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Mitochondrial DNA has a pro-inflammatory role in AMD

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Abstract

Age-related macular degeneration (AMD) is the leading cause of irreversible blindness in the elderly of industrialized nations, and there is increasing evidence to support a role for chronic inflammation in its pathogenesis. Mitochondrial DNA (mtDNA) has been recently reported to be pro-inflammatory in various diseases such as Alzheimer's and heart failure. Here, we report that intracellular mtDNA induces ARPE-19 cells to secrete IL-6 and IL-8, inflammatory cytokines that have consistently been associated with AMD onset and progression. The induction was dependent on the size of mtDNA, but not on specific sequence. Oxidative stress plays a major role in the development of AMD, and our findings indicate that mtDNA induces IL-6 and IL-8 more potently when oxidized. Cytokine induction was mediated by STING (Stimulator of Interferon Genes) and NF-κB as evidenced by abrogation of the cytokine response with use of specific inhibitors (siRNA and Bay 11–7082, respectively). Finally, mtDNA primed the NLRP3 inflammasome and weakly activated it as shown by caspase-1 activation and mature IL-1β secretion. This study contributes to our understanding of the potential pro-inflammatory role of mtDNA in the pathogenesis of AMD.

Graphical abstract

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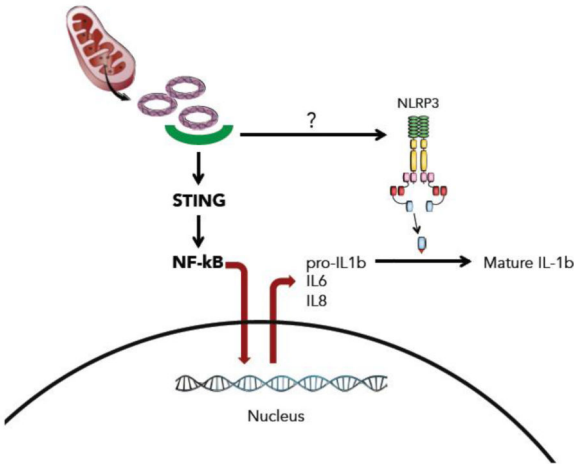

Keywords

Age-related macular degeneration; Retinal pigment epithelium; Mitochondrial DNA; Inflammation; NLRP3 inflammasome

Introduction

Mitochondrial damage and dysfunction are associated with aging and the development of neurodegenerative diseases[1,2], including age-related macular degeneration (AMD)[3,4]. The examination of retinas from patients with AMD has consistently revealed mitochondrial DNA (mtDNA) damage and a decreased capacity for repair[3–7]. More specifically, ultrastructural studies point to a decline in the number and structural integrity of mitochondria in the RPE of aged and AMD eyes[8]. Although it remains uncertain whether this finding is a cause or a consequence of disease, mitochondrial damage can be conceptualized to contribute to the development of AMD by way of increased oxidative stress and deregulated apoptosis and necrosis, which culminate in RPE death[4].

Inflammation is another plausible link between mitochondrial damage and disease. In diverse pathologies that cause cell injury or death, mitochondria can act as a major source of damage-associated molecular patterns (DAMPs)[9]. These are danger signals of various origins that act by engaging intracellular or extracellular pattern recognition receptors (PRRs) in order to stimulate an immune response and cytokine production. Among the proposed DAMPs, mtDNA has received considerable attention due to its immunostimulatory potential when uncontained[9]. In fact, mtDNA release has been reported in various pathologic conditions, including rheumatoid arthritis[10], cardiomyopathy[11], trauma[12], systemic inflammation[13–17], and neurodegeneration[18,19]. Since inflammation is a an emerging major feature of AMD, and given the compromised structural integrity of mitochondria in the RPE of AMD patients, we hypothesized mtDNA release as potentially inflammatogenic in the pathogenesis of AMD.

In particular, mtDNA has been found to induce the pro-inflammatory cytokines IL-6 and IL-8 in vitro and in vivo[12,13,17–20]. Both of these cytokines have been associated with AMD

progression[21–25] and the development of choroidal neovascularization (CNV)[26–28]. In macrophages, mtDNA induces the NLRP3 inflammasome[29,30], which has also been recently reported to be involved in the pathogenesis of AMD[31–33]. NLRP3 activation leads to cleavage of the pro form of caspase-1 into its mature form, with the latter cleaving the pro forms of IL-β and IL-18 into their mature forms, which are subsequently secreted by the cell[34]. Since the RPE is directly involved in the pathogenesis of AMD[35], we thought it meaningful to examine whether mtDNA can induce the pro-inflammatory cytokines IL-6 and IL-8, and the NLRP3 inflammasome in ARPE-19 cells.

In this study, we report that mtDNA stimulates IL-6 and IL-8 secretion as well as priming of the NLRP3 inflammasome in ARPE-19 cells. Those effects were mediated by a common pathway involving Stimulator of Interferon Genes (STING), an adaptor protein that is increasingly being viewed as a central mediator of the DNA sensing pathways[36]. We also report that mtDNA activates the NLRP3 inflammasome, leading to the secretion of IL-1β from ARPE-19 cells. Together, these findings provide a novel mechanistic link between mitochondrial damage and inflammation in AMD based on the inflammatogenic potential of mtDNA.

Materials and Methods

Cell Lines and Reagents

ARPE-19 cells (CRL-2302), THP-1 cells (TIB-202), and RPMI-1640 medium (30–2001) were purchased from the American Type Culture Collection (ATCC) (Manassas, VA). Human primary retinal pigment epithelial cells (hRPE) (194987) were obtained from Lonza (Walkersville, MD). Dulbecco's Modified Eagle Medium (DMEM/F-12) (11330-057), heat-inactivated fetal bovine serum (HI-FBS) (10438-026) and Penicillin-Streptomycin (15140122) were purchased from Life Technologies (Grand Island, NY). HP X-tremeGene transfection agent (06366244001) was obtained from Roche (Indianapolis, IN), recombinant human IL-1α (200-LA-010) from R&D Systems (Minneapolis, MN), and Bay 11–7082 inhibitor (196870) from EMD Millipore (Billerica, MA). Anti-IL-18 antibody (ab137664), anti-IL-6 antibody (ab32530), and anti-TATA binding protein (TBP) (51841) were obtained from Abcam (Cambridge, MA). Anti-IL-1β antibody (MAB201), anti-IL-8 antibody (MAB208), and anti-STING (MAB7169) were purchased from R&D Systems. Anti-caspase-1 (sc-515) was purchased from Santa Cruz Biotechnology (Dallas, TX). Anti-β-tubulin antibody (2128), anti-A20 (5630) and HRP-linked secondary antibodies (7074S, 7076S) were obtained from Cell Signaling Technology (Danvers, MA). ELISA kits for IL-1β (DLB50), IL-18 (7620) and IL-8 (D8000C) were purchased from R&D. Taq Polymerase (M0267S) was purchased from New England Biolabs (Ipswich, MA).

Cell Culture

ARPE-19 cells were maintained in DMEM/F-12 medium supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. THP-1 cells were grown in RPMI-1640 medium supplemented with 10% HI-FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 50 μM 2-mercaptoethanol. All cells were grown in humidified 5% CO2 at 37°C and passaged every 2–3 days.

Preparation of mtDNA

Total DNA was extracted from ARPE-19 cells using QIAamp DNA mini kit (51304) from Qiagen (Valencia, CA) according to the manufacturer's protocol. From the extracted DNA, specific mtDNA fragments were amplified by PCR on Bio-Rad BiCycler (Hercules, CA) using primers and methods from a published protocol[37]. The PCR products were run on a gel to verify specificity before being purified using a PCR purification kit (28104) from Qiagen. The concentration of DNA was then measured using NanoDrop 2000 (ThermoFisher Scientific, Waltham, MA). Total DNA template was undetectable in the final elute as determined by blank controls. To generate oxidized mtDNA, 8-oxo-dGTP (N-2034) from TriLink Biotechnologies (San Diego, CA) was added to the PCR reaction mixture at the same concentration as the other nucleotides. To generate FAM-tagged mtDNA, FAM-tagged primers were used. All primers were ordered from the Massachusetts General Hospital DNA core facility (Boston, MA).

mtDNA Transfection into Cells

ARPE-19 cells were seeded in 24-well plates at a density of $3x10^4$ cells/well in complete DMEM/F12 medium. The following day, IL-1α was added at 5ng/mL to prime the cells. 24 hours later, culture medium was changed to antibiotic-free DMEM/F12 medium supplemented with 1% FBS, and cells were transfected at 70–80% confluence with 1μg/mL mtDNA using HP X-tremeGene agent at a ratio of 1:1 according to the manufacturer's protocol. Transfection agent alone was used as a control for all experiments. 48 hours later, culture media were collected and cells lysed with NP40 lysis buffer (Invitrogen) supplemented with protease inhibitors (Complete Mini, Roche). For THP-1 experiments, 1.5 x 10^6 cells were seeded in each well of a 6-well plate in complete RPMI medium supplemented with 200 nM PMA to allow differentiation into macrophages. 24 hours later, medium was renewed and LPS added at 500ng/mL to prime the cells. 3 hours later, medium was changed to antibiotic-free RPMI medium and transfection of mtDNA was performed as per above. 24 hours later, culture media and cell lysates were collected as per above.

Western Blot

Total protein concentration was determined by DC Protein Assay (Bio-Rad, Philadelphia, PA, US). Equal amounts of total protein (for cell lysates) or culture medium (for secreted cytokines) were loaded on a NUPAGE 4–12% bis-tris polyacrylamide gel (Life Technologies, Grand Island, NJ) and subjected to electrophoresis. The proteins were transferred to a PVDF membrane (Millipore, Billerica, MA), and the membrane was subsequently blocked with non-fat milk and incubated with primary antibodies against IL-8 and capsase-1 at 1:500; IL-18, A20, and TBP at 1:1000; IL-1β STING, IL-6 and β-tubulin at 1:2000. The membrane was then washed and incubated with secondary antibodies conjugated to horseradish peroxidase (Cell Signaling Technology) at 1:10000. The membrane was developed with enhanced chemiluminescence (ECL Select) (GE Healthcare, Wauwatosa, WI). The intensity of protein bands was quantified using the software Image Lab 4.1 (Bio-Rad).

mtDNA Tracking

ARPE-19 cells were seeded on EZ Slides (PEZGS0416, Millipore) at $5x10^4$ cells/well in antibiotic-free medium and allowed to settle overnight. The following day, cells were transfected with 1μg/mL of FAM-tagged mtDNA using HP X-tremeGene agent at a ratio of 1:1 according to the manufacturer's protocol. 24 hours later, cells were carefully washed with PBS before fixation in 4% PFA for 5 minutes. Afterwards, cells were washed again with PBS and mounted using Vectashield mounting medium with DAPI (H-1200) (Vector Laboratories, Burlingame, CA). Slides were visualized on Zeiss Axio Imager M2.

MTT Viability Assay

ARPE-19 cells were transfected with mtDNA in a 96-well plate under the same conditions outlined above. 48 hours later, cells were washed with PBS and incubated for 3 hours with 0.5 mg/mL MTT (Life Technologies) dissolved in PBS. The formed formazan crystals were dissolved by adding 100μL of acidified (0.04 N HCl) isopropanol (Sigma-Aldrich, St. Louis, MO) to each well. Absorbance was read at 590nm using the SpectraMax190 Microplate Reader (Molecular Devices, Sunnyvale, CA).

siRNA experiments

For STING knockdown verification, ARPE-19 cells in 6-well plates were transfected at 70–80% confluence with 20nM of either STING siRNA (Qiagen) or control siRNA in antibiotic-free DMEM/F12 medium using RNAiMAX (Invitrogen) according to the manufacturer's protocol. 48 hours later, medium was renewed and the same treatment was repeated for 48h, after which cell lysates were collected and immunoblotted for STING. For STING knockdown experiments, ARPE-19 cells were seeded in 6-well plates at a density of $2x10^5$ cells/well in antibiotic-free medium. The following day, they were primed with 5 ng/mL IL-1α and transfected with 20nM of STING or control siRNA for 48 hours using the RNAiMAX protocol. Medium was then renewed and mtDNA (1μg/mL) and siRNA (20nM) were co-transfected using HP X-tremeGene reagent at a 1:1 ratio as per manufacturer's instructions. 48 hours later, culture media and cell lysates were collected for analysis.

p65 NF-κB nuclear translocation

ARPE-19 cells were transfected with mtDNA in 100mm dishes under the same conditions outlined above. 24 hours later, nuclei were extracted using a kit (40010) (Active Motif, Carlsbad, CA) and proper nuclear fractionation was verified by western blot with TATA binding protein and b-tubulin. p65 NF-κB was quantified in the nuclear extracts using TransAM NF-κB ELISA (43296) (Active Motif) after compensation for total protein concentration.

Caspase-1 activity

ARPE-19 cells were transfected with mtDNA in glass-bottom 24-well plates under the same conditions outlined above. 24 hours later, FAM-YVAD-FMK (#97) (Immunocytochemistry technologies, Bloomington, MN), a probe specific to caspase-1, was added at the concentration recommended by the manufacturer. After a 2-hour incubation at 37°C and 5%

CO2, Hoechst was added to the wells at 1μg/mL and cells were reincubated for 10 minutes. Cells were washed carefully with PBS and visualized using Zeiss AxioObserver 21.

Statistical analysis

All experiments were performed in triplicate unless otherwise noted. Statistical analysis was performed on JMP Pro software version 11.2.0 from SAS (Cary, NC). Results are expressed as mean ± SEM. Statistical significance for differences between two treatment groups was determined with Student's t-test. One-way ANOVA with Tukey post-hoc correction was used for multiple group comparisons. A p-value of < 0.05 was considered statistically significant.

Results

mtDNA induces IL-6 and IL-8 secretion from ARPE-19

To determine the effect of mtDNA on IL-6 and IL-8 secretion from ARPE-19 cells, a 2kb fragment of mtDNA was amplified by PCR and transfected into ARPE-19 cells. IL-6 and IL-8 were measured in the culture medium 48h post-transfection by western blot. Figure 1A shows that mtDNA induced a 4-fold and a 2-fold increase in the secretion of IL-6 ($p<0.001$) and IL-8 ($p<0.01$), respectively, compared to a transfection agent control. In contrast, neither cytokine was induced when mtDNA was used without transfection agent. This suggests that IL-6 and IL-8 are induced by intracellular mtDNA rather than extracellular mtDNA binding to a cell surface receptor. To confirm this, labeled mtDNA was generated by PCR using FAM-tagged primers, and ARPE-19 cells were treated for 24h with labeled mtDNA with or without transfection agent. Figure 1B shows mtDNA being present in an intracellular cytoplasmic location only when combined with a transfection agent. This finding, in conjunction with the previous result (Fig. 1A), indicates that only intracellular mtDNA can induce IL-6 and IL-8 secretion. To assess whether the observed cytokine response was due to cell death, cell viability was determined using an MTT assay at 48h post-transfection with the same mtDNA dose. mtDNA did not cause significant cell death compared to a transfection agent control, indicating that mtDNA induces IL-6 and IL-8 secretion rather than merely causing cell death and release of cytokines.

Oxidized and larger mtDNA fragments induce a stronger cytokine response

To determine whether the sequence of the mtDNA fragment has any effect on cytokine induction, four 2-kb fragments of mtDNA with different sequences were transfected into ARPE-19 cells. They were found to produce similar inductions of IL-6 and IL-8 (Fig. 2A), indicating that mtDNA induces IL-6 and IL-8 in a sequence-independent fashion. Since AMD has been associated with impaired autophagy[38] and RPE death[39], mtDNA under such conditions may be fragmented and accumulate in the cell in different sizes[40]. Therefore, to determine the effect of the size of mtDNA on IL-6 and IL-8 induction, 3 mtDNA fragments of different sizes were generated by PCR and transfected into ARPE-19 cells. The result shows that larger mtDNA fragments induced stronger IL-6 and IL-8 responses (Fig. 2B). Furthermore, mtDNA has been found to be more oxidized in AMD retinas compared to age-matched controls[41]. To mimic the in vivo situation, oxidized mtDNA was generated by PCR incorporating the modified nucleotide 8-oxoguanine and transfected into ARPE-19 cells.

This resulted in a stronger IL-6 and IL-8 secretion compared to non-oxidized mtDNA (Fig. 2C). Finally, the time course and dose response were evaluated, and both IL-6 and IL-8 were found to be induced in a time and dose-dependent fashion (Fig. 2D and 2E).

mtDNA induces IL-6 and IL-8 secretion through STING

STING, an endoplasmic reticulum transmembrane protein, has been shown to be important in the immune response to foreign DNA in the context of infection, and self DNA in the context of autoimmunity[42]. Our experiments show that it is expressed in ARPE-19 cells and even more so in primary human RPE cells (Fig. 3A), and this expression can be successfully knocked down in ARPE-19 cells with siRNA specific to STING (Fig. 3B). To explore whether STING is involved in mtDNA-induced IL-6 and IL-8 secretion, mtDNA was transfected into ARPE-19 cells where STING had been knocked down with siRNA. The result shows that STING knockdown partially abrogated mtDNA-induced IL-6 (Fig. 3C) and IL-8 (Fig. 3E) secretion, indicating that STING mediates the induction of IL-6 and IL-8 secretion by mtDNA.

mtDNA induces IL-6 and IL-8 secretion via NF-κB

Because the NF-κB pathway has been recently shown to be activated by STING [43,44], we sought to determine whether it is also activated by mtDNA. The levels of NF-κB subunit p65 were measured by ELISA in nuclear isolates of ARPE-19 cells 24h post-transfection with mtDNA. Figure 4B shows that mtDNA induces p65 nuclear translocation 1.4-fold ($p<0.01$). mtDNA also induces the expression of A20 (Fig. 4C), a marker of NF-κB activation. These results together indicate that mtDNA activates the NF-κB pathway. To test whether NF-κB mediates the IL-6 and IL-8 induction by mtDNA, ARPE-19 cells were briefly pre-incubated with different concentrations of an NF-κB inhibitor (Bay 11–7082) before mtDNA transfection. This led to a dose-dependent abrogation of mtDNA-induced IL-6 and IL-8 secretion (Fig. 4D), indicating that the NF-κB pathway is involved in mediating IL-6 and IL-8 induction by mtDNA.

mtDNA primes the NLRP3 inflammasome through STING and NF-κB

Since the NLRP3 inflammasome is primed by NF-κB[34,45,46], we hypothesized that mtDNA could prime the NLRP3 inflammasome. To test this hypothesis, the levels of the pro forms of IL-1β, IL-18, and caspase-1 in ARPE-19 cells transfected with mtDNA were analyzed by western blot. The result shows that mtDNA upregulates pro-IL-1β 2.4-fold ($p<0.05$) and pro-caspase-1 1.4-fold ($p<0.05$) compared to control (Fig. 5A), indicating that mtDNA primes the NLRP3 inflammasome. In contrast, mtDNA did not change the level of pro-IL-18 (Fig. 5A). To test whether the pro-IL-1β induction is mediated by STING and NF-κB, the same inhibition strategy used previously was followed. The result shows that mtDNA induces pro-IL-1β via STING, as evidenced by abrogation of the induction with STING knockdown (Fig. 5B). Also, NF-κB inhibition dose-dependently abrogated the induction (Fig. 5C), indicating that mtDNA induces pro-IL-1β through the NF-κB pathway. Together these findings indicate that mtDNA primes the NLRP3 inflammasome through a STING/NF-κB pathway.

mtDNA and the NLRP3 inflammasome in the RPE

To determine whether mtDNA also activates NLRP3, we performed ELISA for mature IL-1β and IL-18 on the culture medium of ARPE-19 48h post-transfection with mtDNA. Figure 6A shows that, in ARPE-19 cells, mtDNA induces secretion of IL-1β 5.9-fold compared to control ($p<0.01$, $n=4$), but not IL-18. Although these ELISA assays are designed to detect the mature forms of IL-1β and IL-18, the pro forms of these cytokines do cross-react if present in high quantities. For this reason, western blotting was performed yet repeatedly failed to detect any mature form in either cell lysates or culture media. This is not due to technical difficulties on our part since we could detect the mature form of IL-1β in THP-1 cells after mtDNA transfection (Suppl. Fig). The major caspase involved in the maturation of IL-1β is caspase 1. Its activity was assessed in ARPE-19 cells 24h after mtDNA transfection using FAM-YVAD-FMK probe, a molecule that fluoresces when bound to active caspase-1. Imaging showed that cells transfected with mtDNA had increased FAM-YVAD-FMK signal (Fig. 6B), suggesting potential activation of caspase-1 by mtDNA in ARPE-19 cells.

Discussion

AMD is associated with a number of risk factors, including age, smoking, obesity and cardiovascular disease. Despite the uncertainty surrounding the pathophysiology of this disease, the last decade has seen increasing evidence to support a role for mitochondrial dysfunction in its development[4–7]. Compared to age-matched controls, RPE cells of AMD patients have been reported to have a decline in the number and structural integrity of mitochondria[8] as well as a decreased content of electron transport chain proteins[47], indicating impaired mitochondrial biogenesis even at early stages of AMD. Furthermore, damage to mtDNA has been documented in the RPE of AMD patients even before vision loss occurs[5–7], suggesting the potential benefit of early interventions to rescue mitochondrial function in slowing down disease progression. Recent work has also identified a role not only for damaged mtDNA but also for inherited mitochondrial DNA variants[48]. In that study Kenney et al found that J and H cytoplasmic hybrids have significantly altered expression of several nuclear genes involving alternative complement, inflammation and apoptosis pathways. The same group in a separate study[49] has also found that mtDNA variants mediate energy production and expression levels for CFH, C3 and EFEMP1, genes known to be involved in AMD[50–57]. Since altered mitochondrial function is not only present but also likely contributory to AMD progression, we postulated release of inflammatogenic mtDNA as a potential mechanism for disease perpetration.

In fact, a wealth of evidence supports a role for inflammation in the development and progression of AMD[35,58]. Specifically, clinical and genetic studies have revealed the importance of IL-6 and IL-8 in the pathogenesis of AMD. IL-6 is a pleiotropic inflammatory cytokine that can exaggerate immune and inflammatory responses, and its level has been associated with AMD onset[24] and progression[22]. IL-8 is a member of the CXC chemokine family and a potent chemoattractant for neutrophils and monocytes, and IL-8 haplotypes have been found to confer an increased risk of AMD[21,23,25]. In our study, intracellular mtDNA induced IL-6 and IL-8 secretion from ARPE-19 cells, suggesting that mtDNA can

potentially contribute to the inflammatory component of AMD. Both interleukins studied have also been associated with wet AMD, as they have been correlated with CNV and macular thickness in exudative AMD[27,28]. Furthermore, both cytokines are pro-angiogenic and induce VEGF[59–62], a crucial factor in CNV development. In aged RPE cells with compromised mitochondrial integrity, mtDNA molecules that leak into the cytosol may therefore potentiate a local inflammatory microenvironment that furthers progression of AMD and CNV. This phenomenon may be even more prominent in situations where mtDNA degradation is impeded, such as impaired autophagy in the RPE. The latter phenomenon has been associated with AMD[38], and it could cause accumulation of damaged organelles and molecules in the RPE, including mtDNA. Our experiments show that larger mtDNA fragments and higher mtDNA doses cause more IL-6 and IL-8 secretion. These findings suggest that RPE cells in AMD may be even more susceptible to mtDNA-induced damage due to their declining autophagic function and an inability to break down mtDNA into its basic nucleotide constituents.

Oxidative stress has also been implicated in aging and AMD[63], and part of the evidence comes from genetic studies that showed an association between antioxidant enzyme gene polymorphisms and AMD[64,65]. Furthermore, mitochondria are the main cellular source of oxidative stress[66], and mtDNA has been found to be oxidized in AMD retinas[41]. In our study, oxidized mtDNA induced a stronger IL-6 and IL-8 response compared to non-oxidized mtDNA. This suggests that mtDNA may be even more inflammatogenic in AMD patients by virtue of its oxidized status, worsening a vicious cycle of inflammation and damage that progressively leads to mitochondrial failure and death of the RPE. This phenomenon may be particularly pronounced in AMD variants with compromised anti-oxidative defense mechanisms[64,65]. Although our findings indicate that induction of IL-6 and IL-8 was sequence-independent, we cannot exclude the importance of nucleotide composition, especially in oxidative situations where differential nucleotide susceptibility to oxidation will translate into different overall oxidative charges across fragments with different nucleotide constitutions. Studying this phenomenon would also require the use of smaller mtDNA fragments where dissimilar nucleotide compositions are more likely to be found.

Extracellular mtDNA had no effect on either IL-6 or IL-8 secretion, at least within the resolving capacity of western blot. This could be explained by a lack of surface receptors for mtDNA on ARPE-19 cells. Practically all the DNA sensors described to date are intracellular[36], including Toll-like receptor 9 (TLR9), a transmembrane receptor of unmethylated CpG motifs that has been confirmed to be predominantly intracellular rather than on the cell membrane[67,68], including in ARPE-19[69]. Our findings are also in concordance with previous evidence that extracellular mtDNA has no immunostimulatory effect unless used with a molecule that aids its uptake into the cell[20,70]. In other studies, extracellular mtDNA was mildly immunostimulatory in certain cell types, but only at very high concentrations (>10μg/mL)[13,20]. In ARPE-19 cells, extracellular CpG DNA has been shown to induce IL-8 secretion only at the dose of 84μg/mL, almost 2 orders of magnitude higher than the concentration we used, and the authors attributed the effect to CpG DNA that had been internalized by phagocytosis[69].

Although it remains unclear which DNA sensors are involved in sensing intracellular mtDNA, we have identified STING as an important downstream mediator of the inflammatory pathways triggered by mtDNA in ARPE-19 cells as evidenced by STING knockdown experiments. This transmembrane endoplasmic reticulum protein has been clearly demonstrated to take center stage in the response to intracellular foreign and self-DNA[36,42]. Unlike DNA sensors, which exhibit overlapping and redundant functions, STING seems to constitute a common downstream node to most DNA-sensing pathways, a property that might be advantageous in therapeutic efforts to modulate the innate immune defenses of the cell. Our study extends the importance of STING to RPE cells in their inflammatory response to mtDNA. To our knowledge, this is the first report of STING or any DNA sensing function in RPE cells. Such knowledge may prove valuable in understanding the consequences of insufficient autophagy in the face of the high metabolic demands that constantly weigh on the aging RPE. Once activated, STING can in turn stimulate NF-κB[43,44]. Our findings indicate that mtDNA can indeed activate this latter pathway. NF-κB blockade has been shown to inhibit choroidal neovascularization[71,72], highlighting the importance of NF-κB in the progression of AMD. NF-κB also regulates the expression of many genes in RPE cells, including IL-6 and IL-8[73,74], and this is corroborated by our experiments of NF-κB inhibition abrogating the induction of cytokines.

Recent studies have shown NF-κB to be critical for priming of the NLRP3 inflammasome in RPE cells[75]. The NLRP3 inflammasome has recently received widespread attention for its possible association with both dry and wet AMD[31–33], and several stimuli have been shown to trigger its activation in ARPE-19 cells[76–80]. We found that mtDNA primes but does not conclusively activate the NLRP3 inflammasome through a STING/NF-κB pathway as evidenced by upregulation of the pro forms of IL-1β and caspase-1 but minimal upregualtion of the mature form. Pro-IL-18 appeared to be constitutively expressed in our cells, which is in accordance with previous findings in ARPE-19[76,81] and other cell types[34]. Our data suggests a potential low-level activation of the NLRP3 inflammasome by mtDNA in ARPE-19 cells as evidenced by secretion of low levels of mature IL-1β to the culture medium and increased FAM-YVAD-FMK signal. It should be noted however that concerns about the specificity of the latter probe have been raised[82]. More importantly, because of differences and serious deficiencies in AMD experimental models, there is still no consensus as to whether NLRP3 activity is detrimental or protective in the first place. One study suggested a protective effect on CNV through the anti-angiogenic effect of IL-18 using an acute laser-injury CNV model[33], whereas another study suggested a detrimental effect through IL-1β using a VEGF-A-overexpressing mouse[31]. The role of these cytokines in the pathogenesis of AMD remains unclear. Our data unfortunately cannot argue one way or another. The low-level inflammasome activation that we observed could be compensatory and protective, or detrimental, or even just a “spillover” of overall inflammation. In addition, NLRP3 activation in RPE cells is orders of magnitude less than that of inflammatory cells, and it still remains to be answered if a chronic low-level activation of the inflammasome in RPE cells could play a role in the evolution of AMD. One can argue that in contrast to acute inflammatory processes where highly potent inflammatory cells may be needed, AMD and other chronic diseases such as atherosclerosis are characterized by a state of para-inflammation[83,84], a term used to describe a response to stress that is intermediate between

the basal state and inflammation. In our study, we found that mtDNA could induce secretion of the mature form of IL-1β in THP-1 cells[85], a macrophage cell line, indicating that mtDNA can activate NLRP3 in macrophages, consistent with previous reports in bone-marrow-derived macrophages[29,30]. This suggests a role for aged macrophages in licensing NLRP3 activation within the retinal microenvironment when exposed to their own accumulated mtDNA or mtDNA that has been phagocytized from dying retinal cells such as the RPE. Moreover, previous evidence of a direct physical interaction between mtDNA and NLRP3[30] makes the applicabiliy of NLRP3 activation by mtDNA in other cell types more plausible, at least conceptually.

In summary, this study elucidates the pro-inflammatory properties of mtDNA in ARPE-19 cells and suggests inflammation as a plausible mechanistic link between mitochondrial damage and AMD, one in which accumulating inflammatogenic mtDNA in aged RPE cells can create an inflammatory microenvironment that aids in the development and progression of AMD. Our findings also suggest STING and NF-κB as interesting therapeutic targets to explore in the future for the purpose of modulating innate immune defenses in the aging RPE.

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Highlights

- mtDNA induces IL-6 and IL-8 secretion from ARPE-19 cells via a STING/NF-kB pathway
- Oxidized and larger mtDNA is more immunostimulatory
- mtDNA primes and potentially activates the NLRP3 inflammasome in ARPE-19 cells

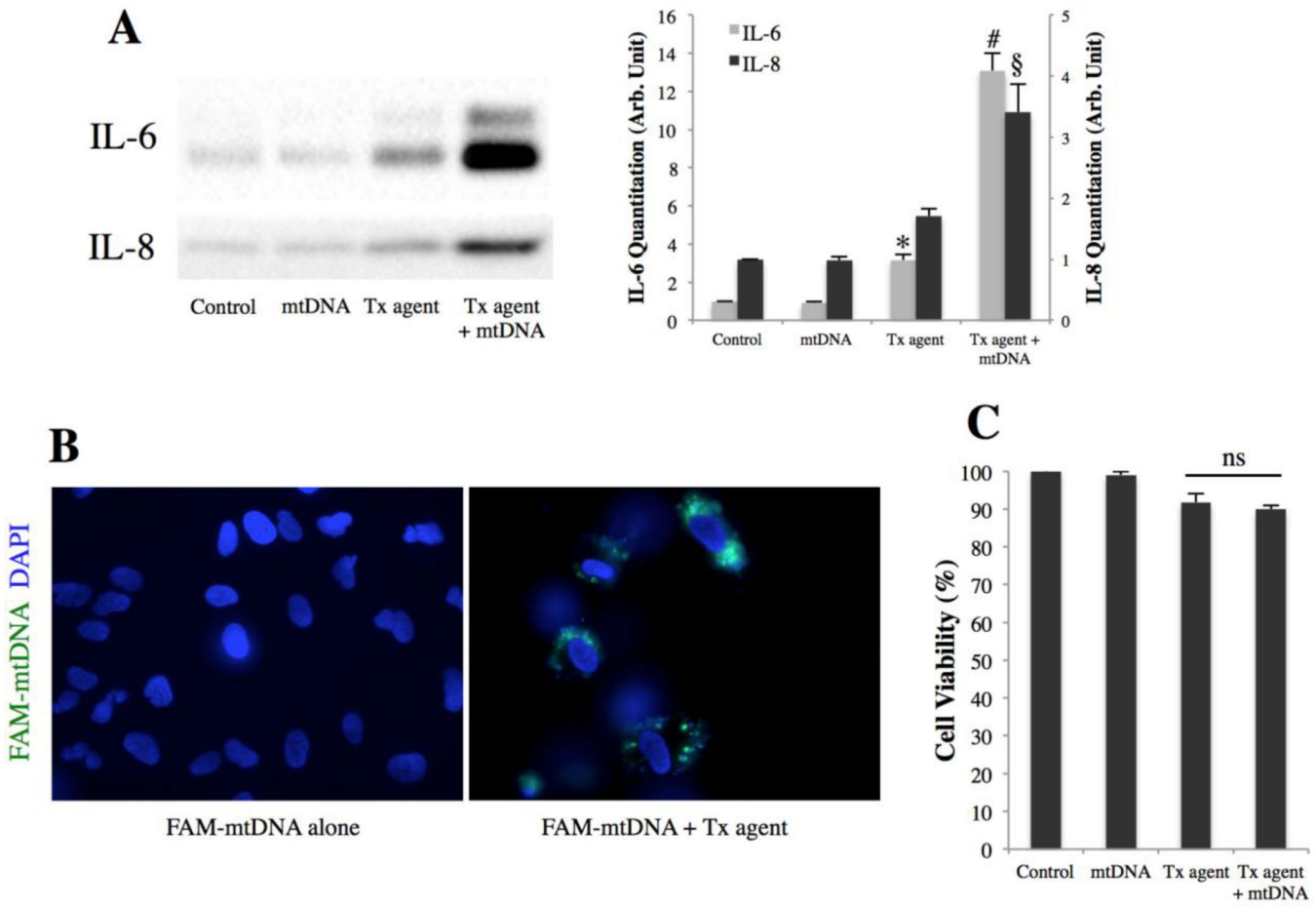

Figure 1. mtDNA induces IL-6 and IL-8 secretion from ARPE-19 cells

(A) IL-6 and IL-8 secretion from ARPE-19 as determined by western blot of culture media 48h post-transfection with 1μg/mL mtDNA. Right, quantitation by densitometry. *$p < 0.05$ versus control and mtDNA groups; #$p < 0.001$ versus all other groups; §$p < 0.01$ versus all other groups.

(B) ARPE-19 cells 24h after treatment with FAM-tagged mtDNA with or without transfection agent. mtDNA in green, DAPI in blue.

(C) ARPE-19 viability determined by MTT 48h post-transfection with 1μg/mL mtDNA.

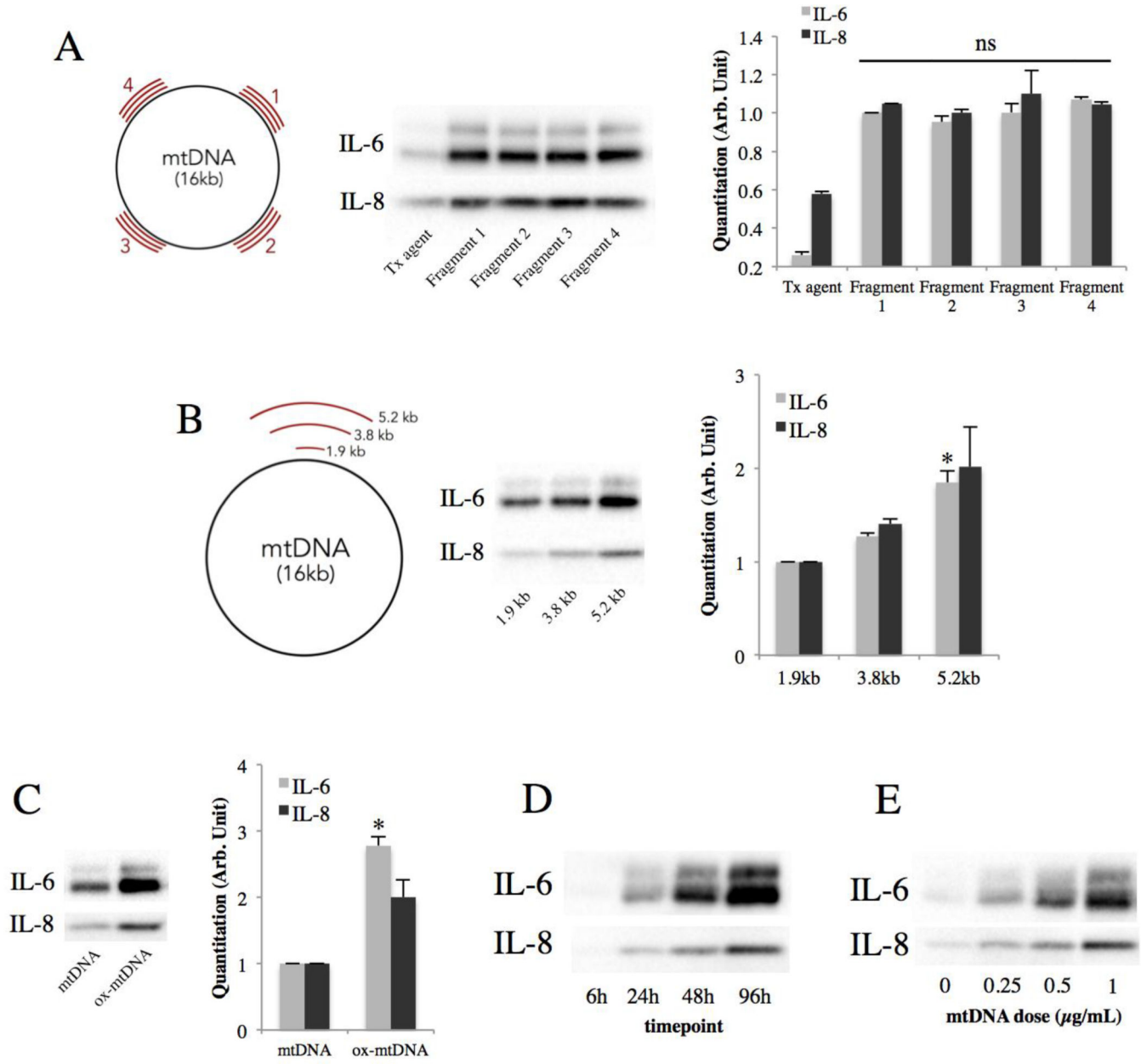

Figure 2. Oxidized and larger mtDNA fragments induce a stronger cytokine response

(A) IL-6 and IL-8 secretion from ARPE-19 cells 48h post-transfection with 1μg/mL of 2-kb mtDNA fragments of different sequences but similar sizes (schematic). Right, quantitation by densitometry.

(B) IL-6 and IL-8 secretion from ARPE-19 cells 48h post-transfection with 1μg/mL of mtDNA fragments of different sizes (schematic). Right, quantitation by densitometry. *$p < 0.05$ versus all other groups.

(C) IL-6 and IL-8 secretion from ARPE-19 cells 48h post-transfection with 1μg/mL of mtDNA or oxidized mtDNA. Right, quantitation by densitometry. *$p < 0.01$ versus control.

(D) Time course of IL-6 and IL-8 induction by 1μg/mL of mtDNA.

(E) Effect of mtDNA dose on IL-6 and IL-8 secretion at 48h.

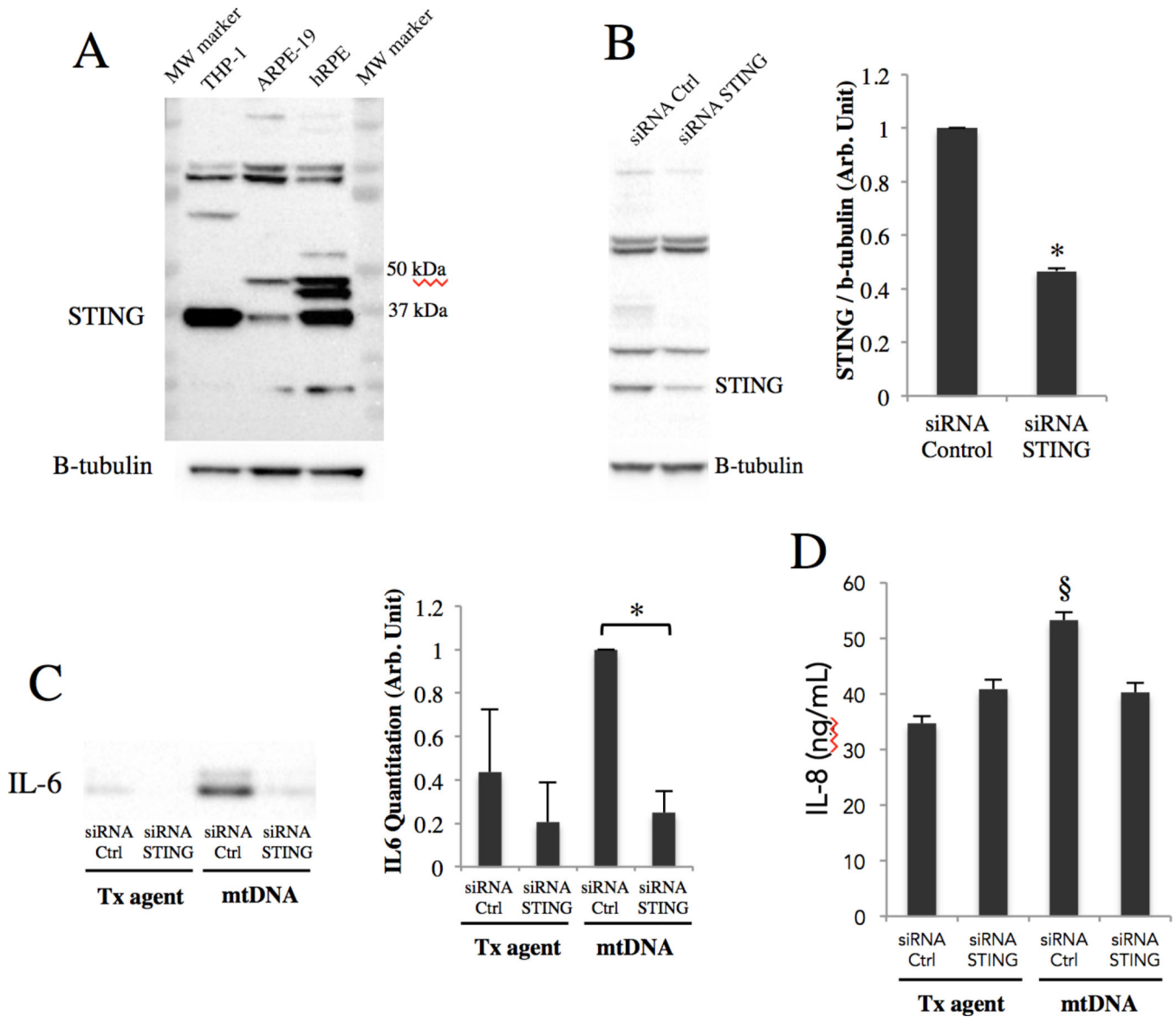

Figure 3. mtDNA induces IL-6 and IL-8 secretion through STING

(A) STING immunoblotting of untreated ARPE-19 and primary human RPE lysates alongside a THP-1 lysate (positive control for STING). 40ug of total protein loaded per lane. Prominent band seen at STING's predicted molecular weight (≈40kDa).

(B) ARPE-19 cells were treated for a total of 96h with 20nM of either STING siRNA or control siRNA and lysates immunoblotted with STING monoclonal antibody. Right, quantitation by densitometry. *$p < 0.001$ versus control siRNA.

(C&D) Along with mtDNA transfection, ARPE-19 cells were pre and co-treated with STING or control siRNA, and culture media were collected at 48h and subjected to western blot for IL-6 (C) and ELISA for IL-8 (D), as western blot did not provide sufficient resolution to study differences in IL-8 levels. *$p < 0.05$; §$p < 0.01$ versus all other groups.

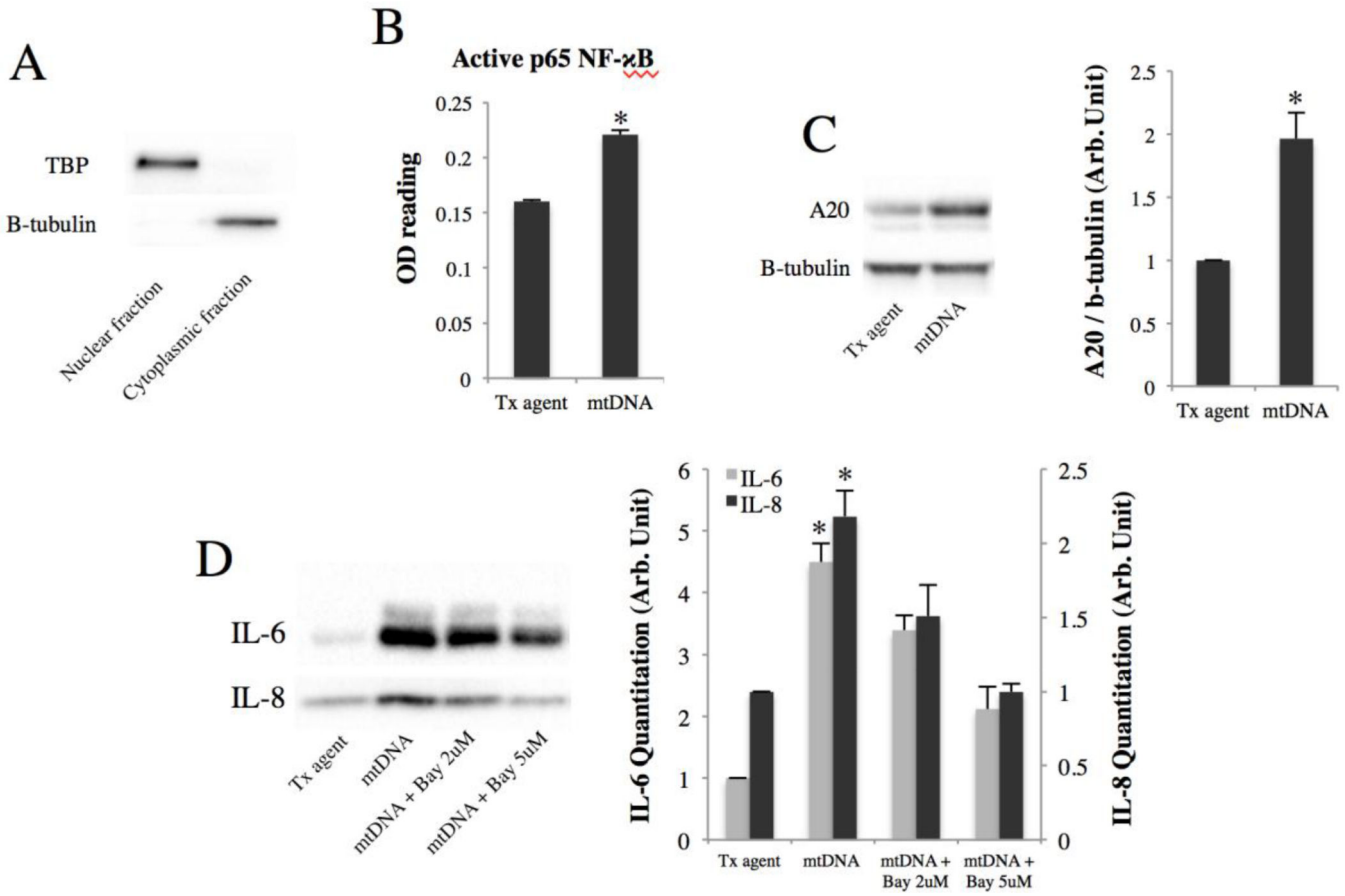

Figure 4. mtDNA induces IL-6 and IL-8 secretion via NF-κB

(A) Nuclear fractionation efficiency verified with tata-binding protein (TBP) and b-tubulin.

(B) p65 NF-κB ELISA performed on nuclear isolates of ARPE-19 cells 24h post-transfection with mtDNA. *$p < 0.01$ versus control.

(C) A20 expression level 48h post-transfection of mtDNA. Right, quantitation by densitometry. *$p < 0.05$ versus control.

(D) ARPE-19 cells were pre-incubated for 30 minutes with an irreversible NF-κB inhibitor (Bay 11–7082) before mtDNA transfection. After 48h, culture media were collected and immunoblotted for IL-6 and IL-8. Right, quantitation by densitometry. *$p < 0.05$ versus control and [mtDNA + Bay 5uM] groups.

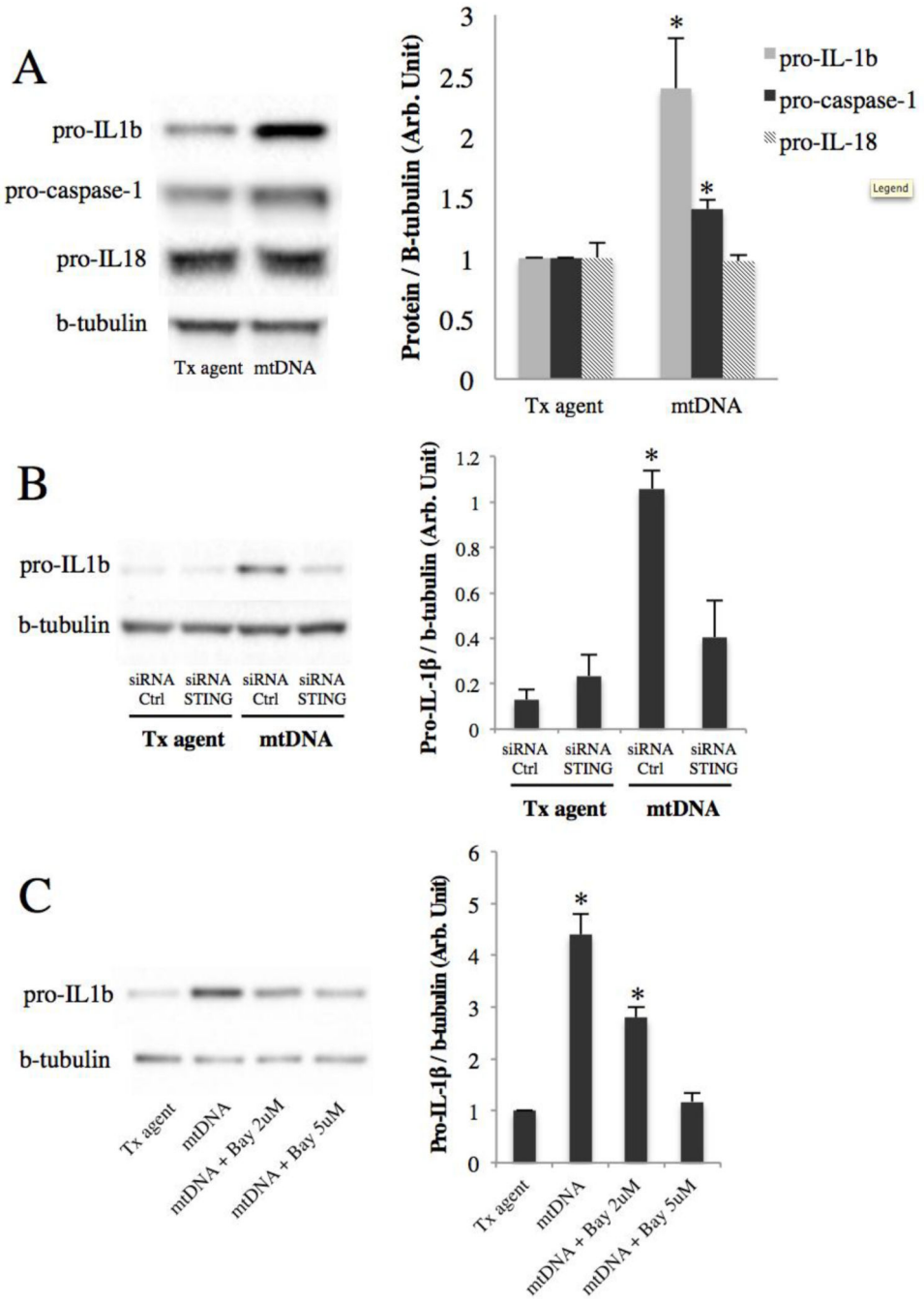

Figure 5. mtDNA primes the NLRP3 inflammasome through STING and NF-κB

(A) Immunoblots of ARPE-19 lysates 48h after mtDNA transfection. Right, quantitation by densitometry. *$p < 0.05$ versus control.

(B) Along with mtDNA transfection, ARPE-19 cells were pre and co-treated with STING or control siRNA, and lysates were collected at 48h and immunoblotted for pro-IL-1β. Right, quantitation by densitometry. *$p < 0.05$ versus all other groups.

(C) ARPE-19 cells were pre-incubated for 30 minutes with an irreversible NF-κB inhibitor (Bay 11–7082) before mtDNA transfection. After 48h, lysates were collected and

immunoblotted for pro-IL-1β. Right, quantitation by densitometry. *$p < 0.05$ versus all other groups.

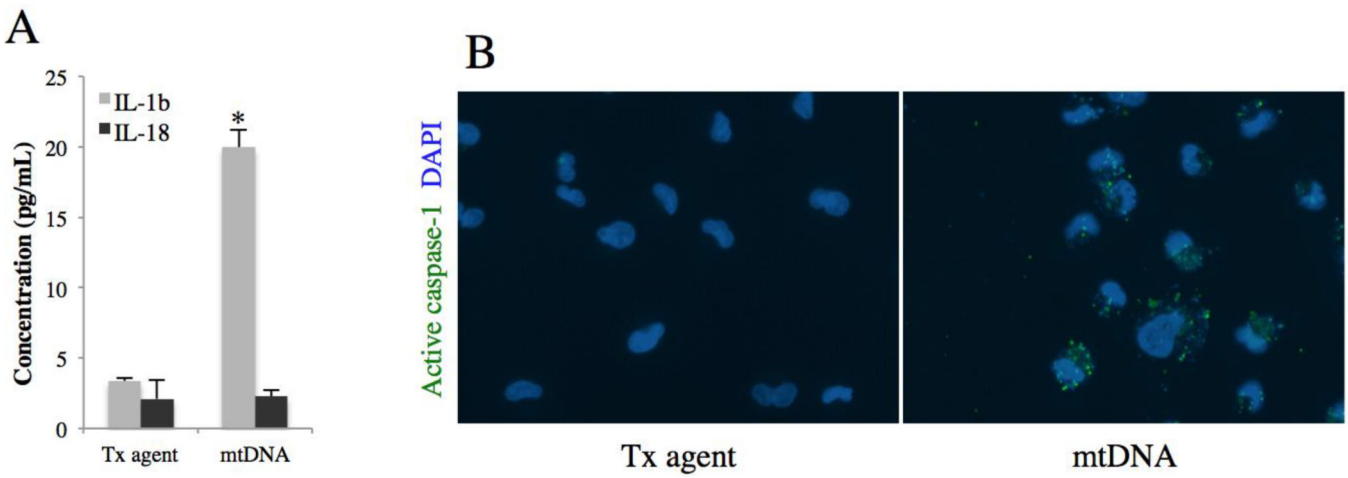

Figure 6. mtDNA and the NLRP3 inflammasome in the RPE

(A) IL-1β and IL-18 ELISA on culture media of ARPE-19 cells 48h post-transfection with mtDNA. Results are mean ± SEM, n=4. *p < 0.01 versus control.

(B) ARPE-19 caspase-1 activity assessed 24h after mtDNA transfection using fluorescent FLICA probe FAM-YVAD-FMK.