Characterizing the Effect of Mitochondria Uptake in Macrophages

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Abstract

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As central hubs for many cellular processes, mitochondria are crucial for cell metabolism and survival. For a long time, it was widely assumed that mitochondria are constrained within the cytoplasm of the cell. However, a decade of research has revealed the possibility of intercellular mitochondria migration, involving the movement of mitochondria from one cell type to unrelated cell types. Energy-intensive cells such as cardiomyocytes and brown adipocytes are subjected to a high rate of ROS (reactive oxygen species) generation. Mitochondria quality control pathways are critical in preserving mitochondrial health and homeostasis to maintain cellular functions. Cardiomyocytes export dysfunctional mitochondria as an alternative method of quality control. Resident macrophages in the environment actively capture and degrade cardiac mitochondria. While this process benefits cardiomyocytes by preserving their mitochondrial function, the biological consequences of acquiring cardiac mitochondria have not been fully characterized in macrophages. Here, we used an *in vitro* system combining primary macrophages and the artificial transfer of cardiac mitochondria to investigate macrophage respiration, proliferation, as well as the morphology and turnover of their endogenous mitochondria after mitochondria uptake.

1 Introduction

The Mitochondrion and Its Intracellular Roles

Having originated from ancient endosymbiotic events, mitochondria are multifunctional, double membrane-bound organelles within many eukaryotic cells. These organelles retained a separate genome, encoding essential respiratory protein complexes and enabling self-replication. Mitochondria are essential for many cellular processes. As the "powerhouses" of the cell, they are responsible for generating the majority of the cell's energy currency, ATP. The respiratory complexes utilize high-energy electrons produced from the breakdown of carbohydrates/fatty acids through glycolysis and the citric acid cycle to generate a proton gradient in the mitochondrial intermembrane space. The chemical gradient is used to drive the synthesis of ATP. This combined process is also known as oxidative phosphorylation. Mitochondria also regulate a crucial intracellular messenger, calcium. Through a selection of membrane proteins, such as the mitochondria uniporter (29) and sodium/lithium/calcium exchanger (NCLX) (30), mitochondria play essential roles in regulating the storage, uptake, and efflux of calcium. The organelle also participates in apoptotic signalling. The release of Cytochrome C, a member of the respiratory chain, initiates the assembly of apoptotic machinery (1). There are also evidence suggesting that mitochondria are associated with other cellular processes such as cell differentiation (2) and migration (3).

The Identification of Mitochondria Migration

For a long time, the functions of mitochondria were primarily associated with intracellular processes, confining their role within the cytoplasm of the cell. In fact, it was widely presumed that cells only obtained mitochondria from parent cells. However, a decade of research revealed increasing evidence supporting the possibility of intercellular or horizontal mitochondria migration. In 2004, Rustom et al.(4) detected the presence of nanotubular structures between two distinct populations of cells cultured together. The group demonstrated a unidirectional transfer of membrane-bound organelles through these nanotubular structures. Later, Koyanagi et al.(5) visualized the transfer of mitochondria in neonatal cardiomyocytes and endothelial progenitor cells using mitochondria-specific stains. Furthermore, a study in 2006 by Spees et al.(6) demonstrated functional roles of mitochondria migration. It was reported that the aerobic respiration of mitochondria-deficient cells can be restored through the migration of mitochondria from nearby cells. The co-culturation of mtDNA defective/depleted A549 cells with hMSCs (human adult nonhematopoietic stem/progenitor cells) generated colonies of A549 cells containing mtDNA from hMSCs, evident with their similar polymorphic sites with the donor (6). These colonies exhibited parental mitochondrial function, with comparable levels of intracellular ATP, and oxygen consumption. These pioneering studies highlighted the capability of mitochondria to stretch their functions far beyond the cytoplasm of their native cell.

The Intercellular Roles of Mitochondria Migration

The current paradigm suggests that the migration of mitochondria serves important functions in both the recipient and donor cells. For example, activated platelets release oxygen-consuming mitochondria, both in the membrane-bound form and as free mitochondria (7). In recipient cells, these exogenous mitochondria upregulate neutrophil antimicrobial responses (7). It has also been shown that mesenchymal stem cells upregulate their wound-healing capacity upon receiving platelet-derived mitochondria (8). In the central nervous system, astrocytes release mitochondrial components to nearby neurons, increasing their viability after focal ischemia (9). Further supporting this notion, the levels of extracellular mitochondria in the cerebrospinal fluid increases after subarachnoid hemorrhage, and having a higher mitochondrial membrane potential in these extracellular mitochondria is correlated with better recovery (10). Support cells, such as mesenchymal stromal cells (MSCs), can also donate their mitochondria to facilitate endothelial cell (EC) engraftment (11). Lin, R. Z. et al. demonstrated functional vessel formation by ECs through MSC-mediated mitochondrial transfer (11). The migration of mitochondria can also promote pathogenic outcomes if malignant cells are the recipients. Cancer cells have been shown to acquire mitochondria from infiltrating lymphocytes, promoting their survival and impairing immune responses (12). A similar notion is found by transferring mitochondria to mtDNA-deficient cancer cells, resulting in the restoration of their tumor-forming potential (13).

For donor cells, the disposition of mitochondria can serve as a powerful mechanism for mitochondria quality control, which is essential for preserving mitochondrial health, homeostasis, and tissue function (38). To support the intense metabolic demand of the heart, cardiomyocytes have a tightly distributed mitochondria network that occupies a large proportion of their total cell volume (37). Oxidative phosphorylation, the process by which mitochondria synthesize ATP through a series of oxidation-reduction reactions, generates reactive oxygen species (ROS) as a deleterious byproduct, driving mitochondrial dysfunction over time (39). Intracellular degradation, via lysosomal pathways, are canonical methods to recycle dysfunctional mitochondria. However, it has been demonstrated that in pathological settings, canonical mitochondria quality control pathways are insufficient (14). Under these circumstances, dysfunctional mitochondria are exported as an alternative method of mitochondria quality control. Cardiomyocytes with loss-of-function mutations in lysosomal proteins demonstrate an increase in the secretion of mitochondria into the extracellular space in vesicles (15). Exported mitochondria are actively captured and eliminated by resident macrophages (17). Interfering with the elimination of exported mitochondria is associated with impaired cardiac energetics and function (17).

Harnessing the Power of Mitochondria Migration

Realizing that the migration of mitochondria serves profound intercellular roles has stimulated interest in leveraging the mechanism for therapeutic purposes. Administering exogenous mitochondria as means of therapeutic strategies are currently under exploration, with several studies demonstrating success in animal and cell models. For example, the administration of autologous mitochondria enhanced oxygen consumption and post-infarct cardiac function in ischemic heart models (23). The direct injection of mitochondria isolated from remote tissues unaffected by ischemia into the ischemic zone improved post-ischemic recovery (24). Additionally, the intra-arterial delivery of exogenous mitochondria after ischemic stroke reduced infarct volume and increased cell viability (25). Clinical success has also been demonstrated. The enrichment of hematopoietic stem cells with maternally derived healthy mitochondria improved aerobic function in patients with single large-scale mitochondrial DNA (mtDNA) deletion syndromes (SLSMDs) (26). Great strides have also been made in engineering methods to improve the therapeutic potential of mitochondria migration. Iron oxide nanoparticles has been used to facilitate the targeted delivery of mitochondria from human mesenchymal stem cells (hMSCs) selectively to diseased tissues (27). In the research field, an array of methods has been developed over the recent years aimed at engineering alternative ways of characterizing mitochondria migration. Detailed protocols to isolate functional mitochondria from tissues and cells have emerged (16), setting the basis for modelling mitochondria migration. Additional tools such as unique genetic inserts for fluorescent proteins, mt-Keima (28) and mito-Dendra2, as well as Rosamine & carbocyanine-based stains, allow for the characterization and visualization of the mitochondrial network. Taken together, these tools enable us to extensively investigate the mechanisms underlying intercellular mitochondria migration.

Unresolved Questions

Despite the unprecedented advances in the field, many questions remain unanswered, preventing us from harnessing the true potential of mitochondria migration to treat notorious diseases. For example, what are the intracellular fates of the incorporated exogenous mitochondria? What are the exact molecular mechanisms responsible for facilitating the migration of mitochondria between cells? What are the essential signals deciding whether a cell will accept or export mitochondria? Will the origin or the state of the mitochondria influence their modulatory effects?

For this study, we would like to characterize the cellular consequences of macrophages acquiring mitochondria released by cardiomyocytes. As described before, cardiomyocytes export their mitochondria as an alternative method of quality control. As the exported mitochondria accumulate, resident macrophages are responsible for their uptake and clearance. Impaired elimination of exported mitochondria results in ventricular dysfunction and metabolic alterations in cardiomyocytes (17). As donor cells participating in intercellular mitochondria migration, it has been well characterized that cardiomyocytes benefit from this process by preserving mitochondrial homeostasis (17). However, as recipients, the biological effects of mitochondria uptake on macrophages remains elusive. In this study, we used an *in vitro* system to investigate the functional effects of mitochondria uptake on macrophage respiration and proliferation. We also assessed the status of their endogenous mitochondria. We first examined the mitophagy of endogenous mitochondria in macrophages after mitochondria uptake. This allows us to characterize the turnover rate of their endogenous mitochondria. We also quantified the morphology of the endogenous mitochondria network, which indicates mitochondria fusion and fission dynamics. Literature has revealed intriguing correlations between the energy demand and mitochondrial fission/fusion events (18). The process also plays a role in cell movement, where mitochondria are actively trafficked to the leading edge of migratory cells, switching between fusion and fission states to provide energy for the nearby cytoskeleton to facilitate cell movement(19). Furthermore, the morphology and distribution of mitochondria alternate during cell cycle progression (20). By investigating the cellular effects of acquiring exported mitochondria from cardiomyocytes, we deduce the potential mechanisms driving the successful elimination of exported mitochondria in macrophages. Leveraging these mechanisms allows us to alleviate symptoms of cardiac stress and dysfunction in cardiac diseases.

2 Methods

Experimental Design:

To examine the effect of exogenous mitochondria uptake, we need to establish an *in vitro* model system that captures the *in vivo* relationship between cardiomyocytes and macrophages. In the heart, tissue macrophages arise from progenitor cells that colonize developing organs during embryonic development (31). Additionally, circulating monocytes derived from hematopoietic stem cells (HSC) in the bone marrow contribute to tissue resident macrophage populations after birth (31,32). This creates a complicated scenario where multiple transcriptionally distinct macrophage populations coexist in the heart (32). The heterogenous nature of cardiac tissue-resident macrophages introduces potential confounding variables that make it difficult to characterize them. To further complicate the matter, during the isolation of tissue-resident macrophages, fragmentation can occur. Conventional methods of isolating cardiac tissue macrophages involves the enzymatic digestion of the tissue, generating single cell suspensions (33). Following antibody staining, flow cytometry is utilized to isolate macrophage subsets (33). During the preparation of single-cell suspensions, macrophages can become fragmented, attaching their remnants to non-macrophage cell types (34). This results in inaccurate representations of the true composition of isolated macrophages (34). Taken together, these characteristics render tissue-resident macrophages impractical to use as a model for high throughput, extensive analysis. To address this, we used bone marrow-derived macrophages (BMDM), which are primary macrophages that arise from bone marrow precursor cells. We can stimulate their differentiation into macrophages with the administration of colony-stimulating factor (M-CSF). Furthermore, deriving macrophages from bone-marrow precursor cells makes them less susceptible to external factors such as animal age and health, allowing for robust characterization. To model mitochondria exportation by cardiomyocytes, we isolated mitochondria from heart tissues using a combination of centrifugation and homogenization techniques (14). In short, as an *in vitro* model system, we provided cardiac mitochondria to bone-marrow derived macrophages to investigate the cellular effects on respiration and proliferation.

We also wanted to characterize the endogenous mitochondria of macrophages after cardiac mitochondria uptake. Live cell imaging methods are useful in visualizing and characterizing the status of the mitochondria network. We needed a fluorescent probe that is specific to the endogenous mitochondria population. Although an array of compounds are available, such as Rosamine & carbocyanine-based stains that recognize membrane potential and accumulates inside mitochondria, it provides limited information about the status of the stained mitochondria. In 2015, Nuo Sun developed a transgenic mouse model, mt-Keima, in which the animal expressed a mitochondria-targeted fluorescent protein (28). Keima is a coral-derived protein that exhibits a few appealing characteristics. It is a lysosomal protease-resistant protein that can be targeted to the mitochondrial matrix with a sequence from COX VIII, allowing for the visualization of the mitochondria network. Mt-Keima is also a ratio-metric fluorescent protein that has a bimodal excitation spectrum. At physiological pH (8), with confocal microscopy, the excitation wavelength peaks at 458 nm. At acidic pH (4.5), the excitation wavelength peaks at 561 nm. The emission wavelength is equivalent at either pH, peaking at 620 nm. Calculating the ratio between the fluorescence at acidic and neutral pH can provide an indication of the mitophagy, the intracellular lysosomal degradation of mitochondria. Mitochondria are alkaline organelles. Mitophagy allocates these organelles inside lysosomes, causing a sharp change in their pH, able to be detected by mt-Keima. We used mt-Keima as a genetic insert to track the endogenous mitochondria population. This allows for the extensive characterization of their morphology and mitophagy to provide important information about mitochondrial fission/fusion dynamics, as well as mitochondria turnover rate. These parameters are associated with a wide range of cellular signals, such as apoptosis and stress (36).

In summary, our experimental design utilizes an *in vitro* model system and a unique fluorescent probe to investigate macrophage respiration, proliferation, as well as the morphology and turnover rate of their endogenous mitochondria after mitochondrial uptake.

Experimental Procedures and Data Analysis:

- **BMDM Isolation and Culture Conditions:**
  The femur and Tibia are removed from an euthanized mouse. After the removal of muscles, the bones are placed in cold DMEM (Dulbecco's Modified Eagle Medium) with a high glucose supplement (GlutaMAX™). The bones are quickly transferred to culture hoods and are flushed with cold 1x PBS (pH 7.4). The ends of the bones are severed and a 23G needle is inserted to flush 5ml of PBS through. The liquid is collected into 50 ml centrifuge tubes through a 40 μm cell strainer. The contents are centrifuged at 400 x g, 5 min at 4 °C. The pellet is then dissolved in 1 ml of ACK lysis buffer to remove erythrocytes. After the removal, the cells are resuspended and plated in GlutaMAX™ DMEM with 10% FBS, 1% P/S, and 0.004% M-CSF supplements in 14.5 cm cell culture dishes for 6-7 days. The media is replaced every two days. The confluency of the cells are checked regularly.

- **Mitochondria Isolation:**
  The intact mouse heart is excised and quickly minced while immersed in cold MSE (70 mM sucrose, 210 mM mannitol, 5 mM MOPS, 2 mM taurine, 1.6 mM carnitine hydrochloride, and 1 mM EDTA, pH 7.2). The minced heart is submerged in MSE and then centrifuged for 5 min at 600 x g at 4 °C. The minced heart is further digested with 0.1 mg/mL of Trypsin for 10 min. After the addition of equal volumes of MSE buffer, with 0.2% fatty acid-free BSA and 0.5 mg/mL trypsin inhibitor and a centrifugation at 1500 x g for 3 minutes, the heart is homogenized in the presence of 4 mL of MSE buffer with 0.2% fatty acid-free BSA, releasing mitochondria into the homogenate. After another centrifugation at 600 x g for 5 minutes, the supernatant is transferred to dolphin tubes and centrifuged at 8000 x g for 10 minutes.The pellet is resuspended in 100 ml of MSE and recentrifuged at 8000 x g for 10 minutes. The final pellet is resuspended in 50 ml of MSE. The concentration of mitochondria is determined by the BCA assay. The isolated mitochondria are evenly split into two fractions, with one kept in 4 °C (isolated mitochondria) until the experiment, and the other fraction going through 5-6 freeze and thaw cycles (damaged mitochondria).

- **Measuring Macrophage Respiration:**
  Macrophage mitochondrial respiration was analyzed using the Seahorse XFe24 analyzer. Prior to the experiment, the cultured BMDMs are detached with 10 ml of 2mM PBS-EDTA and are replated onto Seahorse XFe microplates at the density of 100,000 cells/well. On experiment day, 25 μg / well of isolated mitochondria is added to the culture media. The mitochondria are kept in the media for 3h, or 24 h. The mitochondria are removed at respective time points by washing the cells twice with 1x PBS (pH 7.4), followed by changing the media to Seahorse XF DMEM assay medium, supplemented with pyruvate, glutamine, and glucose. At the in-

dicated time points, the Seahorse XF Cell Mito Stress Test is conducted to measure oxygen consumption rate and basal extracellular acidification rate simultaneously. It involved the sequential injections of ADP, oligomycin A, FCCP, and Rotenone/antimycin A.

- **Measuring Macrophage Proliferation:**
  BMDMs were seeded with a density of 200,000. 25 μg of isolated mitochondria was administered into the culture medium. The isolated mitochondria is removed by washing the cells twice with 1x PBS (pH 7.4), followed by media replacement after 24 hours. The cells are immediately detached with 2mM PBS-EDTA. The cell number is counted with a hemocytometer.

- **Confocal Microscopy:**
  Prior to imaging, the cells are detached with 10 ml of 2mM PBS-EDTA and replated in glass-bottom dishes. Before imaging, the cells are stained with Hoechst (1 ug/mL) for 15 min. The Leica TCS SP8 confocal laser scanning microscope is used to examine fluorescent cells with the HC PL APO CS2 40x immersion objective lens. The fluorescence signals are imaged in two channels, with sequential excitation wavelengths (458 nm, neutral; 561 nm, acidic). Laser power is set to 1% to avoid damaging while maintaining clear visualization. Imaging settings are kept identical between experiment groups and replicates to allow for comparison. Images were selected from randomized fields and acquired with Z-stacking of 5. With the Leica Application Suite X software, captured images are exported and analyzed in ImageJ.

- **Quantifying Macrophage Mitophagy by Confocal Imaging:**
  Image analysis was conducted on the captured confocal images taken from the previous mitophagy analysis in ImageJ. The fluorescence of mt-Keima 458 nm (neutral) and 561 nm (acidic) are thresholded to create binary masks. The threshold parameter is kept identical across all groups to allow for comparison. Using the analyze particles function, the mitochondrial area in the two channels is calculated. The mitophagy index is determined by the ratio between the mitochondrial area at 561 nm and the mitochondrial area at 458 nm.

- **Mitochondrial Morphological Analysis by Confocal Imaging:**
  The ImageJ software is used to analyze mitochondria morphological parameters. Account for all the mitochondria present in the cell, Z-stacked images individually analyzed by every stack. After thresholding, a binary, pixelized mask is created, allowing the software to recognize all the mitochondria structures. Using the analyze particles function, morphological parameters were calculated. The aspect ratio (AR) fits the smallest possible ellipse on the recognized mitochondria structure, to generate a ratio between the length of the major Axis and the minor Axis of the ellipse. Circularity, is calculated by $(Perimeter)/(4\pi * Area)$. Feret's diameter

is calculated by the longest distance between any two points along the selection boundary of the identified mitochondria structure.

- **Assessing Macrophage Mitophagy with Flow Cytometry:**
  Prior to the experiment, macrophages are detached with 10 ml of 2mM PBS-EDTA and replated in 6 well plates. On the experiment day, cells are treated with 25 μg of isolated mitochondria. At respective time points, after the removal of isolated mitochondria via washing with 1x PBS (pH 7.4), the cells are detached with 1 ml of 2mM PBS-EDTA. After centrifugation at 450 x g for 5 minutes, the cells are resuspended in 1 ml of 1x PBS (pH 7.4) with 5% FBS. Subsequent flow cytometry analysis involved the use of two lasers for excitation, with a wavelength of 405 nm and 587 nm. For detecting the emission from the cells, a 605 nm channel (BV605) and a 610 nm channel (mCherry) is used.

3 Results

A double-labeling System to Track and Validate the Uptake and Intracellular fate of Exogenous Mitochondria

Although it has been previously demonstrated that cardiac macrophages take up cardiac-derived mitochondria *in vivo* (16), it is suggested that the process occurs with membrane-bound mitochondria. It is unknown whether bone-marrow derived macrophages will take up exogenous isolated mitochondria in a similar manner. Although it has been previously demonstrated that resident macrophages take up cardiac-derived mitochondria *in vivo* (16), the process has been shown to occur with membrane-bound mitochondria. It is unknown whether bone-marrow derived macrophages will take up exogenous mitochondria in a similar manner. To validate whether Bone-marrow derived macrophages capture exogenous mitochondria present in the environment, we used a dual labelling system to distinguish exogenous and endogenous mitochondria. We treated BMDMs with MitoTracker Deep Red (MTDR), labelling their endogenous mitochondria, and provided them with isolated mitochondria from a mt-Keima animal. This allows us to visualize the two distinct mitochondria populations with confocal microscopy. Additionally, we used this assay to verify whether mt-Keima provides a robust estimation of the underlying levels of mitophagy. We treated BMDMs with 30 μM of Bafilomycin and 1 μM of FCCP (carbonylcyanide-4-trifluoromethoxyphenylhydrazone) for 6 hours. Bafilomycin is a mitophagy inhibitor that dampens the acidification of lysosomes, and FCCP dissipates the mitochondrial membrane potential to increase mitophagy. After incubating the cells with 25 μg of isolated mitochondria for 6 hours, we were able to detect mt-Keima signals inside BMDMs (Fig. 1B), regardless of whether the administration of bafilomycin and FCCP was present. The administration

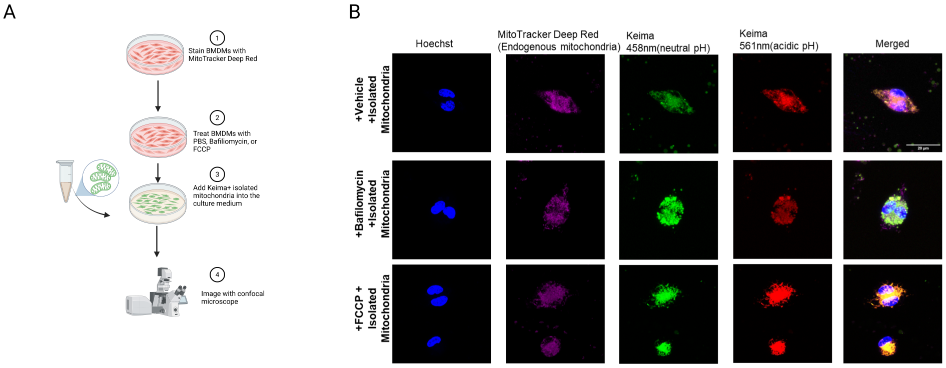


Figure 1: **The assessment of exogenous mitochondria uptake in BMDMs.** (A) Experimental Scheme for *in vitro* validation of exogenous mitochondria uptake by BMDMs ; (B) Represented Z-stacked confocal images of BMDMs displaying mt-keima and MTDR (MitoTracker Deep Red) fluorescence in indicated groups. Scale bar: 20 μm

of bafilomycin triggers a shift in the ratio of Keima 561 nm and Keima 458 nm. The merged images signify a Keima signal ratio that is predominantly in the 458 nm range (Fig. 1B). This signifies that that mitophagy of the exogenous mitochondria has decreased, which is consistent with the effects of bafilomycin. The addition of FCCP triggers an increase in intensity in both the 561 nm and 458 nm keima channels. The overall mt-Keima ratio shifts towards the 561 nm range, as indicated by the merged image (Fig. 1B). This indicates that the mitophagy of the exogenous mitochondria has increased, which is consistent with the effects of FCCP. Together, these results suggest that the *in vitro* uptake of exogenous mitochondria from BMDMs occurs, and mt-Keima provides a reasonable estimation of the underlying levels of mitophagy.

Functional Assessment of Macrophages after Uptake of Cardiac Mitochondria

Past studies have demonstrated that mt-DNA deleted p0 cells can restore their mitochondrial function and aerobic respiration by capturing exogenous mitochondria (8).

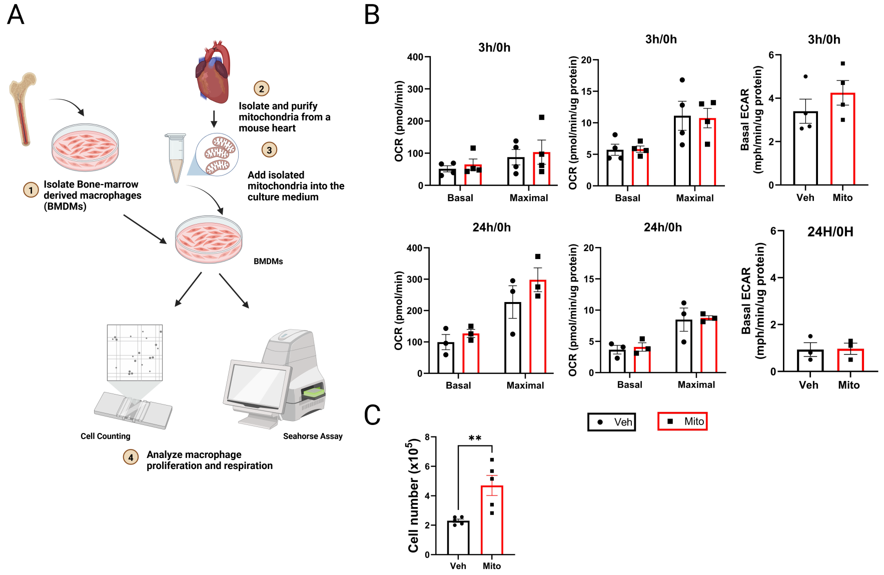


Figure 2: **The Immediate and Delayed Metabolic Consequences of Acquiring Exogenous Mitochondria in BMDMs.** (A) Experimental Scheme for functional assessment of BMDM respiration and proliferation; (B) Bar graphs depicting the relative basal OCR, maximal OCR, and basal ECAR between the vehicle (+mitochondria isolation buffer, MSE) and mitochondria-treated BMDMS at indicated timepoints; (C) Cell proliferation measured at 24 hours; N=4; All data are presented as mean±SEM. Statistical significance was determined by the Mann-Whitney Test.

It remains unknown whether the acquisition of exogenous mitochondria will alter the metabolic profiles of macrophages. Due to the bacterial origin of mitochondria, it is possible that the administration of exogenous mitochondria can activate and shift BMDMs towards the M1 phenotype, which is indicated by the decrease in the oxygen consumption rate (OCR) and the increase in extracellular acidification rate (ECAR) (35). To gain clarity on whether the acquisition of exogenous mitochondria alters the cellular metabolism in macrophages, we conducted Seahorse Cell Mitochondria Stress Tests on BMDMs after providing them with 25 μg of mitochondria purified from heart tissues. We analyzed the basal oxygen consumption rate (basal OCR), which characterizes the oxygen consumption used to meet the cellular ATP demand under baseline conditions. After decoupling the established proton gradient in the mitochondrial intermembrane space with FCCP, we also recorded the maximum respiratory capacity (maximal OCR). FCCP stimulates electron respiratory chain to perform at their maximum capacity. This indicates the highest respiration rate a cell can achieve. Additionally, the extracellular acidification rate (ECAR), reflecting levels of cellular glycolysis, is also determined under basal conditions. To assess both the immediate and delayed consequences of mitochondria uptake, we included two major time points of interest. We treated the cells with purified mitochondria for either 3 or 24 hours and analyzed the cells immediately (3h/0h and 24h/0h). Exogenous mitochondria are removed with PBS washes prior to conducting analysis. At 3h/0h, we did not observe a significant difference in the basal and maximal OCR, as well as the basal ECAR, of BMDMs that received exogenous mitochondria when compared to the vehicle, which received the same volume of mitochondria isolation buffer, MSE (Fig. 1B). Acquiring mitochondria from the environment, or the incorporation of respiratory proteins, could have increased the overall maximal respiration. To account for this, we normalized the data to total cellular protein. After normalization, basal and maximal respiration still did not differ significantly between the vehicle and treatment group (Fig. 1B). Furthermore, basal ECAR, also did not display a significant difference after normalization (Fig. 1B). It has been described that BMDMs have a double time of around 20 hours (22). We analyzed cell proliferation after providing BMDMs with exogenous mitochondria for 24 hours. Cell that received mitochondria demonstrated a significantly higher increase in cell number (Fig. 1C). Similar to the previous timepoint, BMDMs did not demonstrate a significant variation in basal OCR, maximal OCR, and basal ECAR at 24 hours(Fig. 1B). These data suggest that the uptake of exogenous mitochondria promoted macrophage proliferation at 24 hours. However, it did not alter macrophage ATP demand, cellular electron respiratory capacity, and glycolysis.

Assessment of Macrophage Mitophagy of Endogenous Mitochondria after the Uptake of Cardiac Mitochondria

The Seahorse Mitochondrial Stress Test is unable to delineate between the oxygen consumed by the exogenous mitochondria and that consumed by the endogenous mitochondria in the macrophages. Despite not driving a significant

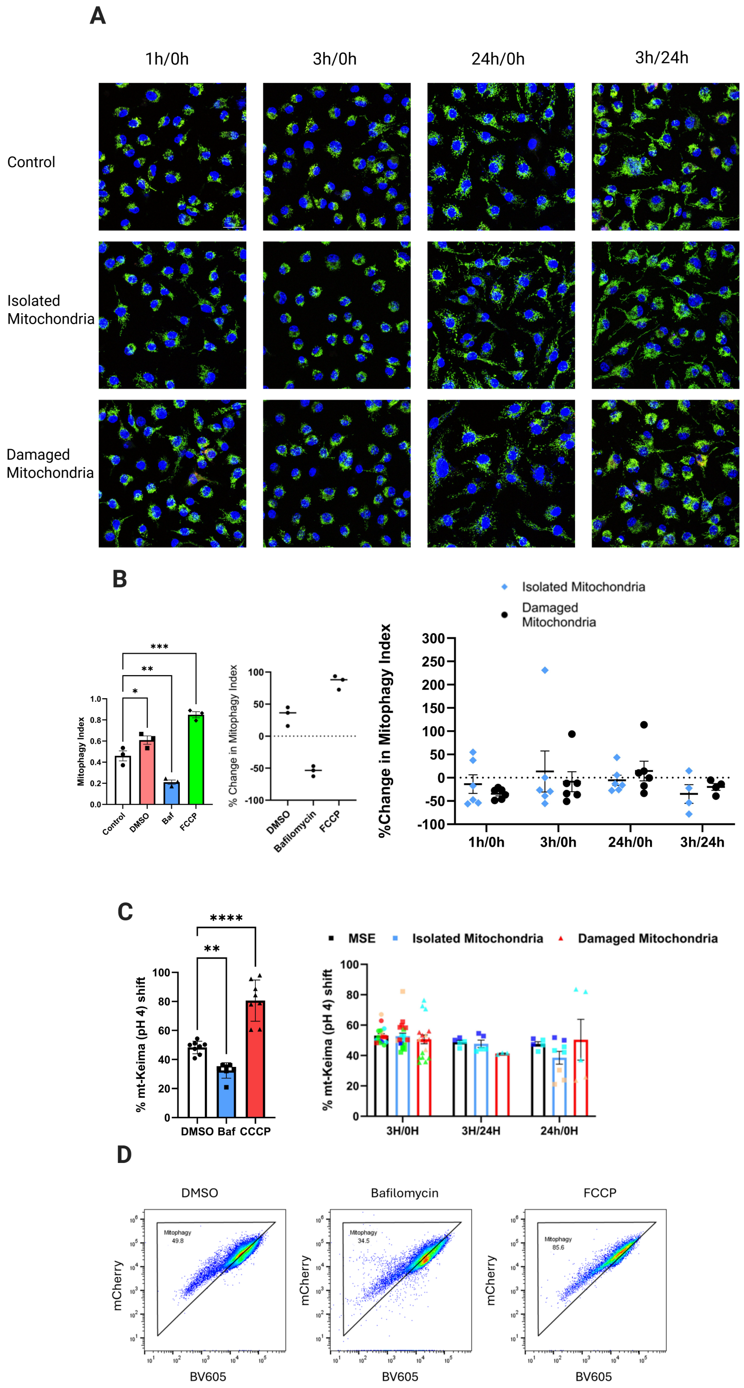
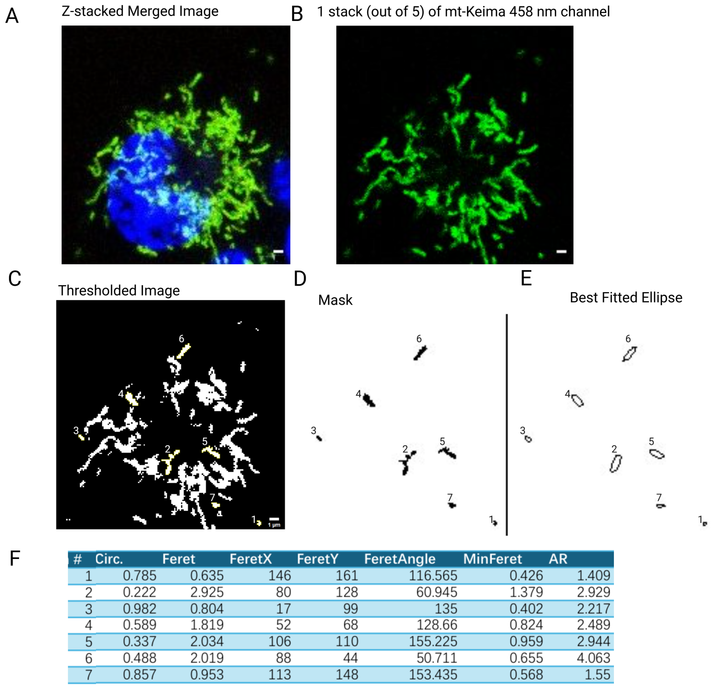


Figure 3: **The acquisition of exogenous mitochondria by BMDMs does not affect the mitophagy of its endogenous mitochondria.** (A) Representive Z-stacked merged confocal images of mt-Keima (458 nm, neutral pH), mt-Keima (561 nm, acidic pH), and Hoechst in the indicated BMDMs. Scale bar: 20 μm; (B) Summary graphs of calculated mitophagy index, the ratio of mt-Keima fluorescence area (561/458) in respective groups and timepoints. N=3. % changes in mitophagy index was generated by comparing the calculated index to the respective controls. All data presented as mean±SEM; Statistical significance for controls (DMSO, Bafilomycin, and FCCP) was determined by ordinary one-way ANOVA. Statistical significance for mitochondria treatment was determined by Dunnett's multiple comparisons test. (C) Summary graphs of % mt-keima (pH 4) shift from FACS analysis. N=3. Ordinary one-way ANOVA was used to determine the statistical significance for controls (DMSO, Bafilomycin, and FCCP) . Dunnett's multiple comparisons test was used to determine the statistical significance of mitochondria treatment. (D) Representative cell-sorting for mt-Keima fluorescence. mCherry: Emission for excitation at 587 nm (mt-Keima acidic), BV605: Emission for excitation at 405 nm (mt-Keima neutral)

alteration in the metabolic profiles of BMDMs, it remains unknown whether the acquisition of exogenous mitochondria has produced other cellular effects. The status of the endogenous mitochondrial network is associated with important cellular signals such as cell cycle progression (19) and apoptosis (36). To gain insight into this, we investigated the mitophagy of endogenous mitochondria in macrophages after cardiac mitochondria uptake. We cultured BMDMs isolated from mt-Keima animals, in which their endogenous mitochondria are intrinsically labelled with the fluorescent protein. Next, we treated these macrophages with 25 μg of isolated mitochondria or damaged (freeze-thawed) mitochondria from the heart. We included similar timepoints as previous Seahorse experiments, with the addition of a timepoint, 3h/24h, meaning the cells were treated with exogenous mitochondria for 3 hours but was not analyzed until 24 hours after the treatment. The lysosomal resistant protein mt-Keima allows for the determination of the underlying rate of lysosomal degradation (mitophagy), as well as the morphology of the endogenous mitochondrial network. These parameters were visualized and quantified using confocal microscopy. Additionally, flow cytometry was utilized to comprehensively analyze the potential changes in the mitophagy of the endogenous mitochondria. Using the analyze particles function in ImageJ (image processing and analysis software), the thresholded mitochondrial area is calculated in the Keima 458 nm (neutral) and 561 nm (acidic) channels. A ratio of the mitochondria area of 561 nm over 458 nm (red/green) was calculated to produce the mitophagy index. We did not find a significant difference in the calculated mitophagy index between the control and the treatment groups across the time-points (Figure 3B). Furthermore, flow cytometry analysis did not highlight any major differences in the overall % of mt-keima (pH 4) shift between the vehicle (MSE) and treatment groups across all measured timepoints (Fig. 3C). Both the image analysis and flow cytometry analysis demonstrated a high variation in groups of cells treated with isolated and damaged mitochondria at 3h/0h and 24h/0h (Fig. 3B, C). The groups treated with isolated and damaged mitochondria for 1h/0h and 3h/24h have a tighter distribution in their measured % change in mitophagy index and % of mt-keima (pH 4) shift. Interestingly, such variation is not present in vehicle groups that received MSE (Fig. 3B, C). The core driver for the described phenomenon remains unclear. The data suggest that the acquisition of exogenous mitochondria does not alter the mitophagy of endogenous mitochondria in macrophages.

The Acquisition of Exogenous Mitochondria Does Not Interfere with the Endogenous Mitochondria Morphology

To further characterize the status of the endogenous mitochondria network after the uptake of cardiac mitochondria in macrophages, we assessed various mitochondria morphological parameters. Mitochondria undergo a set of dynamic events during their life cycles. The continuous events of fusion, fission, and mitophagy will determine the overall morphology, quantity, and distribution of mitochondria in the cell. Mitochondria fission and fusion events are pivotal



#	Circ.	Feret	FeretX	FeretY	FeretAngle	MinFeret	AR
1	0.785	0.635	146	161	116.565	0.426	1.409
2	0.222	2.925	80	128	60.945	1.379	2.929
3	0.982	0.804	17	99	135	0.402	2.217
4	0.589	1.819	52	68	128.66	0.824	2.489
5	0.337	2.034	106	110	155.225	0.959	2.944
6	0.488	2.019	88	44	50.711	0.655	4.063
7	0.857	0.953	113	148	153.435	0.568	1.55

Figure 4: **Demonstration of ImageJ Quantification of Mitochondria Morphological Parameters.** (A) Z-stacked image of the overlapped mt-Keima (458 nm, neutral pH), mt-Keima (561 nm, acidic pH), and Hoechst; Scale bar: 1 μm ;(B) Image depicting one of the planes of the image from the Keima 461 nm channel; (C) A binary image generated after threasholding from (B); (D) Manual selection of mitochondrial objects from (D); (E) Best Fitted ellipse calculated by ImageJ; (F) Quantifications of aspect ratio (AR), Circularity (Circ), and Feret's diameter (Feret) from the selected mitochondrial objects.

to maintain optimal mitochondrial function and to drive cellular processes. In short, fusion events allow for the combination and exchange of mtDNA. In yeast, mutants with fusion defects quickly lose their mitochondrial genome and display impairments in mitochondrial-related parameters such as respiration (21). Fission events could generate mitochondria lacking mtDNA. Without fusion, the amount of mitochondria devoid of mtDNA accumulates, leading to the loss of mitochondrial function over time. Fission events are also pivotal in contributing to mitochondria quality control to eliminate dysfunctional mitochondria. Literature has also suggested intriguing correlations between the energy demand and mitochondrial fission or fusion events (18). The fluorescent microscopy of mt-Keima allows for the visualization of the size, shape and distribution of the labelled mitochondria. It remains unclear whether the acquisition of exogenous mitochondria will affect the morphology of the endogenous mitochondria in BMDMs. To gain a comprehensive understanding, we quantified the structure and morphology of the network of macrophage endogenous mitochondria after the uptake of cardiac mitochondria. Image analysis was conducted on the captured confocal images taken from the previous mitophagy analysis with

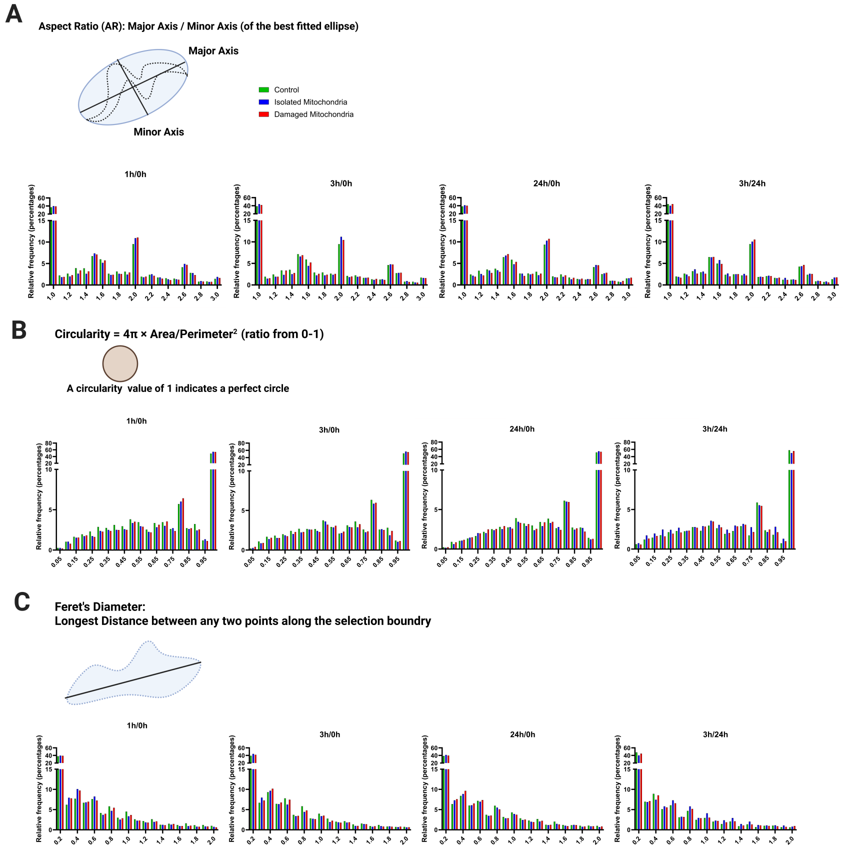


Figure 5: **Quantification of Endogenous Mitochondrial Morphological Parameters after the Acquisition of Exogenous Mitochondria by BMDMs.** Bar graphs depicting the frequency distribution of ImageJ quantification of aspect ratio (A), Circularity (B), and Feret's diameter (C). N=3; Aspect ratio: bin size = 0.1, range = 2; Circularity: bin size = 0.05, range = 0.95; Feret's diameter: bin size = 0.1, range = 1.8

ImageJ. To account for all the endogenous mitochondria present in the cell, every stack was individually analyzed (Fig. 4B). After thresholding, a binary mask is created (Fig. 4C, D), and the software recognizes the mitochondria structures. Using the analyze particles function, the aspect ratio (AR), which is the ratio of the length of the major Axis to the minor Axis of the best-fitted ellipse (Fig. 4E) of the identified mitochondria structure. It is a measurement of mitochondria elongation and roundness (Fig. 4F). The circularity, which is a ratio between area and perimeter, with a perfect circle having the ratio of 1, of the identified mitochondria structure is also calculated to investigate mitochondrial branching (Fig. 4F). Additionally, Feret's diameter, which is the longest distance between any two points along the selection boundary of the identified structure, can provide additional information about mitochondrial elongation and size (Fig. 4F). We have also included similar time points as previous experiments. Across all the timepoints, the analysis did not demonstrate any significant difference in the distribution of AR, circularity and Feret's Diameter in the individual endogenous mitochondria, compared to the controls that did not receive exogenous mitochondria (Fig 5. A-C). Interestingly, around 40 % of the endogenous mitochondria has an aspect ratio of 1 (Fig. 5A), having an identical length of major and minor axis suggests that they are circular. This observation is further confirmed by analyzing mitochondrial circularity, which revealed that 50 % of the endogenous mitochondria population is circular (Fig. 5B). Lastly, Feret's diameter revealed that these mitochondria are also small, with around 40% having a low value for Feret's diameter (Fig. 5C). Taken together, these data suggests that the majority of the BMDMs' endogenous mitochondria is small and circular, and the acqusition of exogenous mitochondria did not perturb their morphology.

4 Discussion

The elimination of dysfunctional mitochondria in cardiomyocytes is critical for preserving cardiac function and health. The disposition of dysfunctional mitochondria to resident macrophages is an effective alternative mechanism for cardiac mitochondria quality control. While beneficial for cardiomyocytes, the functional outcomes for macrophages remain unclear. The overarching goal of this study is to understand the biological effects of cardiac mitochondria uptake on macrophages, with the aim to elucidate the mechanisms that allow macrophages to become crucial members in preserving cardiac function and maintaining cardiac mitochondrial homeostasis. We hypothesized that the acquisition of exogenous mitochondria, due to its bacterial origin, can induce macrophages to adapt a pro-inflammatory role, rapidly increasing their proliferation and altering respiratory profiles. A change in energy demand can also cause profound changes in the underlying network of endogenous mitochondria, shifting the rate of mitophagy and fission/fusion dynamics. With an *in vitro* model, we were able to demonstrate that upon receiving cardiac mitochondria, macrophages does indeed increase their proliferation. However, the uptake of exogenous mitochondria does not drive major perturbations in the respiratory profiles of these macrophages. Additionally, the acquisition of cardiac mitochondria does not alter the mitophagy and morphology of their endogenous mitochondria, which is intriguing since fission and fusion dynamics has been shown to alternate as cells proliferate and progress through the cell cycle (20).

To further examine the effect of exogenous mitochondria uptake, future research can use transcriptional analysis to quantify mitochondria biogenesis, a process involved to increase the number of respiratory mitochondria. As well as the use of electron microscopy to investigate the mitochondrial cristae structure to provide additional insight into the mitochondrial dynamics and morphology.

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