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Original Research

Increased mtDNA Polymorphism Level and Defective Mitophagy are Associated With Proinflammatory Activation in THP-1-Based Cybrids

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Abstract

Background: Mitochondria play a key role in the regulation of inflammatory processes. It is well established that mitochondria contain their own mitochondrial DNA (mtDNA), which displays a substantially higher frequency of polymorphisms compared to the nuclear genome. To maintain mitochondrial functionality, cells employ a quality control mechanism known as mitophagy, which ensures the selective removal of dysfunctional mitochondria. The aim of this study was to investigate the relationship between mitochondrial polymorphisms, the efficiency of dysfunctional mitochondria clearance, and proinflammatory activation of inflammatory cells. **Methods:** In this study, we used cybrids derived from the THP-1 (human monocytic leukemia cell line) that differed in their mitochondrial genome. **Results:** Based on the levels of secreted proinflammatory cytokines (tumor necrosis factor (TNF), interleukin-1β (IL-1β), IL-6, IL-8, and C-C motif chemokine ligand 2 (CCL2)), cybrids were divided into two groups: one exhibiting a low and the other a high proinflammatory response. mtDNA sequencing revealed a higher number of polymorphisms in the high proinflammatory response group. Transcriptome analysis showed that signaling pathways associated with mitophagy were downregulated in cells with a high proinflammatory response. Functional assays confirmed that mitophagy was impaired in this group. **Conclusions:** Increased proinflammatory activity in cybrids correlated with a greater number of mtDNA polymorphisms and with impaired mitophagy. These observations point to an association between mitochondrial genomic variation, reduced mitochondrial quality control, and heightened proinflammatory activation of inflammatory cells. However, the data are associative and do not establish causality. Further mechanistic studies are required to determine whether and how mtDNA polymorphisms and defective mitophagy directly contribute to proinflammatory activation.

Keywords: mitochondria; inflammation; cybrids; mtDNA polymorphisms; mitophagy

1. Introduction

Atherosclerosis is known to be accompanied by localized chronic inflammation. However, the mechanisms underlying the failure of inflammatory resolution and the transition to a persistent, low-grade chronic state in atherosclerosis remain unclear [1,2,3]. Currently, several therapeutic approaches are used to treat atherosclerosis. One of the most widely implemented strategies involves lowering low-density lipoprotein cholesterol levels using statins [4]. However, statin therapy is often associated with significant side effects [5].

An emerging approach to the treatment of cardiovascular diseases (CVD) is anti-inflammatory therapy. The largest study in this area, Canakinumab Anti-Inflammatory Thrombosis Outcomes Study (CANTOS), targeted interleukin-1β (IL-1β), a cytokine predominantly secreted by activated monocytes and macrophages. This intervention reduced recurrent cardiovascular events; however, it was accompanied by a slight increase in mortality due to infectious diseases. The results of this trial demonstrated the high potential of anti-inflammatory strategies in CVD treatment [6]. Nevertheless, the development of more effective and safer therapeutic approaches for atherosclerosis remains necessary.

A promising strategy in anti-inflammatory therapy involves the activation of mitophagy in cells. Mitophagy serves as a mitochondrial quality control mechanism that selectively removes damaged mitochondria [7]. Some mitophagy-associated genes have been suggested as potential therapeutic targets in myocardial infarction [8]. Impaired mitophagy may lead to the accumulation of dysfunctional mitochondria, which can, in turn, exacerbate in-

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flammatory responses [7]. Given that mitochondrial DNA (mtDNA) repair mechanisms are significantly less efficient than those of nuclear DNA, mutations in mtDNA occur more frequently [9]. In this study, our central hypothesis is that impaired mitophagy may facilitate the accumulation of mtDNA polymorphisms due to insufficient removal of dysfunctional mitochondria. Accordingly, the burden of mtDNA polymorphisms is considered not as a direct causal driver, but rather as a potential indirect indicator of compromised mitophagy efficiency. From this perspective, targeting mitophagy is viewed as a promising therapeutic strategy to modulate inflammatory activation. However, prior to elucidating specific molecular mechanisms or establishing causal relationships, it is essential to first define and characterize the association between mitophagy-related processes, mtDNA polymorphism burden, and inflammatory responsiveness.

Thus, the aim of this study was to investigate the relationship between mtDNA polymorphisms, mitophagy, and the inflammatory response of monocytes.

2. Materials and Methods

2.1 Cybrids Culture

To investigate the relationship between the inflammatory response and mtDNA polymorphisms, several immune cell cultures differing in their mtDNA profiles were selected for analysis. Previously, our laboratory successfully generated transmitochondrial cybrids based on the THP-1 monocytic cell line (TIB-202, ATCC, Manassas, VA, USA). These cybrids were produced by fusing mitochondria-depleted THP-1 cells with platelets obtained from patients exhibiting distinct heteroplasmy profiles in their blood cells. Each cybrid thus contained the nuclear genome of THP-1 and the mitochondrial genome of an individual patient [10]. A total of eight cybrids were included in this study: TCN-521, TCI-521, TC-LSM2, TCP-521, TC-520, TC-522, TC-HSMAM1, TC-HSMAM3.

Data on mtDNA polymorphisms in the cybrids are provided in **Supplementary Table 1**.

The cybrids were cultured in RPMI-1640 medium supplemented with glutamine (P0560, PanEco, Moscow, Russia), containing 10% fetal bovine serum (1.1.8.1., Biolot, Saint Petersburg, Russia), 100 U/mL penicillin, and 100 µg/mL streptomycin (A065, PanEco, Moscow, Russia). The culture medium was replaced every three days. During medium changes, a constant cell density of 300,000 cells/mL was maintained. Cells were cultured in a humidified incubator at 37 °C with 5% CO_2.

Cybrids culture were tested for mycoplasma using the MycoReport kit (MR001, Evrogen, Moscow, Russia) according to the study protocol. Mycoplasma testing was performed, and all cell cultures were confirmed negative.

2.2 Assessment of Proinflammatory Activity Upon LPS Stimulation

Cybrids were seeded into sterile 24-well plates at a density of 1×10^6 cells/mL, 1 mL per well, with two wells per condition. Lipopolysaccharide (LPS [L2630, Sigma-Aldrich, St. Louis, MO, USA]) was added to one of the wells at a final concentration of 1 µg/mL. Cells were incubated with LPS for 24 h. After incubation, cells were centrifuged at 300 ×g for 5 min. The supernatants were collected into separate tubes for cytokine panel analysis (TNF, IL-1β, IL-6, IL-8, CCL2) using ELISA. The cell pellets were lysed with ExtractRNA reagent (BC032, Evrogen, Moscow, Russia) and pooled for subsequent RNA extraction and transcriptome analysis.

Enzyme-linked immunosorbent assays were performed in 96-well plates using the DuoSet ELISA Development System reagents (DY510, DY201, DY206, DY208, DY279, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

2.3 RNA Isolation Using the Phenol-Chloroform Method

For RNA isolation, 200 µL of chloroform (8.05.00147, Ekos-1, Moscow, Russia) was added to the cells suspended in ExtractRNA reagent (BC032, Evrogen, Moscow, Russia), followed by gentle mixing by inversion. The mixture was incubated for 10 min at room temperature to allow phase separation. Subsequently, samples were centrifuged at 12,000 ×g for 15 min. The aqueous phase was carefully transferred to new tubes. Then, 600 µL of isopropanol (8.05.00067, Ekos-1, Moscow, Russia) was added, and samples were centrifuged again at 12,000 ×g for 15 min. The supernatant was discarded, and the pellet was washed with 75% ice-cold ethanol. After centrifugation at 12,000 ×g for 15 min, the ethanol was removed, and the pellet was air-dried until visible (approximately 5–7 min). The RNA pellet was resuspended in 22 µL of nuclease-free water (PB207M, Evrogen, Moscow, Russia). To remove contaminating DNA, DNase I (RNase-Free) (M0303, New England Biolabs, Ipswich, MA, USA) treatment was performed using reagents from New England Biolabs, following the manufacturer's protocol. RNA concentration was measured using a NanoPhotometer. The purified RNA samples were stored at –70 °C.

2.4 Preparation and Sequencing of mtDNA Libraries

mtDNA was extracted from cybrids using the QIAamp DNA Blood Mini Kit (51104, QIAGEN, Hilden, Germany), following the manufacturer's protocol. Amplification of mtDNA was performed by long-range PCR using high-fidelity Q5 DNA polymerase (M0491, New England Biolabs, Ipswich, MA, USA). Primer sequences (Evrogen, Moscow, Russia) used for mtDNA amplification: Mt_1 (F: GACGGGCTCACATCACCCCATAA; R: GCGTACGGCCAGGGCTATTGGT), Mt_2 (F: CACACATTCGAAGAACCCGT; R: GTGATTGGCTTAGTGGGCGA).

Amplicon purification was performed using MGI Clean magnetic beads (1000005279, MGI Tech Co., Ltd., Shenzhen, China), after which the amplicons were pooled and subjected to ultrasonic fragmentation using a Covaris S220 instrument. Library preparation was carried out using the MGIEasy Universal DNA Library Prep Set (1000005248, MGI Tech Co., Ltd., Shenzhen, China). Sequencing was performed using paired-end reads on the DNBSEQ-G400 platform (MGI Tech Co., Ltd., Shenzhen, China).

2.5 Bioinformatic Analysis

A total of 16 samples were included in the study. For each cybrid, two conditions were analyzed: untreated control and LPS stimulation. Cybrids were stratified into LowResp and HighResp groups based on their pro-inflammatory activity, comprising 3 matched control-LPS pairs for LowResp and 5 pairs for HighResp. Differentially expressed genes (DEGs) were identified separately within each group by comparing LPS-stimulated samples to the corresponding controls. Group-specific DEGs were then determined, and pathway enrichment analysis was performed for upregulated and downregulated gene sets.

Read quality was assessed using FastQC v0.11.9 (Babraham Bioinformatics, Cambridge, UK). Overrepresented sequences corresponding to regions of human mtDNA were identified, and read duplication was observed. Reads were aligned using the BWA MEM algorithm v0.7.17-r1188 (open-source software developed by Heng Li) with default parameters [11]. Alignment was performed against the revised Cambridge Reference Sequence of the human mitochondrial genome (assembly GRCh38). Deduplication of the aligned reads was carried out using Picard MarkDuplicates v3.0.0 (Broad Institute, Cambridge, MA, USA). The average mapping efficiency was approximately 68%.

SNP calling was performed using MutServe v2.0.0-rc13 (University Hospital Cologne, Cologne, Germany), which accounts for deletions and generates consensus sequences for each sample. The resulting VCF files were annotated using the accompanying rCRS annotation dated August 20, 2020.

The table containing polymorphism data was generated using RStudio 2025.05.0+496 (Posit Software, PBC, Boston, MA, USA) and the tidyverse package [12]. To identify heteroplasmic variants, the data were filtered using a minor allele frequency threshold of ≥0.02.

DEG analysis was performed in DESeq2 v1.38.0 (Bioconductor, Buffalo, NY, USA) using a paired design, where LPS-stimulated samples were compared to matched controls (K) within each cybrid. DEGs were identified using the DESeq2 package for R/Bioconductor [13]. The thresholds were set at $|\log_2\text{FoldChange}| \geq 0.6$ and nominal p-value ≤ 0.05. Nominal P-values were used for the differential expression analysis, while adjusted P-values were used for visualization in the volcano plots (Figs. 2A,B).

Pathway enrichment analysis was performed using the Reactome database via EnrichR (Ma'ayan Lab, Icahn School of Medicine at Mount Sinai, New York, NY, USA) [14].

2.6 Confocal Microscopy-Based Assessment of Mitophagy

To assess mitophagy efficiency via confocal microscopy, cells were stained with 100 nM MitoTracker Green (M7514, Thermo Fisher Scientific, Eugene, OR, USA). After washing, cells were seeded into 6-well plates. A mitophagy activator, 2 µM carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP [C2920, Sigma-Aldrich, St. Louis, MO, USA]), was added to the cells for 2 h. Subsequently, 50 nM LysoTracker Deep Red (L12492, Thermo Fisher Scientific, Eugene, OR, USA) was added for 30 min. After washing, the cells were seeded into confocal imaging dishes (BDD012035, Jet Biofil, Guangzhou, China).

Images were acquired using a Leica DMi8 STELLARIS 5 confocal microscope equipped with an HC PL APO CS2 63×/1.40 OIL objective. MitoTracker Green was excited using a 488 nm laser, and fluorescence emission was detected in the 494–568 nm range. LysoTracker Deep Red was excited with a 638 nm laser, and fluorescence emission was collected in the 643–798 nm range.

The acquired images were processed using CellProfiler software v. 4.2 (Broad Institute, Cambridge, MA, USA). For each experimental condition, three images were analyzed: two single-channel images and one image containing segmented objects (cells, N ≈ 100). Mitophagy level was defined as the average degree of colocalization between mitochondria and lysosomes per cell.

2.7 Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics 27 software (IBM Corp., Armonk, NY, USA). For data with a normal distribution, the paired Student's *t*-test was used. For non-normally distributed data, the Mann–Whitney U test (for unpaired comparisons) and the Wilcoxon signed-rank test (for paired comparisons) were applied. A p-value of <0.05 was considered statistically significant.

3. Results

3.1 High Inflammatory Response of Cybrids With Increased Number of Polymorphisms in mtDNA

To investigate the relationship between the inflammatory response of monocytes and mtDNA polymorphisms, eight cybrid cell cultures with distinct mtDNA heteroplasmy profiles were analyzed. These cybrids had been previously generated by fusing mitochondria-depleted THP-1 cells with platelets from individual patients exhibiting dif-

ferent blood cell heteroplasmy profiles [15]. Each cybrid culture contained the nuclear genome of THP-1 and the mitochondrial genome of a specific patient. The names of the cybrid cultures analyzed are listed below: TCN-521, TCI-521, TC-LSM2, TCP-521, TC-520, TC-522, TC-HSMAM1, TC-HSMAM3.

To assess the proinflammatory response of the cybrids, cells were stimulated with 1 µg/mL LPS for 24 h, and cytokine secretion (TNF, IL-1β, IL-6, IL-8, CCL2) was measured by ELISA.

The results showed significant variability in cytokine secretion among the cybrid cultures (Fig. 1). To characterize this variation, we performed principal component analysis (PCA) on the cytokine secretion data. On the PCA plot, the cybrids segregated into two clusters (Fig. 1A). Kernel Density Estimation revealed a bimodal distribution of cytokine secretion levels, which enabled k-means clustering into two distinct groups. Consequently, the cybrids were classified into two groups differing qualitatively in cytokine secretion: Group 1 exhibited a low inflammatory response (LowResp), whereas Group 2 demonstrated a high inflammatory response (HighResp) (Fig. 1B). The LowResp group included cybrids TCN-521, TCI-521, and TC-520, while the HighResp group consisted of TC-LSM2, TCP-521, TC-522, TC-HSMAM1, and TC-HSMAM3.

Since the cybrids differed solely in their mitochondrial genome, it was hypothesized that the number of mtDNA polymorphisms might be associated with the intensity of the cellular inflammatory response. To investigate this, mtDNA sequencing was performed on the studied cybrids (Table 1). Heteroplasmy levels of the identified polymorphisms are presented in **Supplementary Table 1**. Polymorphisms were defined as mtDNA mutations with heteroplasmy ≥0.02. Polymorphisms associated with diseases or physiological conditions were summarized in **Supplementary Table 1**.

It was found that the HighResp cybrid group harbored a greater number of mtDNA polymorphisms in coding genes and the D-loop region (Table 1) compared to the LowResp group. This observation suggests a possible relationship between mtDNA polymorphism burden and the elevated secretion of proinflammatory cytokines by the cybrids.

3.2 Transcriptomic Analysis of Cybrid Cultures

To elucidate the mechanisms underlying the heightened inflammatory response in certain cybrids, transcriptomic analysis was performed on LPS-stimulated cybrid cultures.

First, DEGs in each cybrid culture following LPS stimulation were identified. DEG data for the LowResp (Fig. 2A) and HighResp (Fig. 2B) cybrids are presented as volcano plots.

Following LPS stimulation, 64 genes were upregulated and 82 genes were downregulated in LowResp cybrids compared with the control. In HighResp cybrids, 319 genes were upregulated and 93 genes were downregulated compared with the control (Fig. 2C).

Pathway enrichment analysis was conducted using the Reactome 2022 database to identify signaling pathways associated with the increased secretory activity observed in the cell cultures. The top 15 most enriched upregulated pathways in HighResp cybrids were predominantly related to cellular inflammatory responses (Fig. 2D).

Analysis of downregulated pathways revealed that Selective Autophagy, Pexophagy, and Macroautophagy pathways were suppressed in HighResp cybrids. Selective Autophagy is involved in targeted autophagic degradation of damaged organelles. Pexophagy specifically mediates the removal of damaged peroxisomes. Macroautophagy facilitates autophagosome formation and the degradation of organelles within lysosomes (Fig. 2E).

Finally, a protein-protein interaction network analysis of DEG-encoded proteins was performed (Fig. 2F). TNF, MYD88, TRAF3, and TRAF6 exhibited the highest number of interactions with other proteins.

Interestingly, 3 out of the 15 enriched signaling pathways were related to autophagy. We hypothesized that alterations specifically in mitophagy efficiency could be associated with the heightened inflammatory response observed in these cells.

3.3 Cybrids Exhibiting High Inflammatory Response Display Reduced Mitophagy Efficiency

We assessed mitophagy efficiency (i.e., inducible mitophagy capacity under acute stress) in the studied cultures by evaluating the colocalization of mitochondria (MitoTracker Green) and lysosomes (LysoTracker Deep Red) following treatment with 2 µM FCCP for 2 h (Fig. 3A,B).

Mitophagy was induced by FCCP in all LowResp cybrids (Fig. 3C). In contrast, 4 out of 5 HighResp cybrids showed no response upon FCCP treatment. Moreover, when comparing the two groups, inducible mitophagy was significantly lower in the HighResp group than in the LowResp group. These results suggest that mitophagy may be associated with the elevated proinflammatory response observed in the cells.

4. Discussion

THP-1-based cybrids exhibited two distinct types of proinflammatory responses to LPS stimulation: low and high. Moreover, a greater number of mtDNA polymorphisms was associated with increased secretion of proinflammatory cytokines. Transcriptomic analysis revealed that cells with a high inflammatory response displayed decreased activity in autophagy-related signaling pathways. Functional assessment of mitophagy efficiency demonstrated that cybrids characterized by a heightened proinflammatory response and an elevated mtDNA polymorphism burden exhibited defective mitophagy.

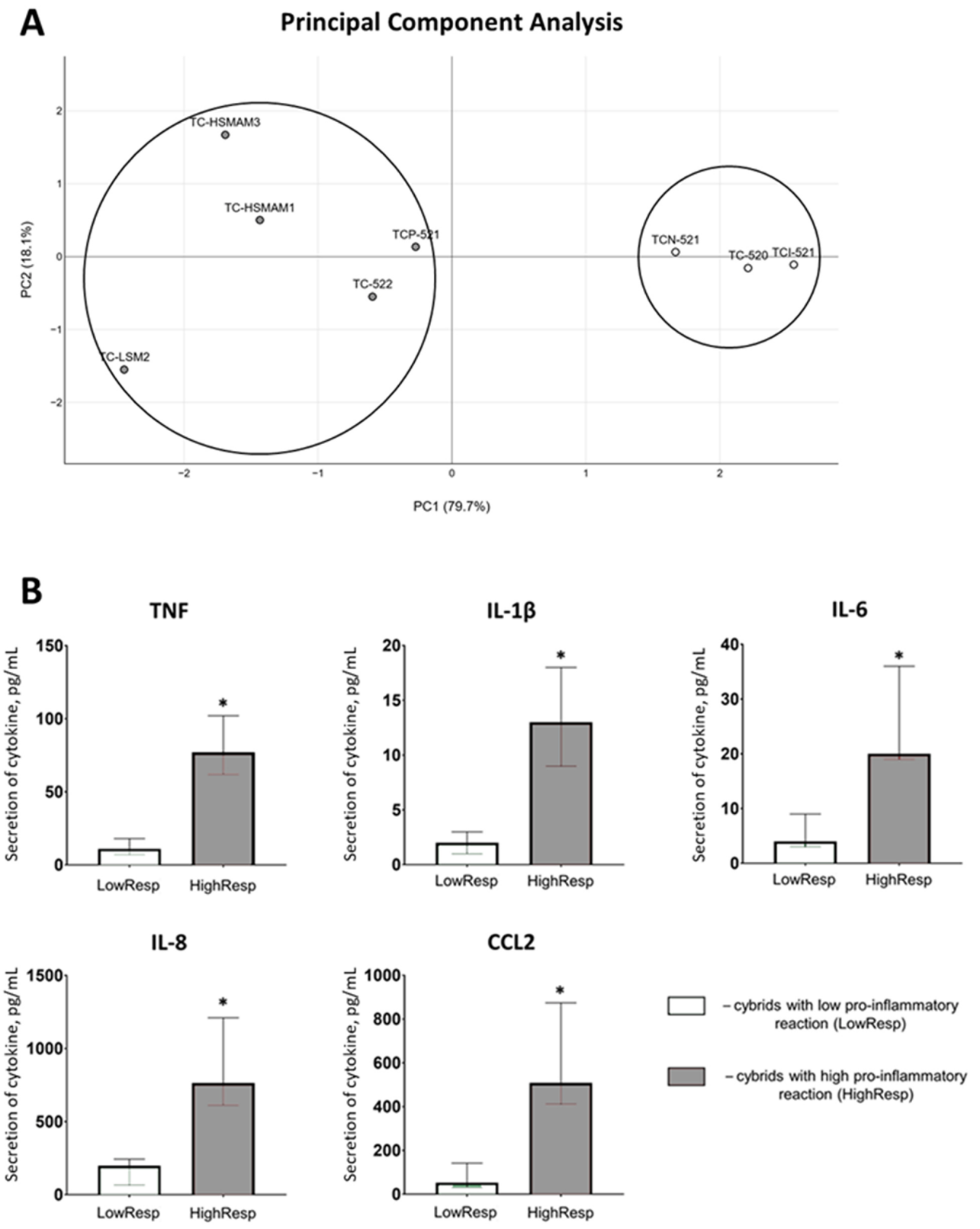


Fig. 1. Cytokine secretion (TNF, IL-1β, IL-6, IL-8, CCL2) by the two cybrid groups. (A) Principal component analysis showing the distribution of individual cybrid cultures. Each point represents an individual cybrid culture. The circles indicate the LowResp and HighResp cybrid groups for visualization purposes. (B) Comparison of cytokine secretion between LowResp and HighResp cybrid groups following LPS stimulation. Data are presented as median [Q1, Q3]. *$p < 0.05$, according to the Mann–Whitney U test.

Table 1. mtDNA polymorphisms in cybrids.

	Type of pro-inflammatory reaction of cybrids							
	LowResp Group			HighResp Group				
	520	TCN	TCI	522	MAM1	MAM3	TCP	LSM2
ATP6				+		+		
CO2						+		
CYB				+		+		
D-LOOP	+	+++		+		++		+++
ND1	+							+++
ND2						+		
ND4					+			+
ND5				++		+		+
ND6	+							
RNR1						+	++	
RNR2				+				+
TD				+				
TL2						+		
Median [95% CI]	0.0 [0.000–0.167]			0.4 [0.200–0.600]				

The column headers represent cybrid names, and the rows correspond to genes in which polymorphisms were detected. The number of "+" symbols indicates the quantity of polymorphisms in each gene per cell culture. Bold "+" denotes polymorphisms previously associated with various pathologies (detailed in **Supplementary Table 1**).

The primary focus of this study was cybrids generated by fusion of mitochondria-depleted THP-1 cells with platelets isolated from patients with atherosclerosis [15]. The cybrids contained a uniform THP-1 nuclear genome and an individual mitochondrial genome derived from each patient.

Currently, cybrids are widely used to study the role of mitochondria in various diseases [16]. Trimmer et al. [17] demonstrated that mitochondria in cybrids derived from patients with Parkinson and Alzheimer diseases exhibited reduced membrane potential compared to cybrids from control subjects. Bellizzi et al. [18] showed that cybrids (143B.TK$^-$ osteosarcoma cells depleted of their own mtDNA and repopulated with foreign mitochondria) differed in basal expression levels of IL-1β and TNFR2. Thus, multiple studies confirm that cybrid models are suitable for investigating the role of mitochondria and specific mtDNA polymorphisms in cellular function [10,19].

Previously, we demonstrated differences among the studied cybrids. Cybrids with impaired mitophagy exhibited elevated basal secretion of proinflammatory cytokines (IL-6, IL-8, IL-1β, TNF, CCL2) and an enhanced response to repeated LPS stimulation, indicating a loss of tolerance mechanisms. In contrast, cybrids with normal mitophagy showed a diminished response upon repeated stimulation, reflecting an ability to regulate inflammation [20]. In the present study, we further characterized the proinflammatory response of cybrids to LPS stimulation. We demonstrated that cybrids differed in their secretion of proinflammatory cytokines, with activation of distinct signaling pathways in response to LPS. Cybrids also differed in mitophagy levels following FCCP treatment. Moreover, cybrids exhibiting a high proinflammatory response displayed defective mitophagy. Collectively, these data suggest that cybrids with defective mitophagy exhibit an exacerbated proinflammatory phenotype.

In the present study, cybrids exhibiting a high proinflammatory response harbored a greater number of mtDNA polymorphisms in genes encoding respiratory chain proteins. Alterations in the structure or expression of these genes may lead to mitochondrial dysfunction and activation of proinflammatory signaling pathways [21]. Previously, mutations in mitochondrial genes associated with impaired respiratory chain function have been described. For example, the heteroplasmic mutation m.10372A>G in the *MT-ND3* gene, identified in patients with sensorimotor axonal polyneuropathy, was accompanied by the formation of paracrystalline inclusions and reduced ATP production in myoblasts [22]. A homoplasmic congenital mutation m.15482T>C in the *MT-CYB* gene, detected in blood samples, has been linked to complex III dysfunction and the development of hypertrophic cardiomyopathy [23]. Polymorphisms 15431G>A and 15674T>C in the *MT-CYB* gene were detected in the blood of COVID-19 patients; these variants altered the structure of cytochrome b and reduced its enzymatic activity [24]. The homoplasmic mutation m.6277A>G in the *MT-CO1* gene led to decreased intracellular electron transfer and proton leakage in Rhodobacter sphaeroides. Moreover, this mutation was associated with tumor development [25]. Shen et al. [26] reported

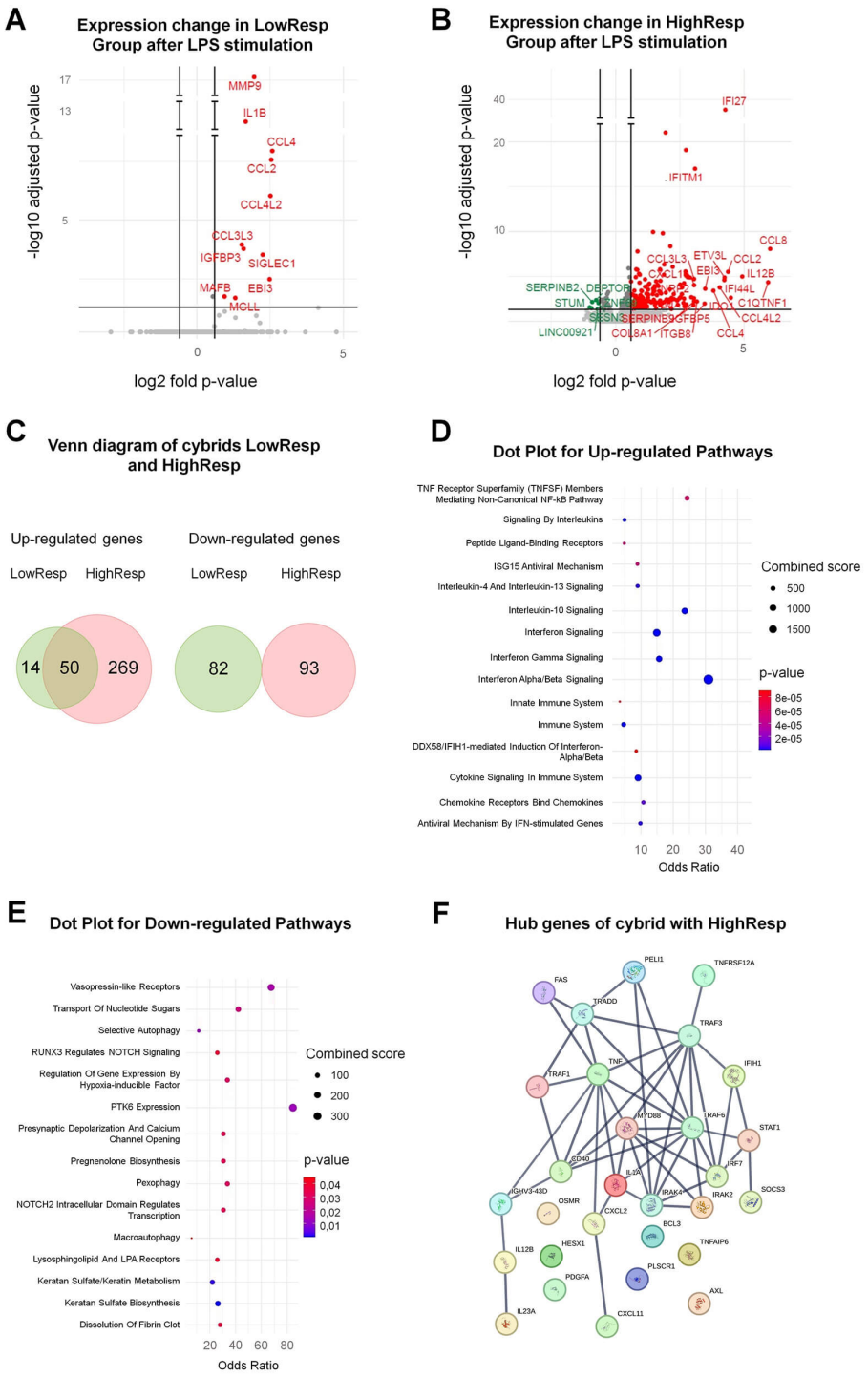
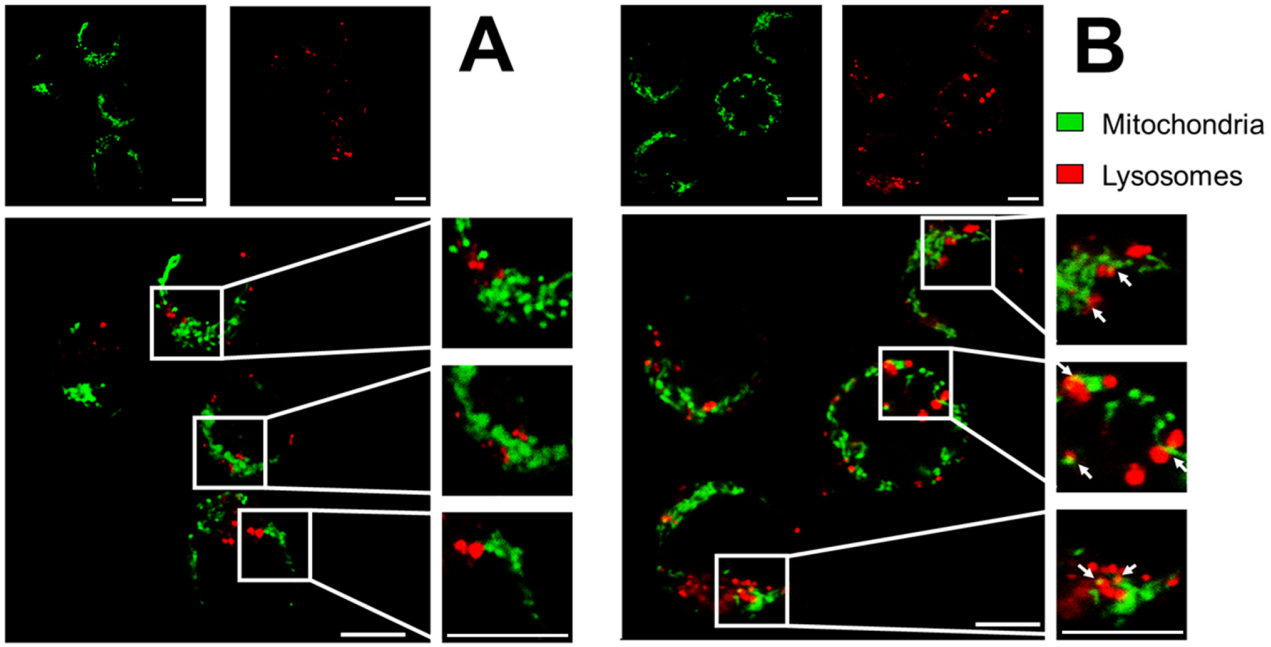


Fig. 2. Differential gene expression and functional pathway analysis in cybrids with distinct mtDNA profiles following LPS stimulation. (A,B) Volcano plots depicting gene expression changes in the two cybrid groups (LowResp and HighResp) after LPS stimulation. The x-axis represents $\log_2$ fold change, and the y-axis shows statistical significance ($-\log_{10}$ adjusted *p*-value). Upregulated genes are highlighted in red, downregulated genes in green. (C) Venn diagrams of up- and downregulated genes in LowResp and HighResp cybrids. The left panel shows unique and shared upregulated genes in both groups; the right panel shows downregulated genes. The green circle corresponds to the LowResp group, the red circle to the HighResp group. (D,E) Pathway enrichment analysis associated with DEGs in HighResp cybrids using Reactome 2022. The color scale indicates p-values, and the dot size corresponds to the combined score ($\log(p\text{-value}) \times z$). (F) Protein-protein interaction network analysis of hub genes in HighResp cybrids based on STRING data. Each node represents a gene. Line intensity corresponds to the interaction confidence score (>0.900).



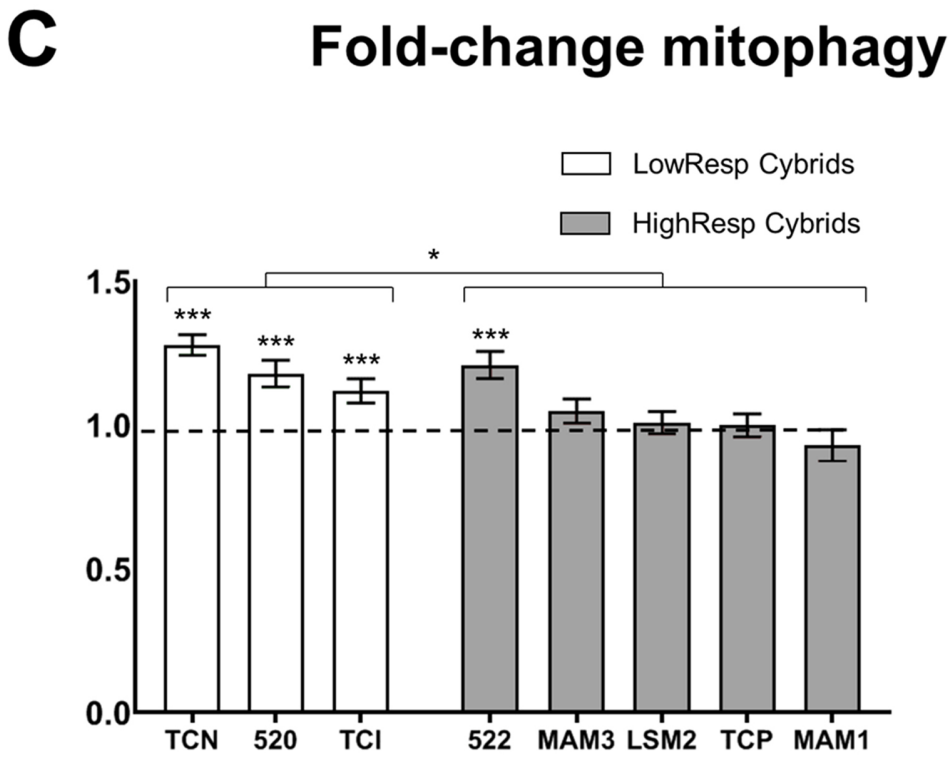


Fig. 3. FCCP-stimulated mitophagy in cybrids.(A,B) Confocal images of cybrids showing basal and FCCP-stimulated mitophagy. The top panels display mitochondria (MitoTracker Green) and lysosomes (LysoTracker Deep Red) separately. The bottom panels show merged channels with colocalization indicated by white arrows. All scale bars = 10 µm. (C) Relative change in mitophagy upon FCCP stimulation in cybrids. The dashed line represents basal mitophagy levels. White bars correspond to the LowResp group, gray bars to the HighResp group. Data are presented as mean ± SE. *** $p < 0.001$, according to the Student's t-test; * $p < 0.05$, by Wilcoxon group analysis. N = 4 (four independent experiments).

a correlation between the presence of the m.6459T>C mutation in blood and reduced ATP levels, decreased mitochondrial membrane potential, increased reactive oxygen species, and elevated apoptotic cell fractions. The congenital heteroplasmic mutation m.9553G>A in the *MT-CO3* gene caused a 59% reduction in complex IV activity in a patient with mitochondrial encephalopathy (mtDNA was isolated from muscle tissue) [27]. Mutations in the *MT-ATP6* gene, including heteroplasmic m.8839G>C and homoplasmic m.9176T>G, resulted in decreased membrane potential, reduced ATP synthesis and hydrolysis, and incomplete assembly of complex V, respectively [28].

Moreover, not only individual point mutations in mtDNA but also the overall mutational burden has been linked to inflammation. It was previously demonstrated that inflamed synovial tissue from patients with rheumatoid arthritis harbored a higher number of mtDNA mutations compared to healthy tissue [29]. In this study, the number of mtDNA mutations correlated directly with cytokine levels of TNF and IFN-γ in synovial fluid. Importantly, this association was independent of patient age [29]. Another study identified a set of unique homoplasmic mtDNA variants – 187 variants found exclusively in patients and 122 exclusively in healthy controls – in individuals with psoriatic arthritis. Furthermore, several missense variants were detected solely in psoriatic arthritis patients [30]. The presence of these variants impaired the functionality and stability of the encoded proteins. Additionally, patients with psoriatic arthritis exhibited elevated levels of oxidative mtDNA damage compared to controls. Taken together, mitochondrial dysfunction caused by mtDNA mutations may contribute to the pathogenesis of psoriatic arthritis [30].

These findings underscore the critical role of mtDNA polymorphisms in mitochondrial function. Even single nucleotide variations within the mitochondrial genome can significantly impact oxidative phosphorylation efficiency, ATP production, mitochondrial membrane potential, and reactive oxygen species generation. Disruptions in these processes not only result in metabolic dysfunction but also activate signaling cascades associated with inflammation. Such proinflammatory responses, in turn, may contribute to the progression of various diseases. On the other hand, inflammation may promote the accumulation of mtDNA mutations. Although we do not discuss this aspect in detail here, it represents a plausible alternative causal direction. However, to date, a causal relationship between inflammation and the emergence of mtDNA mutations has not been definitively established.

Some of the polymorphisms identified in the present study have been previously associated with various pathologies. The germline mutation m.16189T>C in the MT-DLOOP region has been linked to elevated insulin levels and insulin resistance [31]. The heteroplasmic somatic mutation m.3243A>G in the MT-DLOOP, along with the heteroplasmic variant m.16093T>C in peripheral blood mononuclear cells, was associated with increased reactive oxygen species production and decreased ATP levels and mitochondrial membrane potential [32]. The m.309C>T mutation in the MT-DLOOP was associated with gait speed [33]. The heteroplasmic mutation m.310T>C in the MT-DLOOP was inversely correlated with obesity [34]. The homoplasmic germline mutation m.3308T>C in MT-ND1 was detected in a patient with left ventricular hypertrabeculation/noncompaction [35]. The homoplasmic mutation m.4048G>A in *MT-ND1* found in blood was associated with maternally inherited essential hypertension [36]. The heteroplasmic germline mutation m.12622G>A in *MT-ND5* was linked to Leigh syndrome in three brothers [37]. Thus, the increased burden of mtDNA polymorphisms observed in cybrids with a high proinflammatory response may represent a critical factor contributing to mitochondrial dysfunction and exacerbated inflammation.

To elucidate the mechanisms underlying the dysregulated inflammatory response in cybrids harboring a high burden of mtDNA polymorphisms, transcriptomic analysis was performed. As expected, signaling pathways associated with inflammation were upregulated in the high proinflammatory response group. Interestingly, several downregulated pathways were related to autophagy. Functional assessment of mitophagy in the cybrids revealed that cells with a high proinflammatory response exhibited defective mitophagy, as evidenced by the absence of mitophagy induction upon FCCP treatment.

Recent studies increasingly focus on the role of defective mitophagy in the development of chronic diseases [38,39]. For various reasons, the process of removing damaged or dysfunctional mitochondria may become impaired [40,41]. In cases of defective mitophagy, damaged mitochondria persist within the cell instead of being eliminated. This leads to the accumulation of mitochondria with altered structure and function, causing further damage to cellular components and DNA [42]. Damaged mitochondria can release mtDNA into the cytoplasm, which can be recognized by the cell as a stress or danger signal, thereby triggering inflammatory responses as the cell attempts to counteract the perceived threat. Moreover, various receptors, including Toll-like receptors, can interact with mtDNA. This interaction activates intracellular signaling pathways, leading to the activation of transcription factors such as NF-κB and various interferons, ultimately promoting the production of inflammatory cytokines and immune response mediators [43,44,45].

Mitophagy defects can occur at various stages of the process, including the recognition of damaged mitochondria, autophagosome formation, or their fusion with lysosomes. Upon loss or reduction of mitochondrial membrane potential, PINK1 accumulates on the outer mitochondrial membrane and recruits Parkin. The PINK1/Parkin complexes tag defective mitochondria by interacting with LC3B, initiating autophagosome formation. After mem-

brane closure, the autophagosome fuses with a lysosome, forming an autolysosome where the mitochondrion undergoes degradation [46]. It is known that mutations in the *PINK1* and *Parkin* genes can lead to Parkinson disease development. In a clinical study, Borsche et al. [47] demonstrated elevated plasma IL-6 levels in patients with Parkinson disease.

Mitophagy can also be impaired at the stage of autophagosome-lysosome fusion. LAMP-1 and LAMP-2 constitute approximately 50% of all lysosomal membrane proteins. Eskelinen [48] reported that reduced levels of LAMP-1 and LAMP-2 were associated with increased accumulation of autophagic vacuoles, altered lysosomal morphology, and disrupted cholesterol metabolism in mouse embryonic fibroblasts.

Defects in mitophagy can also contribute to the development of inflammatory processes. The relationship between mitophagy and inflammation has previously been demonstrated using mitophagy activators and inhibitors. Zhao et al. [49] modulated pulmonary inflammation in mice by administering either the mitophagy inhibitor 3-methyladenine or the mitophagy activator rapamycin. They found that rapamycin treatment reduced mortality compared to the control group, whereas 3-MA increased mortality. Furthermore, induction of mitophagy with rapamycin decreased the secretion of TNF, IL-6, and CCL2, while inhibition of mitophagy with 3-MA led to increased cytokine secretion [49].

There are relatively few studies on the relationship between mtDNA and atherosclerosis. Previously, mitochondrial haplogroups A and M7a were found to increase the risk of coronary atherosclerosis in Japanese populations [50]. Additionally, Wei et al. [51] demonstrated that mtDNA copy number was inversely correlated with the severity of cardiovascular diseases (including coronary artery disease, myocardial infarction, and degree of stenosis). They also identified certain rare mtDNA variants in the *MT-ATP6* and *MT-COII* genes that were associated with the absence of myocardial infarction and hyperlipidemia, suggesting a protective role [51]. Furthermore, it has been shown that patients with ischemic heart disease exhibit higher levels of mtDNA damage in unstable atherosclerotic plaques [52]. Patients with atherosclerosis also had significantly higher total MutPred load (a method predicting the functional impact of amino acid substitutions reflecting the cumulative potential deleteriousness of all mutations in mtDNA protein-coding genes) compared to controls [53].

Thus, our data are consistent with global studies and confirm the association between mtDNA polymorphisms, mitophagy, and proinflammatory activation of cells.

This study has several limitations. It does not establish causal relationships between the mtDNA polymorphism burden, impaired inducible mitophagy, and enhanced proinflammatory responses. In addition, the molecular mechanisms underlying the observed associations were not investigated. Differences in cybrid phenotypes may therefore be driven not only by mtDNA variation, but also by nuclear–mitochondrial incompatibility or by general mitochondrial stress responses. Notably, signaling pathways associated with cellular stress were elevated in the HighResp group. However, no inflammatory activity was observed under basal conditions. The generation of cybrid cell cultures is a labor-intensive and technically demanding process. Consequently, the present study is limited to a small sample size ($n = 8$). Accordingly, our suggestion that chronic inflammatory diseases may be associated with an increased mtDNA polymorphism burden should be regarded as a hypothesis requiring further investigation. Cybrids were grouped based on their cytokine secretory activity, as cytokine production directly reflects immune cell behavior, in contrast to other intracellular molecular processes. Nevertheless, we acknowledge that alternative grouping strategies (based on mtDNA genotyping or mitophagy activation) could have resulted in different group compositions. The study design does not allow us to assess the individual contribution or functional impact of each mtDNA polymorphism. Therefore, we focused on the overall number of polymorphisms rather than variant-specific effects. The assessment of mitophagy ideally requires the application of multiple complementary approaches, particularly when investigating specific signaling pathways and mechanisms. In the present study, we focused exclusively on the ability of cells to induce mitophagy in response to FCCP treatment. The mechanisms and reasons underlying the lack of response to FCCP in certain cell cultures remain subjects for future investigation.

This underscores the role of the mitochondrial genome in the development of the immune response, as well as the importance of maintaining effective mitophagy to limit excessive cytokine production and prevent chronic inflammation. It is known that certain mitophagy activators, such as urolithin A, alliin, and bergapten, possess anti-inflammatory properties [54,55,56]. Therefore, the search for novel compounds capable of stimulating mitophagy while simultaneously reducing inflammation represents a relevant and promising direction for the development of therapies aimed at normalizing immune responses in various pathologies.

5. Conclusions

The results suggest that an increased number of mtDNA polymorphisms was associated with heightened proinflammatory activity and reduced mitophagy efficiency. These observations are correlative and do not establish whether impaired mitophagy causes the heightened inflammatory response or vice versa. Resolving directionality will require targeted experiments that modulate mitophagy and assess consequent changes in inflammation and mitochondrial status. A plausible interpretation is that defective inducible mitophagy may contribute to accumu-

lation of dysfunctional mitochondria, which in turn can amplify proinflammatory signaling. If this proves causal, activation of mitophagy could represent a promising strategy for developing therapies for chronic inflammatory diseases.

Availability of Data and Materials

Data available from the corresponding author upon reasonable request.

Author Contributions

Writing – original draft: ADZ, NGN; Visualization: ADZ; Methodology: ADZ, SSV; Data analysis: ADZ, DBE; Investigation: ADZ, SSV, YSC; Transcriptome sequencing and analysis: VNS, AVO, DDB; Sequencing analysis of mtDNA: ADZ, NGN, AVO, DDB; Provision of Cybrids: VVS; Conceptualization: NGN, ANO, YEY; Project administration: NGN; Supervision: ANO. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Research Institute of Atherosclerosis (06 May 2024). The study did not involve direct participation of humans or animals; therefore, written informed consent was not applicable.

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Conflicts of Interest

The authors declare no conflicts of interest.

Supplementary Material

A table with a complete list of mtDNA polymorphisms identified in cybrids is presented in **Supplementary Table 1**. Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/FBE49053.

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