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

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ABSTRACT

Mitochondria modulate inflammatory processes in various model organisms, but it is unclear how much mitochondria regulate immune responses in human blood leukocytes. 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 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 (Piasecka 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; Garade et al., 2016; Koshiha 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 implicate 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 around 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 cytokines 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 (Cohort1), 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 #11835055). 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, #L4130) 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 #A68674), 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 1000M. 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 to a 96-well plate at a dilution of 1:500 (100μl/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 μL/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 instrument (Luminex technologies) following the manufacturer instructions were performed using 5-parameter (5-PL) logistic regression.

The EC50 of LPS using increasing concentrations of LPS (ELISA), non-linear curve fits were performed using S-parameter (5-PL) logistic regression. The ECo50 of LPS-induced IL-6 reflects each participant's sensitivity to LPS exposure tested across treatment groups. ECo50 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 with the 2 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 could 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 30min (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 10min 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 were 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: i) 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 + Mitochondrial 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 mitochondria 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 \( P < 0.05 \)). 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 \( P \) values were derived from mixed-effects models. Effect 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 immune 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 3.95% (\( d = -0.29, p = 0.36 \)). Complex III inhibition with Antimycin-A had an anti-inflammatory effect in 55% of individuals, leading to a median reduction 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 stimulation, 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: \( SD_{\text{Rot}} = 19\% \), \( SD_{\text{AntiA}} = 13.2\% \), \( SD_{\text{KCN}} = 20.9\% \), and \( SD_{\text{Oligo}} = 21.6\% \), 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 influence 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 inhibition 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 100% of individuals showing a reduction of 752-fold compared to baseline (Fig. 2E). Inhibition of Complex V with oligomycin led to an overall anti-inflammatory response in 74% of individuals, leading to a median reduction of 3.95% (\( d = -0.29, p = 0.049 \)) (Fig. 2F).

![Diagram of immune response to LPS and mitochondrial inhibitors](image_url)

**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 \( (10 \text{ ng/mL}) \). (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.
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 EC\textsubscript{50} 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 EC\textsubscript{50} (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 (EC\textsubscript{50} = 147.5 ± 48.8, mean ± SEM) compared to women (EC\textsubscript{50} = 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 (Supplemental Table S2). Again, marked inter-individual differences were
noted in stimulated cytokine levels.

We then examined how respiratory chain inhibition influenced levels across this cytokine panel. Overall, all inhibitors altered LPS-stimulated cytokine levels significantly compared to LPS (P < 0.005). Rotenone, Antimycin-A, KCN, and Oligomycin decreased the LPS-induced elevation of most cytokines, but some inhibitors potentiated the release of some cytokines (Fig. 4B, Supplemental Figure S2). In particular, KCN exposure most potently reduced the LPS induction of a large portion of the cytokines (Fig. 4C), including TNF-α whose levels were suppressed by 72% relative to LPS alone (d = 1.59, p = 0.0003).

To explore and visualize the overall effect of mitochondrial inhibition on the inflammatory phenotype, we ran a partial least square discriminant analysis (PLS-DA) with leave-one-out cross-validation (LOOCV). This procedure tests whether all 20 cytokines considered together in the same model contain enough information to distinguish between LPS and LPS + KCN treatments. The model for KCN yielded a prediction accuracy of 83% and produced a reasonable separation of treatments (Fig. 4D, Supplemental Table S3). In comparison, models for other inhibitors did not perform as well (accuracies 55–65%, near chance level), reflecting their smaller effect sizes. In comparison, DEX treatment yielded the most robust cytokine clusters with altered correlation strength across treatment groups. Each square is a correlation with n = 20 individuals.

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 cytokine. 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 mitochondrial 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 cytokines 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), 31 (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, 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 = 0.73, 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), respiratory chain activities, 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 (r² = 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 interindividual 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 extend 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; Spierenberg 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) signaling via dexamethasone (DEX), with mitochondrial modulators and thus
study their interaction. Additionally, recognizing that immune responses come in different types reflected by their different cytokine signatures (Duffy et al., 2014), it is also possible to examine not only the magnitude but also the type of immune response by simultaneously probing multiple pro- and anti-inflammatory cytokines. Compared to isolated cells where systemic factors are removed, whole blood conditions preserve potential physiologically relevant functional interactions among different circulating leukocytes. However, it should be noted that factors other than the immune cells themselves, such as pre-existing circulating cytokines, levels of metabolites, or extracellular mitochondria (Al Amir Dache et al., 2020; Song, 2020) could also influence immune cell responsiveness to LPS stimulation.

We observed large inter-individual differences in magnitude of LPS-stimulated pro-inflammatory cytokytoke levels consistent with prior literature, including for IL-6 and TNF-α (Copeland et al., 2005; Wurpel et al., 2005), indicating that cells from different individuals vary widely in their ability to produce cytokines (high and low responders). We also noted modest associations between stimulated IL-6 and TNF-α and baseline leukocyte cell counts, suggesting that 15–21% of the inter-individual variability in LPS responses could in part be attributed to whole blood cell type composition. This leaves >80% of the variance in cytokine release to be explained by other factors within these cells. While inter-individual variability in immune responses have previously been attributed to polymorphisms in immune responsive genes (Li et al., 2016), sex-hormones (Taneja, 2018), age, or experimental factors such as LPS exposure time and dosage, our findings add mitochondrial respiratory capacity to the list of potential immunomodulators in humans.

In LPS sensitivity assays that examined EC50 of LPS-induced IL-6 levels, we observed only a minor effect of mitochondrial function on leukocyte sensitivity to LPS. Mitochondrial inhibitors caused a small effect size shift towards a less sensitive, or more tolerant state (i.e., high EC50). While mitochondria have been implicated as modulators as well as targets of LPS-induced inflammation in isolated macrophages (Van den Bossche et al., 2016), our data illustrates a potential link between mitochondrial respiratory capacity and LPS sensitivity in human blood leukocytes. We speculate that changes in mitochondrial function could contribute to a small fraction glucocorticoid resistance in humans and animals chronically exposed to stress (Niraula et al., 2018; Walsh et al., 2018), but testing this hypothesis requires further work.

Multiple studies have emphasized the critical balance between pro- and anti-inflammatory cytokines and the relevance of inter-cytokine interactions in health and disease (Cicchese et al., 2018). Here we asked if mitochondrial respiration affected this pro/anti-inflammatory balance and found that inhibition of respiratory chain function in blood changed not only the overall inflammatory cytokine levels, but also the ratios of pro- and anti-inflammatory cytokines. Pro/anti-inflammatory cytokine ratios in humans and mice are indicators of susceptibility to infection and disease risk (Chae, 2018; Andres-Rodriguez et al., 2019). Interestingly, inhibiting Complex IV significantly elevated the pro/anti-inflammatory cytokine ratio, specifically the IL-6/IL-10 and IL-1p/IL-10 ratio indicating an overactive inflammatory response. This effect could possibly result from a disrupted balance of Th1L(IL-6,IL-1p)/Th2(IL-10) cell types since T cells rely on mitochondria for energy and metabolic support during inflammation (Dimitru et al., 2018). Why this effect was specifically induced by inhibition of Complex IV remains unclear but may relate to the role of Complex IV as the ultimate site of oxygen consumption within the mitochondrion. Moreover, Complex I inhibition elevate both IL-1p and IFN-γ levels, which may also more strongly correlated to each other upon Complex I inhibition, indicating that both cytokines may be under regulation of a common signaling factor from mitochondria. Combined, these results demonstrate that alterations in mitochondrial energetics in general and especially perturbation of Complex IV, modifies LPS-related cytokine signatures in blood leukocytes.

We reported previously that acute stimulation of fibroblasts with DEX is sufficient to extrude mtDNA into the cytoplasm (Trumpf 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 mitochondria 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 (Bekkhat and Neigh, 2018; Rainville et al., 2018), as well as in LPS-induced systemic inflammation (Marshall 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 differences 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 immunomodulation by mitochondrial energetics and provides proof-of-concept data that intrinsic inter-individual variation in mitochondrial phenotypes contribute to differences in immune responses in acute inflammation.

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Author contributions

KRK and MP designed the study with input from NR. CT and KRK prepared the IBR protocol. MM and JT collected and processed the samples. KRK performed the stimulation experiments, and MM performed 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbih.2020.100080.

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Figure S1. Correlation between blood cell subtypes at baseline and LPS-stimulated cytokine response. (A) Correlation coefficient (Spearman r) between % baseline cell subtype distribution in and LPS-stimulated IL-6 and TNF-α levels in whole blood from n=13 individuals. Positive correlations are red and negative correlations are blue. (B) Spearman correlation along with linear regression lines showing direction of association between stimulated cytokines (IL-6 and TNFα) and distribution of cell subtypes.
Figure S2. Mitochondrial respiration inhibitors alter LPS-stimulated cytokine response. (A-C) Fold change of LPS-stimulated cytokine levels upon respiration inhibition (green bars) by Rotenone (Complex I), Antimycin A (Complex III) and Oligomycin (Complex V) relative to LPS only (blue bars). The cytokines are ranked from most- to least- suppressed by an inhibitor. Data are mean±SEM of n=19-20 individuals.
Figure S3. Mitochondrial respiration defects alter inter-cytokine associations in leukocytes. (A-C) Effect of mitochondrial respiration inhibition and DEX treatment on inter-cytokine correlations. The P values and $r^2$ are derived from Spearman rank correlation and linear regression respectively. Data presented are from n=19-20 and significant correlations are shown in bold.
Figure S4. Linear regressions (Pearson r) between the age of participants in Cohort 2 and their baseline mitochondrial measures. Age does not correlate with mitochondrial enzyme activities of Complex I, II (SDH), IV (COX), mitochondrial content (CS), copy number (mtDNAcn) and mitochondrial health (MHI). n=37 except mtDNAcn (n=30).