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Immune system cells from COVID-19 patients display compromised mitochondrial-nuclear expression co-regulation and rewiring toward glycolysis

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SUMMARY

Mitochondria are pivotal for bioenergetics, as well as in cellular response to viral infections. Nevertheless, their role in COVID-19 was largely overlooked. Here, we analyzed available bulk RNA-seq datasets from COVID-19 patients and corresponding healthy controls (three blood datasets, N = 48 healthy, 119 patients; two respiratory tract datasets, N = 157 healthy, 524 patients). We found significantly reduced mtDNA gene expression in blood, but not in respiratory tract samples from patients. Next, analysis of eight single-cells RNA-seq datasets from peripheral blood mononuclear cells, nasopharyngeal samples, and Bronchoalveolar lavage fluid (N = 1,192,243 cells), revealed significantly reduced mtDNA gene expression especially in immune system cells from patients. This is associated with elevated expression of nuclear DNA-encoded OXPHOS subunits, suggesting compromised mitochondrial-nuclear co-regulation. This, together with elevated expression of ROS-response genes and glycolysis enzymes in patients, suggest rewiring toward glycolysis, thus generating beneficial conditions for SARS-CoV-2 replication. Our findings underline the centrality of mitochondrial dysfunction in COVID-19.

INTRODUCTION

A significant number of COVID 19 patients develop severe fatal consequences of the respiratory tract infection, attributed to catastrophic inflammatory events, described jointly as a 'cytokine storm'. This inflammatory state is associated with deleterious oxidative stress, and elevated production of reactive oxygen species (ROS) both point toward altered mitochondrial activity (Singh et al., 2020). This is further supported by evidence suggesting a central role for the mitochondria in modulating the activity of the innate immune system (Bordon 2018; Zuo and Wan 2019; Ganji and Reddy 2020). Specifically, once viral genomes are recognized by the innate immune system, the retinoic acid inducible gene I (RIG I) like receptors (RLRs), RIG I, and MDA5, interact with mitochondrial antiviral signaling protein (MAVS). MAVS, in turn, activates the intracellular signaling cascade that induces the transcription of genes encoding type I interferons (Rehwinkel and Gack 2020).

Several lines of evidence underline the mitochondria as a pivotal cellular target during SARS CoV 2 infection: Firstly, the discovery of high sequence similarity between SARS CoV 2 encoded proteins and mitochondrial localized proteins encoded by SARS CoV 1 (3/29 protein coding genes) (Shi et al., 2014; Gordon et al., 2020), which were shown to impact mitochondrial gene expression (Shao et al., 2006) and components of the innate immunity (Burtscher et al., 2020). Secondly, recent extensive computational analyses predicted preferential mitochondrial localization of SARS CoV 2 RNAs and sgRNAs (Wu et al., 2020) and experimental evidence revealed interaction of SARS CoV 2 encoded proteins with the mitochondria (Gordon et al., 2020). Third, recent evidence revealed alteration of mitochondrial dynamics in COVID 19 patients, which is associated with increased fusion, and inhibition of mitochondrial fission (Holder and Reddy 2021). It is thus likely that mitochondrial function is hijacked, and altered, upon SARS CoV 2 infection (Gatti et al., 2020; Singh et al., 2020). Despite the known regulatory involvement of the mitochondria in the immune system, the regulatory response of the mitochondria to SARS CoV 2 infection has been largely overlooked. Recent RNA seq analysis in airway clinical samples and in lung associated cell lines suggested little or no response of mitochondrial gene expression, either in the mitochondrial genome (mtDNA) or in the nucleus, to SARS CoV 2 infection (Miller et al., 2021). This finding, which was reported during the course

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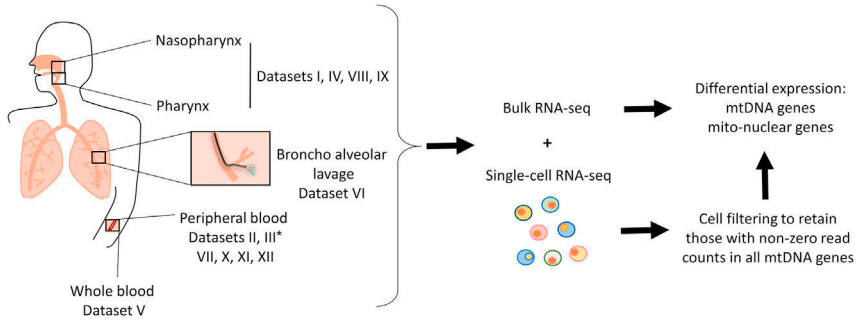


Figure 1. Workflow of this study

Several different publicly available bulk and single-cell RNA-seq datasets from healthy and COVID-19 patients were obtained for analysis (see Table 1 for resources). For scRNA-seq data quality control, cells were filtered to retain only those with read counts in mtDNA encoded-protein genes. Finally, differential expression analysis of mitochondrial genes was performed. *Dataset III was used both for bulk and scRNA RNA-seq analyses, and hence was considered two separate datasets.

of our work, may lead to the conclusion that mitochondrial gene expression and subsequent mitochondrial activities are not significantly affected in COVID 19 patients. However, such findings do not dismiss possible tissue/cell type specific regulatory response of the mitochondria to SARS CoV 2 infection.

Here, by analyzing both bulk and single cells RNA seq (scRNA seq) data in COVID 19 patients as compared to controls we discovered significant reduction in mtDNA gene expression levels in patient samples from blood, in contrast to inconsistent changes in samples from the respiratory tract. Consistent with this finding, scRNA seq analysis also revealed that such altered mtDNA gene expression preferentially occurred in immune system cells, whereas respiratory tract epithelial cells tended to display less prominent alterations. The response of mitochon drial related pathways in the nucleus (such as ROS generation, TCA, glycolysis) is discussed in light of the apparent dependence of SARS CoV 2 replication on glycolysis (Codo et al., 2020), likely on the expense of the mitochondrial oxidative phosphorylation (OXPHOS). Our findings underline a tissue type and cell type dependent negative response of mitochondrial gene expression regulation in COVID 19 patients.

RESULTS

Mitochondrial gene expression in COVID-19 patients is significantly reduced in peripheral blood, but not in the upper respiratory tract

To examine whether SARS CoV 2 infection associates with altered mitochondrial regulation, we assessed mtDNA encoded genes' expression in thirteen publicly available datasets from healthy and COVID 19 pa tients: five bulk RNA seq datasets from naso oropharyngeal (NP/OP) swab (Lieberman et al., 2020; Mick et al., 2020), peripheral blood and whole blood (Bernardes et al., 2020; Levy et al., 2021; Thair et al., 2021), as well as eight scRNA seq datasets from peripheral blood mononuclear cells (PBMC) (Bernardes et al., 2020; Wilk et al. 2020, Wilk et al., 2021; Ren et al., 2021; Stephenson et al., 2021), Bronchoalveolar lavage fluid samples (BALF) (Liao et al., 2020), and nasopharyngeal (NP) samples (Chua et al., 2020; Ziegler et al., 2021) (Figure 1, Table 1 notice dataset numbering, used below).

In consistence with previous findings, differential expression analysis of mtDNA genes in bulk RNA Seq from the upper airway in healthy individuals as compared to patients who were positively diagnosed for SARS CoV 2 (Datasets I, IV) did not show any significant difference in mtDNA gene expression in the patients (Miller et al., 2021) (N 524 SARS CoV 2 patients, N 157 healthy control; see Table 1) (Figure S1). In contrast, analysis of three independent bulk RNA seq datasets from peripheral blood of healthy individ uals and COVID 19 patients (N 34 healthy individuals and N 106 COVID 19 patients; see Table 1 in STAR Methods), revealed significant reduction in the expression of 8/13 and 11/13 mtDNA encoded protein coding transcripts, respectively (Figure 2 and S2, Table S1; Datasets II, V https://doi.org/10.17632/8kd3xjfrh4.1).

We next analyzed bulk RNA seq data (Dataset III) collected from 14 healthy individuals, samples from 13 hospitalized COVID 19 patients, each having up to five samples collected during disease progression, termed pseudotime points (Bernardes et al., 2020). Since the sample size of pseudotime 7 (recovery

Table 1. Datasets sample size and resources

Dataset[a]	Number of SARS CoV 2 patients analyzed	Number of healthy individuals	Reference	Download resource
Upper airway - Dataset I (bulk)	94	103	(Mick et al., 2020)	https://github.com/czbiohub/COVID-19-transcriptomics-pathogenesis-diagnostics-results
Peripheral blood - Dataset II (bulk)	62	24	(Thair et al., 2021)	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE152641
Peripheral blood – Dataset III (bulk *and* single cells)	13	14	(Bernardes et al., 2020)	Bulk RNA-seq: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE161777 scRNA-seq: FastGenomics (https://beta.fastgenomics.org/p/565003)
Nasopharyngeal swab - Dataset IV (bulk)	430	54	(Lieberman et al., 2020)	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE152075
Whole blood - Dataset V (bulk)	44	10	(Levy et al., 2021)	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE171110
BALF - Dataset VI (single cells)	9	3	(Liao et al., 2020)	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE145926
PBMC - Dataset VII (single cells)	8	6	(Wilk et al., 2020)	https://www.covid19cellatlas.org/ (hosted by the Wellcome Sanger Institute) (Ballestar et al., 2020)
NP - Dataset VIII (single cells)	19	5	(Chua et al., 2020)	FigShare: https://doi.org/10.6084/m9.figshare.12436517
NP - Dataset IX (single cells)	29	15	(Ziegler et al., 2021)	Single Cell Portal: https://singlecell.broadinstitute.org/single cell/study/SCP1289/
PBMC – Dataset X (single cells)	19	8	(Wilk et al., 2021)	https://www.covid19cellatlas.org/
PBMC – Dataset XI (single cells)	9	5	(Ren et al., 2021)	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE158055
PBMC – Dataset XII (single cells)	49	23	(Stephenson et al., 2021)	https://www.covid19cellatlas.org/ (hosted by the Wellcome Sanger Institute)

[a]Bulk or Single cells RNA-seq is indicated per dataset.

long term follow up) was very small (N 2), it was excluded from further analysis. The pseudotime points were originally defined based on inflammatory markers and ventilation requirements as follows: pseudo times 1,2 and 3 were considered 'severe', pseudotimes 4 and 5 were considered early/moderate and late convalesces, respectively, and pseudotime 6 were from recovered individuals (see STAR Methods). Notably, to avoid minute sample sizes in bulk RNA seq of Dataset III, we grouped pseudotimes 1, 2 and 3 (all originally defined as 'severe'). Pairwise comparison of gene expression between control and each of the remaining disease trajectory phases revealed significant reduction in the expression of all mtDNA protein coding transcripts in the severe, moderate/early convalescent as well as in late convalescent indi viduals (Figure 2B, Table S1). Interestingly, no significant change was observed in mtDNA protein coding transcript levels while comparing healthy controls to recovered individuals (pseudotime 6; Figure 2B). Hence, our results suggest that mtDNA gene expression is consistently reduced in patients' blood, but not in the respiratory tract, and that such altered expression is likely reversible upon recovery.

Expression of nuclear DNA-encoded OXPHOS, ROS formation, TCA, glycolysis and mitochondria-related viral response genes is generally elevated in COVID-19 patients

As positive co expression of mitochondrial and nuclear DNA encoded OXPHOS genes has been observed across many human tissues (Barshad et al., 2018) we asked whether the reduced mtDNA gene expression in

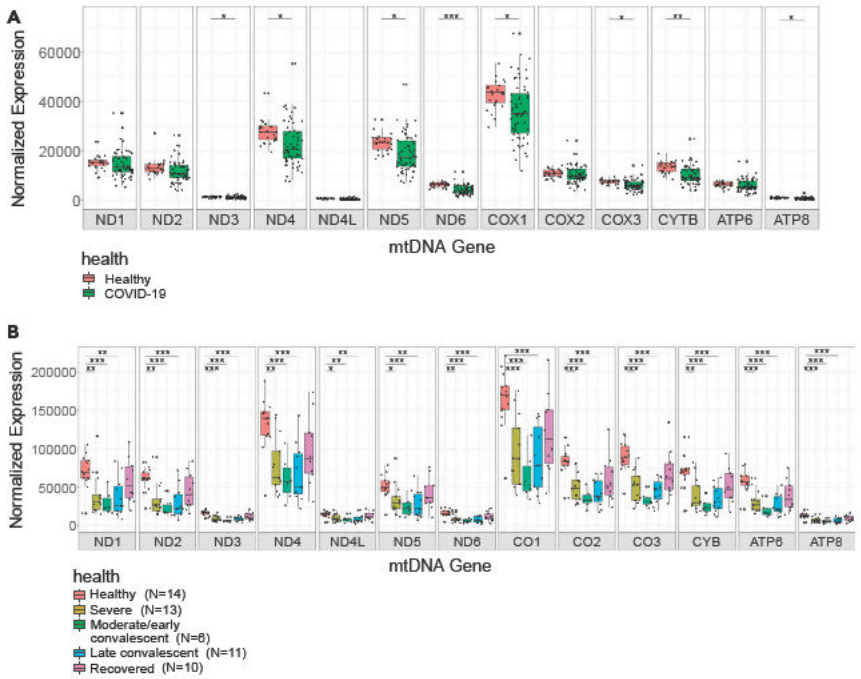


Figure 2. Decreased mtDNA gene expression levels as a feature of COVID-19 in peripheral blood

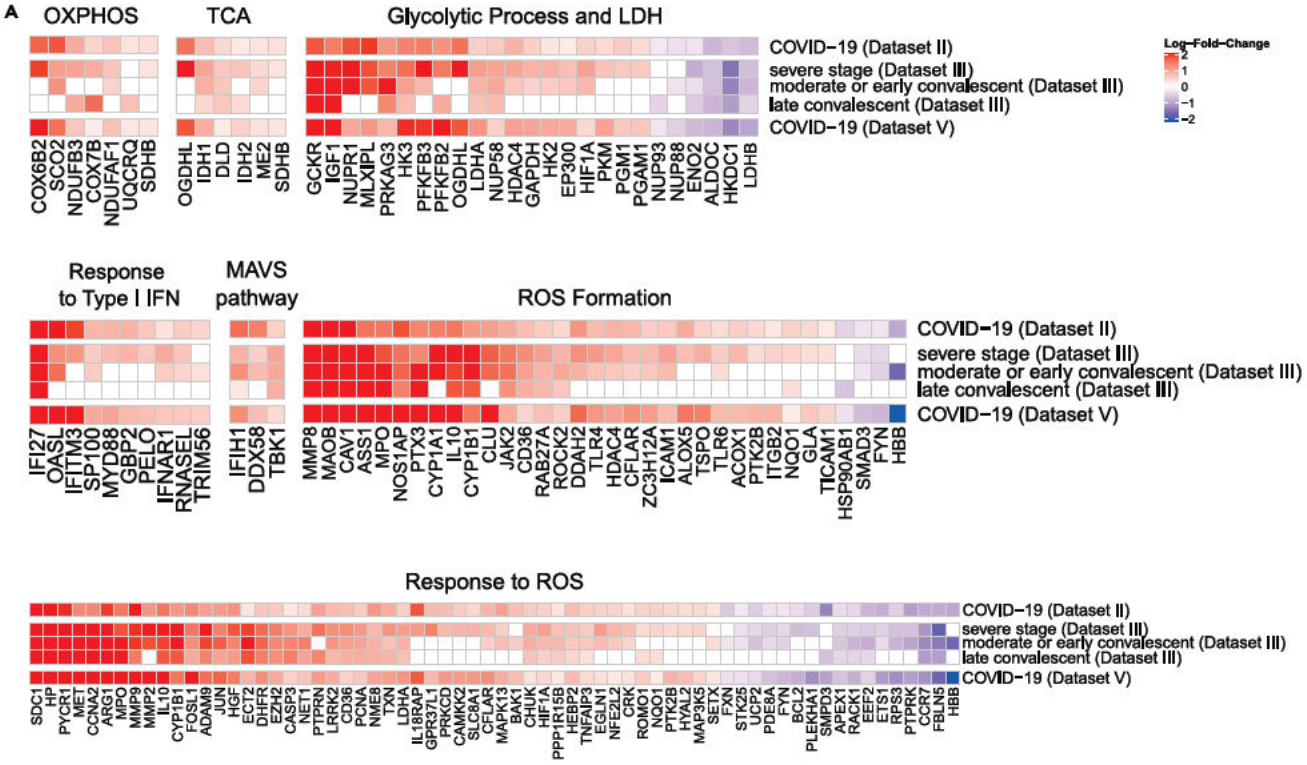
(A) Box plot of bulk RNA-seq analysis in peripheral blood displays lower mtDNA gene expression in COVID-19 patients as compared to healthy controls (Dataset II).

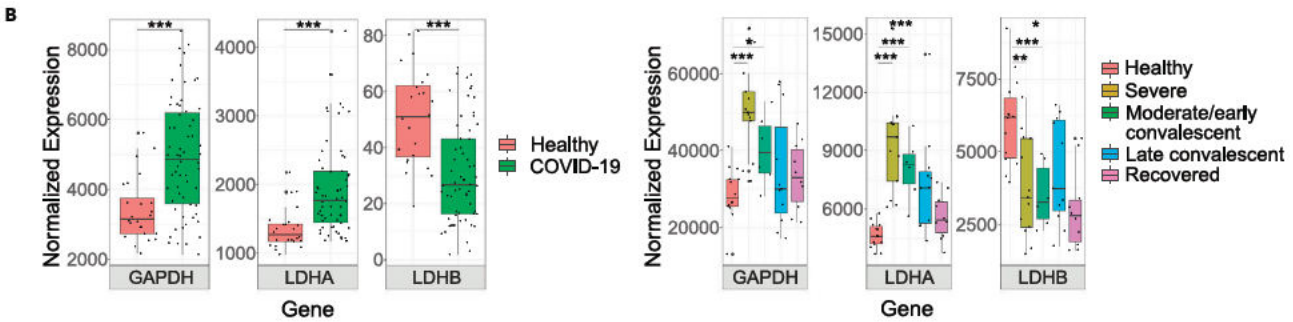
(B) Box plot displaying mtDNA gene expression across COVID-19 pseudotimes as compared to control (Dataset III – bulk RNA-seq). X axis – mtDNA genes, Y axis – normalized read counts, which account for expression levels. Significance: * - $p < 0.05$, ** - $p < 0.005$, *** - $p < 0.0005$.

See also Figures S1 and S2 and Table S1.

COVID 19 patients correlate with changes in nuclear DNA encoded OXPHOS genes and related pathways. To this end, we subjected Datasets II, III and V to assessment of differentially expressed genes involved in the following biochemical pathways: OXPHOS structural and assembly genes, mitochondrial ribosome (Wolf and Mootha 2014; Barshad et al., 2018). We also analyzed the following mitochondria related Gene Ontology (GO) terms: tricarboxylic acid (TCA), glycolysis enzymes including L lactate dehydroge nase, response to type I interferons, mitochondrial antiviral signaling, including genes involved in RLR MAVS pathway (hereby annotated as MAVS pathway; Table S2) (Burtscher et al., 2020; Rehwinkel and Gack 2020), generation and response to reactive oxygen species (ROS) formation, along with ROS scavenging enzymes. Notably, since measurement of the average expression of a given pathway may mask the impact of SARS CoV 2 infection on individual genes, we performed a gene by gene differential expression analysis (while stringently correcting for multiple testing) (Figure 3A). We screened for differen tially expressed genes that were consistently significant in Datasets II, III, and V, and considered only genes whose responses were consistent with at least one disease condition from Dataset III. Notably, while considering Dataset III, we focused on conditions that displayed significantly reduced mtDNA genes expression analysis. The analysis revealed a tendency toward elevated expression among OXPHOS sub units (7 elevated genes), TCA (6 elevated genes) and glycolysis (19 elevated genes, 6 reduced genes) in Datasets II and V and in severe, moderate/early and late convalescent individuals from Dataset III (Fig ure 3A; Table S3). Specifically, while considering glycolysis, expression levels of a key glycolysis enzyme glyceraldehyde 3 phosphate dehydrogenase (GAPDH), were elevated in Datasets II and V as well as in severe and moderate/early convalescent patients of Dataset III (Figure 3B). It is worth noting that gene expression analysis of mitochondrial ribosome subunits revealed a mixed response in patients, with only few statistically significant values (Table S3).

Notably, we found that the expression of two subunits of lactate dehydrogenase (LDH), a key enzyme that regulates pyruvate generation by glycolysis, responded differently in the peripheral blood samples of pa tients versus controls, as follows: The expression of LDHA, a subunit which is more abundant in glycolytic


A
OXPHOS
TCA
Glycolytic Process and LDH
COVID−19 (Dataset II)
severe stage (Dataset III)
moderate or early convalescent (Dataset III)
late convalescent (Dataset III)
COVID−19 (Dataset V)
Log−Fold−Change
COX6B2 SCO2 NDUFB3 COX7B NDUFAF1 UQCRQ SDHB
OGDHL IDH1 DLD IDH2 ME2 SDHB
GCKR IGF1 NUPR1 MLXIPL PRKAG3 HK3 PFKFB3 PFKFB2 OGDHL LDHA NUP58 HDAC4 GAPDH HK2 EP300 HIF1A PKM PGM1 PGAM1 NUP93 NUP88 ENO2 ALDOC HKDC1 LDHB
Response to Type I IFN
MAVS pathway
ROS Formation
IFI27 OASL IFITM3 SP100 MYD88 GBP2 PELO IFNAR1 RNASEL TRIM56
IFIH1 DDX58 TBK1
MMP8 MAOB CAV1 ASS1 MPO NOS1AP PTX3 CYP1A1 IL10 CYP1B1 CLU JAK2 CD36 RAB27A ROCK2 DDAH2 TLR4 HDAC4 CFLAR ZC3H12A ICAM1 ALOX5 TSPO TLR6 ACOX1 PTK2B ITGB2 NQO1 GLA TICAM1 HSP90AB1 SMAD3 FYN HBB
Response to ROS
SDC1 HP PYCR1 MET CCNA2 ARG1 MPO MMP9 MMP2 IL10 CYP1B1 FOSL1 ADAM9 JUN HGF ECT2 DHFR EZH2 CASP3 NET1 PTPRN LRRK2 CD36 PCNA NME8 TXN LDHA IL18RAP GPR37L1 PRKCD CAMKK2 SLC8A1 CFLAR MAPK13 BAK1 CHUK HIF1A PPP1R15B HEBP2 TNFAIP3 EGLN1 NFE2L2 CRK ROMO1 NQO1 PTK2B HYAL2 MAP3K5 SETX FXN STK25 UCP2 PDE8A FYN BCL2 PLEKHA1 SMPD3 APEX1 RACK1 EEF2 ETS1 RPS3 PTPRK CCR7 FBLN5 HBB


B
Normalized Expression
GAPDH
LDHA
LDHB
Gene
Healthy
COVID-19
Healthy
Severe
Moderate/early convalescent
Late convalescent
Recovered

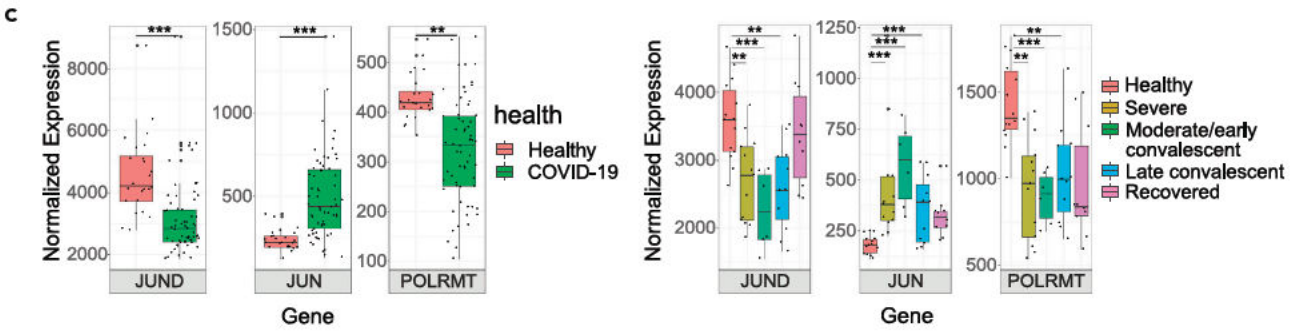

C
Normalized Expression
JUND
JUN
POLRMT
Gene
health
Healthy
COVID-19
Healthy
Severe
Moderate/early convalescent
Late convalescent
Recovered

Figure 3. Expression of nuclear DNA genes involved in mitochondrial function or regulation of mitochondrial gene expression was consistently altered in peripheral blood from patients

(A) Heatmaps of significant differentially expressed genes in mitochondria-related biochemical pathways. Color bar representing the log-fold-change (logFC) of significant and consistent genes' expression in COVID-19 patients. Genes with logFC higher than 0.2 or less than 0.2 are shown. Red: positive logFC, purple: negative logFC.

(B) Box plots of GAPDH, LDHA and LDHB and (C) JUND, JUN (i.e., c-Jun), POLRMT expression levels in Datasets II (left panel) and Dataset III- bulk RNA-seq (right panel) (see Figure S2 for Dataset V). X axis – gene names; Y axis – normalized read counts as in Figure 2. Significance: * - $p < 0.05$, ** - $p < 0.005$, *** - $p < 0.0005$.

See also Figure S2 and Tables S2 and S3 (https://doi.org/10.17632/8kd3xjfrh4.1).

tissues (Read et al., 2001; Porporato et al., 2011), was elevated in patients from Datasets II and V and in severe, moderate/early and late convalescent of Dataset III (Figure 3B). In contrast, the expression of LDHB, a subunit that is more abundant in high OXPHOS tissues (Read et al., 2001; Porporato et al., 2011), was reduced in patients from Datasets II and V and in severe, moderate/early convalescent samples of Dataset III (Figure 3B). As LDH activity governs the choice between generation of pyruvate (which promotes OXPHOS function) versus lactate, these results further support the interpretation that OXPHOS function is likely compromised and rewired to glycolysis in patients versus controls.

Lastly, genes that participate in type I interferon pathway (likely a reflection of the so called mitochondria related cytokine storm), genes involved in MAVS pathway, ROS formation and ROS response, mostly displayed elevated expression in COVID 19 patients in Dataset II, V and in severe, moderate/early and late convalescent samples of Dataset III (Figure 3A). Specifically, there were 10 elevated genes in the IFN1 pathway, and three genes involved in the MAVS pathway. Moreover, the expression of retinoic acid inducible gene I (RIG I), melanoma differentiation associated protein 5 (MDA5) (DDX58 and IFIH1, respectively) and TANK binding kinase 1 (TBK1), was consistently elevated in COVID 19 patients (Figure 3A, Table S3), supporting the increase in type I interferon pathway. Next, most of the significant genes from the ROS formation pathway (30 out of 34) displayed elevated expression in patients; similarly, most ROS response pathway genes (49 out of 66) displayed elevated expression as well. Notably, the expression of certain ROS scavenging enzymes (e.g., Peroxiredoxin 3 (PRDX3) and superoxide dismutase 2 (SOD2) (Yoboue et al., 2018)) was also significantly elevated in COVID 19 patients in 2/3 Datasets (Table S3). Taken together, these findings suggest that along with the apparent rewiring toward glycolysis, COVID 19 patients also suffer from possible oxidative stress.

Identifying candidate regulatory factors to explain changes in mtDNA gene expression in patients

We hypothesized that the reduction of mtDNA gene expression levels in COVID 19 patients could be regulated, rather than spontaneous. To identify candidate regulatory factors that potentially explain such putative downregulation, we assessed differential expression of a set of known and candidate mtDNA regulatory factors of transcription, regulatory factors of mtDNA replication, and nuclear DNA encoded factors with known mitochondrial RNA binding activity (Wolf and Mootha 2014; Cohen et al., 2016), as well as RNA and DNA binding proteins that were recently identified in human mitochondria (Ardail et al., 1993; Fernandez Vizarra et al., 2008; She et al., 2011; Blumberg et al., 2014; Lambertini et al., 2015; Chatterjee et al., 2016) (Table S2). Our analysis revealed that in all peripheral and whole blood bulk RNA seq datasets (Datasets II, III, and V), the expression of JUN (c Jun) was significantly elevated while the expression levels of JunD and of mitochondrial RNA polymerase (POLRMT) were reduced especially in peripheral blood datasets (Datasets II, III) (Figures 3C and S2, Table S3). The reduction in POLRMT expression in patients, the only known mitochondrial RNA polymerase, provides an attractive explanation to the reduction in mtDNA gene expression (Figures 3C and S2). The altered expression of c Jun and JunD is intriguing these are known nuclear gene expression regulators that were also found to bind human mtDNA *in vivo* (Blumberg et al., 2014). Our results suggest that these are candidate regulators of gene expression in both the nuclear and mitochondrial genomes (see Discussion).

Single cells RNA-seq analysis further support reduction in mtDNA gene expression in COVID-19 patients, especially in immune system cells

Our above described analysis of bulk RNA seq data suggest that the response of mitochondrial genes' expression in patients' peripheral blood cells is more consistent than cells coming from the respiratory

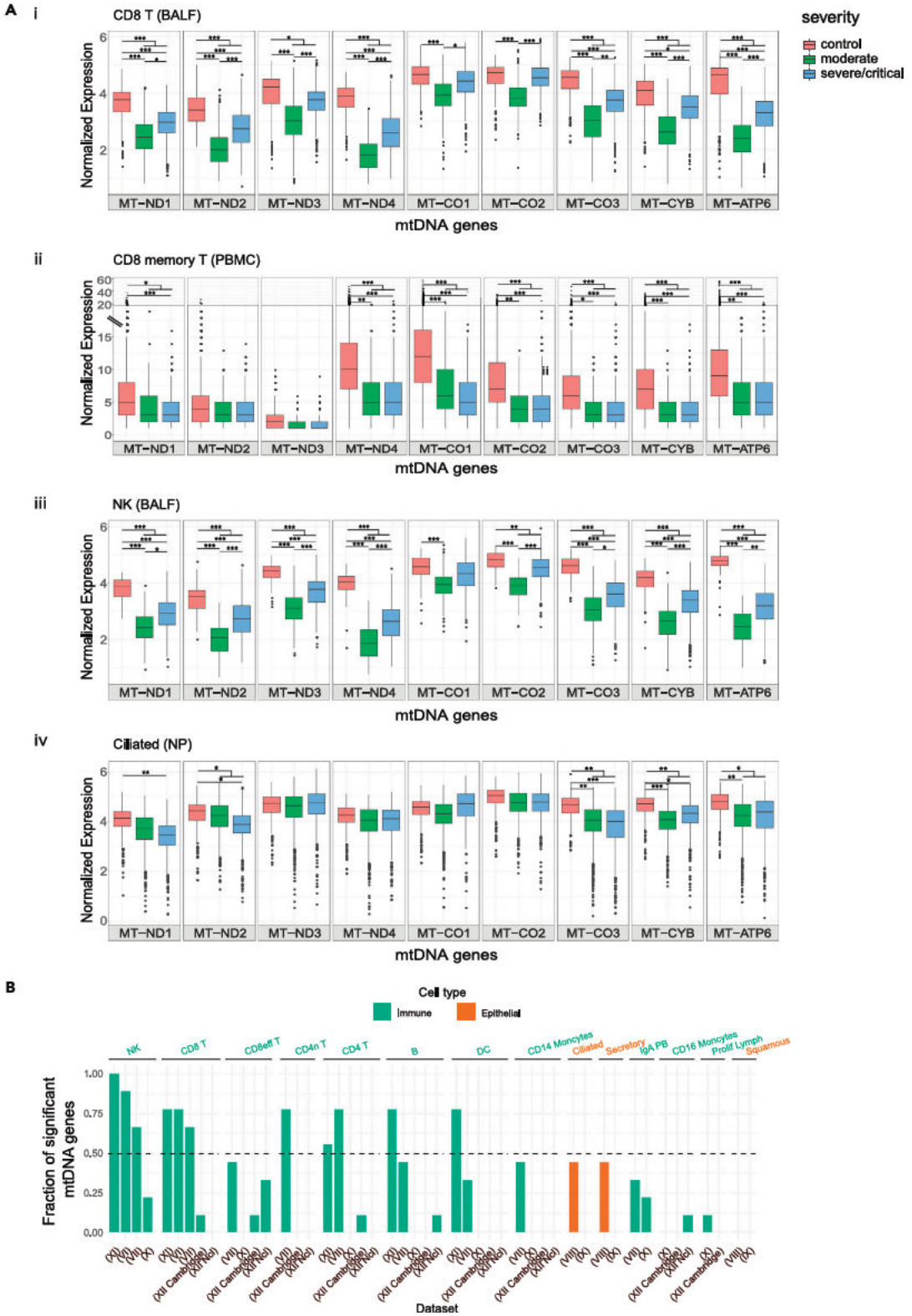


Figure 4. The change in mtDNA genes expression in COVID-19 patients varies among cell types, and is more prominent in immune system cells

(A) Box Plots of mtDNA gene expression from healthy and COVID-19 patients in (i) CD8 T BALF cells (Dataset VI), (ii) CD8 T memory PBMC cells (Dataset VII), (iii) NK BALF cells (Dataset VI) (iv) Ciliated NP cells (Dataset VIII). X axis - gene names; Y axis – normalized UMI (unique molecular identifier) counts.

Figure 4. *Continued*

(B) Bar plot presenting the fraction of significantly altered mtDNA genes' expression per cell type. X axis: cell types present in at least two datasets, Y axis: fraction of mtDNA genes with significantly altered expression. Orange - Epithelial cells, Green – Immune system cells. Dataset numbers are indicated in parenthesis. The dashed line represents a threshold of significance in half of the analyzed mtDNA genes.
See also Tables S1 and S4 (https://doi.org/10.17632/8kd3xjfrh4.1).

tract. As blood cells are enriched by immune system cell types, it is possible that cells belonging to the im mune system went through a more prominent response of mtDNA gene expression to COVID 19, than other cell types. To address this possibility, we analyzed eight scRNA seq datasets including a total of 1,192,243 high quality and annotated cells (Tables 1 and S4). In total, we analyzed cells from 77 critical/se vere COVID 19 patients, 65 COVID 19 patients with a moderate phenotype, 65 healthy individuals and scRNA seq data from the patients studied in Dataset III (above analyzed also for bulk RNA seq in peripheral blood) (see Table 1 in STAR Methods). To avoid technical noise due to known elevated proportions of zero read counts in scRNA seq data (Vallejos et al., 2017), we included in our analysis only cells whose sequencing read counts were higher than zero for each of our analyzed mtDNA genes (Table S4). Notably, since scRNA seq libraries were prepared while enriching for poly(A) mRNAs, we focused our analysis on nine mtDNA genes with known longer 3′ poly(A)(ND1 4, CO1 3, CytB, and ATP6) (Slomovic et al., 2005), and hence reduced the false discovery rates of lower mtDNA gene expression in certain genes. Finally, we considered a finding biologically meaningful if it was observed in at least two independent datasets.

Firstly, in consistence with our analysis of bulk RNA seq from peripheral blood, significantly reduced mtDNA encoded genes' expression was observed in several T cells sub types (Table S1, https://doi.org/10.17632/8kd3xjfrh4.1). Specifically, reduced mtDNA gene expression was consistently observed in CD8+ T cells from patients in BALF samples (Dataset VI) and from CD8 T cells, including CD8 memory T cells, from PBMC (Datasets VII, XI) (Figure 4A, Table S1 https://doi.org/10.17632/8kd3xjfrh4.1). A smaller effect on mtDNA gene expression (measured by the fraction of mtDNA genes which displayed a significant change in patients), yet still reduction in expression, was observed in CD8 effector cells from PBMC (Datasets VII, XII) (Figure 4B, Table S1 https://doi.org/10.17632/8kd3xjfrh4.1). Similarly, significantly reduced mtDNA gene expression in patients was also observed in CD4 T cells from 2/5 PBMC datasets, CD4 memory T cells (Dataset VII) (Figure 4B) and additional sub types of T cells that were represented only in one dataset each, such as CCR7+ T cells and Follicular helper CD4 T (Dataset VI and Dataset XII, respectively) (Table S1). Significantly reduced expression of most mtDNA encoded genes was also observed in patients' NK cells in BALF (Figure 4A), as well as in NK cells from PBMC samples (3/4 datasets) regardless of their severity level (Figure 4B, Table S1 https://doi.org/10.17632/8kd3xjfrh4.1). It is worth noting that in both CD4 T and NK cells we observed a significant reduction in mtDNA gene expression (Dataset X) in moderate patients as compared to healthy individuals. Reduction in mtDNA gene expression was also observed in other cell types from patients such as Monocytes, IgA PB, Dendritic cells (DC), and B cells (PBMC), although with weaker effect, confined to certain datasets (Figure 4B, https://doi.org/10.17632/8kd3xjfrh4.1). Notably, certain cell types showed inconsistent response among the analyzed data sets: mtDNA expression in platelets of COVID 19 patients was significantly elevated only in one out of two datasets (Table S1 https://doi.org/10.17632/8kd3xjfrh4.1) and proliferating T cells and Macrophages displayed a mixed tendency in mtDNA gene expression in patients (Table S1 https://doi.org/10.17632/8kd3xjfrh4.1). As currently we consider significance only for results that were replicated by at least two datasets, the biological importance of the mixed response should be evaluated once additional RNA seq datasets become available. Taken together our analysis strongly suggests a consistent reduction in mtDNA gene expression in COVID 19, especially in immune system cells.

Disease severity does not clearly impact the reduction in mtDNA gene expression in patients

While considering Datasets IV and IX (NP), division into individual cell types belonging to the immune sys tem led to cell sample sizes below our threshold for further analysis. To overcome this problem, we group ed all cell types belonging to the immune system for further comparisons of differential expression between patients and controls; this analysis revealed consistent and significant reduction in mtDNA genes' expression in patients (https://doi.org/10.17632/8kd3xjfrh4.1). Finally, we took advantage of available expression data for B cells and monocytes from scRNA seq in the frame of Dataset III. Such analysis re vealed significantly reduced expression of most mtDNA analyzed genes in CD14 + monocytes (pseudotime points 1,5), in CD16 + monocytes (mainly in pseudotime points 1,4, and 5), and in B cells (mainly in pseu dotime points 1,4) (https://doi.org/10.17632/8kd3xjfrh4.1). Notably, while comparing mtDNA gene

expression of the immune system cell types between the pseudo time points (Dataset III scRNA seq), and between the severity levels (e.g., moderate and critical) in Datasets VI XII (Table S1), no significance was observed between most of the pseudo time points. These results further suggest that the reduced mtDNA gene expression in COVID 19 patients occur in a variety of cell types from the immune system, with apparently inconsistent effect of disease severity.

Respiratory tract harbor immune system cells which display mtDNA gene expression reduction in COVID-19 patients

Our analysis of bulk RNA seq from upper respiratory tract samples did not reveal any significant changes in COVID 19 patients as compared to healthy controls. Nevertheless, it is possible that either (A) mtDNA gene expression did not change in cells from the respiratory tract of the patients, or that (B) the response of mtDNA gene expression in the respiratory tract of patients varies among cell types. Our analysis of scRNA seq imply weaker, yet significantly reduced mtDNA gene expression (i.e., evident only in ND2, ND4, and CO3 transcripts) in epithelial cells from patients' respiratory tract samples (BALF, Dataset VI). This finding contrasts with the observed *stronger* reduction in the expression of most mtDNA genes in T cells and NK cells isolated from the same BALF samples (Table S1). Secondly and similarly, analysis of res piratory tract samples from Dataset VIII revealed significantly reduced expression of four mtDNA genes (ND1, CO3, CYB, and ATP6) in Ciliated, Secretory, and Secretory differentiated epithelial cells in COVID 19 patients, yet none of the genes displayed significant change in Squamous cells from this dataset. However, all epithelial cells (such as Squamous, Ciliated and Secretory) from Dataset IX, except for Goblet cells did not show any significant change in the expression of mtDNA encoded genes (Figures 4A and 4B, Table S1 https://doi.org/10.17632/8kd3xjfrh4.1). Finally, IFNG responsive epithelial cells (IRC), displayed a significant reduction in the expression of only a single mtDNA gene (ATP6) (https://doi.org/10.17632/8kd3xjfrh4.1). These results suggest that whereas cells belonging to the immune system consistently show reduction in mtDNA gene expression, whether isolated from the peripheral blood or from the respiratory tract, such response is weaker, and notably varies among respiratory tract epithelial cells.

Analysis of scRNA-seq databases identify candidate regulators that explain reduced mtDNA gene expression in COVID-19 patients

We next asked whether reduction in mtDNA gene expression in certain cell types from patients is associ ated with expression changes in genes with mitochondrial function encoded by the nuclear genome. To address this question, we performed differential expression analysis of OXPHOS genes and mitochon drial related pathways that were analyzed in the bulk RNA seq datasets (see above). We found that in cell types which displayed reduction in the expression of most mtDNA genes, the significant expression of most nuclear DNA encoded OXPHOS genes showed elevated expression (in all tested Datasets) (Table S3, https://doi.org/10.17632/8kd3xjfrh4.1). Similar to the analysis of bulk RNA seq, our analysis of mitochondria related pathways revealed that such cells displayed mostly elevated expression of genes belonging to glycolysis, response to type I interferon and MAVS pathway genes (https://doi.org/10.17632/8kd3xjfrh4.1).

To further test our hypothesis that the reduced mtDNA gene expression in certain cell types from patient samples reflects downregulation of mtDNA gene expression upon SARS CoV 2 infection, we assessed changes in regulatory factors of mtDNA genes' expression (Table S3) in the scRNA seq datasets from pa tients and controls. This analysis revealed that in consistence with the analysis of bulk RNA seq, significantly reduced expression was observed in JunD in the following cell types: CD8 T cells, T cells, CCR7+ T cells (Dataset VI BALF), NK cells (Datasets VI BALF, XI PBMC), DC, Monocyte (Dataset XI PBMC), CD4n T cells (Dataset VII PBMC), in all immune cells from Dataset VIII NP and in CD14 + Monocytes (Dataset III) (Table S3 in all pseudotimes except for pseudotime 2). Similarly, in consistence with the analysis of bulk RNA seq, the expression of c Jun was elevated in B cells (XI PBMC), mDC, CCR7+ T, all T cells from Dataset VI (BALF) (Table S3) and non classical CD16 + Monocytes (Dataset III; in all pseudotimes except for pseudotime 2). These results support the functional involvement of these two members of the activator protein 1 (AP1) transcription factors family in the regulatory response of mtDNA gene expression to SARS CoV 2 infection.

DISCUSSION

Our results indicate a tissue and cell type dependent reduction in mtDNA encoded gene expression in response to SARS CoV 2 infection. Firstly, bulk RNA seq analysis of samples coming from the peripheral

and whole blood showed such reduction, in contrast to samples coming from the respiratory tract that did not. Secondly, analysis of scRNA seq revealed reduced mtDNA gene expression levels in several tested immune system cells, as compared to little or no reduction of mtDNA gene expression in respiratory tract epithelial cells. These findings support cell type and tissue dependent differences in the magnitude of mitochondrial gene expression regulatory effect in COVID 19 patients. We are tempted to interpret these results, as partially explaining the apparent lack of change in mtDNA gene expression in patients' respira tory tract bulk RNA seq samples: the weaker mtDNA gene expression differences in epithelial cells from patients (as well as in some immune system cells isolated from the respiratory tract) might have masked the difference in mtDNA gene expression in the respiratory tract, but not in blood. Because two out of the five analyzed bulk RNA seq datasets stem from different sequencing platforms (Datasets IV, V), the dif ferences in the impact on mtDNA gene expression could be attributed to the different tissues of origin, although variation in sample collection methods and/or sequencing platforms cannot be excluded. Another parameter that may affect gene expression differences in the mitochondria is mtDNA genetic backgrounds (haplogroups) (Cohen et al., 2016). It would therefore be of interest to study the contribution of haplogroups to gene expression differences between healthy and COVID 19 samples once larger sample sizes become available.

We hypothesized that reduction in mtDNA gene expression in samples from COVID 19 patients stem from a general alteration in OXPHOS gene co regulation. The general tendency toward increased expression in nuclear DNA encoded OXPHOS genes in COVID 19 patients supports our hypothesis, as by and large healthy human tissues display positive co expression of mitochondrial and nuclear DNA encoded members of the OXPHOS machinery (Barshad et al., 2018). This leads to two possible interpretations: the altered coordination of mito nu clear gene expression in COVID 19 patients may (A) reflect a compromised mito nuclear co regulation, and/or (B) controlled rewiring of the OXPHOS machinery to glycolysis. Indeed, we found that COVID 19 patients ex hibited a general increase in the expression of genes encoding glycolysis enzymes, reduced expression of LDHB, and increased expression of LDHA. This is consistent the observed dependence of SARS CoV 2 replica tion in monocytes on active glycolysis (Codo et al., 2020), suggesting that OXPHOS malfunction in COVID 19 patients primarily enables generation of a glycolytic environment, which in turn promotes the cytokine storm. This interpretation is supported by our observed increased expression of genes involved in the IFN pathway, as well as increased expression of genes involved in RIG I like receptors and MAVS pathway as previously shown (Gordon et al., 2020; Rui et al., 2021). The latter might lead to activation of a signaling cascade that positively induces the genes encoding type I interferons (Rehwinkel and Gack 2020). We speculate that our observed consistent alteration in mtDNA gene expression and related pathways enabled the viral induced cytokine storm, and hence is fundamental to the disease etiology. Testing for this possibility requires a future time course controlled experiment in cells (preferably from the immune system) in which gene expression is assessed in time intervals following SARS CoV 2 infection.

We noticed, that cells and tissues that displayed a consistently significant difference in mitochondrial gene expression between patients and controls (in at least two independent datasets), displayed reduction in mtDNA gene expression in patients (both in bulk RNA seq and scRNA seq), regardless of difference be tween sample collection sites and sequencing platforms used. In contrast, elevated mtDNA gene expres sion in patients was inconsistent among databases (such as the observation of Platelets). This finding strongly suggests that SARS CoV 2 affects mitochondrial regulation in a similar manner in different cells. Nevertheless, although the direction of the effect is consistent (e.g., reduction in mtDNA gene expression in COVID 19 patients) the regulatory phenomenon is influenced by cell types, which raises an interesting question as to the role of the affected cell types in the disease etiology.

As mentioned above, our findings indicate that bulk RNA seq from blood, but not from the respiratory tract, showed significant reduction in mtDNA gene expression. Apart from the apparent cell type compo sition, and differences between these two types of samples, extracellular intact cell free active mitochon dria have been found in blood (Al Amir Dache et al., 2020). It will therefore be of interest to measure the number of extracellular mitochondria in both blood and respiratory tract samples from COVID 19 patients as compared to control, and assess their transcriptional signatures. This may add another layer to our understanding of the alteration in mitochondrial gene expression patterns in COVID 19 patients.

To identify first clues for the candidate mechanism underlying reduced mtDNA gene expression in COVID 19 patients we took a candidate gene approach and sought for association between altered

mtDNA gene expression in patients with known regulators of mtDNA transcription, post transcription, and replication. Our findings indicate that in patient peripheral and whole blood samples, reduction in mtDNA gene expression is associated with reduced expression of the mitochondrial RNA polymerase (POLRMT), along with elevated expression of c Jun, and reduced expression of JunD. The reduction in POLRMT expression provides a simple explanation for the reduced mtDNA gene expression in the patients' periph eral blood, but this observation was not clearly identified in single cells. Interestingly, unlike POLRMT, the altered expression of c Jun and JunD was consistently found in both the bulk RNA seq and sc RNA seq datasets, e.g., the expression of c Jun increased and JunD decreased in patients in cell types that showed significantly reduced mtDNA gene expression. This particularly attracted our attention, as c Jun and JunD binding in human mtDNA was identified in certain cell lines (Blumberg et al., 2014), thus suggesting their possible involvement in mtDNA transcriptional regulation. Our identified association of altered c Jun and JunD expression in patient samples suggests that they likely act as a repressor and activator, respectively, of mtDNA gene expression, and that such impact is induced in patients. As c Jun and JunD are well known for their regulatory targets in the nucleus, it will be of great interest to experimentally assess their mecha nism of action while in the mitochondria, their possible involvement in mito nucelar co regulation, and whether their mitochondrial localization is altered in response to SARS CoV 2 infection.

While further considering the mechanism by which mtDNA gene expression was down regulated in COVID 19 patients, one should consider additional factors, such as microRNAs. Indeed, recent microRNA seq GO term analysis of peripheral blood (Li et al., 2020) revealed altered expression of microRNAs in COVID 19 patients that target genes which associate with the mitochondrial matrix. Furthermore, microRNA seq differential expres sion analysis from blood plasma (Farr et al., 2021) revealed that the expression levels of hsa miR 542 5p, a miRNA which likely co localize with human mitochondria (Borralho et al., 2015) and was previously associated with mito chondrial dysfunction (Garros et al., 2017), was significantly reduced in COVID 19 patients. Third, the expression of hsa miR 483 5p which binds and represses the activity of FIS1 (Purohit and Saini 2021), a key mitochondrial fission component (James et al., 2003), was significantly elevated in COVID 19 patients who were treated by sup plemental oxygen. Hence, there is room for future investigation of the role of mitochondrial targeted microRNAs in mtDNA regulation in general, and in COVID 19 in particular.

Along with the observed reduced expression of mitochondrial genes we identified elevated expression of several genes involved in the IFN1 response pathway. This is interesting, as it was previously shown that treatment of mammalian cells with IFN leads to reduction in mtDNA gene expression, thus suggesting a regulatory impact of IFN on mtDNA transcripts (Shan et al., 1990). This offers an attractive explanation for the connection between the cellular response to SARS CoV 2 infection and changes in mitochondrial regulation. As our results indicate that such changes occur in a cell type dependent manner, mostly apparent in immune system cells, it is not surprising that previous analyses of (lung) epithelial cells following SARS CoV 2 infection displayed a rather low response of type I and III interferon (Blanco Melo et al., 2020), and inconsistent changes in mtDNA genes expression (Miller et al., 2021). Hence, it will be of great interest to carefully assess in the future the underlying mechanism by which IFN modulates mitochondrial gene expression regulation.

In summary, we observed reduced levels of mtDNA gene expression in multiple cell types, yet preferentially in cells belonging to the immune system, regardless of collection from blood or from the respiratory tract. This finding, along with apparent opposite gene expression changes in nuclear encoded OXPHOS genes in COVID 19 patients suggest departure from co expression regulation of the mitochondrial and nuclear ge nomes. This interpretation may explain the elevated expression of genes involved in ROS production, which likely reflect cellular response to mitochondrial dysfunction. This change was also accompanied by elevated expression of glycolytic enzymes, especially LDHA, and reduction of LDHB expression. Such findings suggest that upon SARS CoV 2 infection, cells which particularly belong to the immune system, rewire to glycolysis. Analysis of mtDNA gene expression in the unique set of patients who provided samples during COVID 19 dis ease progression suggests that the reduction of mtDNA gene expression is reversible upon recovery. It is thus possible that recovery of mitochondrial function predicts better health conditions of COVID 19 patients, thus underlining the mitochondria as an important drug target to ameliorate patients' health conditions.

Limitations of the study

Our work is based on analysis of gene expression at the RNA level. Nevertheless, expression variability in COVID 19 patients may be also observed at the protein level, which would be interesting to assess, yet

obviously cannot be observed in analysis of RNA seq. Secondly, RNA seq analysis, which corresponds to the steady state RNA levels, cannot decipher whether the observed changes in mitochondrial gene expression in COVID 19 patients were due to transcriptional regulation, RNA decay, or both. Clues to such were identified by observing correlation between the changes in mitochondrial gene expression in patients and potential regulators of mitochondrial transcription (POLRMT, c Jun and JunD). Such associations should be considered with caution until subsequent experimental validations are performed.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.103471.

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AUTHOR CONTRIBUTIONS

HM analyzed the data and participated in writing the manuscript; AZ Analyzed bulk RNA seq data; DM conceived the study, wrote the paper and supervised the analysis.

DECLARATION OF INTERESTS

The authors declare no competing interests. The manuscript is based on analyses of publicly available data. The source of each dataset is mentioned within the STAR Methods section.

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