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.Full Length Article

.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 EC_{50}-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

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.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), evi-.dence suggests that additional mechanisms must influence immune .function, including cellular energetics (Breda et al., 2019).

.Immune responses require a substantial rise in cellular energy de-.mand 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 macro-.phages 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 pep-.tides, 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 psy-.chosocial 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 cyto-.kine 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 systematic 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 knowl-.edge 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-inflam-.matory cytokines and alter multi-cytokine signatures, ii) mitochondrial .function is necessary for glucocorticoid (GC) suppression of pro-.inflammatory cytokines, and iii) baseline mitochondrial energy pro-.duction capacity would in part explain the inter-individual differences in .LPS-driven immune responses. Overall, this work provides initial evi-.dence that mitochondria modulate different aspects of immune re-.sponses 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 thera-.peutic or exercise trial and mitochondrial disease diagnosis. Before the .blood draw, participants completed a brief questionnaire to collect in-.formation 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 glucocor-.ticoid 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 (Thermofisher #11835055). For dose-.dependent lipopolysaccharide (LPS) stimulation, blood was incubated .for 6 h at 37 °C and 5% CO_2 with bacterial endotoxin LPS from *Escher-.ichia 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 #A8674), 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 100uM. 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% CO_2 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 (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 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 back-.ground 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' 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 che-.mokines were measured in plasma using the ProcartaPlex bead Immu-.noassay (ThermoFisher #EPX 200-12185-901) on a Luminex-200 .instrument (Luminex technologies) following the manufacturer's pro-.tocol. 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 incu-.bated 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×*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×*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×*g* for 10 min .at RT. An additional wash was carried out with HBSS at 200×*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 produc-.tion 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 spectrophotomet-.rically 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 polymer-.ase 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 per-.formed i) Spearman rank correlation analysis to test significant .inter-cytokine relationships and Pearson analysis for cytokine- MHI re-.lationships, 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 cy-.tokines 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 cy-.tokines 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 mito-.chondria 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-in- .hibitors 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 3.95% ($d = .-0.29$, $p = 0.36$). Complex III inhibition with Antimycin-A had an anti- .inflammatory effect in 55% of individuals, leading to a median 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: $SD_{Rot} = 19\%$, $SD_{AntiA} = 13.2\%$, $SD_{KCN} = 20.9\%$, .and $SD_{Oligo} = 21.6\%$, 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 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 in- .dividuals 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

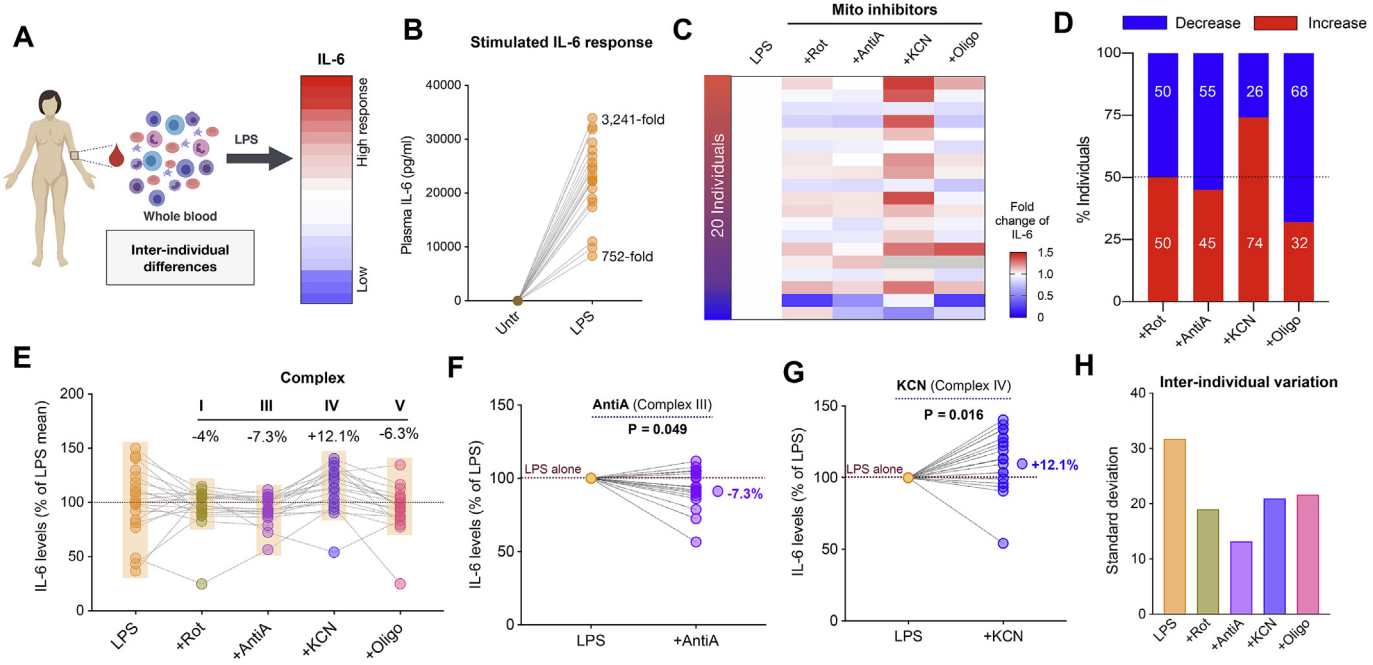

Fig. 1. Inhibition of mitochondrial respiratory capacity causes large inter-individual differences in acute LPS-induced IL-6 levels in human blood. (A) Experimental design illustrating the quantification of cytokine levels from whole human blood (n = 20) before and after LPS stimulation (10 ng/mL). (B) 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.

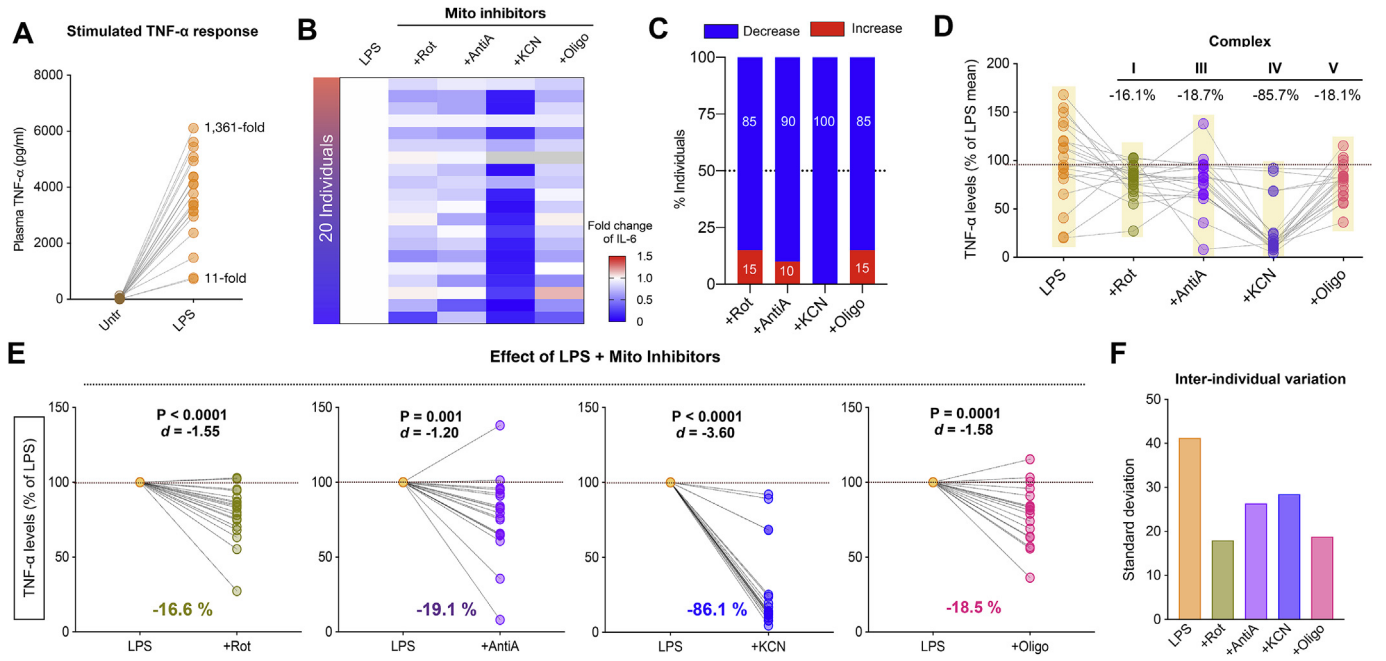

Fig. 2. Inhibition of mitochondrial respiratory capacity reduces LPS-induced TNF-α levels in human blood. (A) Fold change of LPS-stimulated TNF-α levels relative to unstimulated levels. (B) Effect of mitochondrial respiratory chain inhibitors (+Rot for Complex I, +Anti A for Complex III, +KCN for Complex IV and +Oligo for Complex V) on LPS-stimulated TNF-α levels. Missing data are shown in grey. (C) Proportion of individuals showing either increase or decrease in TNF-α levels upon mitochondrial respiratory chain inhibition. (D) TNF-α abundance relative to LPS, illustrating the inter-individual differences across inhibitors (highlighted semi-transparent boxes) where each datapoint is an individual (n = 19–20). Median % change in TNF-α response is shown for each inhibitor. (E) % change in TNF-α levels upon respiratory chain inhibition relative to LPS treatment alone presented with effect sizes (Cohen's *d*) and P values from paired *t*-test. (F) Inhibition of mitochondrial respiration reduces inter-individual variation in stimulated TNF-α levels.

.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 ma-.jority 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 sug-.gest 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

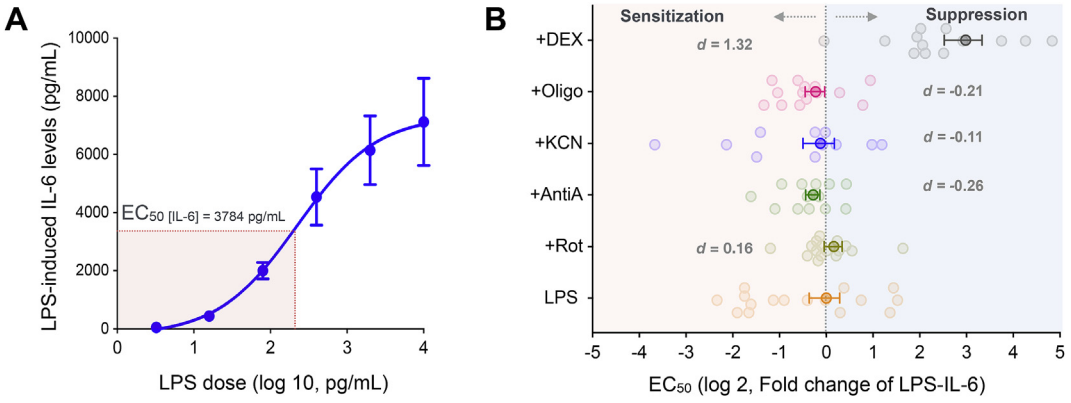

Fig. 3. Sensitivity to LPS exposure is mildly affected by mitochondrial respiratory chain function. (A) Dose-dependent increase in IL-6 levels and LPS EC_{50} (LPS concentration necessary to reach the half-maximal IL-6 levels) measured in whole blood. (B) Effect of mitochondrial respiratory chain inhibitors on EC_{50} relative to LPS alone. Negative values represent sensitization (low EC_{50}) and positive values represent suppression (high EC_{50}) of LPS-response. Effect sizes computed as Cohen's *d*. Data presented are mean ± SEM, n = 20.

.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_{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_{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_{50} = 147.5 ± 48.8, .mean ± SEM) compared to women (EC_{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 stimula- .tory effects of LPS on multiple known inflammatory cytokines, chemo- .kines, and interferons (Fig. 4A), including IL-6 which exhibited the .strongest induction (~2,000-fold) relative to unstimulated levels

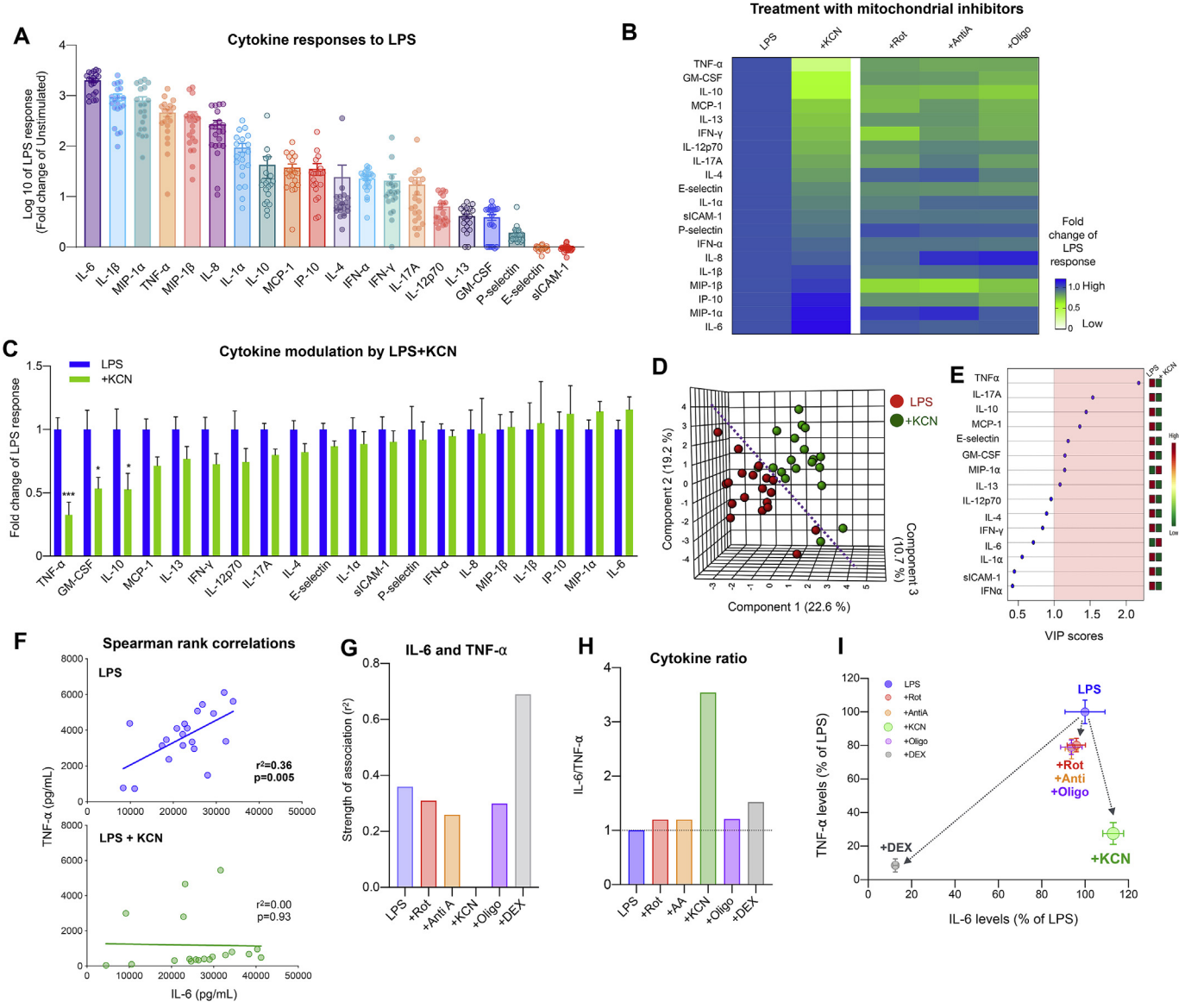

Fig. 4. Inhibition of mitochondrial Complex IV alters pro- and anti-inflammatory cytokine profiles. (A) LPS-stimulated cytokine levels relative to the unstimulated levels. (B) Heatmap illustrating the effect of mitochondrial respiratory chain inhibitors on stimulated cytokine response relative to LPS-only. 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 (r^2) 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.

.(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 eleva- .tion 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 inhibi- .tion 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 of the models tested, consistent with .the large immunosuppressive effects of DEX (see Fig. 6B). Among the 8 .significant cytokines (VIP score >1 in PLS-DA model) that distinguished .the LPS + KCN treatment, TNF-α, IL-17A, IL-10 were the top 3 cytokines, .all downregulated by KCN (Fig. 4E).

.Because TNF-α can regulate the expression of IL-6 (Katz et al., 2004), .and both cytokines are often co-released (Arango Duque and Desco- .teaux, 2014), we next examined the influence of perturbing mitochon- .drial respiratory chain function on these two cytokines. As predicted, .LPS-induced IL-6 and TNF-α levels were significantly correlated upon .stimulation, but KCN treatment abolished this coupling (Fig. 4F,G,I). .While other inhibitors (Complexes I, III and V) caused a 18–20% .reduction in both 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-1 β/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), 31 .(KCN) and 67 (Oligo). Representative cytokine correlations are shown in

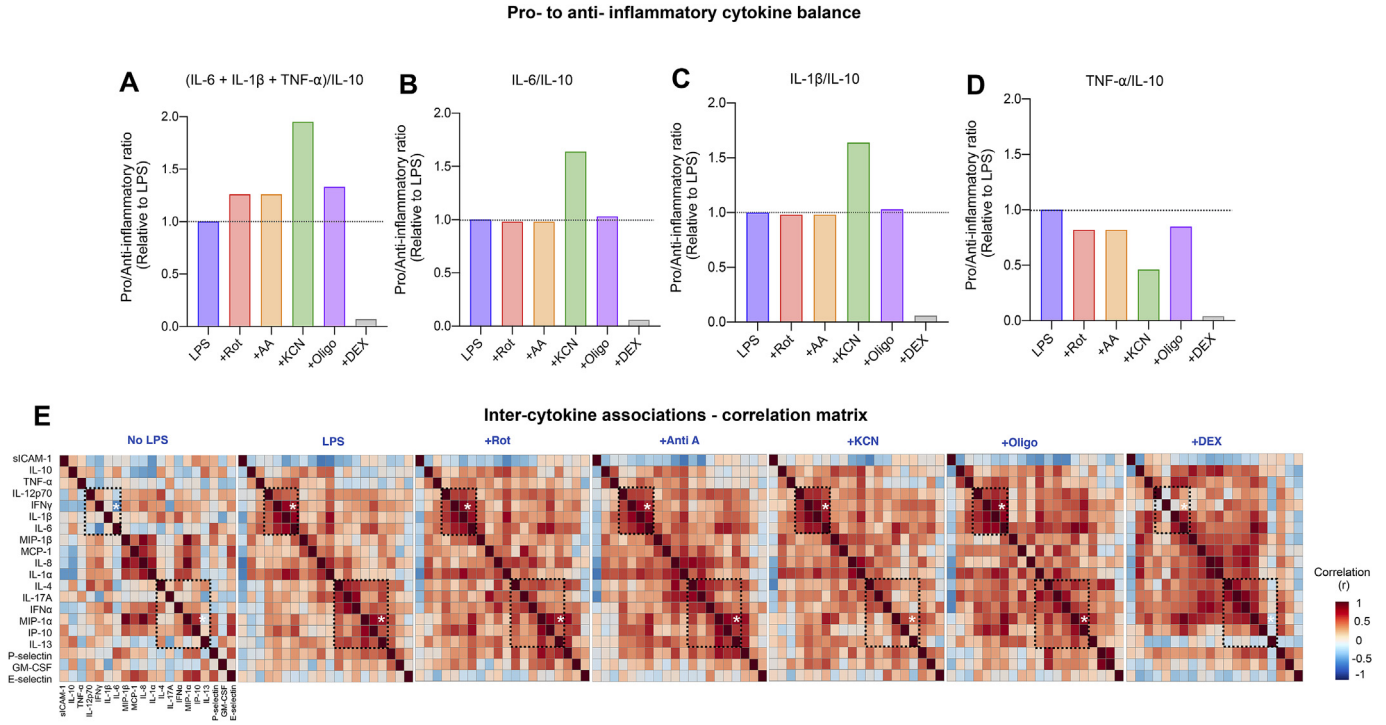

Fig. 5. Perturbation of mitochondrial respiratory capacity modifies cytokine signatures. (A) Effect of mitochondrial respiration inhibitors and GC-mimetic DEX on group-level pro-to anti-inflammatory cytokine ratio, expressed relative to LPS (dotted line). Respiration inhibitors promote a pro-inflammatory state relative to LPS. (B–D) Complex IV inhibition by KCN increases pro-to anti-inflammatory cytokine ratios in IL-6 and IL-1β but decreases in TNF-α. (E) Effect of treatments on inter-cytokine correlations (Spearman rank). Cytokines are rank-ordered based on hierarchical clustering of cytokines from the LPS-group. The dotted regions highlight specific cytokine clusters with altered correlation strength across treatment groups. Each square is a correlation with n = 19–20 individuals.

.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

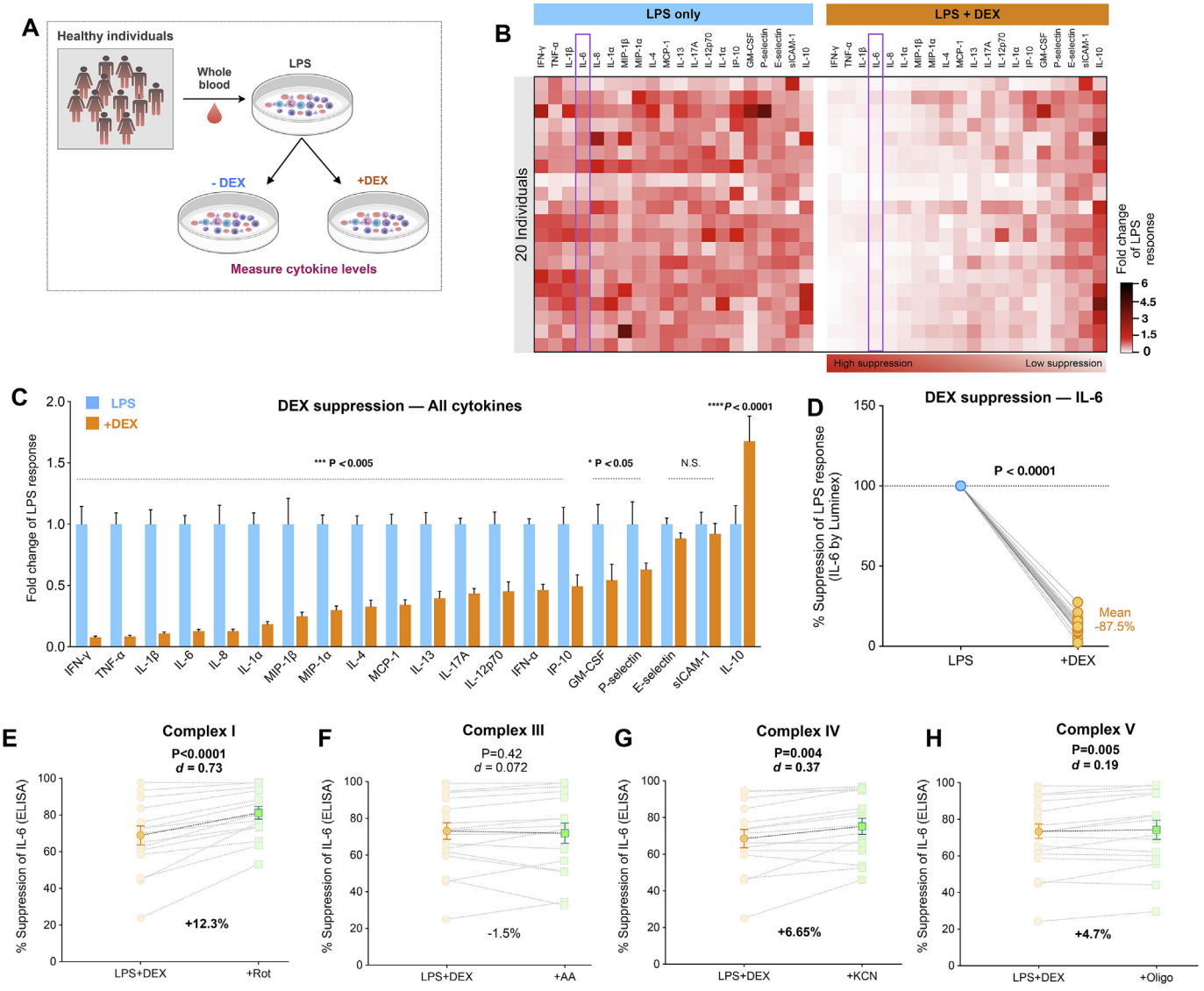

Fig. 6. Glucocorticoid signaling suppresses most LPS-induced pro-inflammatory cytokines and increases IL-10. (A) Experimental design to probe DEX-mediated effects on cytokine signatures. (B) Effect of DEX on LPS-induced inflammatory cytokine responses relative to LPS alone. The cytokines are ordered from most-to least-suppressed by DEX. (C) Fold change in cytokine response to LPS by DEX exposure relative to LPS alone. P values from one-way ANOVA. (D) % DEX suppression of stimulated IL-6, paired *t*-test, n = 19–20. (E–H) Effect of respiratory chain inhibitors on %DEX-suppression. Effect sizes (Cohen's *d*) and % changes in DEX-suppression are presented, with P values from paired *t*-test. Data are means ± SEM, n = 20 except in E-H where n = 15–16.

.bioenergetic capacity accounts for a portion of inter-individual differ-.ences 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 inte-.grated into an index of mitochondrial functional capacity, the MHI .(Picard et al., 2018). In the same individuals, whole blood was stimu-.lated 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 mito-.chondrial measures were correlated with cytokine levels in both un-.treated (no-LPS) and LPS-stimulated conditions, and the magnitude and .direction of the associations between mitochondrial content (citrate .synthase), respiratory chain activities, mtDNAcn, 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 corre-.lated 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 mito-.chondrial 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 mod-.ulates blood cytokine response upon LPS and DEX exposure. Acute .pharmacological inhibition of mitochondrial respiration reduced inter-.individual variation in cytokine levels, altered overall cytokine signa-.tures, 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 in-.flammatory 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 respira-.tory 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 me-.diators of immune processes (Strahler et al., 2015). First, it is possible to .vary the strength of the immune challenge, such as exposure to lipo-.polysaccharide (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 selec-.tive inhibitors, including inhibition of specific respiratory chain com-.ponents. Finally, this approach also makes it possible to combine known .immunomodulators, such as immunosuppressive glucocorticoid (GC)

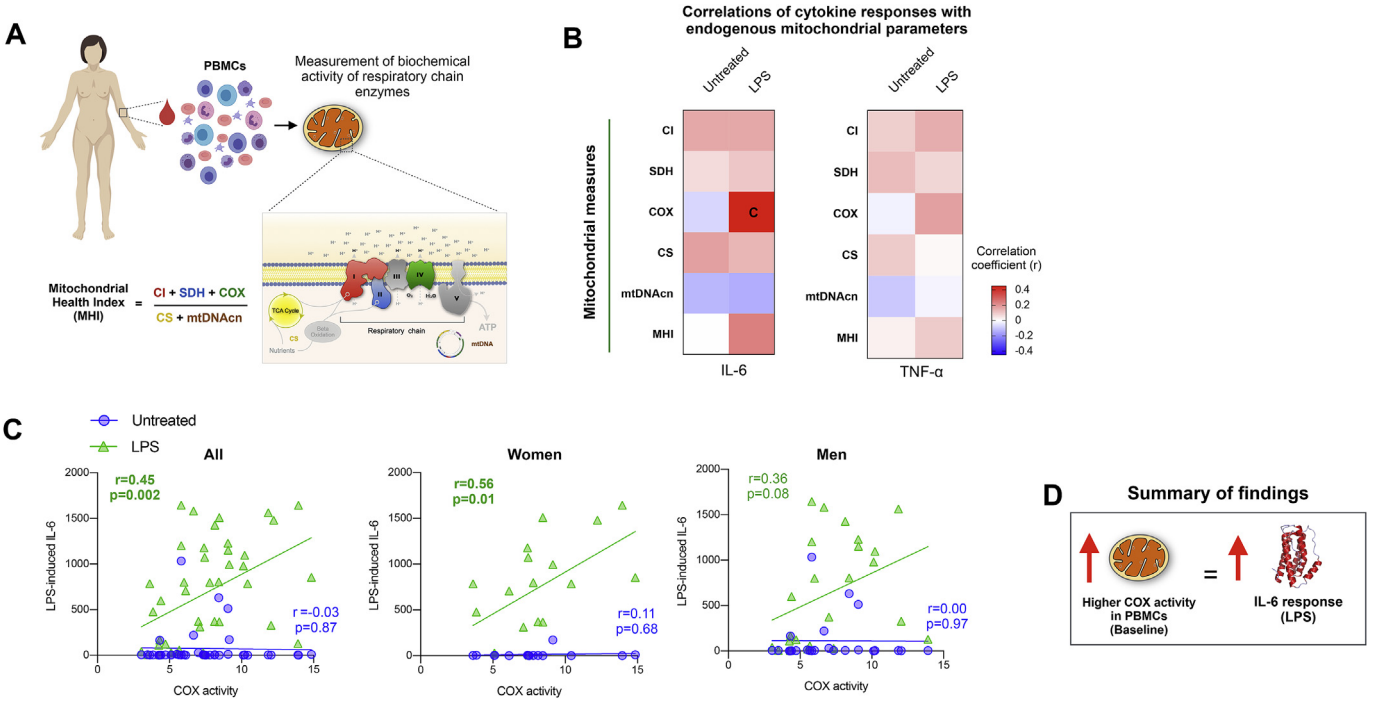

Fig. 7. Associations between intrinsic bioenergetic capacity and cytokine production. (A) Schematic illustrating the measurement of mitochondrial respiratory chain activity, mtDNA copy number (mtDNAcn, and the mitochondrial health index (MHI) from human peripheral blood mononuclear cells (PBMCs). (B) Correlation between baseline mitochondrial measures and baseline and LPS-stimulated IL-6 and TNF-α responses shown as a heatmap with positive correlations (r) red and negative correlations blue. The correlation shown in the box marked with a "c" is shown in the next panel. (C) Correlation between baseline Complex IV (COX) activity measured in PBMCs and IL-6 levels at baseline (untreated) and after LPS stimulation (1 ng/mL) in all participants, and disaggregated by sex. (D) Schematic summarizing the overall correlation between mitochondrial function and cytokine response. Correlation coefficients (r) are derived from Pearson correlation analysis, n = 39–42 (Women = 15-18, Men = 24). *TCA*: tricarboxylic acid cycle, also known as "Krebs cycle". (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

.signaling via dexamethasone (DEX), with mitochondrial modulators and .thus study their interaction. Additionally, recognizing that immune re-.sponses 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 condi-.tions 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 mitochon-.dria (Al Amir Dache et al., 2020; Song, 2020) could also influence im-.mune cell responsiveness to LPS stimulation.

.We observed large inter-individual differences in magnitude of LPS-.stimulated pro-inflammatory cytokine levels consistent with prior .literature, including for IL-6 and TNF-α (Copeland et al., 2005; Wurfel .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 res-.piratory capacity to the list of potential immunomodulators in humans.

.In LPS sensitivity assays that examined EC_{50} 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 .EC_{50}). 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 in-.dicators 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, specif-.ically the IL-6/IL-10 and IL-1β/IL-10 ratio indicating an overactive in-.flammatory response. This effect could possibly result from a disrupted .balance of Th1(IL-6,IL-1β)/Th2(IL-10) cell types since T cells rely on .mitochondria for energy and metabolic support during inflammation .(Dumitru et al., 2018). Why this effect was specifically induced by in-.hibition 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 elevated both IL-1β and .IFN-γ levels, which were 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 (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 multi-.ple 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 dysfunc-.tion 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 mito-.chondria 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 chal-.lenges 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 in-.dividuals 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 pro-.found modulatory effects of mitochondrial respiration on specific cyto-.kines. 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- .ences 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.

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.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

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.Appendix A. Supplementary data

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

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