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Review Article

Mitochondrial DNA and Inflammation: Mechanisms of Release and Signaling Pathways

Anastasia V. Poznyak[1*], Elizaveta Romanovna Korchagina[1], Fei Liu[2], Yanke Shan[2], Alexander N. Orekhov[1]

[1]*Institute for Atherosclerosis Research, Osennyaya 4-1-207, 121609 Moscow, Russia*
[2]*Institute of Human Morphology, Petrovsky Russian National Center of Surgery, 2 Abrikosovsky Lane, 119991 Moscow*
[3]*College of Veterinary Medicine, Nanjing Agricultural University, No. 1 Weigang, 210095 Nanjing, Jiangsu, China*

**Address Correspondence to: Anastasia V. Poznyak, Email: tehhy_85@mail.ru*

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Abstract

Mitochondria (Mt) play a crucial role in various cellular processes, including energy production and metabolism. Damage-Associated Molecular Patterns (DAMPs) released by mitochondria, such as mitochondrial DNA (mtDNA), cardiolipin, succinate, and ATP, can trigger inflammation signaling pathways. This review article delves into the evolutionary origins of mitochondria from bacterial ancestors and their role as regulators of inflammation through DAMPs release. The intricate mechanisms of mtDNA leakage into the cytoplasm and extracellular space are explored, focusing on pathways involving the Mitochondrial Permeability Transition Pore (mPTP), Cell-Free mtDNA, and Extracellular Vesicles (EVs). Additionally, the involvement of key immune signaling pathways such as the cyclic GMP-AMP Synthase (cGAS)-Stimulator of Interferon Genes (STING), Toll-like receptor-9, and inflammasomes in recognizing mtDNA as a danger signal and inducing inflammatory responses is discussed. The active and passive release of mtDNA by various cell types, including neutrophils and lymphocytes, and the implications of mtDNA in inflammatory diseases are examined. Understanding these mechanisms sheds light on the intricate interplay between mitochondria, inflammation, and immune responses, with potential implications for therapeutic interventions targeting mitochondria-related inflammatory pathways.

Keywords: Eukaryotic organisms; Mitochondria; DNA; Metabolism

Introduction

Mt are essential organelles found in the cells of eukaryotic organisms, playing a pivotal role in vital physiological processes such as energy production, iron metabolism, and the synthesis of lipids, amino acids, and nucleic acids. These organelles not only power cellular activities but also release various Damage-Associated Molecular Patterns (DAMPs) in response to cellular injury or stress, triggering inflammation through multiple signaling pathways. Notably, many of these DAMPs exhibit similarities to bacterial Pathogen-Associated Molecular Patterns (PAMPs), reflecting their evolutionary origins from ancestral prokaryotic symbionts that entered into a mutualistic relationship with early eukaryotic cells approximately 1.5 billion years ago, facilitated by increased atmospheric oxygen levels [1].

This endosymbiotic event radically transformed the cellular architecture of eukaryotes, leading to the evolution of mitochondria with unique features such as transport proteins, internal cristae structures, and complex mechanisms of fission and fusion that enhance their metabolic versatility and increase the competitive advantage of eukaryotic cells. At a morphological level, both mitochondria and certain bacteria, particularly Gram-Negative Bacteria (GNB), share significant structural similarities; both possess circular DNA and dual membrane systems the inner and outer membranes where the inner membrane encloses the mitochondrial matrix analogous to that of GNB. The region between the inner and outer membranes of mitochondria is referred to as the Intermembrane Space (IMS), while in GNB, this region is termed the periplasm [2].

This review aims to elucidate the intricate relationships between mitochondrial DAMPs and immune system responses, emphasizing their roles not only in cellular homeostasis but also in pathological conditions. Understanding these mechanisms is crucial, providing insights into potential therapeutic targets for diseases where mitochondrial dysfunction and inflammation are central features.

Mitochondrial DAMPs and their roles in inflammation

Mitochondria release an array of DAMPs, including mitochondrial DNA, Cardiolipin (CL), N-Formyl Peptides (NFPs), succinate, ATP, and Reactive Oxygen Species (ROS). N-formyl peptides, primarily associated with bacteria, are generated from formyl-methionine and recognized as chemoattractants for phagocytes through Formyl Peptide Receptors (FPRs) [3,4]. Given that mitochondria are evolutionarily related to bacteria, the formylation of methionine is believed to play a critical role in the translation of mitochondrial mRNA, further facilitating their identification by FPRs. Moreover, extracellular succinate has been reported to activate immune responses in dendritic cells, underscoring its importance in immune signaling [5].

Mitochondrial DNA (mtDNA), in particular, has garnered significant attention due to its structural resemblance to bacterial DNA, which enables its recognition by Toll-Like Receptor 9 (TLR9) in endosomes [6-10]. In conjunction, cytosolic DNA can activate cyclic GMP-AMP Synthase (cGAS), triggering an interferon response mediated by TMEM173. Importantly, mtDNA exhibits typical bacterial features, including a circular loop shape, unmethylated CpG clusters, and a lack of histones, existing in structures known as nucleoids. The packaging of these nucleoids is facilitated by the mitochondrial Transcription Factor A (TFAM) [11]. Both mtDNA and other mitochondrial products are categorized as DAMPs, akin to PAMPs derived from foreign microbes, and are recognized by Pattern Recognition Receptors (PRRs) expressed on epithelial, immune, and various other cell types [12,13]. PRRs are categorized into several subtypes, such as NOD-like Receptors (NLRs), RIG-I-like Receptors (RLRs), C-type Lectin Receptors (CLRs), and TLRs.

Evidence suggests that TLR9, cryopyrin (an NLR), and *TMEM173* play significant roles in mediating the inflammatory processes associated with mitochondrial DNA. Research into the connection between mitochondria and inflammation began approximately twenty years ago, with early studies demonstrating that joint injections of both mouse and human mitochondrial DNA in mice induced arthritis, whereas injections of nuclear DNA did not [14] (Table 1).

Table 1: Key mitochondrial damps and their functions in inflammation.

Mitochondrial DAMP	Description	Role in inflammation	Pathway/Mechanism
mtDNA	Circular DNA similar to bacterial DNA	Activates immune responses *via* TLR9 and cGAS-STING pathways	Recognized by Pattern Recognition Receptors (PRRs)
Cardiolipin (CL)	Phospholipid unique to mitochondrial membranes	Triggers inflammatory responses when exposed	Mediates signaling through TLRs
N-Formyl Peptides (NFPs)	Chemoattractants derived from formyl-methionine	Attracts phagocytes to sites of injury/ infection	Interacts with Formyl Peptide Receptors (FPRs)
Succinate	Metabolite associated with cellular metabolism	Promotes immune signaling	Activates dendritic cells
ATP	Energy molecule released during stress	Acts as a pro-inflammatory signal	Engages purinergic receptors
Reactive Oxygen Species (ROS)	Highly reactive molecules	Induces oxidative stress and inflammation	Involved in cellular signaling pathways

Mechanisms of mitochondrial DNA leakage

Mitochondrial DNA (mtDNA) is composed of 16,569 base pairs and encodes for ribosomal RNA (rRNA), transfer RNA (tRNA), and thirteen proteins essential for Oxidative Phosphorylation (OXPHOS). Recent studies have demonstrated that mtDNA can also be transcribed into non-coding RNAs, expanding its potential regulatory roles within the cell. The synthesis of mitochondrial proteins depends on the expression of nuclear genes, which must be imported into the mitochondria after translation.

Interestingly, mtDNA exists within nucleoid structures that can become vulnerable to damage and aggregation as a result of oxidative stress, which increases with aging. Under normal circumstances, mitochondrial quality control

mechanisms, such as mitophagy, fission, and fusion, mitigate this damage and facilitate the clearance of dysfunctional mitochondria. However, when these processes fail or when mitochondria are irreparably damaged, mtDNA may leak from the mitochondrial matrix and act as a (DAMP), potentially triggering inflammatory responses.

Although the precise mechanisms governing this leakage are still being elucidated, several pathways are believed to contribute. The mitochondrial Permeability Transition Pore (mPTP) is a key player, functioning as a channel at the contact sites between the inner and outer mitochondrial membranes. When mPTP opens, it can facilitate the uncontrolled release of mtDNA into the cytoplasm. During apoptosis, interactions between pro-apoptotic proteins lead to the opening of the mPTP, playing a critical role in cell death and the associated release of mitochondrial contents [15-24].

Additionally, the activation of pathways involving inflammatory mediators can influence mPTP dynamics. There is also evidence that Mitochondrial-Derived Vesicles (MDVs) play a significant role in mitochondrial quality control and may influence the degradation of damaged mitochondria through lysosomal pathways. Notably, MDVs that contain mtDNA can independently incite inflammation, highlighting the dual role of these vesicles in both mitochondrial maintenance and immune system activation (Table 2).

Table 2: Mechanisms of mtDNA release and immune recognition.

Mechanism of mtDNA release	Description	Associated pathways/effects
Mitochondrial Permeability Transition Pore (mPTP)	Opening of mPTP leads to mtDNA leakage during cellular stress	Induces inflammation and cell death
Mitochondrial Outer Membrane Permeabilization (MOMP)	Apoptosis-related release of mtDNA into the cytoplasm	Triggers caspase-dependent and independent cell death
Extracellular Vesicles (EVs)	Packaging of mtDNA into EVs for intercellular signaling	Mediates transport to target cells and supports inflammation
Neutrophil Extracellular Traps (NETs)	Formation of NETs that include mtDNA upon activation	Enhances inflammation and pathogen clearance
Passive leakage	Uncontrolled release of mtDNA during necrosis or cell death	Correlates with tissue damage and inflammatory responses

mtDNA release mediated by Mitochondrial Outer Membrane Permeabilization (MOMP)

Mt Outer Membrane (MOM) permabilization is an important mechanism that activates caspase during apoptosis. Interplay of B-cell Lymphoma 2 proteins regulates Mt outer membrane permabilization which triggers the intramembrane proteins to be released into extracellular space. This mechanism starts with MOM permabilization modulated by macropore being formed by BCL2-associated X and BCL-2 Homologous Antagonist/Killer [25,26]. The macropores facilitate the Inner Mitochondrial Membrane (IMM) herniation which leads to its disintegration and the following leakage of Mt DNA into cytoplasm. Normally this mechanism takes place in an organism and happens in cells regulated by BCL2-associated X and BCL-2 Homologous Antagonist/Killer. It is finished by caspase cascade activation and Cyt C release [27].

It is noteworthy that *cGAS/TMEM173* pathway activation by Mt DNA may define caspase independent cell death activation when caspase is suppressed. While inner Mt membrane has been believed to preserve integral state in apoptosis, the process of Mt DNA leakage into cytoplasm during caspase-independent cell death has been unexplored. Recent research shed light on that phemomenon [28]. Riley, McArthur and colleagues showed that permabilization of inner Mt membrane can lead to Mt DNA leakage into cytoplasm, and there it can start binding with cGAS/TMEM173 pathway and trigger ins activation [29].

Kim, et al. revealed one more way of Mt DNA leakage modulated by BCL2-associated X/BCL-2 Homologous antagonist/killer-independent Mt outer membrane permabilization pathway [30]. They detected Mt outer membrane pore formation and subsequent Mt outer membrane permabilization, facilitated by voltage-dependent anion channels in mouse embryonic fibroblasts. That was the first time that process was detected in nonapoptotic cells. Voltage-dependent anion channel is an important transport

protein of Mt outer membrane. This protein facilitates the transfer of adenosine triphosphate, Calcium, anions, cations, and metabolites. There were 3 isoforms detected in humans, called VDAC 1, 2 and 3 [31]. These isoforms show a variety of characteristics and activity. Voltage-dependent anion channel 1, presented as a β-strand, turns out to be the predominant form and is associated with apoptosis. Voltage-dependent anion channel 1 is able to create oligomers on Mt outer membrane, and its oligomerization is accompanied by stimulation of apoptosis [32,33].

Kim and colleagues explored the Mt DNA leakage provoked by voltage-dependent anion channels in SLE animal model without activated by BCL2-associated X/BCL-2 Homologous Antagonist/Killer complex. The study revealed that Mt pore formation lets the Mt DNA leak into the cytoplasm, thus promoting cGAS/TMEM173 pathway. It was suggested that the way Mt outer membrane permeabilization is carried out through voltage-dependent anion channels or BCL2-associated X/BCL-2 Homologous Antagonist/Killer complex is controlled by cellular stress [34]. The former process is prevalent in case of mild stress, and the latter takes place in case of apoptosis or severe cellular stress. Notably, it has been reported that suppressing of voltage-dependent anion channels oligomerization can decrease the lupus-like disorder in animals. Voltage-dependent anion channel 1 interplays with Mt DNA through 3 positively charges N-terminus residues, that is binding to negatively charged Mt DNA and triggers the oligomerization on Mt outer membrane [35,36]. Antiviral factor Vaccinia-Related Kinase 2 (VRK-2) is believed to control the voltage-dependent anion channel 1-mediated Mt DNA release into the extracellular space. With a virus present, VRK-2 interacts with voltage-dependent anion channel 1 and triggers its oligomerization. It seems to be stimulating Mt DNA binding to voltage-dependent anion channel 1. The deficit of VRK-2 can negatively affect Mt DNA binding to voltage-dependent anion channel 1 and its oligomerization, and its excessive expression significantly stimulates Mt DNA binding [37].

mtDNA release mediated by mitochondrial Permeability Transition Pore (mPTP)

MPTP is a part of the inner Mt membrane contents. The mPTP open lets metabolites and small molecules be transferred, and proton flux is critical in this case.

In case of an Mt stress, the pore opens and thus triggers a cascade of non-convertible processes which disrupt Mt functioning. Subsequently, ion flux causes excess of Ca^{2+}, pyridine nucleotide depletion, Mt depolarization, suppression of adenosine triphosphate production, which in turn leads to inhibited respiration, metabolic dysfunction, swollen matrix and cellular death [38]. Accordingly, one would expect to detect the participation of mPTP in diseases related to impaired Mt function. Swollen Mt matrix is an important part since it causes the Mt outer membrane to rupture, enhancing the leakage of pro-apoptotic proteins. Mt PTP not only facilitates the metabolite flux, but also creates a pathway for Mt DNA leakage. Garcia and colleagues reported that in case of OS, Mt DNA leaked *via* mPTP in hepatic cells of rats. In that trial Mt were triggered with iron, Ca^{2+} and hydrogen peroxide to augment OS. Subsequently, hydrolysis of Mt have been detected, as well as TBA-reactive substance generation, which are an OS biomarker [39,40].

Even though Mt DNA leakage through mPTP was widely investigated, its underlying processes are still not fully understood. Specifically, it is unclear if the Mt DNA release is active or passive. Available research results allow us to conclude that Mt DNA and other Mt components possibly leak from the cell due to cellular injury that causes the Mt PTP to open. Although, while Mt genome is usually found outside of cell in case of external cellular injury, the connection between the open in mPTP and the Mt DNA leakage is still not fully known [41].

Immune Recognition Pathways [42-50]

cGAS-STING pathway: The cyclic GMP-AMP Synthase (cGAS)-Stimulator of Interferon Genes (STING) pathway plays a significant role in health and disease. Within eukaryotic cells, DNA is typically located in the mitochondria or nucleus. When it is detected in the cytoplasm, it can be recognized as foreign, triggering inflammation, as shown in previous studies. Additionally, leakage of mitochondrial DNA into the cytoplasm has been found to promote inflammatory responses and is implicated in various pathological conditions, such as autoimmune diseases and cancer. Recent studies have highlighted that the Interferon (IFN1) response is a key signaling pathway activated during infections, with the STING molecule playing a central role in mediating this response. For instance, it was demonstrated that STING activation is also influenced by various infectious agents and stress signals,

which can exacerbate or modulate its activity.

While cytoplasmic DNA can activate STING, further investigations have established that the principal ligands for STING are Cyclic Dinucleotides (CDNs) such as c-di-AMP and c-di-GMP, rather than cytoplasmic DNA itself. Emerging evidence shows that certain bacterial infections can directly elevate CDN levels, providing a direct link between microbial infection and innate immune activation. Moreover, it has been shown that cGAS serves as an upstream sensor for STING; when ATP or Guanosine Triphosphate (GTP) are present, cGAS binds to cytoplasmic DNA, leading to the synthesis of cyclic GMP-AMP (cGAMP), which subsequently acts as a ligand to activate STING.

During mitochondrial DNA leakage, cGAS binds to mtDNA and catalyzes the production of cGAMP, serving as a ligand for STING and triggering a conformational change that promotes its activation. Notably, a 2023 study by Liu, et al. [51] provided insights into the structural dynamics of STING upon cGAMP binding, revealing that specific conformational changes are essential for STING oligomerization and subsequent activation. Once activated, STING translocates from the endoplasmic reticulum to the Golgi apparatus, a process facilitated by phosphorylation by TBK1. There, it recruits TBK1 and IκB kinase (IKK), further propagating the signaling cascade.

This cascade results in the phosphorylation of downstream targets, including Interferon Regulatory Factor 3 (IRF3) and IκB-alpha, by activated TBK1 and IKK. Importantly, findings from Zhang, et al. (2023) emphasized that STING can also activate other signaling pathways, influencing not only the nuclear translocation of IRF3 and Nuclear Factor-κB (NF-κB) but also bridging with metabolic pathways that elevate local ATP levels, enhancing the overall immune response. Consequently, these transcription factors induce the expression of type I interferons (IFN1) and pro-inflammatory cytokines, which are critical for an effective immune response and play pivotal roles in modulating the immune landscape in diseases such as cancer and chronic inflammation.

Toll like receptor-9

Toll-Like Receptors (TLRs) are Pattern Recognition Receptors (PRRs) that have been conserved throughout evolution, playing a crucial role in the innate immune system, particularly in recognizing foreign molecules in the Extracellular Matrix (ECM). Currently, ten TLRs have been identified in humans. These receptors are characterized as type I transmembrane proteins, with the N-terminal domain functioning as the ectodomain where Pathogen-Associated Molecular Patterns (PAMPs) and DAMPs bind to activate NF-κB. TLR9, recognized as the first TLR to detect DNA, is predominantly found in the Endoplasmic Reticulum (ER). Upon activation, it is transported to lysosomes, where it recognizes hypomethylated CpG sites in DNA. However, it does not exhibit specificity for pathogenic DNA, as self-DNA can also activate it, a phenomenon that has been previously described. The hypomethylation characteristic of mitochondrial DNA (mtDNA) enhances its resemblance to foreign DNA, allowing for its recognition by TLR9.

The mechanisms governing the transfer of mtDNA to lysosomes and its recognition by TLR9 remain incompletely understood; these processes may be linked to mitophagy or the transfer of mitochondrial-derived vesicles. Recent studies have suggested that mitochondrial stress, such as that induced by oxidative stress or inflammation, can promote the release of mtDNA fragments into the cytoplasm, which may subsequently be targeted to lysosomes *via* specialized vesicles, highlighting a potential role for mitochondrial dynamics in this process [52]. Moreover, significant findings from Lu, et al. (2023) have demonstrated that certain enzymes involved in mitophagy can also facilitate the degradation of mtDNA, thereby modulating TLR9 signaling and the consequent inflammatory response [53].

It has been shown that downstream signaling from TLR9 is mediated *via* MyD88, which activates mitogen-activated protein kinases (MAPKs) and NF-κB, stimulating inflammation or provoking an IFN1 response *via* interferon regulatory factor 7. New research has illustrated that TLR9 activation not only leads to canonical NF-κB signaling but also induces a unique transcriptional program involving the activation of several type I Interferon-Stimulated Genes (ISGs), which play a critical role in modulating the immune response and enhancing antiviral defenses [54]. Furthermore, the interplay between TLR9 signaling and the Mitochondrial Antiviral Signaling (MAVS) pathway has been underlined, suggesting that mtDNA sensing *via* TLR9 can synergistically enhance the overall antiviral response [55].

These advances emphasize the importance of TLR9 not

just as a sensor of microbial DNA but also as a crucial player in recognizing host-derived mtDNA during stress conditions, thereby linking innate immune activation with cellular stress responses [56]. Understanding these pathways further elucidates how TLR9-mediated responses can influence various pathological conditions, including autoimmune diseases and cancer, where self-DNA sensing may contribute to chronic inflammation and disease progression.

Inflammasomes

Inflammasomal activation is a crucial component of the immune system's response to DAMPs and Pathogen-Associated Molecular Patterns (PAMPs). Inflammasomes are multiprotein complexes comprising an Interleukin-1 Converting Enzyme (ICE), adaptor proteins, and receptor proteins. The recognition of foreign molecules and cellular stress is mediated by receptor proteins, which bind to adaptor proteins, initiating the recruitment of pro-caspase-1 for transformation into active ICE. The downstream activities primarily promote inflammation through the cleavage of Gasdermin D (GSDMD) at its N-terminal domain, activating it to bind to membranous phospholipids and form pores, along with cleaving the precursors of interleukin-1 beta and interleukin-18.

Various NLRs and PYHINs can serve as inflammasomal receptor proteins, including several specific types. Recent studies have identified new members of the NLR family, such as NLRP3 and NLRP6, as critical regulators of inflammasome activation in response to mitochondrial DNA (mtDNA). For instance, a study in 2023 highlighted that NLRP3 specifically interacts with mtDNA and initiates a potent inflammatory response in macrophages, indicating that mitochondrial stress can serve as a key trigger for NLRP3 inflammasome activation [57].

Available data suggest that mtDNA may act as endogenous inflammasome agonists. It has been shown that certain receptor proteins can recognize mtDNA and induce inflammasomal activation *via* dual mechanisms: direct recognition through cytosolic receptors and indirect signaling through mitochondrial stress responses. A growing body of evidence has elucidated the structure of a specific protein that binds to mtDNA; this protein contains a HIN-200 domain at its C-terminus capable of recognizing and binding to double-stranded DNA. Additionally, it has been revealed that other sensor proteins, such as cyclic GMP-AMP synthase (cGAS), can also bind to mtDNA, leading to the production of cyclic GMP-AMP (cGAMP) and the subsequent activation of the Stimulator of Interferon Genes (STING) pathway, thus enhancing the inflammatory response [58].

Activation of one particular inflammasome type, however, necessitates multiple signals, such as calcium signaling, reactive oxygen species, lysosomal rupture, and potassium efflux, as indicated by research findings. Recent studies have explored the interplay between these signals, demonstrating that alterations in calcium homeostasis, often triggered by mitochondrial dysfunction, can specifically sensitize cells to inflammasome activation, revealing a complex regulatory network that modulates the response to stress and damage [59].

While the combination of certain inflammasome components appears to associate with mtDNA or oxidized mtDNA (ox-mtDNA), new findings suggest that modified mtDNA is more likely to evoke inflammasome activation, as oxidized forms are recognized as more potent stimuli [60]. However, the nature of this binding whether direct or indirect remains to be clarified. Further investigations are needed to fully understand the molecular interactions and regulatory mechanisms underlying mtDNA-induced inflammasome activation, which may have significant implications for understanding inflammatory diseases and therapeutic interventions targeting these pathways.

Mechanisms of release of mtDNA outside the cells

Mitochondrial DNA was found in plasma, synovia, CSF, serum and other fluids. There is now growing attention to Mt DNA and cell-free Mt-DNA, as they may turn out to be inflammatory markers and mortality predictors. *In vivo*, blood is typically used for Mt DNA concentrations evaluation, since it is easily obtained and tested. Although, the Mt DNA precise source is usually unclear. Available data demonstrate that >90% of cell-free Mt DNA is found in whole Mt in bloodstream, their amount is approximately 105 /mL -106 /mL. Such Mt are typically found in healthy individuals, proving that the majority of circulating Mt DNA does not induce inflammation, and that inflammatory response can only be induced by free Mt DNA [61,62].

Mt DNA may be found outside of cells because of passive leakage out of dead cells or as a result of an active controlled process, e.g., the process of being released by

leukocytes such as neutrophils. While both processes are physiologically significant, the contribution of each is yet to determined [63].

Active release of mtDNA: Extracellular Traps (ETs)

Neutrophils are an important well-characterized source of cell-free Mt DNA. Upon exposure to bacterial pathogen-associated molecular patterns, neutrophils express NETs-neutrophils extracellular traps. NETs are net-like structures made of proteins and chromatin. NETs are responsible for killing bacteria. Deoxyribonucleic acid found in neutrophils extracellular traps is mostly nuclear, however, Mt DNA is found there as well. Mt DNA presence is not random and it has possible inflammatory effects. Neutrophils extracellular traps formation is associated with development of numerous inflammation disorders, such as DM, nonalcoholic steatohepatitis, and trauma [64].

Formation of Neutrophils Extracellular Traps (NETosis) can be of 2 types-vital and suicidal. Whereas the term "NETosis" is recommended to use only when related to cellular death, "vital NETosis" is nonetheless a widely used term which is also used in this study. Suicidal neutrophils extracellular traps formation is featured by destruction of nuclear membrane and plasmid, which takes from five to eight hours. When neutrophils are activates, there is a release of Ca^{2+} from endoplasmic reticulum into cytosol, which leads to production of NADPH-dependent reactive oxygen species [65,66]. Reactive oxygen species cause the destruction of nuclei and granules, letting their components to mix. Myeloperoxidase and Norepinephrine are enzymes found in Peptidyl Arginine Deiminase 4 and azurophilic granules. They induce chromatin de-condensation, and subsequently the membrane disruption lets the neutrophils extracellular traps be released to the extracellular space. And on the contrary, vital neutrophils extracellular traps formation is quick (from five to sixty minutes) and does not imply cellular death. In this process, neutrophils keep structure of nuclear membrane and plasma intact, while reactive oxygen species promote formation of neutrophils extracellular traps. In this form, extracellular traps include only Mt DNA, and in 2009 that form was detected for the first time. Suppressed release of Mt DNA was found after diphenyleneiodonium therapy which suppresses reactive oxygen species [67]. Furthermore, it was reported that neutrophils with reactive oxygen species deficit do not demonstrate Mt DNA release. These discoveries showed that neutrophils extracellular traps formation and subsequent Mt DNA leakage relies on reactive oxygen species being present, rather than on cellular death. McIlroy and colleagues observed formation of neutrophils extracellular traps comprising Mt DNA in trauma subjects [68]. Consistent with the findings of Yousefi and colleagues, the diphenyleneiodonium therapy suppressed neutrophils extracellular traps formation and stopped Mt DNA leakage [69]. This impact of diphenyleneiodonium therapy was affirmed in another trial, in which an elevated Mt DNA leakage was found in subjects with trauma, which provoked neutrophils extracellular traps formation. This mechanism relied on the activation of toll-like receptor 9. It is noteworthy that levels of neutrophils extracellular traps in young individuals turned out to be higher than in older ones. Neutrophils extracellular traps formation seems to be closely connected to the Mt DNA-induced intracellular signaling pathways activation [70]. E.g., in individuals with sickle cell disease blood concentration of cell-free Mt DNA was elevated. This elevation possibly provokes neutrophils extracellular traps formation and cGAS/TMEM173 signaling pathway. The researchers indicated that Mt were retained by sickle cell disease erythrocytes and supposed that it might be the reason for the increased concentration of cell-free Mt DNA. The way of releasing of Mt DNA out of the cell could be explained by 2 mechanisms. The first one implies that Mt DNA is leaking into cytoplasm and then is trapped in vesicles which start fusion with membrane, causing Mt DNA expulsion. The second one suggests that Mt fuse with the membrane which causes the leakage of Mt components out of the cell [71].

Notably, in neutrophils mitophagy is not responsible for clearing injured Mt, there is another pathway for that. Normally, an extrusion of Mt contents out of the cell takes place. On the contrary, oxidized mitochondrial DNA is cleared *via* another process, modulated by Mt DNA/Mt transcription factor A disassembly, subsequent vesicle generation where oxidized Mt DNA is trapped, and finally their transport to lysosomes to be degraded. This process has major consequences. In systemic lupus erythematosus subjects, neutrophils demonstrated a disruption of this process which causes insufficient clearance of oxidized Mt DNA and results in oxidized Mt DNA aggregation in Mt after which it leaks while binding to Mt transcription factor A. While oxidized Mt DNA is able to induce plasmacytoid dendritic cells activation, this results in significant amount

of IFN1 being produced [72].

DNA-based ETs may be generated by eosinophils to catch and kill foreign microorganisms. The first trial that revealed the eosinophilic ability to generate ETs displayed that cells stay healthy after formation of an ET, and Mt DNA is then released. The released DNA was identified as Mt DNA *via* analyses with application of microscopy and molecular biology. Those structures are called eosinophilic ETs. An ET identifies a foreign microorganism, limits its mobility and is supposed to kill it. Extracellular deoxyribonucleic acid enzymatic degradation leads to eosinophilic inability to achieve the pathogen death [73]. The eosinophilic extracellular traps possibly have Mt origin, but this theory is yet to be validated. Lately trials have demonstrated that DNA contained in eosinophilic extracellular traps was nuclear. Hereby, whereas EET DNA is probably not dangerous for foreign microorganisms, it is essential for eosinophil-mediated antibacterial effect. The EET mechanism also relies on the reactive oxygen species generation, e.g., ETs of mast cells and neutrophils. A release of Mt DNA has also been detected in large granular lymphocytes, B lymphocytes and T lymphocytes as a reaction to ODNs.

The purpose of Mt DNA extracellular traps is still unexplored. Lymphocytes and eosinophils comprise Mt and a little number of Mt DNA. This gives rise to doubt that extracellular traps composed only of Mt DNA may maintain normal function. Moreover, in context of lymphocytes, cell-free Mt DNA is not connected to lytic enzymes, which implies that it only has an inflammatory purpose [74].

Active release of mtDNA mediated by Extracellular Vesicles (EVs)

Another active cell-free Mt DNA source is EV. This is a complex of particles which is secreted into organism fluids and is necessary for intercellular communication. Whereas division of extracellular vesicles into groups is still debated, there are 3 agreed categories: MV (microvesicles), AB (apoptotic bodies), and exosomes. MV are extracellular vesicles secreted by plasma membranes, their size being under 1 μm. AB are extracellular vesicles that constitute the end product of cellular death, their size being one to five μm. Exosomes extracellular vesicles which are produced inside cells. Exosomes have the size of thirty to hundred and fifty nm, with lipid double-layer structures secreted by cells to intercellularly transport molecules [75,76]. Exosomes comprise nucleoacids and proteins obtained from inside the cells. At first, it was believed that extracellular vesicles release was just a process of cell waste removal. Although, it has been revealed that extracellular vesicles are essential for cell-to-cell communications, as they function as transfer cargo of various messengers, such as proteins, deoxyribonucleic acid, ribonucleic acid. It was reported that extracellular vesicles release is a highly controlled process. In clinical practice, EVs are now the subject of increasing interest as potential novel therapeutic approach. Many host traits were reported to participate in the extracellular vesicles production, such as Rab guanosine triphosphatase family members-Rab-27 and Rab-35 [77].

Mt DNA and other Mt contents are typical cargo of extracellular vesicles. Mt DNA packing in vesicles is another pathway which was discovered some time ago by Guescini and colleagues [78]. They reported that *in vitro* Mt DNA migration is carried out with assistance of myoblasts, astroglia and glioblastomic cells. Sansone, et al. later conducted a study in a breast cancer model and validated the Mt genome release through extracellular vesicles in stromal cells and following transport to malignant cells. The process of Mt DNA packaging into extracellular vesicles has not been fully elucidated. Although, the researchers assumed that the genome transport constitutes a cancer cell protective mechanism which is supposed to help maintain the metabolic functioning and avoid metabolic quiescence caused by treatment. *In vitro*, the mitochondrial genome packing into EVs has been shown in cellular culture of a placenta [79]. Notably, extracellular vesicles concentrations of Mt DNA were elevated in presence of APLAs that were shown to elevate pre-eclampsia risk. While this mechanism was not fully understood, the researchers hypothesized that the APLAs therapy leads to Mt ruptures and the following release of Mt genomic material that may be packaged into extracellular vesicles. Moreover, it was discovered that placental extracellular vesicles enhance ECs activation *via* toll-like receptor 9-mediated signaling pathway. Elevated release of Mt genome has also been detected in alcohol-related neutrophilia [80]. This elevation provoked neutrophils activation, thus facilitating the development of alcoholic hepatic damage. This was confirmed in animal models, where administration of mitochondrial DNA-rich particles provoked the development of neutrophilia. The researchers explained this discovery by toll-like receptor 9

activation, since the above-mentioned impact has not been detected in animal model with toll-like receptor 9 deficit. Ye and colleagues demonstrated elevated concentration of mitochondrial genome-carrying exosomes in plasma of CHF subjects [81]. Exosomes have been absorbed by cells, stimulating inflammation, that enhanced the production of pro-inflammatory interleukin-1 beta and interleukin 8, and promoting activation of toll-like receptor 9-nuclear factor kappa B pathway. The degree of the immune response has been found relying on the mitochondrial DNA copies, and the inflammation was shown to be suppressed after toll-like receptor 9 suppressor therapy [82,83].

Later researches showed that the release of Mt cargo in extracellular vesicles may be carried out by various types of cells as a reaction to pro-inflammation stimulus. Interestingly, Mt proteins are also found in extracellular vesicles where no inflammation stimuli are present. The purpose of the Mt cargo is not fully understood, although, researchers hypothesized that Mt genome packing in extracellular vesicles may constitute a mechanism of Mt transport which may be useful for Mt maintaining their function, or to rescue impaired Mt functioning in numerous pathological conditions [84,85].

Recent research determined factors that control the traffic of Mt contents, such as Mt genome, into extracellular vesicles. Mt are able to generate vesicles that are called Mt-derived vesicles, for transfer of Mt contents to organelles. Todkar, et al. demonstrated that Mt-derived vesicles are not only essential for MitAP, but also necessary for transferring of Mt contents to extracellular vesicles, in a mechanism which relies on proteins OPA-1 and SNX-9. While injured Mt is present, that could possibly comprise proinflammatory molecules, Parkin suppresses the pathway and starts targeting Mt-derived vesicles to be degraded by lysosomes, hereby avoiding the release of damage-associated molecular patterns [86].

Passive release of mtDNA

Passive leakage of mitochondrial DNA is mostly detected in case of cellular death, necrotic or apoptotic. Whereas the term “passive” is typically used to refer to the Mt DNA release that takes place after cellular death, it is important to mention that this mechanism is still controlled. A number of processes can mediate the Mt presence outside cells or their leakage during cellular necrosis or apoptosis [87].

In the process of apoptosis, cellular fragmentation in apoptotic bodies facilitates removal of apoptotic cells and helps avoid undesirable inflammatory response against self-antigens. As was previously mentioned, ABs are large extracellular vesicles and they comprise deoxyribonucleic acid, ribonucleic acid, and proteins, such as Mt proteins and Mt genomic material.

Despite earlier findings, it was established that apoptotic bodies formation is a highly controlled mechanism mediated by numerous factors, such as *ROCK-1* and *PANX-1* [88]. Contents of apoptotic bodies was found to be utilized for intercellular communications. One of apoptotic bodies features if heterogeneity, and a part of them comprise uninjured but non-functional Mt. Whereas apoptotic bodies have similar surface biomarkers with their original cells, those biomarkers may possibly be used to identify apoptotic bodies origin, and subsequently, origin of Mt and Mt genome detected inside them [89,90].

Nonetheless, during apoptosis, a cell may release undamaged Mt in an active manner, questioning the theory that they can be released exclusively through apoptotic bodies. Notably, actively released Mt by an apoptotic cell was found to be a pro-inflammatory feature. Uninjured Mt may be recruiting neutrophils and, upon absorption by macrophages, may trigger inflammasome activation through cryopyrin. In the same manner, Mt may be released in the process of necroptosis-controlled necrosis process-although not induce inflammation.

In the process of necrosis, passive leakage was mostly researched in pathophysiological conditions resulting in acute injury, such as trauma, *IRI*, sepsis, or chronic disorders, e.g. viruses, *RI*, tumors, degenerative nerve diseases [91,92]. In the majority of these disorders, the levels of circulating cell-free Mt DNA were found to directly correlate with the degree of necrosis or injury. This might lead to a conclusion that Mt DNA is leaking in a free manner into the extracellular space through a rupture in cellular membrane caused by a trauma. Panel studies in which levels of cell-free Mt DNA were observed after damage validated that version.

Moreover, this finding that Mt genome evaluated outside of a cell is connected to Mt damage-associated molecular patterns, supports the theory that in these clinical conditions a release of Mt DNA and other Mt contents is passive and

unregulated, and not selective and controlled. Passive release was also detected in cases when there was no macroscopic damage. Intense exercise enhances cell-free Mt DNA release, which concentrations in the extracellular space are elevated right after the exercise. There is also a possibility that cell-free Mt DNA was released in a passive manner because of the cell necrotic process, although that theory is yet to confirm [93,94].

Limitations

While the research surrounding mitochondrial DAMPs in disease has made significant strides, several limitations warrant consideration. One of the foremost challenges arises from the variability inherent in experimental models. Much of the work has utilized animal models or *in vitro* systems, which may not accurately reflect human physiology. This discrepancy can complicate the translation of findings into clinical relevance, as differences in mitochondrial biology between species can lead to divergent outcomes.

Furthermore, the immune system's response to mitochondrial DAMPs is highly complex and context-dependent. This variability complicates the interpretation of results, as different cell types may respond differently based on the disease state, microenvironment, and other influencing factors. The lack of standardization in the methods for measuring and quantifying mitochondrial DAMPs, such as mtDNA, further exacerbates this issue. Variability in techniques and protocols among studies can result in inconsistencies and hinder comparative analyses.

Additionally, while there is a strong association between mitochondrial DAMPs and various diseases, the precise causal relationships remain to be clearly delineated. It is critical to avoid over-interpreting correlational data without robust mechanistic insights to support these findings. The therapeutic potential of targeting mitochondrial DAMPs presents exciting prospects; however, substantial challenges exist. These include the risk of off-target effects and the necessity for precision in delivery systems to maximize efficacy while minimizing toxicity.

Lastly, many of the existing studies are cross-sectional in nature, offering merely a snapshot of the role of mitochondrial DAMPs at specific points in time. Longitudinal studies will be essential to elucidate the dynamic changes in mitochondrial signaling throughout disease progression and in response to treatment. Addressing these limitations will be crucial for advancing our understanding of mitochondrial DAMPs and developing effective therapeutic strategies (Table 3).

Table 3: Role of immune cells in mtDNA release.

Immune cell type	Mechanism of mtDNA release	Description of role	Implications for inflammation
Neutrophils	NETosis	Release of mtDNA in Neutrophil Extracellular Traps (NETs)	Enhances inflammation and pathogen clearance
	Active release	Can release mtDNA *via* Reactive Oxygen Species (ROS) production	Contributes to inflammatory responses in various diseases
	Passive leakage	mtDNA leakage during cell death or necrosis	Indicative of tissue damage and inflammatory status
Lymphocytes	Apoptosis	Release of mtDNA during apoptotic cell death	May modulate immune responses
	Activation	Active release of mtDNA in response to stimuli (e.g., TLR activation)	Triggers inflammatory pathways
Eosinophils	Extracellular Traps (ETs)	Formation of eosinophil extracellular traps potentially containing mtDNA	Targets and limits the spread of pathogens
Macrophages	Phagocytosis of damaged cells	Uptake of mtDNA from necrotic or apoptotic cells	Can activate inflammasomes and contribute to inflammatory signaling
	Cytokine release	Secretion of inflammatory mediators following mtDNA sensing	Sustains the inflammatory response
Dendritic cells	mtDNA sensing	Recognize mtDNA *via* intracellular receptors (e.g., TLR9)	Initiates adaptive immune responses and cytokine release

Implications of mitochondrial DAMPS in disease and future perspectives

Mitochondrial dysfunction and the subsequent release of mitochondrial DNA (mtDNA) have been linked to a variety of inflammatory and autoimmune diseases, highlighting the importance of understanding these processes in therapeutic development. The unregulated release of mtDNA into the extracellular space acts as a potent DAMP that can activate

various immune responses, contributing to pathologies such as Systemic Lupus Erythematosus (SLE), Rheumatoid Arthritis (RA), and Inflammatory Bowel Disease (IBD). The recognition of mtDNA by PRRs such as TLR9 and the cyclic GMP-AMP synthase (cGAS) pathway initiates inflammatory signaling cascades, resulting in increased production of pro-inflammatory cytokines and type I interferons [95-112].

In the context of autoimmune disorders, the presence of mtDNA in serum correlates with disease activity and severity, suggesting that mtDNA can serve as a biomarker for these conditions. Additionally, the potential for therapeutic interventions targeting mitochondrial dysfunction presents a novel avenue for treatment. Strategies aimed at enhancing mitophagy, preventing oxidative stress, or modulating the immune response to mtDNA could mitigate the inflammatory processes driven by mitochondrial DAMPs.

Looking ahead, research into the specific mechanisms regulating mtDNA release and its impact on immune modulation is essential for developing targeted therapies. Exploring the interactions between mtDNA and various PRRs may unveil new pathways that can be manipulated for therapeutic benefit. Furthermore, advancements in drug delivery systems that target mitochondria could enhance the effectiveness of treatments aimed at correcting mitochondrial dysfunction.

The role of mtDNA as a potential biomarker in clinical settings warrants further investigation, as it may enable the stratification of patients based on disease severity or the likelihood of therapeutic response. As our understanding of mitochondrial DAMPs evolves, we may discover innovative interventions that harness their signaling properties, offering hope for more effective management of inflammatory and autoimmune diseases.

Conclusion

Mitochondria are integral to cellular function and inflammation regulation through the release of DAMPs such as mtDNA. This review has highlighted the critical role of mitochondrial DAMPs in modulating immune responses and their significant implications for various inflammatory diseases. Understanding the nuanced roles of mitochondria not only enriches our knowledge of cellular signaling but also reveals potential therapeutic targets for inflammatory conditions. Continued research into mitochondrial biology and the pathways governing DAMP release offers exciting opportunities for innovative therapeutic strategies. By elucidating the mechanisms that drive mitochondrial dysfunction and the subsequent release of pro-inflammatory signals, we can advance our efforts in managing conditions associated with mitochondrial dysregulation and improve patient outcomes. As we deepen our insight into the interplay between mitochondria and the immune system, we pave the way for novel interventions that can harness or mitigate the effects of mitochondrial DAMPs in disease.

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