Full Length Article

Mitochondrial respiratory capacity modulates LPS-induced inflammatory signatures in human blood

Kalpita Rashmi Karan, Caroline Trumpf, Marlon A. McGill, Jacob E. Thomas, Gabriel Sturm, Vincenzo Lauriola, Richard P. Sloan, Nicolas Rohleder, Brett A. Kaufman, Anna L. Marsland, Martin Picard.

ABSTRACT

Mitochondria modulate inflammatory processes in various model organisms, but it is unclear how mitochondrial behavior modulates LPS-induced inflammatory cytokines. Here, we examine the effect of i) experimental perturbations of mitochondrial respiratory chain function, and ii) baseline inter-individual variation in leukocyte mitochondrial energy production capacity on stimulated cytokine release and glucocorticoid (GC) sensitivity. In a first cohort, whole blood from 20 healthy women and men was stimulated with increasing concentrations of the immune agonist lipopolysaccharide (LPS). Four inhibitors of mitochondrial respiratory chain Complexes I, III, IV, and V were used (LPS + Mito-Inhibitors) to acutely perturb mitochondrial function, GC sensitivity was quantified using the GC-mimetic dexamethasone (DEX) (LPS + DEX), and the resultant cytokine signatures mapped with a 20-cytokine array. Inhibiting mitochondrial respiration caused large inter-individual differences in LPS-stimulated IL-6 reactivity (Cohen’s d = 0.72) and TNF-α (d = 1.55) but only minor alteration in EC50-based LPS sensitivity (d = 0.21). Specifically, inhibiting mitochondrial Complex IV potentiated LPS-induced IL-6 levels by 13%, but inhibited TNF-α induction by 72%, indicating mitochondrial regulation of the IL-6/TNF-α ratio. As expected, DEX treatment suppressed multiple LPS-induced pro-inflammatory cytokines (IFN-γ, IL-6, IL-8, IL-1β), TNF-α by >85% and increased the anti-inflammatory cytokine IL-10 by 80%. Inhibiting Complex I potentiated DEX suppression of IL-6 by a further 12% (d = 0.73), indicating partial mitochondrial modulation of glucocorticoid sensitivity. Finally, to examine if intrinsic mitochondrial respiratory capacity may explain a portion of immune reactivity differences across individuals, we measured biochemical respiratory chain enzyme activities and mitochondrial DNA copy number in isolated peripheral blood mononuclear cells (PBMCs) from a second cohort of 44 healthy individuals in parallel with LPS-stimulated IL-6 and TNF-α response. Respiratory chain function, particularly Complex IV activity, was positively correlated with LPS-stimulated IL-6 levels (r = 0.45, p = 0.002). Overall, these data provide preliminary evidence that mitochondrial behavior modulates LPS-induced inflammatory cytokine signatures in human blood.

1 Introduction

Chronic inflammation is a hallmark of multiple health disorders that challenge modern medicine (Rea et al., 2018; Ferrucci and Fabbri, 2018). Experimental pre-clinical studies indicate that pro-inflammatory cytokines, especially when their levels are chronically elevated, may directly contribute to disease onset or progression, particularly for heart disease (Hann et al., 1998), sepsis (van der Poll et al., 2017), neurodegeneration (Chitnis and Weiner, 2017), autoimmune disorders like rheumatoid arthritis (Panga and Raghunathan, 2018), and the aging process.
.process itself (Rea et al., 2018). Inflammation can also be acutely induced by psychosocial stress (Marsland et al., 2017). But little is known about the basis for inter-individual differences in inflammatory reactivity – why do some individuals produce large amounts of pro-inflammatory cytokines, while in response to the same stimulus others exhibit more modest or qualitatively different immune responses? Although a fraction of the inter-individual variability in human immune response is attributed to genomic variation (Piarescia et al., 2018), evidence suggests that additional mechanisms must influence immune function, including cellular energetics (Breda et al., 2019).

Immune responses require a substantial rise in cellular energy demand supplied in large part by mitochondria (Hotamisligil, 2017), which can contribute to inflammatory responses in many ways. The rise in energy demand fuels a number of intracellular processes such as biosynthesis of macromolecules, gene expression, protein synthesis – including cytokines – and their exocytosis, metabolic reprogramming, and other signaling processes essential for the acquisition of specific immunological phenotypes (Fox et al., 2005). This includes pro- and anti-inflammatory macrophage differentiation (Huang et al., 2014) and lymphocyte activation (Chapman et al., 2020). Mitochondria also serve as a signaling platform for various innate immunological signaling pathways in macrophages and non-immune cells such as fibroblasts (Chandel, 2015; Garaude et al., 2016; Koshiba et al., 2011). Both innate and adaptive immune responses and their associated intracellular signaling pathways (West et al., 2011; Weinberg et al., 2015) are also under regulation of reactive oxygen species (ROS) generated by electron transfer within the respiratory chain Complexes I and III (Breda et al., 2019). On the other hand, Complex II is involved in activating macrophages via succinate (Tannahill et al., 2013) whereas Complex IV as a critical controller of oxygen flux can regulate anti-viral signaling (Zhao et al., 2012; Li et al., 2006). Mitochondria can also release immunogenic components including their mitochondrial DNA (mtDNA), small peptides, and ATP into the cytoplasm and extracellular space, which are sensed as “bacteria-like” damage associated molecular patterns (DAMPs) that engage canonical innate immune cascades (Meyer et al., 2018). The release of immunogenic circulating cell-free mtDNA (ccf-mtDNA) (Strahler et al., 2015; Boyapati et al., 2017) in response to acute psychosocial stress (Trumpff et al., 2019a, 2019b; Lindqvist et al., 2016) and elevated levels in psychopathology (Lindqvist et al., 2018) also implicates mitochondria in the stress-immune axis in humans. Studies have also found correlations between baseline pro-inflammatory cytokine levels and measures of cellular energetics (Boeck et al., 2018), mitochondrial disease and increased risk of sepsis in children (Eom et al., 2017), and between mitochondrial respiration and T-cell activation in a mouse model of mitochondrial disease (Tarasenko et al., 2017). But whether mitochondria in circulating immune cells influence immune reactivity to acute challenge has not been examined in healthy individuals.

To begin examining this question, we first systematically reviewed the literature for studies reporting associations between mitochondrial function and cytokine response in human health and disease. Our analysis showed that few studies provided indirect correlative evidence for some cytokines (e.g., TNF-α, IL-1β) but a lack of evidence for certain cytokines (e.g., IL-6, IFN-γ). The key findings from the systematic review are presented in Supplemental Table 1, highlighting the gap in knowledge about the influence of mitochondrial function on inflammatory cytokines in humans.

Here, we hypothesized that in healthy human blood: i) inhibiting mitochondrial respiration would exaggerate LPS-induced pro-inflammatory cytokine responses and alter multi-cytokine signatures, ii) mitochondrial function is necessary for glucocorticoid (GC) suppression of pro-inflammatory cytokines, and iii) baseline mitochondrial energy production capacity would in part explain the inter-individual differences in LPS-driven immune responses. Overall, this work provides initial evidence that mitochondria modulate different aspects of immune responses in human leukocytes.

2. Methods

2.1. Participants

For the main study (Cohort 1), a total of 20 healthy adults (age 24–70 years, mean age = 33) were recruited from the Columbia University Irving Medical Center area. Recruitment was by flyers and via email/phone communications. Informed consent was obtained in compliance with guidelines of the Institutional Review Board of the New York State Psychiatric Institute. Exclusion criteria included pregnancy, cognitive deficit, flu or seasonal infection 4 weeks prior, involvement in a therapeutic or exercise trial and mitochondrial disease diagnosis. Before the blood draw, participants completed a brief questionnaire to collect information on their sex, age, ethnicity, health condition and medication.

A total of 60 mL of blood was collected by venipuncture in the antecubital fossa. Whole blood was processed within 10–15 min after collection for LPS-stimulation, mitochondrial inhibitors, and glucocorticoid suppression experiments as well as for total blood cell count. Complete blood count (CBC) was performed on 13 participants and included proportions of white blood cells (WBC), red blood cells, platelets, and differential WBC counts using an automated hematologic analyzer (XN-9000 Sysmex systems), yielding the percentage of total WBC that are neutrophils, lymphocytes, monocytes, eosinophils, and basophils.

A second study (Cohort 2) of 44 healthy nonsmoking, no exercise, sedentary, adults (age 20–45 years, mean age = 32) was conducted to test if baseline mitochondrial function measured directly in isolated PBMCs was correlated with LPS-stimulated inflammatory cytokine levels. These subjects were part of the ‘Exercise and Inflammation Study’ recruited from the Columbia University and Medical Center/New York Presbyterian Hospital community. Only the baseline (prior to exercise intervention) time point was used in this study. Recruitment was done by flyers posted throughout the Medical Center and electronic bulletin boards. The study was approved by the institutional review board (IRB) #6956R (Formerly #5948) and was registered at ClinicalTrials.gov: NCT01335737.

2.2. LPS-stimulation, mitochondrial inhibitors, and glucocorticoid suppression

For LPS stimulation experiments, 16 mL of whole blood was collected in vacutainers with sodium heparin (BD #67878) and was diluted with 1x RPMI without Phenol red (Thermo Fisher #11835050). For dose-dependent lipopolysaccharide (LPS) stimulation, blood was incubated for 6 h at 37 °C and 5% CO2 with bacterial endotoxin LPS from Escherichia coli (Sigma-Aldrich, #L2880) at increasing concentrations ranging from 3.2 pg/mL to 10 ng/mL per well, in a 96-well tissue culture plate (Eppendorf, #30730127). In all whole blood experiments, samples were centrifuged twice at 4 °C, with a first spin at 1,000g for 5 min followed by a second spin at 2,000g for 10 min to obtain cell-free plasma, which was stored at -80 °C for subsequent cytokine quantification. In Cohort 2, 1 ng/mL LPS (Sigma-Aldrich #L4130) was used to stimulate heparinized blood for 4 h at 37 °C. Plasma was collected post centrifugation at 2,040 ×g for 5 min and stored at -20 °C for subsequent cytokine quantification (Sloan et al., 2018).

For mitochondrial respiration perturbation experiments, inhibitors of Complex I-Rotenone (Sigma-Aldrich #R8875), Complex III-Antimycin A (Sigma-Aldrich #A6874), Complex IV-Potassium Cyanide (KCN) (Sigma-Aldrich #201810) and Complex V-Oligomycin (Sigma-Aldrich #75351) were used at final concentration of 100 nM except KCN which was used at 100 μM. The inhibitors were dissolved in DMSO and co-treated with LPS for 6 h. Inhibitor concentrations were selected based either on our preliminary results (Trumpff et al., 2019b) or previous reports, Rot (Worth et al., 2014), Anti A- (van Raam et al., 2008), KCN- (Jang et al., 2016), Oligo- (Ehinger et al., 2016) and DEX- (Alm, 2012). In glucocorticoid suppression experiments, cortisol-mimetic...
.Dexamethasone (DEX, Sigma-Aldrich #D4902) was co-incubated with LPS and whole blood in a 96-well culture plate at a final concentration of .100 nM. Each plate included an untreated control for baseline measures that was incubated at 37°C and 5% CO2 for 6 h. Plasma was collected and stored as described above for subsequent cytokine assessments.

.2.3. Cytokine assays

.2.3.1. IL-6 ELISA

To assess IL-6 levels in response to increasing dose of LPS, sandwich ELISA method was used following instructions provided by BD OptEIA IL-6 ELISA kit (BD #555220) with minor modifications. Briefly, the capture Ab against IL-6 was coated on a 96-well plate at a dilution of 1:500 (100ul/well) and was incubated overnight at 4°C. The coated plate was washed with 1x wash buffer and blocked with serum for 1 h at room temperature (RT). The wells with Ab were aspirated followed by 3 washes with the wash buffer. A diluent containing serum from the manufacturer was added to the wells marked for standards and samples after which the plate was incubated for 2 h at RT. Plasma samples were diluted twice with the provided diluent in the assay. Detection Ab-Streptavidin HRP conjugate (100 µg/well) was added to each well at 1:500 dilution following 5 washes and the plate was incubated for 1 h at RT. A substrate solution was added after 7 washes to each well and incubated for 30 min in dark. The reaction was interrupted by a stop solution and the plate was immediately read in a micro-plate reader (SpectraMax M3 Molecular Devices) at 450 nm and 570 nm. The background OD at 570 nm was used as internal optical control across wells in a plate. A standard curve was generated from each assay to extrapolate the unknown plasma IL-6 concentration from the linear range of the standards. The final concentration was obtained by correcting for sample dilution factor and batch variation. To control for batch variation, the same plasma sample was run on each plate. The detection sensitivity for IL-6 was 4.7 pg/mL and the intra- and inter-assay CV were <10%. To derive absolute cytokine concentrations in the plasma and to determine EC_{50} of LPS using increasing concentrations of LPS (ELISA), non-linear curve fits were performed using 5-parameter (5-PL) logistic regression. The EC_{50} of LPS-induced IL-6 reflects each participant’s sensitivity to LPS exposure tested across treatment groups. EC_{50} values could not be determined from the dose-response curve for 4 individuals.

.2.3.2. Multiplex cytokine array

Cytokine signatures including 20 inflammatory cytokines and chemokines were measured in plasma using the ProcartaPlex bead immunoassay (ThermoFisher #EPX 200-12185-901) on a Luminex-200 instrument (Luminex technologies) following the manufacturer’s protocol. IL-6 and TNF-α levels in unstimulated and LPS-stimulated plasma were obtained from the Luminex assay for downstream analyses. Briefly, plasma samples collected from LPS, LPS + Inhibitors and LPS + DEX experiments were batched for the 20-plex assay. Each batch of samples were run on the control plasma samples to identify any batch effects between plates. These batch controls were LPS-treated blood to ensure detectable cytokine levels. Samples were diluted 1:5 with the assay diluent prior to the assay and run in duplicates. Standards and samples were prepared. Magnetic beads coated with Abs were added to the 96-well plate and washed with a magnetic plate washer. Samples and standards were added to the respective wells with the beads and incubated for 120 min on an orbital shaker at room temperature. Plates with beads were washed at the end of the incubation and detection Ab was added to the wells and incubated for 30 min followed by washes and incubation with streptavidin for 30 min. Beads were washed and resuspended in a reading buffer. The plates were read, and data was acquired in a Luminex 200 analyzer (Luminex, USA). Data QC and visualization was performed using xPONENT software v 4.2 and files were exported for statistical analyses. The assay sensitivities for all the 20-cytokines can be found on the manufacturer website. A detailed description of inflammation measures performed in Cohort 2 is described elsewhere (Sloan et al., 2018).

.2.4. Mitochondrial enzyme activities and mitochondrial DNA copy number (mtDNAcn)

To quantify mitochondrial respiratory capacity and mitochondrial DNA content in circulating leukocytes, peripheral blood mononuclear cells (PBMCs) were isolated from 44 individuals in Cohort 2. Blood (8.5 mL x 5) was collected in acid citrate dextrose (ACD-A) tubes (VWR #VT4606). Blood was centrifuged at 500 x g for 15 min at RT and platelet-rich plasma removed. Hank’s Balanced Salt Sodium (HBSS) without phenol red, calcium and magnesium (Life Technologies, #14175103) was added to replace the removed plasma. PBMCs were then isolated by density gradient separation by layering the diluted blood over 4 mL of Ficoll Paque Plus (VWR, #95021-205) in 15 mL conical tubes and centrifuged at 400 x g for 30 min (no brake) at RT. Total PBMCs at the Ficoll interface were collected in a 50 mL conical tube, diluted 1:1 in HBSS and pelleted by centrifugation at 500 x g for 10 min at RT. An additional wash was carried out with HBSS at 200 x g for 10 min to maximally deplete platelets. Cells were re-suspended in HBSS and counted on the Countess II FL Automated Cell Counter (Thermo Fisher Scientific, AMQAF1000) in a 1:1 ratio of cells to trypan blue and stored at -80°C until measurements were taken. The biochemical activity of mitochondrial enzymes was measured and integrated into a composite index of mitochondria energy production capacity, the mitochondrial health index (MHI) (Picard et al., 2018). Briefly, mitochondrial enzymes were selected and results interpreted on the basis of: 1) their known biological function (markers of energy production capacity or mitochondrial content); 2) their robustness in a microplate format designed for high throughput; 3) their ability to respond metabolic and biological stressors; and 4) the knowledge that the subunits that compose them are encoded by either the mitochondrial or nuclear genomes. Thus, enzymatic activities were spectrophotometrically quantified for citrate synthase (CS), cytochrome c oxidase (COX, Complex IV), succinate dehydrogenase (SDH, Complex II), NADH-Ubiquinone Oxidoreductase (Complex I) and expressed per million cells. In parallel, mtDNA and nuclear DNA abundance were quantified by TaqMan-based multiplex quantitative real-time polymerase chain reaction (qPCR) to normalize for cell number and calculate mtDNA copy number (mtDNAcn) as described in (Picard et al., 2018).

.2.5. Statistical analysis

All statistical analyses were performed using GraphPad Prism v8.2 and MetaboAnalyst v3.0 (Xia and Wishart, 2016). To identify emergent cytokine patterns after mitochondrial respiration inhibition, we performed i) Spearman rank correlation analysis to test significant inter-cytokine relationships and Pearson analysis for cytokine-MHI relationships, ii) Linear regression analysis to test significant associations between cytokines, and iii) Hierarchical clustering using Ward algorithm and Euclidean distance measure to identify similar responsive cytokine groups. We also performed Partial Least Square Discriminant Analysis (PLS-DA) modeling and ranked cytokines based on variable in projection (VIP) score for the first PLS-DA component, yielded a hierarchy of cytokines most useful in distinguishing treatment effects.

In Cohort 1, Spearman rank correlations were used due to small sample size and non-normally distributed data to assess the strength of the association between the variables, including correlation among cytokines pre- and post-LPS stimulation, LPS + Mito-Inhibitors, and LPS + DEX treatments. In Cohort 2, we performed linear regressions between mitochondrial measures in PBMCs and both unstimulated and LPS-stimulated cytokine levels to quantify the strength and direction of their associations. The association between age and measures of mitochondrial content and function in Cohort 2 were also quantified using linear regressions.

To examine intra-individual differences from pre-to post-LPS
. treatment, with mitochondrial inhibitors, and with DEX treatment, we . used pairwise comparisons two-tailed T-test (significance set as P < .05). To detect group differences across untreated, LPS + Mito-inhibitors and LPS + DEX, relative to the LPS measures, one- or two-way . ANOVAs were used. Missing data were handled using mixed effects . models with post-hoc analysis. Adjusted P values were used for multiple . comparisons and F values were derived from mixed-effects models. Ef- . fect sizes (Cohen’s d) were calculated from t-test and ANOVA results .(Cohen, 1988).

3. Results

3.1. Inhibiting mitochondrial respiratory chain function influences LPS- . induced IL-6 and TNF-α levels

To examine immune responses across participants, we first exposed . whole blood to 10 ng/mL LPS and measured IL-6 levels at 6 h (Fig. 1A). . LPS exposure significantly (p < 0.0001) elevated IL-6 levels by 752- to . 3,241-fold compared to baseline (Fig. 1B), demonstrating robust im- . mune response to LPS challenge.

We then examined how each individual responded to the inhibition . of various respiratory chain components, including Complexes I, III, IV, . and V (Fig. 1C) and computed the proportion of individuals in whom . mitochondrial inhibitors had either a pro- or anti-inflammatory effect . relative to their LPS-only levels (Fig. 1D). Complex I inhibition with . Rotenone elevated IL-6 levels in half of the participants but reduced IL-6 . levels in the rest with a non-significant median reduction of 3.93% (d = .0.29, p = 0.36). Complex III inhibition with Antimycin-A had an anti-. inflammatory effect in 55% of individuals, leading to a median reduc-. tion of IL-6 by 7.3% (d = -0.66, p = 0.049) (Fig. 1E and F). In contrast, . Complex IV inhibition by KCN was mainly pro-inflammatory where 74% . of the individuals showed an elevated IL-6 level relative to LPS alone and . a median increase of 12.1% (d = 0.88, p = 0.016) (Fig. 1E,G). On the . contrary, inhibiting Complex V with oligomycin led to an anti- . inflammatory response in 68% of with an overall 6.3% reduction . (Fig. 1E) in IL-6 levels compared to LPS alone (d = -0.41, p = 0.22).

Consistent with substantial inter-individual differences in cytokine . levels previously reported (Damsgaard et al., 2009; Rohleder, 2014), the . between-person standard deviation (SD) was 31.8% after LPS stimula-. tion, reflecting substantial inter-individual differences in IL-6 responses. . Interestingly, all respiratory chain inhibitors made participants respond . more similarly to one another, evident from significantly reduced . inter-individual differences (Fig. 1H). The group standard deviation . after inhibition were: SDRot = 19%, SDAntia = 13.2%, SDKCN = 20.9%, . and SDOligo = 21.6%, representing about half of LPS only condition. . These results suggest that mitochondrial respiratory capacity may . contribute to inter-individual differences in LPS-induced IL-6 responses . and that inhibiting mitochondrial respiratory chain function can influ- . ence both the magnitude and direction of LPS-induced inflammation in . human leukocytes.

We then extended the same analysis to the cytokine TNF-α (Fig. 2) . and found that LPS exposure significantly elevated TNF-α levels by 11- to . 1361-fold (p < 0.0001) compared to baseline (Fig. 2A). Inhibition of . respiratory chain Complexes I, III, IV, and V at the specific LPS dose of . 10 ng/mL had an overall anti-inflammatory effect on LPS-stimulated . levels (Fig. 2B), where 85–100% individuals showed decreased TNF-α . levels with respiratory chain inhibitors (Fig. 2C), in all cases with large . effect sizes. Complex I inhibition decreased TNF-α levels in 85% of . individuals by a median 16.1% (d = -1.55, p < 0.0001). Complex III in-. hibition had an anti-inflammatory effect in 90% of individuals, leading . to a median reduction of 18.7% (d = -1.20, p = 0.001). Interestingly, . Complex IV inhibition led to a robust anti-inflammatory response in .

Fig. 1. Inhibition of mitochondrial respiratory capacity causes large inter-individual differences in acute LPS-induced IL-6 levels in human blood. (A) . Experimental design illustrating the quantification of cytokine levels from whole human blood (n = 20) before and after LPS stimulation (10 ng/mL). (B) Fold change of LPS-stimulated IL-6 levels relative to the unstimulated levels. (C) Effect of mitochondrial inhibitors on stimulated IL-6 levels relative to LPS alone: + Rotenone (Rot) for Complex I, + Antimycin (Anti) A for Complex III, + KCN for Complex IV and + Oligomycin (Oligo) for Complex V. Missing data are shown in grey. (D) Proportion of individuals showing either elevated (pro-inflammatory) or reduced (anti-inflammatory) IL-6 levels in response to each mitochondrial inhibitor. (E) IL-6 levels plotted relative to the mean LPS response (100%) where semi-transparent boxes illustrate reduced inter-individual variability in mitochondrial inhibition. Each data point is a participant and lines indicate blood from the same individual treated with different inhibitors. Median % change is shown for each inhibitor relative to LPS. (F-G) % change in IL-6 levels for LPS + AntiA (Complex III inhibition) and LPS + KCN (complex IV inhibition) relative to LPS-stimulated levels shown for each participant. Median changes are indicated relative to LPS alone with effect sizes (Cohen’s d) and P values from paired t-test. (H) Inhibition of mitochondrial respiration reduces inter-individual variability, quantified by the group standard deviation.
100% of individuals with a median decrease of 85.7% in the TNF-α (d = -.3.60, p < 0.0001). Similarly, inhibiting Complex V led to an anti-inflammatory response of TNF-α levels in 85% of individuals with an overall reduction of 18.1% compared to LPS alone (d = -1.58, p = 0.0001) (Fig. 2D-E). And as for IL-6, respiratory chain inhibitors reduced inter-individual variation in TNF-α (Fig. 2F).

To examine if cell type composition in whole blood may have contributed to inter-individual differences in IL-6 and TNF-α response to LPS, we correlated stimulated IL-6 and TNF-α levels with the proportion (% of total cells) obtained from complete blood counts available from a subset of participants (Supplemental Figure S1A). As expected, the majority of leukocytes were neutrophils and lymphocytes, together composing 81–93% of all cells. Both IL-6 and TNF-α levels tended to be positively correlated with neutrophil count (r = 0.18–0.43) and negatively associated with lymphocyte count (r = -0.17 to -0.51). Stimulated IL-6 levels were also negatively correlated with eosinophil count (r = -0.68, p = 0.01, n = 13) whereas stimulated TNF-α levels were negatively correlated with basophil count (r = -0.61, p = 0.03, n = 13) (Supplemental Figure S1B). There was no correlation of stimulated cytokine levels with baseline monocyte count. These observations suggest that variable cell type proportions at baseline may in part contribute to stimulated cytokine levels in whole blood and call for future studies in isolated cell populations.

### 3.2. Minor influence of mitochondrial respiratory chain function on LPS sensitivity

We next sought to determine if mitochondrial modulation influenced...
.immune cells’ sensitivity across a range of LPS concentrations (3.2 pg/mL to 10 ng/mL). Increasing LPS concentrations caused a dose-dependent increase in IL-6 response fitted with a sigmoidal function (Fig. 3A). Again, there were large variations in LPS-sensitivity across individuals (CV = 88.6%) (Fig. 3B). The median LPS EC50 for IL-6 was .90 pg/mL (range 3.2–10 pg/mL) across 20 individuals. Inhibiting mitochondrial respiratory chain complexes led to only small alterations in the LPS-sensitivity. Complex I inhibition sensitized cells to LPS by 20% (d = 0.16) whereas inhibiting Complexes III, IV and V suppressed LPS-induced IL-6 levels by 30%, 60% and 38% respectively (d = -0.26, -0.11, -0.21 respectively, all N.S.) (Fig. 3B). In contrast, as expected from glucocorticoid suppression, DEX treatment decreased sensitivity as illustrated by a 7.9-fold higher LPS EC50 (d = 1.32, p = 0.001). Finally, although our dataset was not powered to examine sex differences, men tended to show about half the sensitivity to LPS (EC50 = 147.5 ± 48.8, mean ± SEM) compared to women (EC50 = 71.2 ± 19.4; d = -1.02, p = 0.32).

3.3. Inhibition of mitochondrial complex IV alters acute inflammation induced cytokine signatures

To assess the effect of mitochondrial respiration capacity on pro- and anti-inflammatory cytokine signatures, we simultaneously measured the levels of 20 cytokines in LPS-treated samples (LPS conc. at 10 ng/mL) ± mitochondrial respiratory chain inhibitors. This confirmed the stimulatory effects of LPS on multiple known inflammatory cytokines, chemokines, and interferons (Fig. 4A), including IL-6 which exhibited the strongest induction (~2,000-fold) relative to unstimulated levels.

Fig. 4. Inhibition of mitochondrial Complex IV alters pro- and anti-inflammatory cytokine profiles. (A) LPS-stimulated cytokine levels relative to the unstimulated levels. (B) Heatmap illustrating the effect of mitochondrial respiratory chain inhibitors on stimulated cytokine response relative to LPS-only. Cytokines order is based on LPS + KCN-group. (C) KCN-specific effects on stimulated cytokine response relative to LPS. P values from two-way ANOVA with Dunnett multiple comparison. *P < 0.05, **P < 0.005. (D) Partial Least square discriminant analysis (PLS-DA) model derived from cytokine response of each individual with or without KCN. (E) Cytokines are ranked by the variable in projection (VIP) reflecting their contribution to group separation in the overall model. By convention, VIP scores > 1 are considered significant. (F) Effect of KCN on the association between stimulated IL-6 and TNF-α levels compared to LPS alone. Strength of association (r²) and P values are obtained from linear regression. (G) Effect of all inhibitors on the correlation between IL-6 and TNF-α levels. (H) Modulation of the IL-6/TNF-α ratio by inhibitors, expressed relative to LPS alone (dotted line). (I) Bi-plot illustrating the effect of inhibitors on TNF-α and IL-6 levels, expressed relative to LPS alone. Data shown in (C) and (I) are mean ± SEM, n = 19–20.
.LPS-induced IL-6 and TNF-α levels). KCN (Complex IV) reduced .TNF-α levels by 72% but increased the IL-6 by 13% (Fig. 4C). As a result, .KCN treatment increased the IL-6 to TNF-α ratio by 3.5-fold, whereas .other mitochondrial inhibitors only modestly increased the ratio by .0.2–0.5-fold (Fig. 4H). KCN also reduced IL-10 levels by 47% (p = 0.17) .compared to mean LPS levels whereas Complexes I, III and V inhibitors .reduced IL-10 levels by 24%, 26% and 28% respectively (Supplemental .Figure S2A-C). These results suggest that KCN and its target respiratory .chain complex, Complex IV, has a particularly strong influence on the .pro- and anti-inflammatory cytokine levels in whole blood leukocytes.

.3.4. Inhibiting mitochondrial respiratory chain alters cytokine signatures

. To further examine cytokine signatures, we first explored the ratios .of pro- and anti-inflammatory cytokines. To generate a cumulative pro/.anti-inflammatory index, we included IL-6, TNF-α, and IL-1β as pro-.inflammatory cytokines and used IL-10 as the anti-inflammatory cyto-.kine. As a proof-of-concept, relative to LPS alone, DEX robustly .decreased the pro-/anti-inflammatory index, consistent with its potent .anti-inflammatory effects. In contrast, inhibiting any of the mitochon-.drial respiration complexes increased this index by 20-100% (Fig. 5A), .tilting the balance towards a pro-inflammatory state. Individual cytokine .ratios such as IL-6/IL-10, IL-1β/IL-10, and TNF-α/IL-10 were also .differentially affected by respiratory chain inhibitors (Fig. 5B–D), with .KCN consistently showing the most robust immune modulatory effect.

. We next systematically examined the inter-cytokine correlations, .visualized as correlation matrices that reveals their co-regulation .(Fig. 5E). At baseline before LPS stimulation, only 20 pairs of cyto-.kines were correlated to an appreciable degree (r > 0.5) and after LPS .addition, 28 pairs of cytokines were correlated. In contrast, when we .pharmacologically inhibited mitochondrial respiration complexes, the .inter-correlated cytokine pairs increased to 42 (Rot), 51 (Anti A), 51 .(KCN) and 67 (Oligo). Representative cytokine correlations are shown in
Supplemental Fig. 3A-C. Together with the simple cytokine ratios, these results highlight the co-regulation of several pro- and anti-inflammatory cytokines, and the respiration chain complex-specific influence on cytokine signatures.

3.5. Effects of glucocorticoid signaling on inflammatory signatures

We then extended this multi-cytokine approach to examine the specific effects of GC-mediated anti-inflammatory signaling (schematic Fig. 6A). DEX significantly suppressed all well-known LPS-stimulated pro-inflammatory cytokines by 70-90%, reported in the order of most (p < 0.0001) to least suppressed (p < 0.05) cytokines IFN-γ, TNF-α, IL-1β, IL-6, IL-8, IL-1α, MIP-1β, and MIP-1α in Fig. 6B and C. In contrast, DEX had no effect on cell adhesion proteins like P-selectin, E-selectin and sICAM-1 (N.S.), and rather upregulated the anti-inflammatory cytokine IL-10 by 60% (p < 0.0001) (Fig. 6C). The range of DEX-mediated suppression of LPS-induced IL-6 levels was 72-97% across individuals with an average suppression of 87% (Fig. 6D).

Given that glucocorticoid signaling influences mitochondrial behavior (Psarra et al., 2005; Du et al., 2009), and that mitochondria modulate inflammatory cytokine production, we reasoned that a portion of the anti-inflammatory action of DEX may involve mitochondria. Therefore, we tested if mitochondrial respiratory capacity modulated DEX-mediated suppression of IL-6 response to LPS. We extracted the % DEX suppression for IL-6 in each participant and compared it to the % suppression after inhibition of mitochondrial respiration by various Complex inhibitors (Fig. 6E–H). In doing so, we found that inhibiting Complex I augmented DEX suppression of IL-6 levels by 12.3% (d = .073, p < 0.0001, n = 15) whereas Complex III inhibition had almost no effect on %DEX suppression (d = 0.072, p = 0.42, n = 15). Additionally, inhibition of Complex IV and V potentiated IL-6 suppression by 6.6% (d = 0.36, p = 0.004, n = 15) and 4.7% (d = 0.19, p = 0.005, n = 15) respectively.

3.6. Associations between intrinsic mitochondrial respiratory capacity and cytokine responses

We next hypothesized that intrinsic leukocytes mitochondrial...
.bioenergetic capacity accounts for a portion of inter-individual differences in cytokine responses. We therefore measured respiratory chain enzymatic activities for Complexes I, II, IV, citrate synthase, and mtDNA copy number in an independent cohort (Cohort 2, n = 44) of women and men in whom a sufficient number of peripheral blood mononuclear cells (PBMCs) could be isolated to enable reliable measure of mitochondrial function (Fig. 7A). Individual mitochondrial metrics were also integrated into an index of mitochondrial functional capacity, the MHI (Picard et al., 2018). In the same individuals, whole blood was stimulated with LPS (1 ng/mL) and IL-6 and TNF-α levels quantified after 4 h.

To approach this question from an unbiased perspective, all mitochondrial measures were correlated with cytokine levels in both untreated (no-LPS) and LPS-stimulated conditions, and the magnitude and direction of the associations between mitochondrial content (citrate synthase), mitochondrial respiratory capacity of leukocytes, mtDNA copy number, and the MHI were visualized as a heatmap (Fig. 7B). The majority (72%) of correlations were positive (chance would be 50%), suggesting that individuals with higher mitochondrial content and function produce more cytokines, particularly after LPS stimulation. In particular, baseline Complex IV (COX) activity (marked ‘C’ in the IL-6 heatmap) was positively correlated with LPS-stimulated IL-6 levels (r = 0.45, p = 0.002, n = 44). These associations were generally similar with TNF-α, but of lower magnitude. Both women and men showed pronounced positive correlation between baseline COX activity and stimulated IL-6 (Fig. 7C), but the effect size was larger in women (r = 0.56, p = 0.01, n = 18) than men (r = 0.36, p = 0.08, n = 24). Overall, these findings suggest that inherent mitochondrial respiratory capacity of leukocytes may account for 10–30% of the variance in immune reactivity across individuals. These data cannot rule out possible sex-differences in these associations.

In sensitivity analyses examining the association between the age of participants and various measures of mitochondrial behavior in PBMCs, age was not associated with mitochondrial content or functions ($r^2 = 0.00–0.03$, N.S.) (supplementary Figure S4).

.4. Discussion

This study examined how mitochondrial respiratory capacity modulates blood cytokine response upon LPS and DEX exposure. Acute pharmacological inhibition of mitochondrial respiration reduced inter-individual variation in cytokine levels, altered overall cytokine signatures, but only mildly modulated sensitivity to glucocorticoid signaling in Cohort 1. Complex IV activity in isolated leukocytes was positively correlated with LPS-stimulated plasma cytokine levels in Cohort 2. Together, these results suggest that intrinsic mitochondrial respiratory capacity may explain a fraction of inter-individual differences in inflammatory cytokine responses to LPS. Largely, our findings in human blood extent the scientific literature on mitochondria’s role in acute inflammation by providing initial evidence that mitochondrial respiratory capacity influences not only cytokine levels but also the cytokine signatures produced by blood leukocytes in humans.

Experimentally examining immune responses in vitro from human blood has several advantages that allow the isolation of potential mediators of immune processes (Strahler et al., 2015). First, it is possible to vary the strength of the immune challenge, such as exposure to lipopolysaccharide (LPS), a component within the cell wall of Gram-negative bacteria that stimulates various cell types to release IL-6, IL-1β, TNF-α, IL-8, and other pro-inflammatory cytokines (Mosher et al., 2006; Spierenburg et al., 2018). Second, the in vitro approach allows the manipulation of different aspects of mitochondrial function with selective inhibitors, including inhibition of specific respiratory chain components. Finally, this approach also makes it possible to combine known immunomodulators, such as immunosuppressive glucocorticoid (GC)
.Dex is sufficient to extrude mtDNA into the cytoplasm (Trumpff et al., 2019b) along with evidence that a subtype of GR (gamma) resides in mitochondria and regulates ATP production (Morgan et al., 2016). Since circulating levels of GCs in acute and chronic stress can be modulated by the hypothalamic-pituitary-adrenal (HPA) axis via GR activation (Perrin et al., 2019), we explored the possibility that inhibiting mitochondrial respiration would alter GC sensitivity and its ability to suppress the IL-6 response. Notably, GCs significantly suppress LPS-stimulated production of cytokines by upregulating anti-inflammatory mediators like IL-10 (Mann et al., 2019). Accordingly, DEX strongly downregulated multiple pro-inflammatory cytokines. Interestingly, inhibiting mitochondrial respiration in addition to DEX further suppressed cytokine release – in other words, inhibiting mitochondrial respiration potentiated the immunosuppressive properties of DEX. These results suggest that GC signaling in immune cells may involve mitochondria either directly, or indirectly through some aspects of cellular energetics or metabolic signaling.

We also tested whether inherent mitochondrial functional capacity of leukocytes can explain inter-individual differences in LPS-mediated cytokine responses in people. In Cohort 1 experiments with inhibitors, we find that inhibition of respiratory chain function decreased the release of several cytokines, particularly TNF-α and IL-10, whereas IL-6 was modestly increased by Complex IV inhibition. In Cohort 2, we found that higher intrinsic COX activity in isolated PBMCs was correlated with higher stimulated IL-6 levels, and to a lesser extent TNF-α. These results are in part contrary to our first hypothesis that mitochondrial dysfunction would increase cytokine release and suggest a more nuanced view of mitochondrial signaling in specific cytokine pathways. Complementary to our finding in Cohort 2, compared to patients with robust TNF-α response to LPS, immunoparalyzed pediatric sepsis patients with LPS-stimulated TNF-α levels ≤200 pg/mL also had lower mitochondrial respiratory capacity in PBMCs (Weiss et al., 2019). Generally, our combined results indicate that pharmacological perturbations of mitochondrial respiratory function influence cytokine responses (Cohort 1), and that baseline measures of PBMCs mitochondrial respiratory chain capacity are associated with cytokine release, support the conclusion that a fraction of inter-individual variation in cytokine response may be influenced by mitochondrial behavior within human leukocytes.

One interpretation of these findings is that higher mitochondrial energy production capacity at baseline, particularly Complex IV activity, may enable more vigorous acute cytokine production in healthy adults. In summary, associations between mitochondrial measures and cytokine responses are pertinent to understand immune responses to acute challenges like LPS. This study calls for both replication and validation in large and diverse cohorts as well as in primary immune cell-subtypes.

### 4.1 Limitations

This study specifically included healthy individuals to examine the role of mitochondrial respiration in LPS-induced inflammation in an ex vivo whole blood model. Although the within-subject experimental design allowed us to test individual-specific effects of mitochondrial respiratory chain activity despite inter-individual differences in cytokine responses, there are several limitations of the approach. First, whole blood arguably better reflects the physiological cellular mixture in human circulation than isolated and purified cell preparations, but individuals show different proportions of immune cell types that likely differentially produce specific cytokines. Thus, studies in isolated cell types may produce slightly different results and reveal even more profound modulatory effects of mitochondrial respiration on specific cytokines. Second, the sample size in both cohorts is relatively small and precludes definite conclusions about inter-individual and sex-related differences in cytokine behavior. Similarly, while various studies have reported sexual dimorphism in mitochondrial function (Ventura-Clapier et al., 2017), in psychological stress driven endocrine-immune function (Bekhbat and Neigh, 2018; Rainville et al., 2018), as well as in...
.LPS-induced systemic inflammation (Marsland et al., 2017), the .sex-stratified correlations between mitochondrial function and cytokine .response in Cohort 2 should be interpreted with caution. Studies with .larger sample size are needed to establish whether functional differences .between the mitochondria of women and men contribute to sex differ-en ces in stress-immune signaling.

5. Conclusion

Collectively, our results demonstrate that experimental manipulation of mitochondrial respiratory chain function, particularly Complex IV, mildly exaggerates LPS-induced IL-6 levels, markedly reduces TNF-α .levels, and more generally alters multi-cytokine signatures. We also show that mitochondrial bioenergetics moderately influence sensitivity to GC-mediated IL-6 suppression, providing additional evidence that .mitochondria modulate different aspects of immune responses, and .possibly how immune cells are influenced by endocrine factors. This .study in human blood extends in vitro work demonstrating immuno-modulation by mitochondrial energetics and provides proof-of-concept.data that intrinsic inter-individual variation in mitochondrial pheno-types contribute to differences in immune responses in acute .inflammation.

.Funding source

This work was supported by the Wharton Fund to RPS and MP, and .NIH grants GM119793 to MP, MH119336 to MP, ALM and BK.

.Author contributions

KRK and MP designed the study with input from NR. CT and KRK.prepared the IRB protocol. MM and JT collected and processed the .samples. KRK performed the stimulation experiments, and MM per-formed the MHI experiments. VL and RPS provided additional samples .and participant information. KRK performed analyzes. GS, ALM and BAK .provided critical comments on the manuscript. KRK, CT and MP drafted .the manuscript. All authors contributed to the final version of the .manuscript.

.Declaration of competing interest

The authors declare no conflict of interest.

.Acknowledgements

We thank the participants who contributed to the study, Sloan Kra-kovsky and Carlos Acosta for their help with sample collection, as well .as Carla Basulto and Atif Towheed for useful discussions about this work. .We acknowledge the assistance of the Columbia Stem Cell Initiative .(CSCI) and Columbia Center for Advanced Lab medicine (CALM).

.Appendix A. Supplementary data

Supplementary data to this article can be found online at https://do .i.org/10.1016/j.bbbeh.2020.100080.

.References

Al Amir Dache, Z., et al., 2020. Blood contains circulating cell-free respiratory competent mitochondria. Faseb. J. 34 (3), 3616–3630.
.Alm, J.J., et al., 2012. Transient 100 nM dexamethasone treatment reduces inter- and .intraindividual variations in osteoblastic differentiation of bone marrow-derived .human mesenchymal cells. Tissue Eng. C Methods. 18 (9), 658–666.
.Andres-Rodriguez, L., et al., 2019. Immune-inflammatory pathways and clinical changes .in fibromyalgia patients treated with Mindfulness-Based Stress Reduction (MBSR): a .randomized, controlled clinical trial. Brain Behav. Immun. 80, 109–119.
.Arango Duque, G., Descoteaux, A., 2014. Macrophage cytokines: involvement in .immunity and infectious diseases. Front. Immunol. 5, 491.
.Bekhbat, M., Neigh, G.N., 2018. Sex differences in the neuro-immune consequences of .stress: focus on depression and anxiety. Brain Behav. Immun. 67, 1–12.
.Van den Bosche, J., et al., 2016. Mitochondrial dysfunction prevents repolarization of .inflammatory macrophages. Cell Rep. 17 (3), 684–696.
.Boecı, Ç., et al., 2018. The association between cortisol, oxytocin, and immune cell .mitochondrial oxygen consumption in postpartum women with childhood .maltreatment. Psychoneuroendocrinology 96, 69–77.
.Boypati, R.K., et al., 2017. Advances in the understanding of mitochondrial DNA as a .pathogenic factor in inflammatory diseases. F1000Research vol. 6, 169.
.Breda, C.S.N., et al., 2019. Mitochondria as central hub of the immune system. Redox .Biol. 26, 101255.
.Chae, B.S., 2018. Pretreatment of low-dose and super-low-dose LPS on the production of .in vitro LPS-induced mitochondrial mediators. Toxicol. Res. 34 (1), 65–73.
.Chappe, N.S., 2015. Evolution of mitochondria as signaling organelles. Cell Metab. 22 (2), 204–206.
.Chapman, N.M., Boothby, M.R., Chi, H., 2020. Metabolic coordination of T cell .activation and expression. Nat. Rev. Immunol. 20, 55–70.
.Chinini, T., Weiner, H.L., 2017. CNS inflammation and neurodegeneration. J. Clin. Invest. .127 (10), 3577–3587.
.Cicchese, J.M., et al., 2018. Dynamic balance of pro- and anti-inflammatory signals .controls disease and limits pathology. Immunol. Rev. 285 (1), 147–167.
.Cohen, J., 1988. Statistical Power Analysis for the Behavioral sciences., 2nd. Hillsdale, N.J.: L. Erlbaum Associates.
.Copeland, S., et al., 2005. Acute inflammatory response to endotoxin in mice and .humans. Clin. Diagn. Lab. Immunol. 12 (1), 60–67.
.Darrynaard, C.T., et al., 2009. Whole-blood culture is a valid low-cost method to measure .monocyte cytokines - a comparison of cytokine production in cultures of human .whole-blood, mononuclear cells and monocytes. J. Immunol. Methods 340 (2), .95–101.
.Du, J., et al., 2009. Dynamic regulation of mitochondrial function by glucocorticoids. .Proc. Natl. Acad. Sci. U. S. A. 106 (9), 3543–3548.
.Duffy, D., et al., 2014. Functional analysis via standardized whole-blood stimulation .systems defines the boundaries of a healthy immune response to complex stimuli. .Biology 40 (3), 436–450.
.Dumitru, C., Kabat, A.M., Maloy, K.J., 2018. Metabolic adaptations of CD4(+) T cells in .inflammatory disease. Front. Immunol. 9, 540.
.Ehinger, J.K., et al., 2016. Cell-permeable succinate prodrugs bypass mitochondrial .complex I deficiency. Nat. Commun. 7, 12217.
.Eom, S., et al., 2017. Cause of death in children with mitochondrial diseases. Pediatr .Neuro. 66, 82–88.
.Ferrucci, L., Fabbri, E., 2018. Inflammaging: chronic inflammation in ageing, .cardiovascular disease, and frailty. Nat. Rev. Cardiol. 15 (9), 505–522.
.Fox, C.J., Hammerman, P.S., Thompson, C.B., 2005. Fuel feeds function: energy .metabolism and the T-cell response. Nat. Rev. Immunol. 5 (11), 844–852.
.Garn Jude, J., et al., 2016. Mitochondrial respiratory-chain adaptations in macrophages .contribute to antibacterial host defense. Nat. Immunol. 17 (9), 1037–1045.
.Hann, D.M., et al., 1998. Measurement of fatigue in cancer patients: development and .validation of the Fatigue Symptom Inventory. Qual. Life Res. 7 (4), 301–310.
.Hotamisgil, G.S., 2017. Inflammation, metabolism and immunometabolic .disorders. Nature 542 (7640), 177–185.
.Huang, S.C.-C., et al., 2016. Impairment of mitochondrial respiration following ex vivo .cytotoxicity in peripheral blood mononuclear cells. Clin. Toxicol. 54 (4), .303–307.
.Katz, M.R., Irish, J.C., Devins, G.M., 2004. Development and pilot testing of a .psychosocial intervention for oral cancer patients. Psycho Oncol. 13 (9), .642–653.
.Kohsbi, T., et al., 2011. Mitochondrial membrane potential is required for MAVS- mediated antiviral signaling. Sci. Signal. 4 (158), ra7.
.Li, Y., et al., 2006. Cytochrome c oxidase subunit IV is essential for assembly and .respiratory function of the enzyme complex. J. Bioenerg. Biomembr. 38 (5–6), .283–291.
.Li, Y., et al., 2016. A functional genomics approach to understand variation in cytokine .production in humans. Cell 167 (4), 1089–1110 e14.
.Lindqvist, D., et al., 2016. Increased plasma levels of circulating cell-free mitochondrial .DNA in suicide attempters: associations with HPA-axis hyperactivity. Transl .Psychiatry 6 (12), e971.
.Lindqvist, D., et al., 2018. Circulating cell-free mitochondrial DNA, but not leukocyte .mitochondrial DNA copy number, is elevated in major depressive disorder. .Neuropsychopharmacology 43 (7), 1557–1564.
.Mann, E.H., et al., 2019. High-dose IL-2 shows a glucocorticoid-driven IL-17(-/+)IL-10(-/+) .memory CD4(+) T cell response towards a single IL-10-producing phenotype. J .Immunol. 202 (3), 684–693.
.Marsland, A.L., et al., 2017. The effects of acute psychological stress on circulating and .stimulated inflammatory markers: a systematic review and meta-analysis. Brain .Behav. Immun. 64, 208–219.
.Meyer, A., et al., 2018. mitochondria: an organelle of bacterial origin controlling .inflammation. Front. Immunol. 9, 536.
.Morgan, D.J., et al., 2016. Glucocorticoid receptor isoforms direct distinct mitochondrial .programs to regulate ATP production. Sci. Rep. 6, 26419.
.Mother, C.E., Danoff-Burg, S., Brunker, B., 2006. Post-traumatic growth and psychosocial .adjustment of daughters of breast cancer survivors. Oncol. Nurs. Forum 33 (3), .543–551.
.Niraula, A., et al., 2018. Corticosterone production during repeated social defeat causes 
.monoocyte mobilization from the bone marrow, glucocorticoid resistance, and 
.neurovascular adhesion molecule expression. J. Neurosci. 38 (9), 2328–2340.
.
.Panga, B., et al., 2018. A mitochondrial health index sensitive to mood and caregiving 
.stress. Biol. Psychiat. 84 (1), 9–17.

.Van der Poll, T., et al., 2017. The immunopathology of sepsis and potential therapeutic 
.targets. Nat. Rev. Immunol. 17 (7), 407–420.
.
.Paap, M., et al., 2005. Glucocorticoid receptor isoforms in human hepatocarcinoma 
.HepG2 and SaOS-2 osteosarcoma cells: presence of glucocorticoid receptor alpha in 
.mitochondria and of glucocorticoid receptor beta in nucleoli. Int. J. Biochem. Cell 
.Biol. 37 (12), 2544–2558.
.
.Van Raam, B.J., et al., 2008. Mitochondrial membrane potential in human neutrophils is 
maintained by complex III activity in the absence of supercomplex organisation.

.PLoS One 3 (4), e2013.
.
.Rainville, J.R., Tsyglakova, M., Hodas, G.E., 2018. Deciphering sex differences in the 
.immune system and depression. Front. Neuroendocrinol. 50, 67–90.
.
.Rea, I.M., et al., 2018. Age and age-related diseases: role of inflammation triggers and 
.cytokines. Front. Immunol. 9, 586.
.
.Rohleder, N., 2014. Stimulation of systemic low-grade inflammation by psychosocial 
.stress. Psychosom. Med. 76 (3), 181–189.
.
.Sloan, R.P., McKinley, P.S., Bartels, M., et al., 2018. Aerobic exercise training and 
.inducible inflammation: results of a randomized controlled trial in healthy, young 
.adults. J. Am. Heart Assoc. 7 e010201. PMID: 30371169.
.
.Song, X., et al., 2020. Existence of circulating mitochondria in human and animal 
.peripheral blood. Int. J. Mol. Sci. 21 (6), 2122.
.
.Spierenburg, E.A.J., et al., 2018. Stability of individual LPS-induced ex vivo cytokine 
.release in a whole blood assay over a five-year interval. J. Immunol. Methods 460, 
.119–124.
.
.Strahler, J., Rohleder, N., Wolf, J.M., 2015. Acute psychosocial stress induces differential 
.short-term changes in catecholamine sensitivity of stimulated inflammatory cytokine 
.production. Brain Behav. Immun. 43, 139–148.
.
.Tanaka, V., 2018. Sex hormones determine immune response. Front. Immunol. 9, 1931.
.
.Tannahill, G.M., et al., 2013. Succinate is an inflammatory signal that induces IL-1β 
.through HIIP1e. Nature 496, 230.
.
.Taranenko, T.N., et al., 2017. Cytochrome c oxidase activity is a metabolic checkpoint 
.that regulates cell fate decisions during T cell activation and differentiation. Cell 
.Metabol. 25 (6), 1254–1268 e7.
.
.Trumpf, C., et al., 2019. Predictors of ccf-mtDNA reactivity to acute psychological stress 
.identified using machine learning classifiers: a proof-of-concept. Psychoneuroendocrinology 107, 82–92.
.
.Trumpf, C., et al., 2019. Acute psychological stress increases serum circulating cell-free 
.mitochondrial DNA. Psychoneuroendocrinology 106, 268–276.
.
.Ventura-Clapier, R., et al., 2017. Mitochondria: a central target for sex differences in 
.pathologies. Clin. Sci. (Lond.) 131 (9), 803–822.
.
.Walsh, C.P., et al., 2018. Development of glucocorticoid resistance over one year among 
mothers of children newly diagnosed with cancer. Brain Behav. Immun. 69, 
.364–373.
.
.Weinberg, S.E., Sena, L.A., Chandel, N.S., 2015. Mitochondria in the regulation of innate 
.and adaptive immunity. Immunity 42 (3), 406–417.
.
.Weiss, S.L., Zhang, D., Bush, J., et al., 2019 Nov 20. Mitochondrial Dysfunction Is 
.Associated with an Immune Paralysis Phenotype in Pediatric Sepsis. published online 
.ahead of print Shock. PMID: 31764621.
.
.West, A.P., Shadel, G.S., Ghosh, S., 2011. Mitochondria in innate immune responses. Nat. 
.Rev. Immunol. 11 (6), 389–402.
.
.Worth, A.J., et al., 2014. Inhibition of neuronal cell mitochondrial complex I with 
.rotenone increases lipid beta-oxidation, supporting acetyl-coenzyme A levels. J. Biol. 
.Chem. 289 (39), 26895–26903.
.
.Wurfel, M.M., et al., 2005. Identification of high and low responders to 
.lipopolysaccharide in normal subjects: an unbiased approach to identify modulators 
.of innate immunity. J. Immunol. 175 (4), 2578–2587.
.
.Xia, J., Wishart, D.S., 2016. Using MetaboAnalyst 3.0 for comprehensive metabolomics 
data analysis. Curr. Protoc. Biotechnol. 55, 14 10 1–14 10 91.
.
.Zhao, Y., et al., 2012. COX5B regulates MAVS-mediated antiviral signaling through 
.interaction with ATG5 and repressing ROS production. PLoS Pathog. 8 (12), 
e1003086.