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Mito-Omics and immune function: Applying novel mitochondrial omic techniques to the context of the aging immune system

Ana R. Silverstein , Melanie K. Flores , Brendan Miller , Su-Jeong Kim , Kelvin Yen , Hemal H. Mehta , Pinchas Cohen *

Leonard Davis School of Gerontology, University of Southern California, Los Angeles, CA, USA

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ABSTRACT

Recent advancements in genomic, transcriptomic, proteomic, and metabolomic techniques have prompted fresh inquiry in the field of aging. Here, we outline the application of these techniques in the context of the mitochondrial genome and suggest their potential for use in exploring the biological mechanisms of the aging immune system.

1. Introduction

The Whole-Omics approach of GWAS, transcriptomics, proteomics, and metabolomics have been utilized to unravel the biology of aging. Whole-Omics tools have mostly been applied to nuclear genetics, but the same tools can be applied to mitochondrial genetics, which we have termed "Mito-Omics." Mito-Omics is an invaluable tool for newly proposed age-associated diseases, such as immune dysfunction. Unlike general omics techniques, Mito-Omics considers variances in the number of copies of mtDNA per cell (i.e., heteroplasmy), mitochondrial genetic code, and haplogroup architecture of the mitochondrial genome. However, with its emergence, Mito-Omics has unique limitations that must be addressed. In this review, we will introduce the key components of Whole-Omic analysis, discuss how to address emerging challenges when transitioning to Mito-Omics, emphasize how Mito-Omics can be applied to experimental paradigms involving age-associated diseases, and propose the future application of Mito-Omics in studying the aging immune system.

2. Advancements in Whole-Omics analysis

2.1. From GWAS to MiWAS

Next generation sequencing was a paradigm shifter for not only the aging field but life sciences in general. With the ability to sequence individual human genomes, population geneticists have been able to identify novel genomic variants that associate with certain diseases and conditions. One such analytical method is Genome-Wide Association Study (GWAS), an experimental protocol designed to identify associations between genetic variants and traits of interest in a given population. Since its development, GWAS has been used to identify novel single nucleotide polymorphisms (SNPs) that map back to genes involved in the pathology of many diseases of interest [1–3]. Most GWAS pipelines have used SNP-based-arrays to generate millions of genotypes, but high-throughput next generation whole genome sequencing can now be used to identify extremely rare SNPs in regions of the genome that have historically been missed (e.g., introns, small open reading frame microproteins, etc.) [4]. GWAS has indeed identified genome variants that associate with disease, but GWAS is mostly focused on nuclear genes, overlooking an opportunity for biological analysis that lies within the mitochondrial genome.

The maternally inherited mitochondrial genome (mtDNA) consists of a subset of genes that, although small in number, are mighty

* Corresponding author. 3715 McClintock Ave, Los Angeles, CA, 90089, USA.
E-mail address: hassy@usc.edu (P. Cohen).

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in their contributions to proper cell function. Collectively, the compact mtDNA encodes 13 proteins, 22 tRNAs, 2 rRNAs, and a growing list of microproteins [5]. Together, these genes actively regulate cellular respiration and energy metabolism [6,7]. Due to both its high susceptibility to oxidative damage and its absence of effective DNA repair mechanisms, mtDNA is prone to much higher rates of somatic mutation than the nuclear genome [6]. These mutations often lead to mitochondrial dysfunction, making them an important genetic contributor to many diseases of aging [8,9]. However, the extent to which inherited mtDNA SNPs (mtSNPs) contribute to disease risk remains unclear.

By adapting the GWAS experimental design to target mtDNA it is possible to identify novel mtSNPs that associate with diseases, especially diseases with a metabolic pathology (e.g., Alzheimer's disease, diabetes, etc.); we have named this experimental approach Mitochondrial-wide Association Study (MiWAS) [9,10]. Since most SNP-based-arrays only capture roughly a hundred mtSNPs, implementing whole mtDNA sequencing may reveal a set of mtSNPs that have previously remained unidentified, expanding our considerations for biological contributors to disease [10]. Nevertheless, challenges specific to mitochondrial genetics leave uncertainty in the findings of MiWAS.

One MiWAS challenge is mitochondrial heteroplasmy. Hundreds of copies of mtDNA are present in each cell, with variances in the number and types of mtDNA mutations present within each copy of the mitochondrial genome (i.e., heteroplasmy). Due to heteroplasmy, it can be difficult to assess the overall impact of a mtSNP on cell function, as the identified mutation may only be present in some but not all mitochondrial genomes, and thus may only be affecting some but not all mitochondria within a cell [11,12]. MiWAS analysis does not account for mitochondrial heteroplasmy, although deep sequencing techniques have recently been designed to detect low occurring heteroplasmy frequencies, detecting heteroplasmic variations in mtDNA at frequencies as low as 0.2% [13].

Another limitation of MiWAS is controlling for genetic ancestry. While the standard for nuclear GWAS is to control for genetic ancestry via nuclear DNA principal component analysis, groups that have conducted MiWAS have controlled specifically for genetic ancestry using nuclear principal component analysis (PCA), mtDNA PCA, or direct haplogroup comparisons. There is no standard to control for the mitochondrial genetic background during MiWAS. Nonetheless, we recently used MiWAS to identify a SNP in the D-Loop of mtDNA that predisposes Hispanics to cataracts [14]. We found that carriers of the alternative allele MitoG228A were five times more likely to develop cataracts than non-carriers, with over 80% of identified carriers developing cataracts in their lifetime. We included both mtDNA and nucDNA PCA in our MiWAS and showed that the effect of MitoG228A was significant during both mtDNA and nucDNA correction. It has been reported that mtSNPs associate with many age-related diseases including Alzheimer's disease (AD) [15–17], Parkinson's disease (PD) [18,19], diabetes [20] and various cancers [21–23].

Additionally, due to the haplogroup architecture of mtDNA, there are many mtSNPs that are almost always found together with other mtSNPs [24]. It is, therefore, difficult to determine which mtSNP in a haplogroup is driving the associative effect using just MiWAS alone. Therefore, while GWAS and MiWAS are important diagnostic techniques for identifying gene variants of interest, they provide just one piece of the puzzle. To better identify the impact of a polymorphism on disease pathology, other omic analysis techniques should also be used. (Fig. 1).

2.2. Validating with mitochondrial transcriptomics

Mitochondrial transcriptomics can complement MiWAS. In many studies, it is suggested that mtDNA variants may influence gene-gene interactions [22], and change gene expression patterns [16,21]. Transcriptomic analysis is used to identify expression of genes involved in disease pathology and measure novel microprotein transcript expression. More specifically, analyzing the transcriptome reveals important information about the functional elements of identified genes of interest [25], allows for the annotation of previously unidentified genes [26], and also enables the quantification of variations in transcript expression under different conditions [27].

RNA-sequencing has substantially improved successful analysis of both the nuclear and mitochondrial transcriptome. Mitochondrial transcriptomics has become increasingly important for understanding the role of mitochondrial function in disease pathology. However, when utilizing mito-transcriptomics techniques, the structure and replication behavior of mtDNA must be taken into account. One important evaluation method for mtDNA that addresses its unique structure involves identifying the strand-specific transcription patterns of genes of interest [28]. Within its structural arrangement, the mtDNA has genes encoded on both the heavy and light-strand of its genome. Collectively, mtDNA contains 28 genes on the heavy-strand, and 9 on the light-strand. Therefore, when evaluating mtDNA transcription patterns, distinguishing between strands may play an important role in identifying its unknown genetic contributors to disease [29]. To evaluate this pattern, sequence markers or adapters can be used distinguish one strand from another when compiling transcriptomic information [28,30]. Applying these methods to the mitochondrial genome helps confirm the location of genes of interest, further enhancing our understanding of mtSNP expression patterns. Such methods might be used to identify the transcriptome of small open reading frames (smORFs) that encode for mitochondrial-derived peptides, which are bioactive peptides involved in cellular metabolism. The use of high-throughput sequencing methods has also improved our understanding of mitochondrial heteroplasmy when analyzing the mitochondrial genome. Overall, RNA-sequencing has substantially improved our ability to address the unique characteristics of the mitochondrial genome, prompting a more accurate and substantial understanding of the interactions between mtDNA and disease pathology.

Additionally, while the field of mitochondrial epigenetics is still emerging, there is evidence that mtDNA undergoes methylation [31], and that this can affect the expression of mitochondrial-derived peptides [32].Through transcriptomic analysis, the effects of certain mtSNPs and functional modifications on transcription patterns can be assessed, and the functional pathways and molecular interactions related to mtSNPs of interest can be identified [33].

Transcriptomic techniques were recently used to understand the role of methylation of the D-Loop of mtDNA in AD pathology [34]. Methylation is an important regulator and of gene expression, via expression inhibition. This study analyzed mtDNA from APP/PS1 transgenic mice, a commonly used mouse model with mutations associated with early-onset AD. It was determined that APP/PS1 mice had significant decreases in levels of methylation in the D-loop region and increases in levels of methylation in the 12 S rRNA region of mtDNA. Thus, decreased methylation of the D-loop may play a pivotal role in early-onset AD pathogenesis. This study is one of few that explore this potential correlation, further expanding on an important potential contributor to AD development.

Utilizing emerging techniques in transcriptomic analysis to understand the role of mtDNA transcript levels in disease pathogenesis is of increasing importance for many age-related diseases. However, to holistically assess changes in gene expression, both transcript levels and protein levels must be measured; to do so,

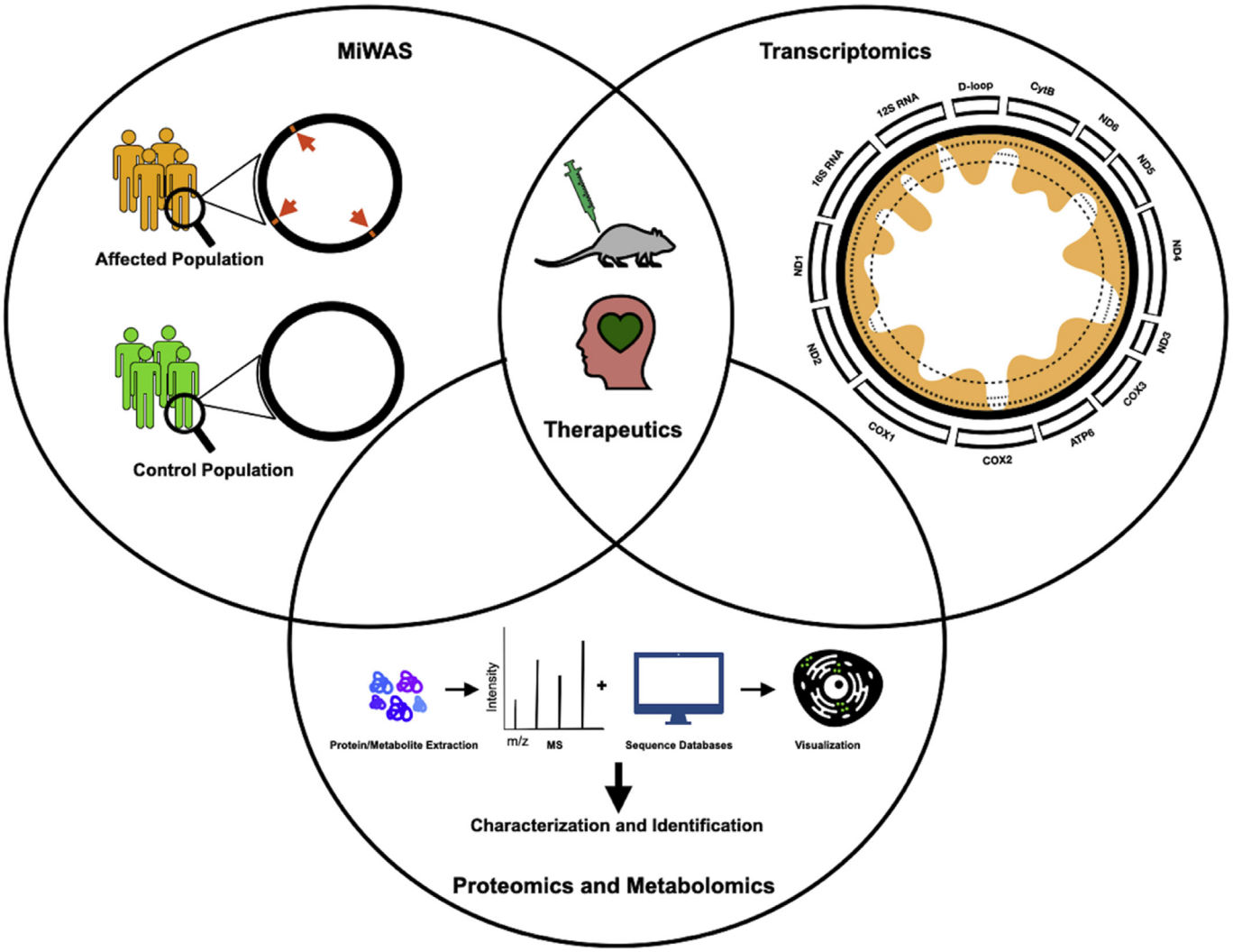

Fig. 1. The Mito-Omics Interface. The interface between mito-genomics (such as MiWAS), mito-transcriptomics, mito-proteomics, and mito-metabolomics proves most effective in developing targeted therapeutic and prevention methods for many diseases of aging.

proteomic analysis is required.

2.3. Expanding with mitochondrial proteomics

Expanding on the findings from the transcriptome, proteomic analysis uses both computational and experimental techniques to identify proteins of interest, distinguishing between coding and non-coding regions of the genome [35]. Just as transcriptomics confirms differential transcript expression associated with disease, proteomics can be used to confirm differential protein expression associated with disease. Thus, proteomic techniques, such as mass spectrometry (MS) and immunofluorescent imaging, can further expand on the biological significance of these proteins of interest by analyzing differences in protein activity, protein-protein interactions, and overall protein function under different conditions. Identifying and analyzing the biological function of these proteins of interest from genome variants can prove helpful in developing biomarkers for disease diagnosis and detection, as well as in developing and testing novel therapeutics for disease treatment and prevention [36]. The use of MS and protein visualization techniques such as immunofluorescent staining and imaging has expanded our ability to identify and characterize proteins, visualize protein-protein interactions, and assess overall protein function. For use in proteomics, MS techniques combine highly sensitive protein fragment labelling, scanning, and signal detection, with sequence information made available in sequence databases to precisely characterize proteins of interest [37,38]. The introduction of MS has been an invaluable tool for proteomic analysis of nuclear encoded proteins.

These proteomics techniques have also been used to efficiently identify novel mitochondrial-encoded proteins associated with the mitochondrial proteome. However, mito-proteomics presents many challenges unique to the mitochondrion. One important challenge is distinguishing nuclear-encoded and mitochondrial-encoded microproteins (i.e., mitochondrial-derived peptides) that have high sequence homology. Another challenge is enrichment protein samples for small molecular weight peptides such as microproteins. The Saghatelian lab has pioneered the proteomic approach for microprotein discovery [39]. They have reported hundreds of novel microproteins derived from nuclear DNA. Additional groups have also reported novel microproteins that even localize to mitochondria [40–43].

Additionally, while applications of MS and computational techniques to the mitochondrial genome has drastically improved

detection of proteins within the mitochondrial proteome, challenges still remain [44–47]. First, the mitochondrion's complex structure of four sub-compartments (i.e., outer membrane, intermembrane space, inner membrane, and matrix) requires enrichment procedures such as fractionation, solubilization, and detergent treatments, all of which entail an enormous amount of optimization that might vary from lab to lab [48]. Second, since roughly 10% of the human proteome remains uncharacterized, proteins identified in certain mitochondrial compartments are likely to have unknown functions, making interpretation difficult, especially in a clinical context [49]. Third, in addition to mitochondrial sub compartment enrichment, special enrichment of mitochondrial microproteins and larger proteins with complex biophysics (e.g., hydrophobicity) is also needed [50]. Recently, refined proteomic sample preparations have been used to enrich microproteins, which have led to the identification of thousands of novel microproteins that have been missed for decades.

Recently, MS was used to identify a set of mitochondrially associated proteins involved in the development of Alzheimer's Disease (AD) [51]. Mitochondria-associated endoplasmic reticulum membranes (MAM) were isolated from brain tissue samples of APP/PS1 and age-matched control mice, and proteins were identified and characterized using liquid chromatography tandem mass spectrometry (LC-MS/MS). Using MS, 128 proteins with mitochondrial, endoplasmic reticulum, or ribosomal localization patterns were shown to significantly change in abundance. The results suggest that significant alteration of the MAM proteome may be involved in early amyloid-beta accumulation associated with AD, emphasizing the importance of studying MAM proteins when researching early stage AD.

Information extracted from MS can then be combined with results from protein visualization techniques to better characterize protein activity and function in the cell. Immunofluorescent imaging and co-immunoprecipitation are two important tools that can be used for protein visualization. By tagging proteins of interest with fluorescent labels, protein binding partners and protein-protein interactions can be observed, either by fluorescent microscopy or by measuring immunoprecipitation results. In one example, immunofluorescent imaging and immunoprecipitation were used to characterize the microprotein MIEF1 [39]. The use of immunofluorescent imaging confirmed MIEF1 localization to the mitochondrial matrix. Results from immunoprecipitation and western blot experiments confirmed that MIEF1 interacted with other mitochondrially associated proteins such as the LYR proteins and its subunits, which are involved in core mitochondrial complexes. From these results, MIEF1 has been characterized as a mitochondrial microprotein that interacts with the mitochondrial ribosome and plays an important regulatory role in mitochondrial genome translation. When MS and other protein visualization and computational techniques are combined, whole-proteomic analysis can be made possible.

Collectively, through whole-proteomic analysis techniques, the biological function and activity of mitochondrial-specific proteins can be identified. When combined with MiWAS and mito-transcriptomics, mito-proteomics provides valuable information about the biological mechanisms contributing to mitochondrially associated diseases, including many diseases of aging.

2.4. Combining with mitochondrial metabolomics

Metabolomics is another important component of Whole-Omic analysis. The study of metabolomics introduces a new molecular component to our analysis of biological systems by measuring the molecular compounds produced by metabolic processes. Understanding the functional role of these molecular compounds, termed metabolites, gives key insights into the pathology of many diseases of aging, specifically those with metabolic etiology [52]. Common metabolomic analytical techniques include, Nuclear Magnetic Resonance (NMR), Liquid Chromatography- Mass Spectrometry (LC-MS), and MS.

NMR spectroscopy is a high-throughput, nondestructive, nonbiased, and easily quantifiable analytical method that allows for high quality identification of novel metabolic compounds. Among its many benefits, the nondestructive nature of NMR allows for metabolite quantification in both living and nonliving samples [53]. However, NMR has proven to be a less sensitive metabolite identification method, and thus is often used in tandem with the highly sensitive LC-MS and MS analytical techniques. A combination of these analytical methods can be used to identify novel metabolic biomarkers and have proven to be valuable tools for incorporating metabolomic data into the promising field of precision medicine. In one such application, researchers utilized the results from metabolomic analysis to identify changes in metabolic enzymes associated with alterations in various oncogenes [54]. Applying similar techniques to the context of the mitochondrial metabolome may also prove to be beneficial in designing novel therapeutics to address many diseases of aging.

Mitochondria play a vital role in many metabolic processes. Analyzing the mitochondrial metabolome is an important part of developing a holistic understanding of the role of mitochondrial function in age-related diseases. More specifically, mitochondrial metabolomics may further refine our understanding of the underlying biology involving mitochondrial peptides [55]. We recently applied a mito-metabolomics approach to assess the effect of two mitochondrially-derived peptides (MDPs), humanin (HNG) and small-humanin-like peptide 2 (SHLP2), on metabolic pathways in mice models. Results showed that treatment with HNG or SHLP2 led to a reduction in the production of many metabolic intermediates involved in the glutathione cycle and sphingolipid pathways, which have been identified as important metabolic pathways associated with cancer and tumor development, and aging, diabetes, and obesity, respectively [7]. Identifying the effects of these MDPs on key metabolic pathways not only improves our understanding of the mitochondrial role in disease pathology, but also highlights the potential therapeutic function of MDPs that may be utilized in the future. However, as with mitochondrial proteomics, effectively utilizing common metabolomic analytical tools for mitochondrial metabolome analysis will primarily require their improved sensitivity, so that all metabolites can be properly identified and characterized. When combined with genomics, transcriptomics, and proteomics, mitochondrial metabolomics will further contribute to the future design of novel therapeutics involved in treating many diseases of aging.

2.5. Mito-Omics and immune function

2.5.1. Immune dysfunction is a symptom of aging

A recently proposed contributor to many diseases of aging is immunosenescence; a term coined for the observed dysregulation in immune system function with age. The accumulation of oxidative damage with age may inhibit proper immune function, leading to drastic changes in important immunological mechanisms such as the inflammatory response, the production of antibodies, and the proper development and function of immune cells [56–59]. To date, age-related changes in the inflammatory response, better termed "inflammaging", and age-related declines in both the innate and adaptive immune response have been observed. With defects in immune function, aging populations are less responsive to vaccines [59], more vulnerable to severe pathogenic infection [58], and at a higher risk of developing many diseases of aging [57].

In response to pathogens, defects in the aging immune system leave populations at risk of more frequent and severe infections, in particular to viral infections such as COVID-19 [60]. Dysfunction in both innate and adaptive immune cell signaling pathways have shown increased susceptibility to pathogenic infection [61,62]. More specifically, age-related changes in the function of natural killer (NK) cells, and in the number, function, and diversity of T cells and B cells may alter host susceptibility [57].

AD and cancer are two examples of many aging related diseases that are heavily impacted by the inability of immune cells from both the innate and adaptive immune systems to function properly. Aberrant innate immunity poses as a key contributor to AD disease progression and initiation. Dysfunction in innate immune system-mediated actions have been shown to contribute and drive AD pathogenesis [63]. Genetic evidence presented in an AD specific GWAS reveals associations between genes linked to innate immunity and AD. Further, pro-inflammatory signaling via toxic amyloid-oligomers that were present in the AD brain were also shown to contribute to synapse deterioration and memory impairment [64] Impaired immune function also contributes to cancer development. Immunosenescence causes defects in naive memory T-cell populations that impair the immune system's ability to mount responses against tumor cells [65]. Additionally, terminally differentiated $CD8^+$ T cells with diminished functionality exhibit increased cytokine production which contributes to a proinflammatory state that may stimulate tumor development [66].

The immune system network is intricate and complex, and thus many contributors to immunosenescence remain undiscovered. However, novel cellular mechanisms associated with immune function are frequently being exposed. Many of these mechanisms link back to the powerhouses of the cell, the mitochondria.

2.5.2. Role of mitochondria in immune function

Mitochondria play a key role in regulating the immune system, and thus potentially play an important role in immune dysfunction and associated age-related diseases. Many innate immune system pathways are stimulated through mtDNA. After cell damage, mtDNA is released and directly activates Toll-like receptor 9 (TLR9), leading to increased transcription of pro-inflammatory cytokines such as IL-6, TNF-α, IL-1β, and MMP-8 [67]. Cytosolic mtDNA also plays a key role in activating Nod-like receptor 3 (NLRP3), stimulating caspase-1 and facilitating IL-1β and IL-18 maturation and proinflammatory cell death of sentinel cells in the innate immune system [68]. Further, a mitochondrial derived peptide (MDP) known as MOTS-c [69] has been shown to reduce inflammation by inhibiting cytokines such as TNF-α and IL-6 while simultaneously promoting an anti-inflammatory response. MOTS-c stimulates IL-10 as well as signal transducer and activators of transcription 3 (STAT3) and aryl hydrocarbon receptor (Ahr) which inhibit NF-κB expression and proinflammatory cytokine production [70]. Another mitochondrial peptide, humanin, also has anti-inflammatory effects [71,72]. In adaptive immunity, mitochondria are also necessary for maintaining Regulatory T cell (T_{reg} cells) function. T_{reg} cells are essential for maintaining self-tolerance in the adaptive immune system [73,74]. T_{reg} cells require mitochondrial complex III to preserve proper T-cell suppression and regulate expression of genes involved in T_{reg} function [73]. Mitochondrial components thus act to both stimulate and preserve proper immune function (Fig. 2). Age-associated damage to these mitochondrial components of interest may be a critical contributor to observed immunosenescence.

2.5.3. Applying Mito-Omics to immune function

Proper immune system function is a vital contributor to healthy aging. The critical role that immune dysfunction may play in disease pathology is important to consider. Applying Whole-Omics and Mito-Omics analysis techniques to the context of the immune system will be crucial for the future design of novel therapeutics for many associated disorders, including neurological disorders such as Alzheimer's Disease, metabolic disorders such as diabetes, and various cancers. Current applications of Whole-Omic analytical techniques on the study of immune function have already proven the importance of applying the Whole-Omic approach to this field.

As with other traits of interest, GWAS can be an important diagnostic tool for identifying genetic contributors to immune dysfunction, and related diseases. Due to the diversity and complexity of the phenotypic presentation of immune system, identifying SNPs specifically associated with immune dysfunction can prove to be more challenging. However, the GWAS approach is still incredibly relevant to immune system research, and recent efforts at immune phenotyping has made more in-depth profiling of the immune system possible. A recent study utilized the GWAS approach and to outline the genetic architecture of the adaptive immune system [75]. The study used GWAS to assess the presence of 54 immune phenotypes in a sample of 489 individuals. From this analysis, the group identified eight genome variants associated with adaptive immune phenotypes involving the maturation and differentiation of B, T, and Th2, Th17, and $T_{reg,}$ cell subsets, all of which are important contributors to disease pathogenesis. While applications of GWAS to assessing immune function are still emerging, studies such as this one reveal the benefit of including the GWAS approach in Whole-Omic analysis when analyzing immune-related diseases.

Transcriptomics has made substantial contributions to the advancement of immunological research. Advances in RNA-sequencing techniques and computational tools have been of particular benefit to characterizing the components of both the innate and adaptive immune system. More specifically, the introduction of single-cell RNA-sequencing (scRNA-seq) has proven to be revolutionary for the field of immunology [76]. scRNA-seq is a highly sensitive sequencing tool that can accurately quantify gene expression from a small amount of starting material within the cell. When applied to the immune system, scRNA-seq can identify previously uncharacterized subpopulations of immune cells, exposing new contributors to the complex network that is involved in proper immune function. Recent use of single-cell transcriptomics has unveiled a novel immune pathway that may contribute to supercentenarian survival, revealing a potentially key contributor to healthy immune aging [77]. PBMCs from seven supercentenarian donors and five controls were collected and sequenced using scRNA-seq. From this sequencing data, a unique subset of CD4 T cells were characterized as cytotoxic with a unique expression profile and were found to be abundant in supercentenarians but not in controls. The abundance of these cytotoxic CD4 T cells could prove to be beneficial for preventing tumor development and severe viral infection, which both commonly increase in frequency with age. Even more recently, transcriptomics has played an important role in characterizing the effects of SARS-CoV-2 infection on immune function [78]. Transcriptomic analysis of blood from COVID-19 patients exposed the unique transcription profile of SARS-CoV-2 infection, elucidating some of the potential molecular mechanisms that impact SARS-CoV-2 severity. We recently analyzed the mito-transcriptomic profile of various SARS-CoV-2 infected cell lines, and found that SARS-CoV-2 also induces unique expression patterns of the mitochondrial transcriptome in many cell environments [79].

Proteomic analysis has also proven important to understanding immune function. Understanding the complex protein network required for a healthy immune system is crucial for identifying the cellular pathways involved in immune dysfunction. The use of mass

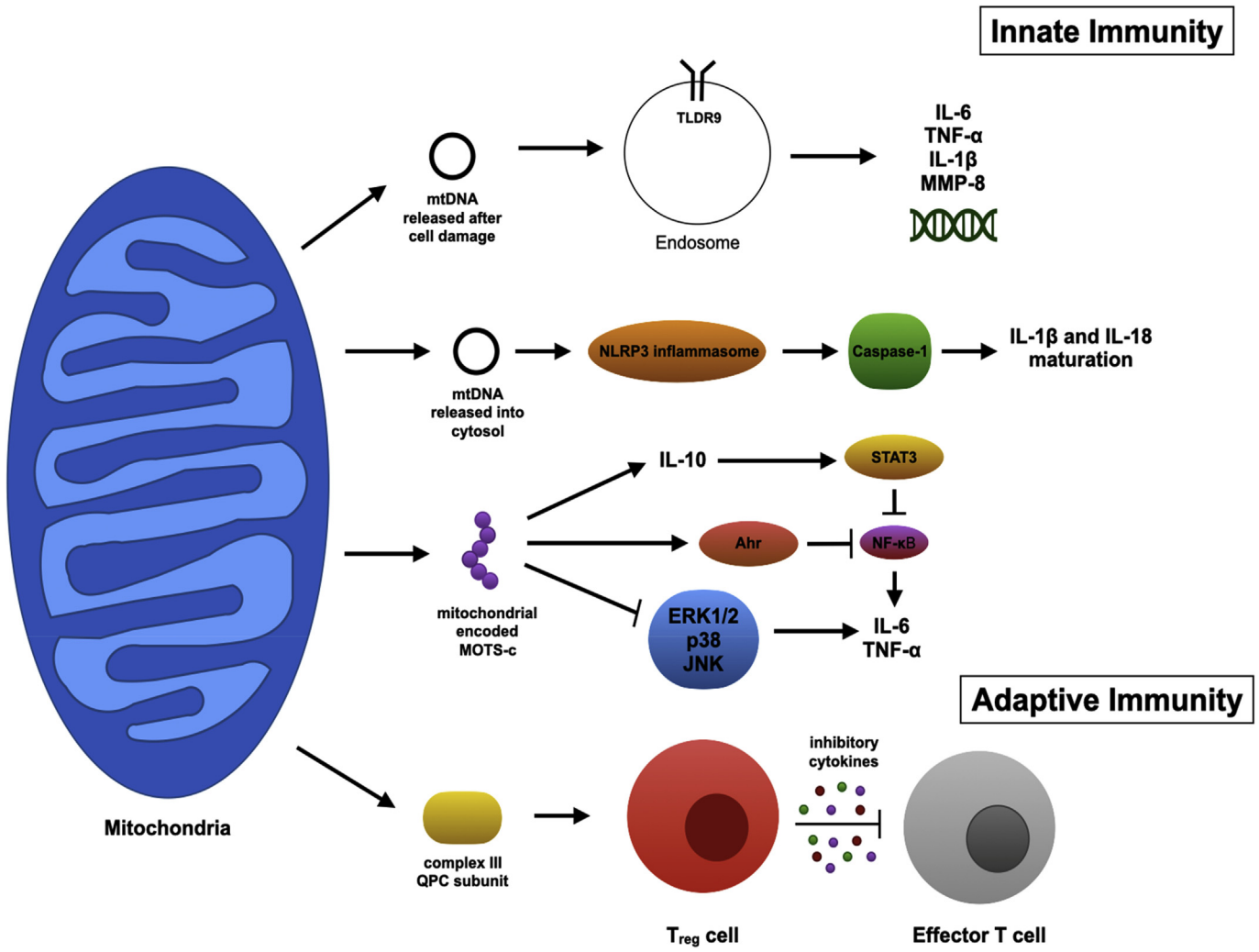

Fig. 2. Mitochondrial Regulation of the Immune System. Toll-like receptor 9 (TLDR9) in the endosome is stimulated by mitochondrial DNA (mtDNA) after cell damage and leads to the increased production of pro-inflammatory cytokines including IL-6, TNF-α, IL-1β and MMP-8. The Nod-like receptor 3 (NLRP3) inflammasome is activated by mtDNA released into the cytosol. The NLRP3 inflammasome activates caspase-1 which cleaves IL-1β and IL-18 into their mature forms. Mitochondrial derived peptide (MDP) MOTS-c has an anti-inflammatory effect. MOTS-c suppresses phosphorylation of three major kinases in MAPK signaling; ERK 1/2, p38 and JNK, which leads to decreased levels of pro-inflammatory cytokines IL-6 and TNF-α. MOTS-c also stimulates IL-10, an anti-inflammatory cytokine that activates signal transducer and activators of transcription 3 (STAT3) and prevents expression of NF-κB and the subsequent production of pro-inflammatory cytokines. NF-κB expression is also inhibited by MOTS-c stimulation of aryl hydrocarbon receptor (Ahr). Mitochondrial complex III subunit QPC is required for Regulatory T cell (T_{reg} cell) suppression of effector T cells in the adaptive immune system.

spectrometry has revealed important information regarding the role of protein secretion and activity in regulating a healthy immune system. More specifically, MS-based proteomic analysis characterizes the unique intracellular behavior required for proper immune function and can also identify changes in protein expression and localization patterns during viral infection [80,81]. Characterizing proteins involved in immune function and identifying protein behavior under viral attack is a promising step towards novel vaccine and therapeutic treatment design. One recent study used MS-based proteomic analysis to understand the signaling relationship involved in stimulation by the proinflammatory cytokine, IL-2 [82]. Among its many regulatory roles, IL-2 is important for stimulating CD8 cytotoxic T cell differentiation and proliferation. Proteomic analysis of cells with inhibited Il-2 stimulation identified many immunomodulatory roles of IL-2, specifically in regulating the expression of metabolic proteins that are essential for proper CD8 T cell differentiation and function. Understanding the process by which proinflammatory cytokines like IL-2 regulate the intracellular network of the immune system gives key insight into potential targets of future therapeutics for chronic inflammatory disorders.

While transcriptomics and proteomics have made substantial contributions to advancements in the field of immunology, the introduction of metabolomic analysis to understanding immune function is still relatively new. However, metabolic approaches have becoming increasingly more common in immunological research. The emergence of metabolomics in immunology will continue to expand our understanding of the intricate intracellular network involved in regulating immune function. Considering the role of metabolites in regulating the key cellular processes involved in the immune system has the potential to reveal important biomarkers of immune response, expand our understanding of immune cell metabolism, and expose the effects of metabolic compounds on immune cell function [83]. Recently, metabolomic analysis was used to understand the effects IFN-gamma and LPS stimuli on macrophage metabolism [84]. The findings of this study identified key metabolic pathways involved in regulating the immune response associated with macrophages. Understanding the

role of metabolic compounds in macrophage activation and function is vital when addressing inflammation and immune function. While this study showed initial identification of many different metabolic compounds potentially involved immune regulation, it also exposes a new direction for immunological research that is just beginning to emerge.

Applying Mito-Omics techniques to the context of the aging immune system is a novel proposal. However, given the previous use of many Whole-Omic analysis techniques in analyzing immune function, and the previously identified pathways for which the mitochondria contribute to immune function, there is vast potential for Mito-Omic tools to be applied to the study of immunosenescence. Our recent application of mito-transcriptomics analysis to the context of SARS-CoV-2 infection emphasizes the emerging value of Mito-Omics applications in studying the immune system. Thus, future use of the Mito-Omics approach in studying immune function may illuminate novel aspects of immune system regulation. These applications could prove beneficial in developing groundbreaking therapeutics for many diseases of aging for which immune function is involved, and possibly in treating the phenomenon of immunosenescence itself.

3. Conclusions and future directions

Recent advances in Whole-Omic technologies have enhanced our understanding of biological mechanisms contributing to many diseases of aging. Expanding these techniques to consider mtDNA for Mito-Omics will enable a more extensive analysis of the biological contributors to age-related diseases. Advancements in specialized genomic, transcriptomic, proteomic, and metabolomic techniques for mtDNA analysis that account for its unique structure and function will be vital for accurately identifying its contributions to age-related diseases, including the aging immune system.

While much about immunosenescence and its functional contributors have been revealed, large information gaps in the biological mechanisms contributing to observed immune dysfunction still remain. Importantly, mitochondrial contributions to immune function and dysfunction are just beginning to be established. Mito-Omic evaluation methods will play a critical role in revealing the mitochondrial specific mechanisms contributing to immune dysfunction.

However, such progress will not come without challenges: considering the haplogroup architecture of mtDNA when conducting genomic analysis will help identify the true biological drivers of a trait of interest; new protein enrichment techniques for mass spectrometry will improve mito-proteomic analysis, enabling the identification of novel microproteins and metabolites associated with mtDNA; and mtDNA protein and microprotein visualization will help identify localization patterns of proteins of interest. Addressing these challenges, among others, will improve key aspects of Mito-Omics and expose novel contributors to diseases of aging in the process.

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CRediT authorship contribution statement

Ana R. Silverstein: Writing - original draft, Visualization. **Melanie K. Flores:** Writing - original draft, Visualization. **Brendan Miller:** Writing - original draft, Writing - review & editing. **Sujeong Kim:** Writing - review & editing. **Kelvin Yen:** Writing - review & editing. **Hemal H. Mehta:** Writing - review & editing. **Pinchas Cohen:** Supervision, Writing - review & editing.

Declaration of Competing interests

Pinchas Cohen is a stockholder and consultant for CohBar Inc.

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