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Accumulation and autofluorescence of phagocytized rod outer segment material in macrophages and microglial cells

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Purpose: To explore the ability of macrophages and microglial cells to phagocytize rod outer segments (ROSs) in a cell culture and characterize the resulting lipofuscin-like autofluorescence (LLAF).
Methods: Either regular or modified ROSs or ROS components (11-cis-retinal, all-trans-retinal, lipids) were fed to macrophages and microglial cells for 4 days. Afterwards, autofluorescence was detected by fluorescence-activated cell sorting (FACS) at two different wavelengths (533 nm and 585 nm), and the cells were imaged by confocal and electron microscopy. Fluorescein isothiocyanate (FITC)-labeled ROSs were added to macrophage and microglial cell cultures for 1–24 h to determine the kinetics of phagocytosis in these cell lines.
Results: Feeding with different ROSs or ROS components led to a significant increase in LLAF in both microglia and macrophages. The 4-hydroxynonenal (HNE)-modified ROSs gave rise to the highest increase in LLAF at both 533 nm and 585 nm. Application of 11-cis-retinal or all-trans-retinal resulted in higher LLAF at 585 nm, compared to application of 9-cis-retinal or liposomes. Fluorescein isothiocyanate-labeled ROSs co-localized well with lysosomes in both types of cells. HNE-modified ROSs were phagocytized more rapidly by both types of cells, compared to unmodified ROSs. Electron microscopy demonstrated inclusion bodies containing whorls of membranes in all types of cells fed with ROSs.
Conclusions: Both macrophages and microglia have the ability to phagocytize ROSs, and this results in increased autofluorescence. Oxidation of ROSs results in faster phagocytosis, higher levels of LLAF, and the appearance of more inclusion bodies inside the cells. Results from the present study suggest that both types of cells accumulate lipofuscin-like material under physiologically relevant conditions. Such accumulation could interfere with their ability to clear cellular debris and could be part of the pathogenetic mechanism for age-related macular degeneration and other lipofuscinopathies.

Lipofuscin is a polymorphous substance consisting of granular yellow-brown pigment granules composed of lipid-containing residues of lysosomal digestion, which are autofluorescent and accumulate in many tissues during senescence [1]. Excessive accumulation of lipofuscin could compromise essential cell function and, therefore, contribute to many age-related diseases, including age-related macular degeneration (AMD) [2]. There is clear clinical and pathological evidence for the age-dependent accumulation of lipofuscin in the retinal pigment epithelium (RPE). Recently, some studies reported that microglial cells and macrophages are also involved in the process of lipofuscin accumulation [3]. Specifically, the authors reported that subretinal microglia containing autofluorescent granules accumulated in an age-dependent manner in the subretinal space of adult normal mice and the number of autofluorescent microglial cells was higher compared to the number of autofluorescent cells at the same age. The autofluorescence emission fingerprints were similar between these cells and RPE cells that accumulate lipofuscin. Similarly, another recent report found accumulation of autofluorescent subretinal macrophages in aging ccl-2 knockout mice that have some phenotypic features resembling human AMD [4]

The ability of RPE cells to phagocytize the tips of rod outer segments (ROSs) as part of the outer segment daily renewal process is very important for the normal functioning of the retina and has been reported and discussed widely [5]. However, very little is known about the ability of either macrophages or microglia to phagocytize and degrade ROS material. Nor is there much known about the contributions of different natural components of ROSs to lipofuscin-like autofluorescence (LLAF) from these cells. Therefore, we examined the phagocytic ability of macrophages and microglia for unmodified and modified ROSs and the resulting change in LLAF in a cell culture model. An accumulation of undegraded ROS material and a proportional increase in LLAF was observed in both types of cells.

METHODS

Murine macrophage and MyD/Trif DKO microglial cell cultures and treatment: The macrophage cell line was the immortalized mouse macrophage cell line A3.1A [6]. The microglial cell line was derived from immortalized microglial cells from MyD88/TRIF double KO, which consists of microglial cells that retain their morphological and functional

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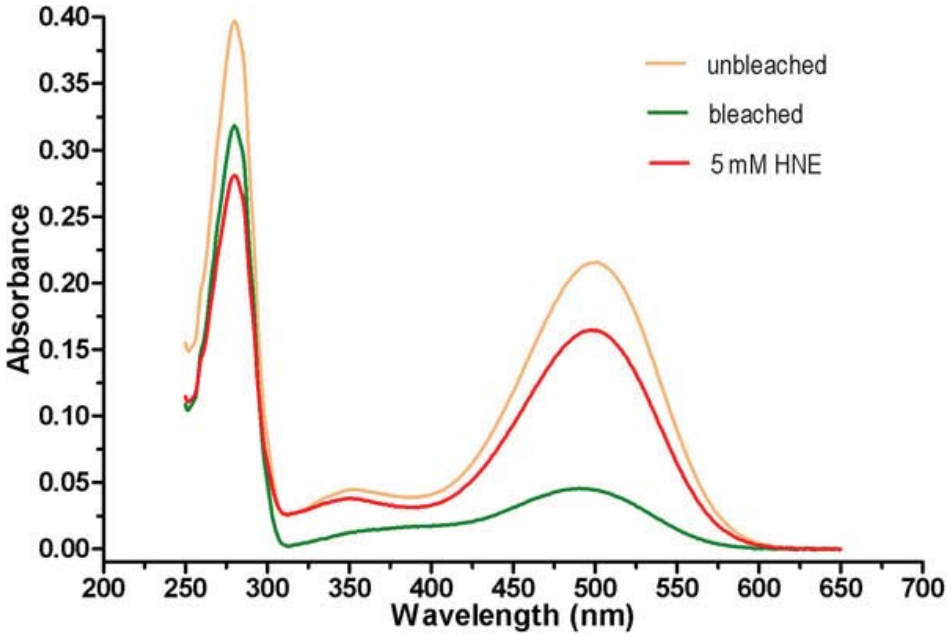

Figure 1. Spectrum of rhodopsin in rod outer segments. The characteristic rhodopsin absorbance at 498 nm was used to quantitate pigment yields. Note the absorbance difference at that wavelength between bleached rod outer segments (ROSs; green trace) and unbleached ROSs (orange trace). The modified rod outer segments (HNE-ROSs) at 5 mM (red trace) showed decrease in absorbance compared to unbleached ROSs.

characteristics [7]. The cells were grown in high glucose Dulbecco s Modified Eagle Medium (DMEM, Cellgro/ Mediatech, Manassas, VA) supplemented with 10% heat-inactivated fetal calf serum (FCS, Sigma-Aldrich, St. Louis, MO), 1% penicillin/streptomycin (Gibco, Grand Island, NY), 1% HEPES, 1% non-essential amino acid solution (NEAA), and 1:1,000 ciprofloxacin at 37 °C in the presence of 5% CO_2. Cells cultured in 10 cm plate were trypsinized and plated in 24 well plates or an 8-well chamber slide at a confluent density of $1.66\times10^5/cm^2$. ROSs were obtained from Invision BioResources (Seattle, WA) and isolated by a method similar to the one described by Papermaster [8]. All ROS preparations were incubated on a shaker at room temperature, overnight. After an additional culturing for 3 days, different types of ROS at 2 µg/ml were added every day for 4 days. The types of ROS were unbleached ROSs (prepared in the dark), bleached ROSs (exposed to 700 lux white light for 1 h), and 4-hydroxynonenal (HNE)-modified ROSs (see below). Additionally, several ROS components were added separately: 11-cis-retinal (10 µM, National Eye Institute, Bethesda, MD), all-trans-retinal (10 µM, Sigma-Aldrich, St. Louis, MO), 9-cis-retinal (10 µM, Sigma-Aldrich). Liposomes were prepared as described below and fed at concentration of 10 µM.

Modification of rod outer segments: The 4-hydroxynonenal (Cayman Chemical, Ann Arbor, MI) was prepared as previously described [9]. ROSs were incubated with 5 mM HNE at room temperature overnight on a shaker. Unbound HNE was removed by repeated washes in PBS 1× strength solution (derived from 10× solution by dilution with distilled deionized water, PBS 10×, Fisher BioReagents, BP3994; Fisher Scientific, Pittsburg, PA). The protein content of ROS preparations was measured using a BioRad BC kit (Bio-Rad, Hercules, CA). Modified ROSs were stored at −80 °C until use.

Extrusion of liposomes: Phosphatidylethanolamine (PE, Avanti Polar Lipids, Alabaster, AL) and phosphatidylcholine (PC, Avanti Polar Lipids) were mixed at a ratio of 60%:40% to a final concentration of 10 mM. The phosphatidylethanolamine used was 1,2-didocosahexaenoyl-sn-glycero-3-phosphoethanolamine (22:6 PE), and the phosphatidylcholine used was 1,2-dioleoyl-sn-glycero-3-phosphocholine (18:1 [Δ9-Cis] PC). The solvent was dried out by argon at room temperature and 1x PBS was added. Liposomes were extruded with Avanti Mini-Extruder (Avanti Polar Lipids) to extrude liposomes to an average diameter of 100 nm. Extruded vesicles were stored at 4 °C for 3–4 days.

Flow cytometry: Cells were cultured in 24-well plates and incubated with different components as described above (see Murine macrophage and MyD/Trif DKO microglia cell cultures and treatment). Cells were repeatedly washed, detached with trypsin, and analyzed on a C6 flow cytometer (Accuri Cytometers, Ann Arbor, MI). A gate was set to exclude cell debris and cell clusters, and 10,000 gated events were recorded. Experiments were performed in triplicates. Two channels were used: the FITC/GFP channel (excitation laser wavelength, 488 nm; detection filter wavelength, 533/30 nm) and the PE/PI channel (excitation laser wavelength, 488 nm; detection filter wavelength, 585/40 nm). For each condition and cell type, the fluorescence detected from the control samples was averaged, and the fluorescence detected from the test samples was expressed as a fraction of the averaged control value. Thus, the values are presented as a ratio (autofluorescence ratio [AF ratio]) compared to the fluorescence recorded from the control.

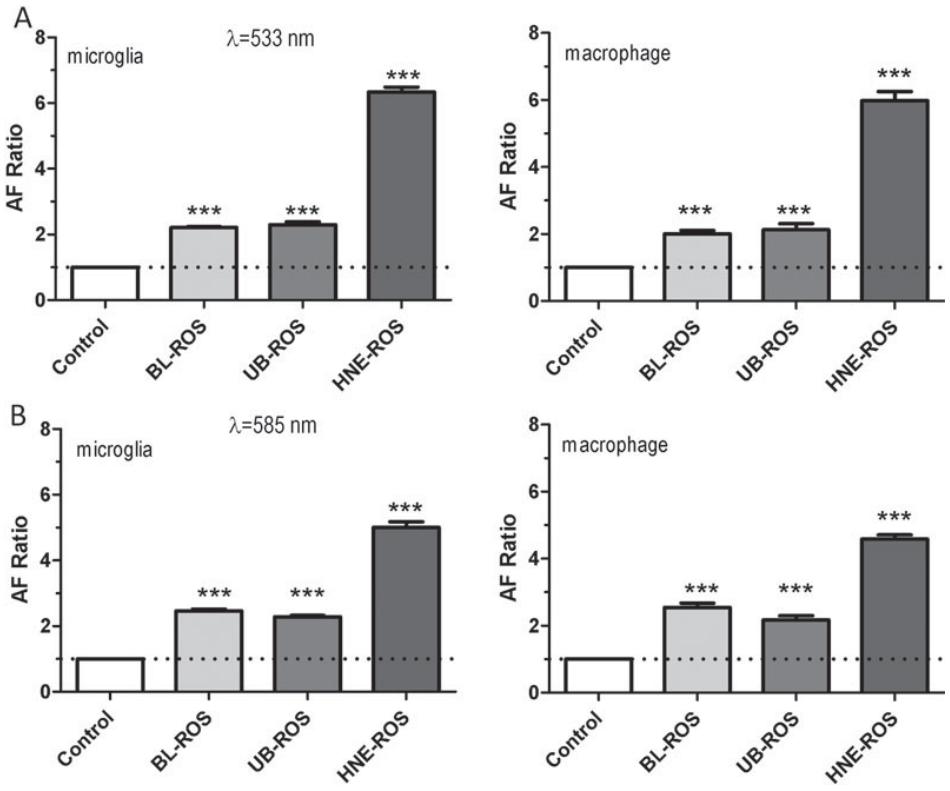

Figure 2. Autofluorescence of macrophages and microglial cells after 4 day feeding with different rod outer segments (ROSs). **A**: This is a fluorescence-activated cell sorting (FACS) analysis of the fluorescein isothiocyanate (FITC) channel (detection filter, 533/30 nm) of the microglial cells (left panel) and macrophage cells (right panel) fed with different ROSs. Note the large increase of AF in cells fed with modified rod outer segments (HNE-ROSs) compared to the other two groups. **B**: FACS analysis in the PE channel (detection filter, 585/40 nm) of the microglial cells (left panel) and macrophage cells (right panel) fed with different ROSs. Note the slight increase in the bleached ROS group and the decrease in HNE-ROSs compared to the corresponding AF registering at 533 nm. Abbreviation key: BL-ROSs=bleached rod outer segments; UB-ROSs=unbleached rod outer segments. Each bar reflects the average value obtained from nine samples. Asterisks indicate statistical significance (one sample *t*-test; ***=p<0.001).

Fluorescence labeling of lysosomes in cultured microglial cells and macrophages: One mM LysoTracker Red DND-99 (Molecular Probes, Junction City, OR) stock solution was diluted to 75 nM in the growth medium. Macrophage and microglial cell cultures were maintained in the LysoTracker Red DND-99-containing medium for 2 h and then replenished with fresh medium. The 561 nm wavelength was used to identify and observe the lysosomes by confocal microscopy.

Fluorescent labeling of rod outer segments: Two mg/ml stock solution of FITC (Molecular Probes) in 0.1 mol/1 sodium bicarbonate at pH 9.0–9.5, was prepared under dim red light, filter-sterilized, and stored in aliquots at −20 °C. The FITC stock was added to the ROS solution (final concentration, 10 µg/ml), and incubation was continued for 1 h at room temperature in the dark. The FITC-stained ROSs (FITC-ROSs) were pelleted in a microcentrifuge (4 min at 5,000× g) and resuspended in growth medium.

Incubation of cultured macrophages and microglial cells with fluorescein isothiocyanate rod outer segments: After culturing for 3 days, macrophage and microglial cells were fed with FITC-ROSs at 4 µg/cm^2 and incubated at 37 °C from 1 h to 24 h. At the end of the incubation time, unattached FITC-ROSs were removed and the cells were washed. To quench external bound ROSs, samples were incubated with 0.4% trypan blue for 10 min.

Confocal microscopy: Cells were cultured in eight-well microscopy glass slides (Lab-Tek Chamber Slide, Nunc, Langenselbold, Germany) and treated with different ROSs as described. After 4 day feeding or 1 day feeding of FITC-ROSs, cells were repeatedly washed to remove noninternalized ROSs, fixed with 4% paraformaldehyde (PFA), stained with 1 µg/ml Hoechst Stain solution H6024 (Sigma-Aldrich) for 5–7 min, and mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Confocal microscopy was performed with a Solamere Technology Group CSU10B Spinning Disk Confocal System that consisted of a Yokogawa CSU10 spinning disk confocal scan head attached to a Nikon TE2000-E2 inverted microscope (Nikon Instruments, Melville, NY) with a custom acousto-optic tunable filter (AOTF)-controlled laser launch with 405 nm, 488 nm, 561 nm, and 636 nm lasers.

Electron microscopy: Cells were cultured in six-well plates and incubated with different ROSs as described previously. The cells were then fixed by adding 1 ml of 2.5% glutaraldehyde (v/v) in 0.75 M Na phosphate buffer (pH 7.2) to each of the wells in the culture plate and allowed to fix overnight at 4 °C. The next day, the fixed samples were washed three times in 0.75 M Na phosphate buffer (pH 7.2). The cells were then scraped off the bottom of the wells with a soft plastic spatula, collected in a microfuge tube, pelletted, briefly rinsed in DH_2O, and postfixed for 1 h in 1% osmium

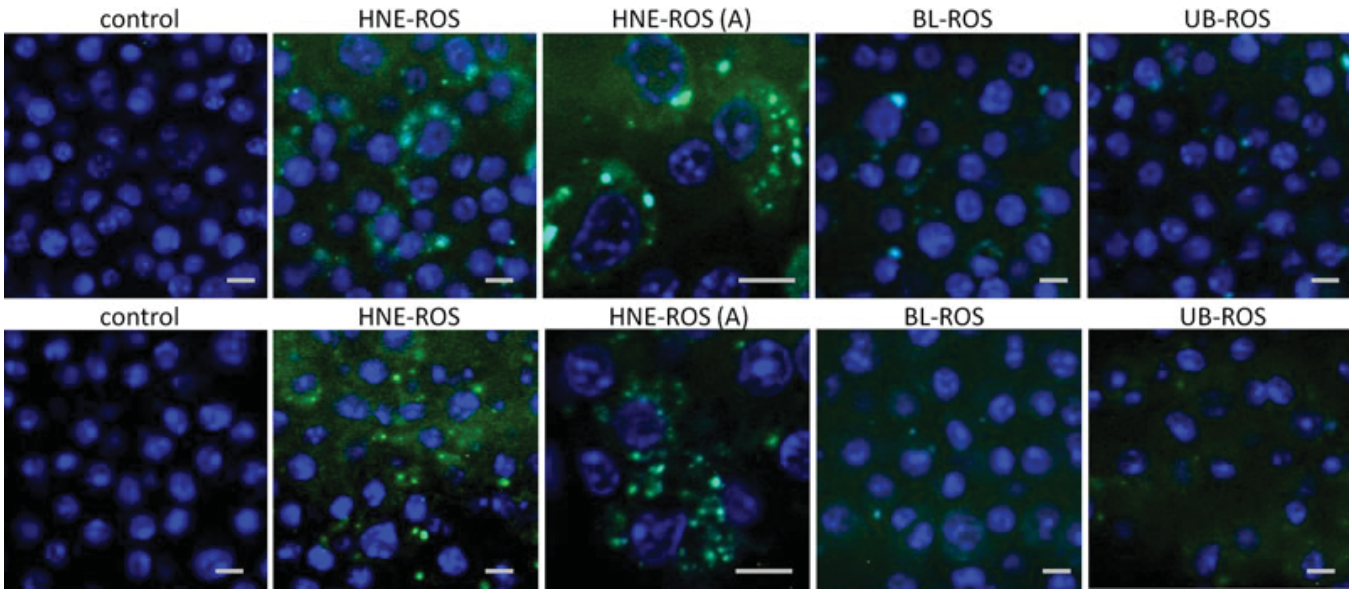

Figure 3. Confocal microphotographs of macrophages and microglial cells after 4 day feeding with different rod outer segments (ROS). Shown are laser-scanning confocal micrographs of macrophages and microglial cells fed with different ROSs. Microglial cells fed with no ROSs (control), bleached ROSs, unbleached ROSs, and HNE-modified ROSs are presented on the top row. Macrophages tested under the same conditions are presented on the bottom row. Each group demonstrates different extents of green-yellow autofluorescence at the FITC channel (excitation, 488 nm; detection, 530 nm). Panels marked as "HNE-ROS (A)" (middle panels) indicate microphotographs of cells fed with HNE-modified ROSs taken at an original magnification of 400×. All other photographs were taken with original magnification 200×. Scale bar=10 μm. Abbreviations are the same as in Figure 2.

tetroxide (w/v) in DH_2O. The fixed cells were then washed again in the DH_2O and dehydrated through a graded ethanol series of 20% increments, before two changes in 100% ethanol. Samples were then infiltrated first with two changes of propylene oxide and then in a mixture of 50% propylene oxide/50% SPIpon 812-Araldite epoxy resin and left overnight to infiltrate. The following morning, the cell pellets were transferred through three changes of fresh SPIpon 812-Araldite epoxy resin and finally embedded in molds filled with the same resin and polymerized for 48 h at 70 °C. The epoxy blocks were then trimmed, and ultrathin sections were cut on a Reichart-Jung ultramicrotome (Leica Microsystems, Buffalo Grove, IL) using a diamond knife. The sections were collected and mounted on copper support grids and contrasted with lead citrate and uranyl acetate. The samples were examined on Philips CM 10 and FEI Tecani 12 BT (FEI, Hillsboro, OR) transmission electron microscopes using a 80 kV accelerating voltage. Images were captured using a Gatan TEM CCD camera (Gatan, Pleasanton, CA).

RESULTS

Autofluorescence changes from feeding with modified and unmodified rod outer segments: Bleaching the ROSs for 1 h resulted in a rhodopsin content decrease of approximately 80%, compared to unbleached ROSs. In contrast, modifying the ROSs with HNE resulted in only an approximately 25% decrease in rhodopsin content (Figure 1). When macrophage or microglial cells were incubated daily with either bleached, unbleached, or HNE-modified ROSs at doses of 2 μg/cm^2 for 4 days, increased LLAF was observed by FACS at both wavelengths (Figure 2). The LLAF in cells fed with HNE-modified ROSs increased around 6–7 fold, compared to a twofold increase in bleached or unbleached ROSs at 533 nm (Figure 2A). Interestingly, LLAF in cells fed with bleached ROSs increased slightly at 585 nm, while the LLAF of cells fed with either unbleached or HNE-modified ROSs decreased slightly at the same wavelength, compared to the change at 533 nm (Figure 2A,B). In general, the LLAF generated by adding HNE-modified ROSs was approximately two to three times higher, compared to either adding bleached or unbleached ROSs, and the difference was highly statistically significant for every condition ($p<0.0001$; Mann–Whitney test).

Meanwhile, confocal microscopy demonstrated the formation of yellow-green inclusions in cells when incubated daily with different ROSs for 4 days (Figure 3). The inclusions were most numerous and prominent in cells fed with HNE-modified ROSs (Figure 3). This was confirmed, through transmission electron microscopy, by the presence of numerous inclusion bodies containing membrane swirls in cells fed with bleached, unbleached, and HNE-modified ROSs (Figure 4, Figure 5, and Figure 6).

The contribution of different components of rod outer segments to the formation of autofluorescence: The composition of ROSs is varied, including retinoids (11-cis-retinal, all-trans-retinal), proteins (e.g., opsin), phospholipids

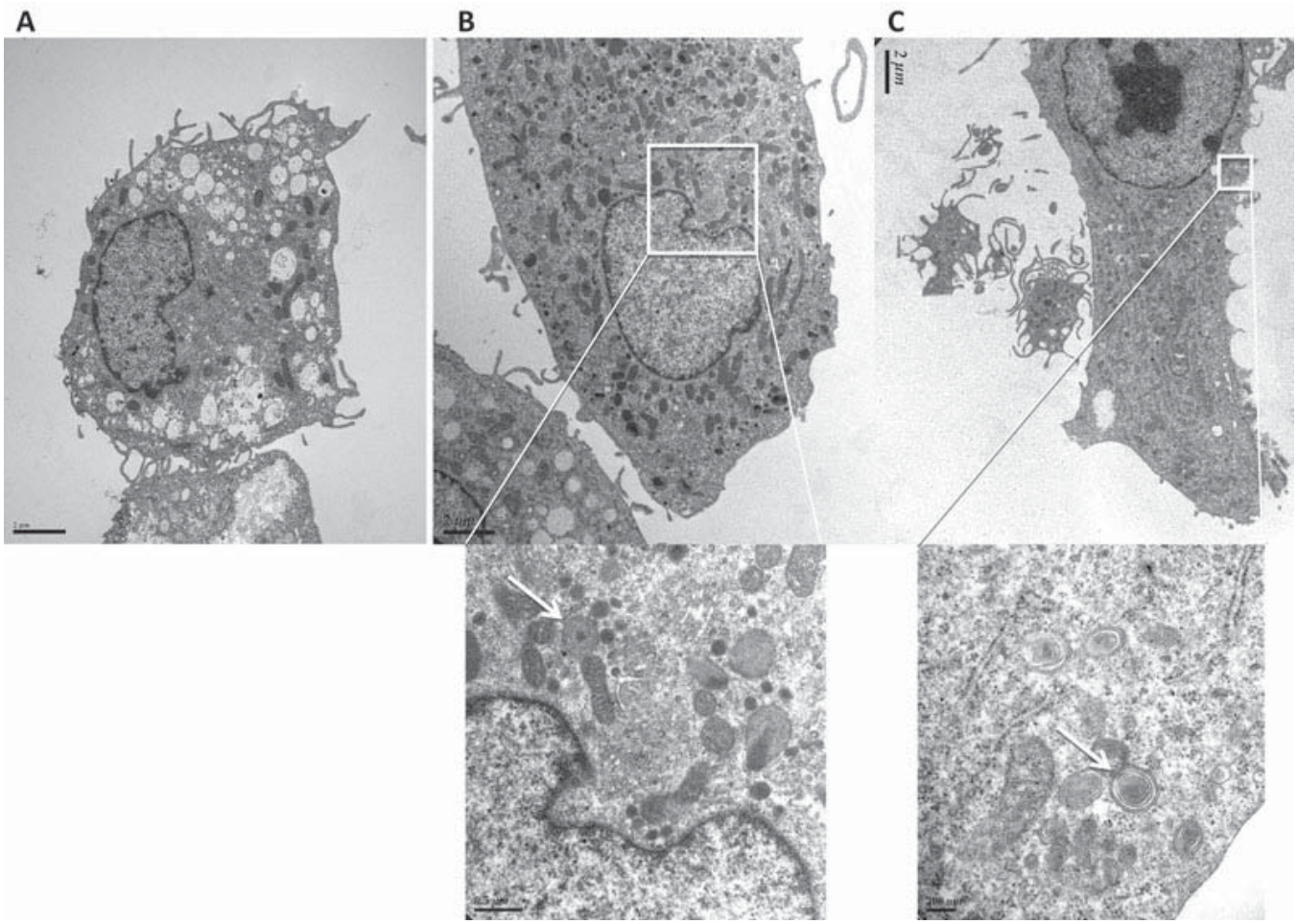

Figure 4. Transmission electron microscopy of microglial cells after 4 day feeding with ROSs. **A**: Electron micrographs of microglial cells (control). **B**: Electron micrograph of microglial cells after 4 day feeding with bleached ROSs. Magnification: 6,000×. A higher magnification of the intracellular inclusion body region is presented in the micrograph below (magnification 26,000×). **C**. Electron micrograph of microglial cells after 4 day feeding with unbleached ROSs. Magnification: 4,200×. A higher magnification of the intracellular inclusion body region is presented in the micrograph below (magnification 43,000×). White arrows indicate intracellular inclusion bodies in the higher magnification photographs in **B** and **C**.

(phosphatidylethanolamine [PE], phosphatidylcholine [PC]), and so forth. Previous work has focused on in vitro formation of fluorophores from oxidized lipid-protein complexes or combinations of PE and all-trans-retinal in RPE cells [9-11]. However, studies examining the parallel or competitive contribution of the different ROS elements in the formation of fluorophores from various components are lacking for RPE cells, as well as for macrophages or microglia. Therefore, we tested the effect of feeding bleached, unbleached, and HNE-modified ROSs and compared the contribution of different components of ROSs by FACS. Among retinoids, LLAF induced by applying 11-cis-retinal was slightly higher, compared to LLAF induced by feeding with all-trans-retinal and 9-cis-retinal, especially at 585 nm (Figure 7). Feeding with liposomes containing PE and PC (6:4 ratio) induced relatively low LLAF, compared to the retinoid.

Phagocytosis of rod outer segments by macrophages and microglial cells: To further investigate the finding of increased LLAF in cells fed with HNE-modified, unbleached and bleached ROSs, we performed a phagocytosis assay in these three groups. FITC-stained ROSs appeared inside the lysosome and co-localized well with it after 24 h incubation in macrophage and microglial cells. The rate of phagocytosis was considerably higher in both macrophages and microglial cells fed with HNE-modified ROSs, compared to the levels of phagocytosis observed in the other two groups (Figure 8 and Figure 9).

DISCUSSION

The presence of both macrophages and microglial cells has been documented in human and other mammalian retinas and subretinal spaces under normal and diseased conditions. For example, microglial cells are ubiquitous in humans, being

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