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Mitochondrial DNA alterations in aged macrophage migration inhibitory factor-knockout mice

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ABSTRACT

The age-induced, exponential accumulation of mitochondrial DNA (mtDNA) deletion mutations contributes to muscle fiber loss. The causes of these mutations are not known. Systemic inflammation is associated with decreased muscle mass in older adults and is implicated in the formation of sporadic mtDNA deletions. Macrophage migration inhibitory factor knockout (MIF-KO) mice are long-lived with decreased inflammation. We hypothesized that aged MIF-KO mice would have lower mtDNA deletion frequencies and fewer electron transport chain (ETC) deficient fibers.

We measured mtDNA copy number and mutation frequency as well as the number and length of ETC deficient fibers in 22-month old MIF-KO and F2 hybrid control mice. We also measured mtDNA copy number and deletion frequency in female UM-HET3 mice, a strain whose lifespan matches the MIF-KO mice.

We did not observe a significant effect of MIF ablation on muscle mtDNA deletion frequency. There was a significantly lower mtDNA copy number in the MIF-KO mice and the lifespan-matched UM-HET3 mice compared to the F2 hybrids, suggesting the importance of genetic background in mtDNA copy number control. Our data do not support a definitive role for MIF in age-induced mtDNA deletions.

1. Introduction

Macrophage migration inhibitory factor (MIF), one of the first identified cytokines, is an important component of innate immunity that localizes macrophages to sites of inflammation (Bloom and Bennett, 1966; Bucala, 1996). MIF is a soluble factor produced by activated T lymphocytes (David, 1966) and in mouse anterior pituitary cells, where it is released upon stimulation with lipopolysaccharide (LPS), leading to systemic effects (Bernhagen et al., 1993). MIF has a number of pro-inflammatory functions (reviewed in (Calandra and Roger, 2003)). In macrophages, MIF induces TNF-alpha secretion (Calandra et al., 1994). Co-injection of LPS and MIF potentiates endotoxemia, and administration of anti-MIF antibodies ablates this effect, protecting mice from LPS, and decreasing TNF-alpha levels (Bernhagen et al., 1993). MIF is expressed in both immune cells and non-immune tissues, including the pancreas, pituitary gland, heart, and skeletal muscles (Claria et al., 1991; Miyatake et al., 2014). The level of MIF expression does not appear to vary between muscle groups (Miyatake et al., 2014). MIF is implicated in the exacerbation of a wide range of inflammatory diseases, including colitis, atherosclerosis, and pancreatitis (Nishihira and Mitsuyama, 2009); and may act to counterbalance the anti-inflammatory and immunosuppressive effects of glucocorticoids (Fingerle-Rowson and Bucala, 2001). MIF knock out mice (MIF-KO) are resistant to lethal doses of lipopolysaccharide or *Staphylococcus aureus* enterotoxin B and have lower tumor necrosis factor alpha responses after challenge (Bozza et al., 1999). MIF-KO mice have increased susceptibility to numerous pathogens including infections with *Leishmania major* and *Salmonella typhimurium* (Koebernick et al., 2002; Satoskar et al., 2001). IL-6 and IκBα expression increase between 4 and 24 months in WT mice, but there is no increase with age

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in these inflammatory markers in 24mo MIF-KO mice (Xu et al., 2016). Thus, inhibiting MIF's pro-inflammatory effects has been a promising clinical approach in protecting patients from septic shock and other inflammatory diseases (Cvetkovic and Stosic-Grujicic, 2006).

In addition to its role in inflammation, MIF influences diverse molecular processes important for cellular homeostasis including glucose and fat metabolism, mitochondrial respiration, cell cycle regulation, and autophagy (Preau et al., 2013; Toso et al., 2008) (Hagemann et al., 2007; Xu et al., 2016). The abundance of MIF is elevated, at old age, in certain long-lived mouse strains and after calorie restriction (CR) (Hardman et al., 2005; Miller et al., 2002; Mizue et al., 2000; Welford et al., 2006). Although MIF levels in human serum do not significantly change with age, MIF mRNA expression was significantly elevated in the livers of long-lived Snell dwarf and growth hormone receptor KO mice, while MIF mRNA expression was significantly elevated in the liver, heart and skeletal muscle of CR mice (Miller et al., 2005, 2002). These findings are consistent with the hypothesis that MIF is necessary for longevity and the lifespan-extending effects of CR. The upregulation of MIF in caloric restriction and long-lived strains of mice contrasts with the reduced inflammation in these models (Gonzalez et al., 2012; Masternak and Bartke, 2012). MIF is, however, pleiotropic with additional roles beyond inflammation including involvement in glucose metabolism, the hypothalamic-pituitary axis, NLRP3 activation, atherosclerosis and cancer that likely affect lifespan (Harper et al., 2010; Harris et al., 2019).

To test the role of MIF as a modulator of CR, MIF-KO mouse lifespan was examined under both *ad libitum* and CR conditions (Harper et al., 2010). The MIF-KO mice lived longer than wild-type (WT) mice and calorie restricted MIF-KO mice had further extended lifespans. Since genetically matched mice were not available to serve as the MIF-WT control, the control mice were F2 hybrids between C57BL/6 and 129/SvJ. The F2 hybrids would contain a mixed background of C57BL/6 and 129 genes in equal proportions, but would not have a genetic background identical to the MIF-KO mice. The lack of a genetically-matched, littermate control complicated the interpretation of the lifespan effects.

Low-grade chronic inflammation has emerged as a possible mechanism of cellular aging and age-induced diseases (Fougere et al., 2017; Franceschi and Campisi, 2014). Inflammation plays an important role in epigenetic modifications of cytokine expression, oncogenes, and tumor suppressor genes, leading to chronic inflammatory diseases and carcinogenesis (Grivennikov and Karin, 2010). Systemic inflammation is strongly associated with skeletal muscle aging and is considered a major contributor to sarcopenia – the age-related loss of skeletal muscle mass and function (Rosenberg, 1997); reviewed in (Dalle et al., 2017). Despite evidence that chronic inflammation contributes to aging and age-induced diseases, we have a poor understanding of the molecular mechanisms underlying how chronic inflammation drives aging at the molecular and cellular level in skeletal muscle.

Life-long inflammation has been implicated in mitochondrial DNA (mtDNA) damage, possibly leading to generation and subsequent accumulation of clonally-expanded mtDNA deletions (Fig. 1) (Lopez-Armada et al., 2013; Tschopp, 2011; van Beek et al., 2019). MtDNA mutations may arise from an inflammatory insult, such as reactive oxygen species (ROS), that directly cause double-strand breaks in mtDNA. With age, somatically derived mtDNA deletions clonally accumulate within a subset of individual cells(Herbst et al., 2007; Schwarze et al., 1995; Wanagat et al., 2001). Age-induced mtDNA deletions are large, located primarily in the mitochondrial major arc and disrupt multiple genes encoding protein subunits necessary for oxidative phosphorylation. When the intracellular abundance of deletion-containing mtDNA genomes exceeds 90%, the electron transport chain (ETC) function is disrupted and cells lose cytochrome c oxidase (COX) activity prior to undergoing cell death (Cheema et al., 2015; Herbst et al., 2013). COX-negative, ETC-deficient cells have been detected in the brain, heart, kidney, and skeletal muscle of aged mammals including humans, where they contribute to the cellular phenotypes and tissue degeneration of aging (Baris et al., 2015; Ekstrand et al., 2007; Herbst et al., 2016; McKiernan et al., 2007; Wanagat et al., 2001). Apoptosis and necrosis, induced by mtDNA deletions, result in persistent cycles of mitochondrial damage, cell death, and inflammation (Cheema et al., 2015; Krishnan et al., 2008).

The precise etiology of mtDNA deletions is not fully understood. We hypothesized that reactive oxygen and nitrogen species (RONS) generated by inflammation-linked processes trigger mtDNA damage, leading to the accumulation of clonally-expanded mtDNA deletions. Growing evidence suggests that chronic upregulation of pro-inflammatory mediators, like TNF-α, IL-1β and IL-6, are induced during aging (Visser et al., 2002). ROS-induced formation of mtDNA double strand breaks is thought to generate somatic mitochondrial deletions (Krishnan et al., 2008). In neurodegenerative diseases like multiple sclerosis, inflammation is thought to cause damage to mtDNA and, through repair processes and clonal expansion, produce mtDNA deletions in neurons (Campbell et al., 2011).

The possible role of MIF in affecting lifespan and the opportunity to examine the effect of inflammation on mtDNA deletion frequency led us to measure the abundance of mitochondrial DNA deletions and ETC deficient fiber number in 22-month old MIF-KO mice. We hypothesized that a life-long decrease in skeletal muscle inflammation in MIF-KO mice would reduce mtDNA deletion frequency and resulting formation of ETC deficient fibers. We measured mtDNA deletion frequency using digital PCR, and ETC deficient fiber abundance by histochemistry. We did not find significant changes in mitochondrial genetics or enzymatic abnormalities between 22-month-old MIF-KO and WT mice of either sex. Our data argue against a role for MIF-mediated inflammation in mtDNA deletions in skeletal muscle.

2. Materials and methods

2.1. Animals

Breeding and husbandry details for the MIF-KO and control mice have been previously described (Harper et al., 2010; Miller et al., 2007)). The MIF-KO allele is on a mixed C57BL/6 and 129/SvJ background. As genetically matched non-MIF-KO mice were not available, F2 hybrids of C57BL/6 and 129/SvJ, which approximate the mixed background of the MIF-KO mice were used as MIF + controls. Female four-way cross genetically heterogeneous UM-HET3 mice (Miller et al., 2014) approximate the lifespan of female MIF-KO mice, with median lifespans of 895 days versus 896 days, respectively. A predetermined cross-sectional cohort of mice from the Harper et al. (2010) lifespan study was euthanized at 22 months, an age in this strain at which ~5-15% of females have died spontaneously, minimizing selection bias

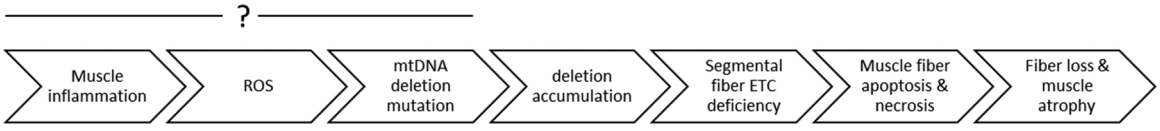

Fig. 1. Model for contribution of inflammation to mtDNA deletion-induced cell loss. Life-long chronic, low-grade muscle inflammation includes the release of cytokines, such as MIF, and chemokines that induce the production of reactive oxygen species (ROS), which result in macromolecular mitochondrial damage including generation of mtDNA deletion mutations. Accumulation of mutations leads to segmental biochemical defects in muscle fibers, triggers apoptosis, macrophage recruitment, and necrotic cell death.

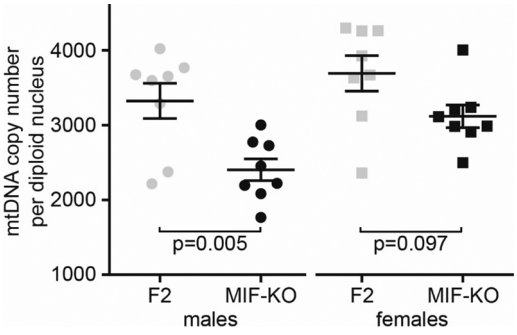

Fig. 2. Male MIF-knock out mice have lower mtDNA copy numbers in the quadriceps compared to the F2 control at 22 months of age. There is no significant differences in mtDNA copy numbers in the females. Individual data points are plotted and the bars denote the mean ± SEM. Eight mice of each sex were analyzed per genotype.

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2.2. MtDNA copy number and mtDNA deletion frequency by digital PCR

Frozen muscle samples were powdered under liquid nitrogen using a mortar and pestle. Approximately 25 mg of powdered muscle was used in DNA isolation as previously described (Herbst et al., 2017). Quality control of the DNA was performed with UV spectrometry and gel electrophoresis. A 5-prime nuclease cleavage assay and chip-based digital PCR (dPCR) was used to quantitate copy numbers for nuclear DNA (nDNA), total mtDNA, and mtDNA deletions with specific primer/probe sets for each as previously described (Herbst et al., 2017). Samples were diluted to the manufacturer's recommended target range (200 to 2000 copies per microliter) in 17.5 µL reactions using QuantStudio 3D Digital PCR Master Mix v2 (ThermoFisher; Waltham, MA) and loaded onto Quantstudio 3D digital PCR 20 K Chip (Version 2, ThermoFisher; Waltham, MA). Final primer and probe concentrations were 900 nM and 250 nM, respectively. Digital PCR cycling conditions were Taq-polymerase activation at 95 °C for ten minutes, 40 cycles of denaturation at 94 °C for 30 s and annealing/extension at 60 °C for two minutes. MtDNA copy numbers per microliter and the threshold were determined using QuantStudio 3D Analysis Suite Cloud Software (Version 3, ThermoFisher; Waltham, MA). Direct quantitation of the major arc deletions by dPCR used the same cycling conditions but for 60 cycles. DPCR quantitation of all samples and for all targets was performed on blinded samples.

2.3. Histological analyses of ETC deficient fibers

Following euthanasia, quadriceps muscles were dissected from the mice, bisected at the mid-belly. One half was embedded in optimal cutting temperature compound (Sakura Finetek) and both halves were then flash frozen in liquid nitrogen and stored at −80 °C. A minimum of one hundred 10 µm thick consecutive transverse cross-sections were cut with a cryostat at −17 °C and placed on Probe-On-Plus slides (ThermoFisher). Slides were stored at −80 °C until needed. At 100 µm intervals, a group of three serial sections was stained for (1) cytochrome c oxidase (COX), (2) succinate dehydrogenase (SDH), and (3) dual stained, first for COX and then for SDH as previously described (Herbst et al., 2016). In the dual-stained slide, ETC deficient fibers appear blue on a brown background, which facilitates the identification of ETC deficient fibers. The individually stained COX and SDH sections allow confirmation of the COX-negative/SDH hyper-reactive phenotype of ETC deficient fibers. ETC-deficient fiber counts and cross-sectional area of the quadriceps were obtained from digital images. The absolute number of ETC deficient fibers (COX-/SDH++) was determined by following each fiber throughout the 100 serial sections in both control and treated mice. We normalized ETC deficient fiber number to the volume of muscle tissue (i.e., the volume density of ETC deficient fibers), which was calculated from the muscle cross sectional area and number of sections examined as previously described (Wanagat et al., 2001). Normalization to muscle volume permits comparison between muscle of different sizes and fiber number. Researchers were blinded to the treatment groups.

2.4. Statistical analyses

Data were analyzed using GraphPad Prism (Version 7, GraphPad Software). All data with normal distribution were presented as means ± SEM. Two-way ANOVA was used to identify sex and genotype effects for mtDNA copy number, mtDNA deletion frequency, and ETC deficient fiber number. P-values reported for individual comparisons were derived using Sidak's multiple comparison approach. Comparisons across sex and genotype were not interpreted. The fraction of mice with any ETC deficient fibers was also tested by chi-square analysis. Kruskal-Wallis testing was used to compare ETC deficient segment length.

3. Results

3.1. Skeletal muscle mtDNA copy number in 22-month old MIF-KO mice versus F2 hybrid controls

We used digital PCR to quantitate mtDNA copy number from quadriceps muscle homogenates. Sex and genotype both had significant effects on mtDNA copy number, but there was no significant interaction between the factors (sex $p = 0.0106$; genotype $p = 0.0008$; interaction $p = 0.3872$; Fig. 2). In 22-month old muscle homogenates, there was no significant difference in mtDNA copy number between the F2 control male and female mice. Copy number was significantly lower in the male MIF-KO mice where there was a 28% decrease ($p = 0.0054$). The female MIF-KO mice also had a 16% lower mtDNA copy number ($p = 0.097$).

3.2. MtDNA mutation frequency

After determining mtDNA copy number, we again used digital PCR to quantitate the absolute number of mtDNA deletions in the samples. MtDNA deletion frequency is the ratio of mtDNA deletions to total mtDNA copy number. There were no significant effects of sex or genotype on mtDNA deletion frequency (sex $p = 0.1120$; genotype $p = 0.695$; interaction $p = 0.3107$; Fig. 3). MtDNA deletion frequency was 60% lower in females as compared to males at 22 months but this difference did not reach significance. Similarly, deletion frequency was 65% lower on average in male MIF-KO and 47% lower in female MIF-

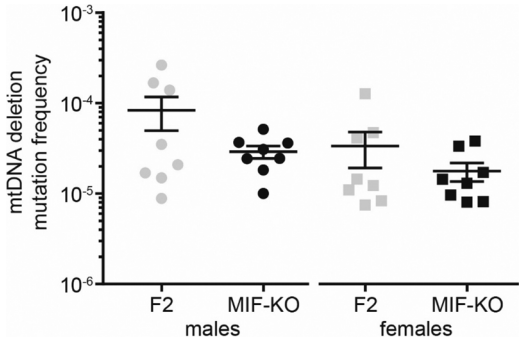

Fig. 3. MtDNA deletion frequency in MIF-KO is not different from the F2 control in quadriceps muscle at 22 months of age. Individual data points are plotted and the bars denote the mean ± SEM. Eight mice of each sex were analyzed per genotype.

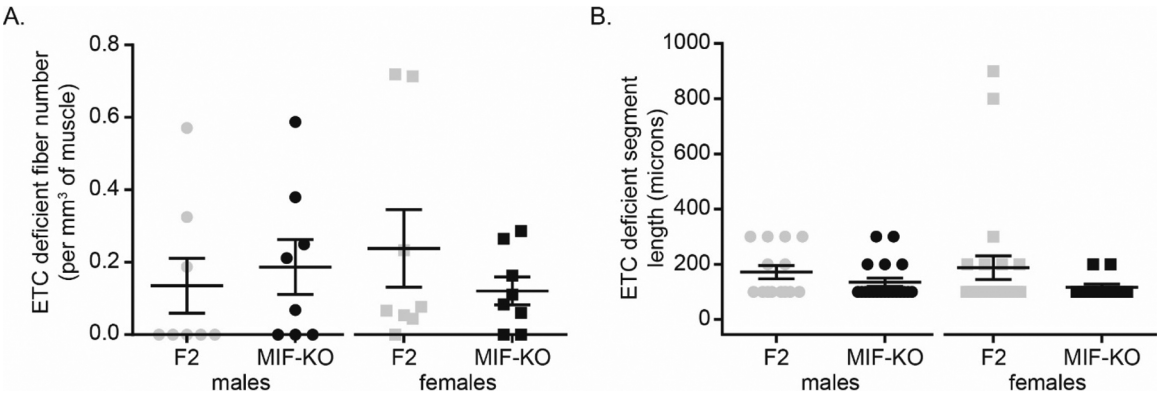

Fig. 4. Number and length of electron transport chain (ETC) deficient fibers are not different between MIF-KO and F2 control mice in 22mo quadriceps muscle. **A**. ETC deficient fiber number was quantitated by volumetric histochemistry of muscle sections. Individual data points are plotted and the bars denote the mean ± SEM. Eight mice of each sex were analyzed per genotype. **B**. ETC deficient segment length is the number of sections within which the cytochrome *c* oxidase activity is negative multiplied by the interval of analysis (100um). Individual ETC deficient segment lengths are plotted from all the mice and the bars denote the mean ± SEM. There were between 12 and 24 fibers in each group.

KO compared to the F2 controls but neither of these was statistically significant.

3.3. ETC deficient fiber abundance and ETC deficient fiber length in MIF-KO mice

Using *in situ* histochemistry, we counted the number of ETC deficient fibers identified in 100 serial sections taken from each OCT embedded quadriceps muscle. The number of ETC-deficient fibers identified across 1000 microns varied from 0-11. Many mice at 22 months of age had zero ETC deficient segments within the volume of sampled quadriceps (i.e., 1000 microns along the length of the muscle). There were no significant effects of sex or genotype on ETC deficient fiber number at this age (Fig. 4**A;** sex $p = 0.8153$, genotype $p = 0.6781$, interaction $p = 0.2882$). A Chi-square test of the hypothesis that groups differed in the proportion of mice lacking any ETC deficient fibers did not produce a significant effect ($p = 0.6211$ and 1.000 for males and females, respectively). Similarly, there was no effect of genotype ($p = 0.3920$) on the length of the ETC deficient segments in the affected muscle fibers (Fig. 4B). Dunn's non-parametric multiple comparison test did not identify any significant differences among the four groups.

4. Discussion

In this study, we investigated the role of inflammation in age-induced mitochondrial mutations in skeletal muscle by using MIF-KO mice, which have reduced systemic inflammation (Calandra and Roger, 2003). As compared to F2 control mice, the 22mo MIF-KO mice have a similar ETC deficient fiber abundance and only a trend toward lower mtDNA deletion frequency that was not statistically significant. These findings suggest that, at this age, the MIF gene does not significantly influence mtDNA deletion frequency. At 22-months of age, survival of the MIF-KO and F2 female mice is 95% and 85%, respectively (Harper et al., 2010). At a similar percent survival in other aging rodent models, both the abundance of ETC deficient fibers and the frequency of mtDNA deletions is entering its exponential phase (Herbst et al., 2017). At this age, many mice in the study had zero ETC deficient fibers within the volume of tissue examined. For this reason, our data set may have limited implications for older ages when mitochondrial defects are more abundant in mice.

The significant difference in mtDNA copy number between MIF-KO mice and F2 controls and the trend toward a lower deletion frequency in the longer-lived MIF-KO mice can be interpreted in two ways. One interpretation is that MIF ablation alters mtDNA replication and stability, affecting copy number and deletion accumulation. Alternatively, as the F2 mice are only approximate controls for the MIF-KO (i.e., they are not on an identical genetic background), there may be other alleles that alter mitochondrial biology (Harper et al., 2010). For example, the CHCHD10 gene (responsible for coordination of cristae junctions in the mitochondrion) is located ˜75 kb downstream from MIF on chromosome 10; mutations in CHCHD10 are linked to mitochondrial myopathy with mtDNA deletions as well as frontotemporal dementia, amyotrophic lateral sclerosis, and Parkinson's disease (Rubino et al., 2018). Mitochondrial DNA copy number and regulation thereof involves numerous proteins (Clay Montier et al., 2009). Greater than 90 genes have been identified in Drosophila that affect mtDNA copy number (Fukuoh et al., 2014). Many of these genes are master regulators of metabolism (e.g., PGC-1alpha, NRF-1, NRF-2, PPARgamma, etc.) and these pathways interact with numerous behavioral traits and environmental factors to affect mtDNA copy number. Finally, aging itself affects muscle mtDNA copy number (Herbst et al., 2017). Another potential confounder is that littermate controls, which would account for *in utero* and nursing effects, were not available for the MIF-KO mice. Thus, it is not surprising that statistical differences in mitochondrial DNA copy number were observed between the two strains.

Harper et al. (2010) suggested a genetic difference other than the MIF allele might underlie the lifespan observations in the MIF-KO mice. To better interpret the influence of the mouse genetic backgrounds, we

Table 1
MtDNA copy number and deletion frequency in different strains of female mice.

Mouse strain	Median lifespan (days)	MtDNA copy number[a] (per diploid nucleus)	MtDNA deletion frequency[a] ($x10^{-5}$)
MIF-KO	895[b]	3118 ± 151	1.77 ± 0.41
F2 control	774[b]	3691 ± 238	3.36 ± 1.44
UM-HET3	896[c]	3127 ± 199	1.58 ± 0.51

[a] Values are from 22month old mice and shown as mean ± SEM.
[b] (Harper et al., 2010).
[c] (Miller et al., 2014).

compared mtDNA copy number and deletion frequency of the MIF-KO females and F2 controls with 4-way cross (UM-HET3) MIF-intact females of the same age, i.e., 22 months (Table 1). The female MIF-KO and 4-way cross UM-HET3 mice share a mixed background, have strikingly similar median lifespans, mtDNA copy numbers, and deletion frequencies. The findings in this longevity matched control suggest that MIF ablation is not strongly affecting mtDNA quality at this age and support the interpretation that there are alleles differing between the mice, e.g. CHCHD10, that could affect mtDNA genome integrity and to a lesser extent mtDNA deletion frequency.

Although our data do not support the hypothesis that MIF increases mtDNA deletion frequency, they do not rule out the possibility that inflammation is involved in mtDNA deletion induced pathology. Further studies will be required to examine the interactions among inflammation, aging, and mtDNA deletions. As an alternative to the hypothesis in Fig. 1, mitochondria with mtDNA deletions may increase ROS production (Wanagat et al., 2001), upregulate the expression of pro-inflammatory cytokines such as IL-6, IL-1alpha, CSF1, and CCL11 (Herbst et al., 2013), and thus the ETC deficient fibers themselves may be a source of muscle inflammation. Necrotic ETC deficient fibers recruit macrophages and activate the complement membrane attack complex (Cheema et al., 2015). Release of oxidized mtDNA itself is a known activator of the NLRP3 inflammasome (Zhong et al., 2018) and c-GAS/STING (West et al., 2015). This perspective suggests a role for mtDNA deletion accumulation in chronic, age-induced inflammation.

In summary, our data do not support a direct role for MIF in mtDNA deletion frequency. It is unclear if inflammation is causal to the age-induced increase in mtDNA deletion frequency or follows from it, and is unrelated by any direct causal chain. Additional research is needed to decipher the complex relationship between inflammation and mitochondrial DNA deletions.

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