence of large numbers of neutrophils. Such instances were described in the synovial fluid of rheumatoid joints, in acute peritonitis and pneumococcal pneumonia [6, 7], as well as in experimental lung inflammation in mice induced by LPS [8].

Nonactivated neutrophils have the shortest half-life among leukocytes and are constitutively committed to apoptosis [29, 30]. However, in the current study, we report that some neu-

trophils remained viable in pure neutrophil preparations that were maintained in prolonged culture conditions, without adding external stimuli, such as growth factors or cytokines. During the first hours in culture, fresh neutrophils took up granules and/or microparticles released from other neutrophils, a process that may promote neutrophil activation. Recent evidence suggests that microparticles carrying substantial amounts of intact mRNA and micro-RNA can deliver packaged information. These microparticles can propagate activation and alter the expression of genes in neighboring and distant cells, potentially exerting novel role in homeostasis and diseases [31, 32].

From 3 days in culture and on, a small number of cells were still viable and expressed phagocytic functions. They were

Figure 7. Effects of 3-MA and BafA1 treatment on G ϕ development. Light-microscopy photomicrographs of G ϕ formation in 7-day PMN cultures. (A–C) Treatments were added at Time zero in culture. (A) DMSO, (B) 3-MA (5 mM), and (C) BafA1 (30 mM). (D–F) Treatments were added at 48 h in culture. (D) DMSO, (E) 3-MA, and (F) BafA1. Representative photomicrographs from three experiments.



characterized by large vacuoles and contained engulfed cellular residues at various stages of disintegration. Therefore, the development of these cells was followed by confocal and light microscopy and by time-lapse photography.

A number of steps were undertaken to characterize the phenotype G ϕ and their functions and to validate their neutrophilic origin. First, G ϕ expressed the specific neutrophilic marker CD15 and had characteristic azurophilic CD63⁺ granules [33], and CD66b⁺-specific granules [34, 35]. Second, after elaborate FACS purification, the purified CD15⁺/CD63⁺/CD66b⁺ cells also developed into G ϕ , similar to the Ficoll-isolated neutrophils. Third, monocytes and monocyte-derived M ϕ , cocultured with neutrophils, were CD15- and CD66b-negative. Jointly, these findings indicate that G ϕ could not have developed from contaminating monocytes and attest to the neutrophilic origin of the G ϕ . However, it is not clear if they may have developed from neutrophil precursors and/or may have transdifferentiated or undergone reprogramming into M ϕ -like cells, as was described previously by Araki et al. [12]. It should be noted, however, that only a small number of G ϕ were detected in PMN/monocyte cocultures. This may indicate that the absence of a proper clearance by M ϕ /DC is critical for the development of G ϕ , as we have demonstrated in pure neutrophil cultures.

To determine whether G ϕ expressed additional fundamental characteristics of neutrophils, their phagocytic ability and ROS production associated with soluble stimuli and ingested particles were investigated [35]. Phagocytosis and ROS production were prominent functions of all G ϕ . Apart from internalizing apoptotic neutrophil debris, G ϕ also had engulfed latex beads, opsonized zymosan, and taken up oxLDL (Fig. 4) and generated ROS. Moreover, PMA, which is one of the most established soluble stimuli activating the respiratory burst in neutrophils, also increased ROS production in G ϕ (Fig. 5).

Phagocytes are equipped with multiple types of specialized receptors to recognize their targets. The CD68 scavenger receptor, which is expressed on M ϕ , is thought to affect foam cell formation following uptake of modified LDLs [36]. Apart from M ϕ , CD68 is expressed by a variety of other cell types. In neutrophils, intracellular CD68 localization was identified mainly in the primary granules of neutrophils [37]. But, CD68 gene and protein expression was also demonstrated by real-time RT-PCR, and its uptake by neutrophils was excluded [38]. Thus, the expression of CD68 in freshly isolated neutrophils was also confirmed in this study using immunofluorescence and flow cytometry. Moreover, flow cytometric analysis in freshly isolated neutrophils showed that CD68 was only localized intracellularly but not on the cell surface of neutrophils (Fig. 3G), confirming earlier data on the intracellular localization of CD68 in neutrophils [37]. Moreover, although monocytes, as well as fresh neutrophils, expressed CD68 (Fig. 3F and G and Supplemental Fig. 3D), only G ϕ internalized oxLDL (Fig. 4C and D) and generated ROS after a short oxLDL treatment (Fig. 5). In a preliminary series of experiments (Supplemental Fig. 2E and F), we demonstrated that G ϕ expressed the Nox2 subunit gp91phox. This suggests that G ϕ , like neutrophils, express the primary ROS-generating enzyme, Nox. Thus, taken together, CD68 expression, uptake of oxLDL, and

subsequent increased ROS production indicate that G ϕ may participate in atherogenic processes.

The development of G ϕ in long-term culture conditions has led us to assume the existence of mechanisms that support neutrophil activation after ingesting neutrophil remnants and microparticles and thereby, promote survival mechanisms.

Autophagy is one of the well-established fundamental mechanisms associated with cell survival [15]. The LC3/Apg8 is a key protein in autophagosome formation and the most widely used autophagy-related protein. It comprises a number of family members, including LC3B, which has two isoforms—LC3I and LC3II. Without activation, the cytoplasmic, soluble LC3I isoform is expressing a diffused pattern that is recognized by confocal microscopy. Following stimulation, it is converted into LC3II, a membrane-bound protein, associated with the autophagosomal membrane, and is identified by confocal microscopy by its punctuated expression [20]. LC3II is the only known protein that is associated specifically with autophagosomes and not with other vesicular structures [18, 20, 24].

In the present study, the formation of LC3B aggregates was followed in long-term neutrophil cultures (Fig. 6A–F). After 2 days in culture, some Annexin-V-positive neutrophils coexpressed LC3B, having a mixed apoptotic and autophagic phenotype, as described by Maiuri et al. [17]. However, during the following days, only Annexin-V-negative G ϕ with LC3B-coated vacuoles and LC3B redistribution into punctuated structures were observed [24, 39]. An intensive LC3B aggregation and accumulation were also noted during zymosan and oxLDL internalization. These findings indicate the involvement of an autophagy-related mechanism for oxLDL uptake, as well as for zymosan phagocytosis in G ϕ , as described previously for human neutrophils ingesting opsonized *Escherichia coli* [20]. It should be noted that LC3B was also expressed in the nuclei of G ϕ , but unlike in the cytoplasm, there were no differences in nuclear LC3B expression between untreated and oxLDL-treated G ϕ (Fig. 6D and E). Although LC3 is thought to function primarily in the cytosol, at sites of autophagosome formation, its distribution in the nucleoplasm was also observed in various cell lines [21, 22]. Also, potential nuclear LC3B staining is indicated in the product information. Additionally, the nuclear pool has not been specifically studied, and the mechanisms by which LC3 shuttles between the cytoplasm and nucleoplasm are currently unknown. Furthermore, it was suggested that the enrichment of soluble EGFP-LC3 in the nucleus is maintained independently of active nuclear transport or induction of autophagy [22]. Thus, the presence LC3B in the nucleus of G ϕ confirms earlier studies in other cell types.

To ascertain whether the development of G ϕ was autophagy-dependent, we used two well-established inhibitors of the autophagic pathway that differed in the mode of action, namely, 3-MA and BafA1. 3-MA is a class III PI3K inhibitor that inhibits protein degradation, prevents autophagy at the earliest stages of the autophagosome formation [23], and decreases the level of LC3B-II significantly [26]. BafA1 is a specific inhibitor of vacuolar type that inhibits the acidification of organelles containing enzymes, such as lysosomes and endosomes, and prevents autophagosome maturation by blocking the fusion with lysosomes. Both inhibitors abolished the formation of G ϕ , par-

ticularly if they were added after 48 h in culture, when LC3B-coated vacuoles and redistribution of LC3B into punctuated structures were noted. However, the addition of 3-MA to neutrophil cultures at Time 0 had no effect on G ϕ development, likely as 3-MA is not stable in aqueous solution at physiological pH for more than 24 h (as indicated in product information). Unlike 3-MA, adding BafA1 at Time 0 partially inhibited G ϕ formation, but like 3-MA, it abolished G ϕ formation completely when added after 48 h in culture (Fig. 7). Collectively, these findings support the involvement of the autophagy machinery in the development of G ϕ in these culture conditions.

The current study presents a number of challenging questions that should be addressed further: (1) as G ϕ have not yet been described in vivo, the question arises as to whether they do exist in physiological or pathological conditions; yet, it could be assumed that in some instances, because of the morphologic similarities, when describing large, vacuolated phagocytic M ϕ in vivo, they could be, in fact, G ϕ that were not fully characterized. It should be emphasized, however, that activated neutrophils containing large phagosomes enclosing neutrophil remnants were shown previously in a mouse model of LPS-induced lung inflammation [8]. Giant cell morphology resembling G ϕ was also described in Niemann-Pick disease and to a lesser extent, in Gaucher's disease ("lipid storage disease"). Interestingly, both types of pathologies belong to a family of lysosomal storage diseases [40]. (2) Although neutrophils are considered of limited plasticity and specialization, recent studies suggested otherwise [28]. For instance, in studies conducted on mice, immature and mature neutrophils were differentiated into a previously unrecognized hybrid population with a prolonged lifespan. These hybrid cells exhibited dual features and functionality of neutrophils and DCs when cultured with GM-CSF or upon recruitment to sites of inflammation or infection [41, 42]. These findings challenge the classical view of neutrophils as terminally differentiated cells by depicting their extraordinary plasticity. Thus, as described in the present study, G ϕ may represent a subset of phagocytic neutrophils or neutrophil progenitors, which ingest neutrophil remnants, and this may trigger their activation and promote survival mechanisms via LC3 and other molecules. (3) Whether the remarkable survival ability of G ϕ is beneficial or detrimental, particularly in terms of atherogenesis, is also a critical question [28]. In a recent study, the presence of MPO-positive neutrophils was reported in 72% of ruptured plaques [43]. These latter findings suggest a possible role for activated neutrophils in atherosclerosis and thus, may support a potentially atherogenic role for G ϕ .

Nonetheless, we should acknowledge a methodological limitation in this study. As a result of the low numbers of G ϕ obtained in culture and the importance of their morphological identification, the main experimental approach undertaken was based on confocal, light, and time-lapse microscopy.

In summary, this study describes for the first time the ability of some of the neutrophils maintained in prolonged culture conditions without external stimulants to transform into long-lived giant cells with phagocytic activity, and autophagy is at least partially responsible for this transformation. Once transformed into G ϕ , taking up oxLDL and consequently, generat-

ing ROS, this could imply a potential role in atherogenicity and other pathological conditions. However, further work is needed to determine the potential significance of these G ϕ to inflammatory/anti-inflammatory processes in vivo.

AUTHORSHIP

L.D., S.B., and L.L. were involved in the hypothesis delineation, designed the experiments, and wrote the paper. L.D., S.B., and A.P. performed the experiments. L.D., S.B., A.P., and L.L. analyzed the data.

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DISCLOSURES

The authors declare no conflict of interest.

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