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Imaging macrophages in trehalose with SIMS

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Abstract

Phagocytosis is a major component of the animal immune system where apoptotic cellular material, metabolites, and waste are safely processed. Further, efficient phagocytosis by macrophages is key to maintaining healthy vascular systems and preventing atherosclerosis. Single-cell images of macrophage phagocytosis of red blood cells, RBCs, and polystyrene microspheres have been chemically mapped with TOF-SIMS. We demonstrate here cholesterol and phosphocholine localizations as relative to time and activity.

Keywords

TOF-SIMS; Phagocytosis; Macrophage; Trehalose; Lyophilization

1. Introduction

Macrophage metabolism provides an essential disposal service to the immune system and decreases cholesterol congestion in the arteries [1,2]. Imaging multiple membrane lipids of macrophages at different stages of the phagocytic process would provide new insight into lipid activity. By comparing the m/z 369.37⁺ cholesterol signal in these macrophages to the more ubiquitous phosphocholine (PC) signals, such as m/z 184.07⁺ and m/z 86.10⁺, basic plasma membrane lipid response to phagocytosis can be observed. It has been shown that lipid heterogeneity in the macrophage membrane depends on the identity and size of the object being processed [1–3]. Phagocytosis of large particles necessitate lipid donation from subcellular organelles to the plasma membrane [1]. Lipid constituents in the membrane are heterogeneous as a function of time following/incident to phagocytosis [2–5]. It is now known that membrane cholesterol increases in macrophages progressively as they contact and process LDL droplets [2,5].

2. Experimental

2.1. Materials

Murine J774 macrophages were obtained from American Type Culture Collection (Manassas, VA). Erythrocytes, or red blood cells (RBCs), were a kind donation from the experimentalist. RBCs were opsonized with monoclonal anti-glycophorin A,B (Sigma, St. Louis, MO). Macrophages and RBCs were treated/cultured in Dulbecco's PBS buffer, fetal bovine serum, and 1640 RPMI cell culture media, all purchased from Mediatech Inc. (Heron, VA). Trehalose dihydrate and glycerol were purchased from Sigma (St. Louis, MO) and VWR (West Chester, PA), respectively. Both cell dyes, DID (1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate) and DII (1,1'-dioctadecyl-3,3,3',3'-

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tetramethylindocarbocyanine perchlorate) were obtained from Invitrogen Corp. (Eugene, OR). For membrane cholesterol enhancement experiments, Aquaplex[®], β -Cyclodextrin with cholesterol, was acquired from CTD Inc. (High Springs, FL). Polystyrene beads for alternative phagocytic experiments, were purchased from Duke Scientific (Palo Alto, CA). All materials listed were used without further purification. Cells were freeze-dried (FD) in 50–100 mM trehalose, with 0.3% glycerol, overnight at $\sim 10^{-6}$ Torr. After lyophilisation, the overlayer of dried sugar was removed by a stream of dry nitrogen gas. To prevent ambient hydration, the FD cells were quickly introduced into the SIMS vacuum chamber.

All spectra and images were acquired on a TOF-SIMS instrument, detailed previously [6]. Briefly, images were taken with a 20–25 keV Au⁺ primary beam, delivering ≤ 400 pA at the stage. The beam impinged the surface at approximately 40° to normal, was pulsed at ~ 70 ns, and delivered mass resolutions ($m/\Delta m$) of ≥ 2000 for most peaks of interest with delayed extraction.

2.2. Methods

For culturing or dyeing, all cells were incubated at 37 °C and 6% CO₂. Macrophages were cultured onto Si shards coated with 100 Å of Au. For dyeing experiments, macrophages and RBCs were incubated for ≤ 60 min at 15–25 $\mu\text{g/ml}$ DII or DID. With consent from the University, the experimentalist's RBCs were collected with a sterilized sharps puncture and rinsed with PBS prior to dyeing with DII/DID or opsonization with anti-glycophorin. To significantly increase receptor-mediated attachment and phagocytosis, RBCs were opsonized with anti-glycophorin for approximately 20 min in PBS or serum-free media [7]. Polystyrene microspheres were readily phagocytosed without any treatment. Therefore, $\sim 10^3$ beads were rinsed and suspended in ~ 500 μl PBS with 50–100 mM trehalose, then pipetted directly to ~ 500 μl buffer/media solution of macrophages.

After allowing ≤ 10 min for RBC/bead attachment or ≥ 30 min for advanced phagocytosis, the macrophages were rinsed vigorously in cold PBS to remove free RBCs and particulate. Cells were then rinsed and immediately FD in 50–100 mM trehalose and 0.2–0.3% glycerol distilled water solution. To increase cholesterol content, some macrophages were treated with 250 μM β -cyclodextrin with cholesterol in serum-free media for 1 h prior to the addition of RBCs. To avoid ambient hydration, cells were quickly transferred to the SIMS instrument vacuum chambers.

For dual staining experiments, phagocytosing and non-phagocytosing macrophages were dyed in separate flasks for approximately 60 min. Immediately after staining, both flasks were scraped or agitated vigorously to dislodge cells and 2 ml aliquots from each were added to a separate flask containing several Au-coated Si shards.

3. Results

Explained previously, phagocytosis is known to effect lipid placement in the macrophage outer membrane. As illustrated in Fig. 1 pristine TOF-SIMS images can be generated with this sugar protocol delivering lateral resolutions well below a micron. Using the 20–80% criterea scale, lateral resolution for the macrophage in Fig. 1 is less than 400 nm. At this resolution in this cell, the rolling topography of the cell membrane, consistent with ‘membrane ruffling’, can be seen in the total ion image in Fig. 1. Membrane ruffling is observed periodically for healthy cells with optimal beam focusing, and the elimination of ambient hydration following FD. Membrane ruffling, caused by intercellular actin filaments pushing outward on the membrane, is a common phenomenon observed in macrophages [8].

SIMS images were generated at the earliest stages of phagocytosis, where 7 μm polystyrene (PS) beads and RBC material were first attached to the macrophage membrane. The large microspheres shown in Fig. 2 were exposed to macrophages only long enough to facilitate attachment prior to lyophilization. Multiple beads were attached to most cells and several other images of PS bead phagocytosis (not shown) were taken with similar results. The incident angle of the focused, 200 nm 20 keV Au^+ beam, together with the curved topography in this sample, create a shadowing effect with a factor of $25\times$ lower signal coming from the lee of the cell and beads. Interestingly though, a thin line of phosphocholine m/z 184.1⁺ is observed circumnavigating the bead in direct contact with the macrophage, indicated by a double-headed arrow. The line of phosphocholine wraps completely around the bead, not just the beam-facing region—negating simple topographical considerations. An inorganic artifact, unique to the beads at m/z 142.9⁺, is used as a tag for exposed beads. Notice that beads not associated with the macrophage and not completely layered in lipid display this inorganic signal distinctly. This further confirms that the upper bead, intimately associated with the macrophage, is entirely lipid coated. White, single-headed arrows indicate the beads with no lipid coating at the top of the image, and a bead beginning to be engulfed at the bottom of the image. A thin lipid process, above the fully engulfed bead, “reaching out” towards the uncoated bead in the upper left of the image is circled. This observation is complimentary to fluorescent studies where a transitory “phagocytic cup” from the macrophage membrane extends out to surround particulate in the first few minutes of phagocytosis [8,9]. The phagocytic cup, therefore, is an extension of the macrophage plasma membrane that reaches out, attaching and wrapping particulates in lipid before pulling them into the macrophage for digestion.

Separate experiments were conducted where RBCs dyed with DII were added to macrophages and lyophilized after 7 min incubation. Positive SIMS images show an intense 5–10 μm localization from the DII molecular ion m/z 833.8⁺, $\text{C}_{59}\text{H}_{97}\text{N}_2^+$, attached to a single macrophage. Seen in Fig. 3, the dyed RBC material is distinctly imaged, resting on top of a polarized macrophage. A normalized line scan shows a decrease in m/z PC 86.1⁺, where DII intensifies. Normalization was calculated by dividing the PC signal by the ratio of the total ion image to eliminate topographical effects. Deficiency of the common membrane PC lipid signal indicates that the particulate is possibly a DII agglomerate. As seen in Fig. 3, image analysis and normalization to total counts shows a decreasing, yet definitive, lipid presence. Results observed in Fig. 3, were typical of attached RBC images. The thin lipid layer is again consistent with the “phagocytic cup” as the DII precipitate would have no PC to contribute. An RBC on the other hand, would reasonably contribute PC to the image, eliminating the 30% PC decrease shown in the line scan. Therefore, the macrophage is expected to be in the early stages of phagocytosis of a solid DII particulate. No other lipid or lipid pattern is detected. The absence of the expected cholesterol localization [4] may be due to blockage of the primary beam from imaging the active region by the attached particulate.

Finally, a preliminary experiment was conducted where macrophages were treated with β -cyclodextrin and cholesterol to increase membrane cholesterol [10]. After macrophages were exposed to β -cyclodextrin with cholesterol for 1 h, dyed RBCs were incubated and phagocytosed for 45 min. Seen in Fig. 4, an 8–10 μm , cholesterol localization was observed with a single cell. The normalized cholesterol m/z 369.4⁺ $\text{C}_{27}\text{H}_{45}^+$ signal in the observed region maintained a $4\times$ increase over base levels. The m/z 369.4⁺ signal was normalized, or taken as a ratio to the total ion signal, to eliminate topographical effects. The absence of dye signal suggests that the RBC was either fully internalized or possibly separated from the macrophage upon fracture after lyophilization. Previous SIMS experiments of macrophages enhanced with β -cyclodextrin showed increased membrane cholesterol, but no distinct domains. However, as seen here with phagocytic cells a distinct cholesterol domain is evident. Also seen in Fig. 4, PC fragment ion m/z 184.1⁺ also shows localization with the region of interest (ROI), but at 33% lower counts than cholesterol m/z 369.4⁺—unique for single-cell SIMS imaging. After

45 min of exposure to RBCs, the increased cholesterol [10] and absence of any DII signal, may indicate a site of complete or advanced phagocytosis. Subsequent experiments to enhance cholesterol with other additives, such as acetylated LDL, have proven unsuccessful to date. Additional investigations into the use of β -cyclodextrin to enhance cholesterol for SIMS imaging are underway.

4. Conclusion

Cellular phagocytosis initiates dynamic lipid behavior in macrophage membranes and is crucial to a healthy immune system. With its chemical “snapshot” capabilities, SIMS may be the ideal methodology to compliment fluorescence for time-lapse imaging of phagocytosis. By stepwise imaging of the phagocytic process, we aim to elucidate membrane mechanisms related to this system.

Acknowledgments

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Fig. 1. A positive SIMS $80\ \mu\text{m}^2$ image of a single polarized macrophage, lyophilized in trehalose. Imaged at 256×256 pixels, 20 summations or shots/pixel, 100 pA. (L) Total ion image and relative intensity bar; (R) PC headgroup fragment, $m/z\ 86.1^+$, $\text{C}_5\text{H}_{12}\text{N}^+$ 0–2 cts.

Fig. 2.

Optical and positive SIMS images of macrophages exposed to 7 μm PS beads for several minutes. SIMS images taken with a 20 keV Au^+ primary ion beam. (Top left) Optical image of macrophages attached to PS beads on a 100 $\text{\AA}$ Au-coated glass slide, $\sim 300 \mu\text{m}^2$ fov. (Top right) Total Ion SIMS image with relative intensity bar, $100 \mu\text{m}^2$ fov, 15 summations, 200 pA. (Bottom) Overlay of m/z 142.9 $^+$, PS microsphere surface artifact, red; and m/z 184.1 $^+$, PC headgroup fragment $\text{C}_5\text{H}_{15}\text{NPO}_4^+$, green, both 0–1 cts. Single headed arrows identify m/z 142.9 $^+$ signals from bead surfaces. Double headed arrow indicates an engulfed (lipid-coated) bead. A thin lipid process, consistent with the “phagocytic cup” reaching out towards an uncoated bead is circled.

Fig. 3. Positive 25 keV Au^+ SIMS images of macrophages briefly incubated with dyed (DII) RBCs. Fov $180 \mu\text{m}^2$, 13 summations. (A) Total ion image overlaid with intense DII, m/z 833.8⁺, $\text{C}_{59}\text{H}_{97}\text{N}_2^+$ signal, circled. (B) Combined PC signals of $\text{C}_5\text{H}_{12}\text{N}^+$ and $\text{C}_5\text{H}_{15}\text{NPO}_4^+$, at m/z 86.1⁺ and m/z 184.1⁺, respectively 0–2 cts. (C) Overlay of DII, circled, and PC signals. (D) Mass spectra range for DII molecular ion. (Bottom) Line scan for PC and DII over the area indicated under white bar.

Fig. 4. Positive SIMS image of a single macrophage treated with β -cyclodextrin and exposed to opsonized RBCs for 45 min. Imaged with 20 keV Au^+ , at $95 \mu\text{m}^2$ fov, and 40 summations. (Top) Total positive ion image. (Lower left) PC headgroup fragment m/z 184.1⁺, 0–1 cts. (Lower right) Cholesterol signal m/z 369.4⁺ $\text{C}_{27}\text{H}_{45}^+$ localization, 0–1 cts. Cholesterol counts 50% greater than PC in region of interest.