ORIGINAL ARTICLE

Mitochondrial Dynamics in SARS-COV2 Spike Protein Treated Human Microglia: Implications for Neuro-COVID

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Abstract

Emerging clinical data from the current COVID-19 pandemic suggests that ~40% of COVID-19 patients develop neurological symptoms attributed to viral encephalitis while in COVID long haulers chronic neuro-inflammation and neuronal damage result in a syndrome described as Neuro-COVID. We hypothesize that SAR-COV2 induces mitochondrial dysfunction and activation of the mitochondrial-dependent intrinsic apoptotic pathway, resulting in microglial and neuronal apoptosis. The goal of our study was to determine the effect of SARS-COV2 on mitochondrial biogenesis and to monitor cell apoptosis in human microglia non-invasively in real time using Raman spectroscopy, providing a unique spatio-temporal information on mitochondrial function in live cells. We treated human microglia with SARS-COV2 spike protein and examined the levels of cytokines and reactive oxygen species (ROS) production, determined the effect of SARS-COV2 on mitochondrial bio-genesis and examined the changes in molecular composition of phospholipids. Our results show that SARS- COV2 spike protein increases the levels of pro-inflammatory cytokines and ROS production, increases apoptosis and increases the oxygen consumption rate (OCR) in microglial cells. Increases in OCR are indicative of increased ROS production and oxidative stress suggesting that SARS-COV2 induced cell death. Raman spectroscopy yielded significant differences in phospholipids such as Phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylethanolamine (PE) and phosphatidylcholine (PC), which account for ~80% of mitochondrial membrane lipids between SARS-COV2 treated and untreated microglial cells. These data provide important mechanistic insights into SARS-COV2 induced mitochondrial dysfunction which underlies neuropathology associated with Neuro-COVID.

Keywords SARS-COV2 · Microglia · Neuro-inflammation · Oxidative stress · Mitochondrial dysfunction · Neuro-COVID

Introduction

The clinical presentation of COVID-19 is associated with significant neurological symptoms and neuropathologies such as acute ischemic stroke, meningitis/encephalitis, anosmia, neuromuscular diseases, epileptic seizures, focal neurological deficits as well as neuropsychological abnormalities such as depression, delirium, and psychosis (Al-Dalahmah et al. 2020; Beyrouti et al. 2020; Dixon et al. 2020; Oxley et al. 2020; Paterson et al. 2020; Pero et al. 2020; Poyiadji et al. 2020; Steardo et al. 2020). Emerging data suggests that 85% of COVID-19 long-haulers have multiple neurological symptoms (Clark et al. 2021). Pathological mechanisms that underlie these neurological manifestations include induction of hypoxia, sepsis, severe systemic inflammation and the cytokine storm. Chronic neuro-inflammation and neuronal damage results in a syndrome described as "Neuro-COVID". SARS-COV2 has been isolated from brain tissue with edema and neuronal degeneration. Electron microscopic analysis confirms the localization of SARS-COV2 in neurons, astroglia and microglia.

SARS-COV2 uses the S1 spike protein to attach the virion to the cell membrane by interacting with the hosts

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ACE2 receptor (Wrapp et al. 2020). The neurovirulence of SARS-COV2 is attributed to the expression of the ACE2 receptor by brain microvascular endothelial cells that line the blood brain barrier (BBB), and facilitate the entry of the virus in the brain (Baig et al. 2020). Microglia, neurons and astrocytes also express ACE2 receptors making them a potential target of SARS-COV2.

Microglia are the resident immune cells of the Central Nervous system (CNS) and represent 5–20% of the adult brain. Microglia have the capacity to migrate, proliferate and phagocytize. Under physiological conditions, microglia exist in their "resting" state, however on exposure to a pathogen, microglia transition into an activated state and quickly mobilize to the site of injury to initiate an innate immune response and therefore they are widely used to study neuroinflammation (Zhao et al. 2018; Zhou et al. 2020). Microglia are involved in neuroprotective and neurotoxic responses in the CNS in response to SARS-COV 2 infection (Hanisch and Kettenmann 2007; Farfara et al. 2008; Harry and Kraft 2008; Bachiller et al. 2018; Fatoba et al. 2020). The severe cytokine storm in COVID -19 patients is associated with increased serum levels of proinflammatory cytokines and increased BBB permeability due to cytokine-induced damage (Jahani et al. 2020; Daniels et al. 2014). Cytokines activate microglia and astrocytes and activate microglia to secrete inflammatory mediators, inducing altered neuroplasticity, and other neuropathologies resulting in neuro-cognitive impairment (Fu et al. 2020; Liddelow et al. 2017). SARS-COV2 induces a mitochondrial-dependent intrinsic apoptotic pathway, leading to the activation of caspase-9 followed by initiation of executioner caspases-3/7 (Imre 2020; Bertheloot et al. 2021; Lee et al. 2020); SARS-COV2 spike protein manipulates mitochondrial mechanisms to evade the host immune response and may alter mitochondrial functions leading to enhanced ROS production, perturbed signaling, and blunted antiviral defenses of the host. We speculate that SAR-COV-2 induces mitochondrial dysfunction and activation of the mitochondrial-dependent intrinsic apoptotic pathway, resulting in microglial and neuronal apoptosis. We examined the effect of a recombinant SARS-COV2 spike protein and heat inactivated SARS-COV2 on cytokine production, ROS /NOS production, mitochondrial bioenergetics, and the possible mechanisms that may underlie SAR-COV2 induced mitochondrial dysfunction in human microglial cells. Our results showed that microglia treated with SARS-COV2 release an increased amount of pro-inflammatory cytokines suggesting that SARS-COV2 induces an increased inflammatory response and cytokine dysregulation. SARS-COV2 also induces an increased level of oxidative stress, HIF-1α production and inflammasome activation, contributing to both mitochondrial dysfunction and increased ROS production. Additionally, we used Raman spectroscopy for prognostic evaluation of mitochondrial function in SARS-COV2 treated microglial,

which yielded real time information on SARS-COV2 induced changes in protein & lipid composition, stress response, nuclear division, respiratory activity and cell apoptosis. Our data provide important mechanistic insights into SARS-COV2 induced mitochondrial dysfunction which underlies neuropathology associated with Neuro-COVID. Understanding the pathophysiology behind the neurological symptoms associated with COVID-19 patients will allow development of targeted therapeutics for patients with Neuro-COVID.

Material and Methods

Cell Culture

Human Microglia (HMC3) were obtained from ATCC (Cat # ATCC® CRL-3304™) and grown in Eagle's Minimum Essential Medium (EMEM) (Cat #ATCC® 30–2003™) supplemented with 5% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin. Cultures were maintained at 37^0C in a humidified 5% CO_2 in air incubator. We treated human microglia (HMC3 -ATCC) with (0.5 µg/ml) of recombinant spike protein from SARS-COV2, Wuhan-Hu-1 (Cat # NR-52308; Lot: 70,034,410 BEI resources Inc) which is described as a recombinant form of the spike (S) glycoprotein from SARS-COV2, Wuhan-Hu-1 (GenPept: QJE37812) which was produced by transfection of purified plasmid in SF9 insect cells using a baculovirus expression system and purified by nickel affinity chromatography or 5 µl/ml of heat inactivated SARS-COV2, Isolate USA-WA1/2020, (Cat # NR-52286, Lot: 70,033,548; Pre-Inactivation Titer by TCID50 Assay in Vero E6 Cells $= 1.6 \times 10^5$ TCID50 per mL, BEI resources Inc) for 24–48 h followed by cell viability assays, gene expression analyses, quantitation of mitochondrial respiration, ROS quantitation. The Raman spectroscopic analysis for these cells was done at an 3 h time point, post treatment which enables examining early changes in SARS-COV2 induced cell apoptosis.

Cell Viability Assay

Microglia (100,000 cells) were treated with 0.5 µg/ml of SARS-COV2 spike protein or 0.5 µl/ml of heat inactivated SARS-COV2 isolate for 48 h and cell viability was measured using the CCK-8 assay kit (Dojindo Molecular Technologies, Inc., Rockville, MD). The assay uses a water-soluble tetrazolium salt, WST-8, which is reduced by dehydrogenase activity in cells to produce a yellow-color formazan dye soluble in the tissue culture media and is optical density(OD) quantitated using a spectrophotometer at 450 nm. The amount of the formazan dye generated by the dehydrogenase activity in cells is directly proportional to the number of living cells.

RNA Extraction

Microglia (100,000 cells) were treated with 0.5 μg/ml of SARS-COV2 spike protein or 5 μl/ml of heat inactivated SARS-COV2 isolate for 48 h followed by extraction of cytoplasmic RNA by the acid guanidinium-thiocyanate-phenol–chloroform method using Trizol reagent (Invitrogen-Life Technologies, Carlsbad, CA). T he amount of RNA was quantified using a Nano-Drop ND-1000 spectrophotometer (Nano-Drop™, Wilmington, DE), and isolated RNA was stored at –80 °C until used.

Real Time qPCR

500 ng of total RNA extracted as outline above, was used for the First-Strand cDNA Synthesis Kit (GE Healthcare, Piscataway, NJ), according to the manufacturer's instructions. One microliter of the resultant cDNA from the RT reaction was employed as the template in PCR reactions using well validated PCR primers obtained from the RealTimePrimers.com, that include the pro-inflammatory cytokines IL-8, IL-1β and TNF-α. Primers were provided as a 20 μl solution containing both forward and reverse primers at a final concentration of 10 μM in 10 mM Tris–HCl (pH 7.5) and 0.1 mM EDTA, which were diluted with RNAase/DNAse free water as needed prior to use. We used the SYBR® Green master mix that contained dNTPs, MgCl2, and DNA polymerase (Bio- Rad, Hercules, CA).The final primer concentration used in the PCR was 0.1 μM. PCR conditions were as follows: 95 °C for 3 min, followed by 40 cycles of 95 °C for 40 s, 60 °C for 30 s, and 72 °C for 1 min; the final extension was at 72 °C for 5 min. Gene expression was calculated using the comparative CT method. The threshold cycle (Ct) of each sample was determined, the relative level of a transcript (2ΔCt) was calculated by obtaining ΔCt (test Ct − GAPDH Ct), and transcript accumulation index (TAI) was calculated as $TAI = 2^{-\Delta\Delta CT}$ (Livak and Schmittgen 2001).

Apoptotic Cell Quantitation using the Caspase-3/7 Detection Reagent

CellEvent™ Caspase-3/7 Green Detection Reagent (Cat #: C10423 Thermo Fisher Scientific) enables the examination of Caspase-3/7 activation in live cells, and is a four-amino acid peptide (DEVD) conjugated to a nucleic acid-binding dye with absorption/emission maxima of ~502/530 nm. The DEVD peptide sequence is a cleavage site for caspase-3/7, and the conjugated dye is non-fluorescent until cleaved from the peptide and bound to DNA. Upon Caspase-3/7 activation in apoptotic cells, the DEVD peptide is cleaved, enabling the dye to bind to DNA and produce a bright, fluorogenic response. The reagent is added to microglial cells grown to 70% confluence in a petri dish with a glass bottom, treated with 0.5 μg/ml of SARS-COV2 spike protein for 24 h, then incubated for 30 min, and visualized using the EVOS® FL Cell Imaging System (Life Technologies, Grand Island, NY). Apoptotic cells with activated caspase-3/7 exhibit bright green nuclei, while cells without activated caspase-3/7 exhibit minimal fluorescence.

Measurement of ROS- a General Oxidative stress Indicator

ROS was quantitated using CM-H2DCFDA reagent from Invitrogen™ (Cat # C6827). CM-H2DCFDA is a chloromethyl derivative of H2DCFDA, useful as an indicator for ROS generation in cells and a general oxidative stress indicator. CM-H2DCFDA passively diffuses into cells, where its acetate groups are cleaved by intracellular esterases and its thiol-reactive chloromethyl group reacts with intracellular glutathione and other thiols and subsequent oxidation yields a green fluorescent product that can be quantitated using fluorescent microscopy imaging (Ex/Em: ~492–495/517–527 nm). Microglial cells were plated on glass-bottom petri dishes and cells grow to 80% confluency. Cell were then treated with 0.5 μg/ml of SARS-COV2 spike protein for 24 h following which cells were washed with 1X PBS, following which 5 μM of CM-H2DCFDA (freshly prepared in HBSS) was added and cells incubated for 30 min in dark CO2 incubator. After 30 min, cells were washed with PBS and the ROS production was quantitated immediately by measuring the green fluorescence using the EVOS® FL Cell Imaging System (Life Technologies, Grand Island, NY).

Immunofluorescent Staining

Microglia were grown to 70% confluence in a glass petri dish and treated with 0.5 μg/ml of SARS-COV2 spike protein for 24 h. Standard immunofluoresecent staining procedures were followed. Cells were fixed for 10 min at RT using 4% formaldehyde, followed by permeabilization with ice-cold 90% methanol. Cells were then washed in 1X phosphate buffered saline (PBS) and treated with the following primary antibodies, Anti-ACE2 (E-11); mouse monoclonal (Cat # sc-390851, Santa Cruz Biotechnology, Inc.), Anti-HIF-1α mouse monoclonal (3C144); (Cat # sc-71247, Santa Cruz Biotechnology, Inc.), NOS (pan –NOS antibody, Cat #2977, rabbit polyclonal, Cell signaling technologies), TNF alpha antibody [TNF706] (Cat # GTX108585; mouse monoclonal, Gene Tex), and NLRP3 antibody (Cat # GTX00763, rabbit polyclonal, Gene Tex). The secondary antibodies used include fluorescence labeled Alexa Fluor® 647 goat anti-rabbit IgG (H + L), Alexa Fluor®488 Anti-Rabbit Secondary Antibodies, and Alexa Fluor® 488 rabbit anti-mouse IgG, all obtained from Thermo Fisher Scientific, Grand Island, NY.

DAPI (Cat #D1306 Thermo Fisher Scientific) was used to stain nuclei. The expression levels of ACE2, HIF-1α, NOS, TNFα and NLRP3 in SARSCOV-2 treated microglia were compared to an untreated control, and fluorescence imaging was done using the EVOS® FL Cell Imaging System (Life Technologies, Grand Island, NY). The intensity of the fluorescent signal was quantitated using the ImageJ software (National Institutes of Health, Bethesda, MA, USA).

Mitochondrial Staining in Microglia using the MitoTracker™ Probe

Microglial cells (5000cells/ 35 mm dish) were plated on a glass bottom petri dish filled with culture medium, once attached, cells were treated with 0.5 µg/ml of SARS-COV2 spike protein for 24 h, pre-warmed (37 °C) staining solution containing MitoTracker™ Red CMXRos dye (Thermo Fisher Scientific) was added and cells were incubated for 15 min at 37 °C, after staining was complete, staining solution was replaced with fresh pre-warmed media or buffer and MitoTracker™ Red CMXRos dye staining was imaged using the EVOS® FL Cell Imaging System (Life Technologies, Grand Island, NY). The intensity of the fluorescent signal was quantitated using the ImageJ software (National Institutes of Health, Bethesda, MA, USA).

Mitochondrial Respiration

Microglial mitochondrial energetics was determined using the XFe24 extracellular flux analyzer (Seahorse Bioscience, North Billerica, MA). Microglial cells were seeded in a Seahorse 24-well tissue culture plate at an optimized concentration of 50,000 cells per well. Cells were treated with 0.5 µg/ml SARS-COV2 spike protein or 5 µl/ml of heat inactivated SARS-Coronavirus 2, isolate for 24 h. Basal OCR and extracellular acidification rate (ECAR) were detected in the presence of physiological concentrations of 10 mM glucose, 1 mM pyruvate and 2 mM glutamine. Next, mitochondrial ATP dependent respiration, uncoupled respiration, and non-mitochondrial respiration were assessed after the addition of 1 µM oligomycin, 1.5 µM carbonylcyanide-p-trifluoromethoxyphenylhydrazone (FCCP), and the combination of 0.5 µM each of antimycin A and rotenone, respectively. All experiments were performed using 5 wells per treatment.

BioLegend's LEGENDplex™ Cytokine Assay

We used the LEGENDplex™ Cytokine assay (BioLegend San Diego, CA) to determine the levels of cytokines in the culture supernatants of the microglial cultures treated with 0.5 µg/ml of SARS-COV 2 recombinant Spike protein. The LEGENDplex™ Cytokine assay is a bead-based immunoassay where the soluble analyte is captured between two antibodies. Beads are conjugated with a specific antibody on the surface which serves as the capture bead for that particular analyte. When a selected panel of capture beads are mixed together and incubated with an unknown sample containing target analytes, each analyte will be bound by its specific capture bead. After washing, biotinylated detection antibodies are added and each detection antibody will bind to its specific analyte bound on the capture beads, thus forming capture bead-analyte-detection antibody sandwiches. Streptavidin–phycoerythrin (SA-PE) is subsequently added, which will bind to the biotinylated detection antibodies, providing fluorescent signal with intensities in proportion to the amount of bound analyte. For each bead population, the PE signal fluorescence intensity is then quantified using a flow cytometer. The concentration of a particular analyte is determined based on a known standard curve using the LEGENDplex™ data analysis software.

Non-invasive Optical Nanoscopy

Changes in mitochondrial morphology were observed using the 3D Cell Explorer (Nanolive, Nexus Scientific LLC). We performed non-invasive and label free imaging and the 3D cell images were generated with Nanolive software based on the refractive index measured. Nanolive images were produced using a $60 \times$ microscopy objective and class 1 low power laser with wavelength of 520 nm. Nanolive imaging captured the real time response of mitochondria to drug treatment at high frequencies without experimental artefacts proving information about mitochondrial morphology and cell health. We obtained nanolive images of microglia treated with 0.5 µg/ml of SARS-COV2 spike protein or 5µl/ml of heat inactivated SARS-Coronavirus 2, isolate for 3 h and observed morphological changes in microglia.

Raman Spectroscopy in Microglia

Raman spectra were acquired by a commercial Raman microspectroscope (HORIBA XploRA PLUS). Our Raman spectrometer is equipped with a 1024×256 TE air cooled CCD chip (pixel size 26 microns, temp -60 °C). HORIBA LabSpec6 software was used for data acquisition, fluorescent background removal, baseline correction, and peak fitting. We used $20 \times$ Olympus immersion objective, 532 nm excitation laser wavelength at 100% power, 1800 gr/mm grating for spectra acquisition. Entrance slit was set at 50 µm, producing an approximate spectral resolution of 4 cm − 1.1 We used a stage top incubator chamber (IBIDI Nanolive Heating System/Temperature Controller/Gas Incubation System) mounted on a three-dimensional translation stage on the microscope to keep the environment around samples in a humidified atmosphere (5% CO2, 95% air) at 37 °C for

live cells Raman imaging. The chamber allows continuous monitoring of the spectral features of SARS-COV2 treated microglial cultures for the 3 h duration of the experiment.

Statistical Analysis

Data was analyzed using Graph Pad Prism 5.0 (GraphPad Software La Jolla, CA). Results are expressed as mean $\pm$ SD and for comparisons between control and treated groups, the "t-test" was used for parametric data and Mann–Whitney test for nonparametric data. A p value was < 0.05 was considered as a statistically significant difference. A minimum of n $=5$ Raman hyperspectral datasets were obtained per sample, following which, the fluorescence background signal was subtracted from the Raman spectra at each pixel in the image by a modified polyfit fluorescence removal technique.

Results

Effect of SARS-COV2 on Cell Viability of Microglia

Figure 1A shows 100% viability in microglial cells treated with 0.5 µg/ml of SARS-COV 2 recombinant Spike protein or 5 µl/ml of heat inactivated SARS-Coronavirus 2, isolate at 24 h and a 95% and 85% cell viability corresponding to a 5% and 14% decrease (p $=$ NS) in microglia treated with SARS-COV2 spike protein and heat inactivated SARS-Coronavirus 2, isolate at 48 h post treatment, both indicating that both treatments did not result in any significant cell toxicity at the exposure time and concentrations used in the in-vitro assays.
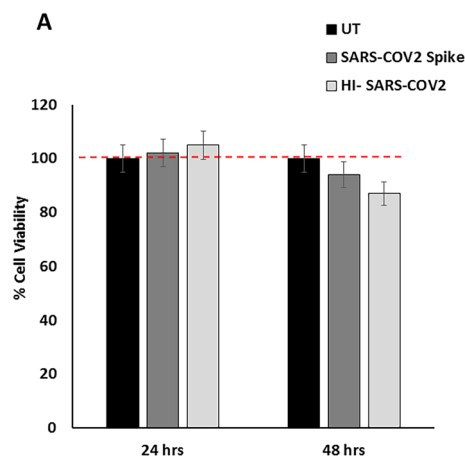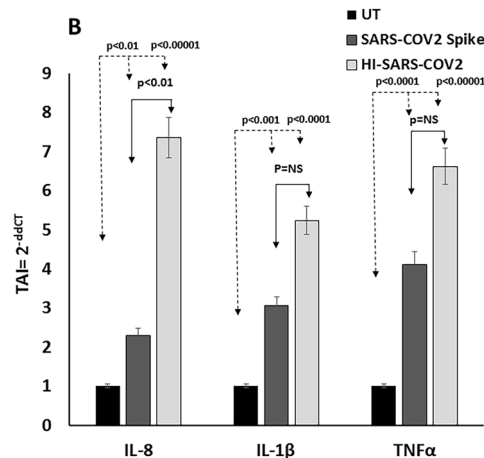
Effect of SARS-COV2 on the Gene Expression of Pro-Inflammatory Cytokines in Microglial Cells

Our data (Fig. 1B) shows that treatment of microglia with SARS-COV 2 Spike protein for 48 h leads to a significant increase in the gene expression levels of the pro-inflammatory cytokines IL-8 (TAI $= 2.3 \pm 0.21$, 1.3-fold increase $p < 0.01$), IL-1β (TAI $= 3.05 \pm 0.19$, 2.05-fold increase $p < 0.001$), TNF-α (TAI $= 4.12 \pm 0.23$, 3.12-fold increase $p < 0.0001$) as compared to the untreated control (TAI $= 1.0 \pm 0.08$). Treatment with the HI SARS-COV2, isolate for 48 h also significantly increased the gene expression levels of IL-8 (TAI $= 7.36 \pm 0.52$, 6.3 fold increase $p < 0.00001$), IL-1β (TAI $= 5.24 \pm 0.33$, 4.2 fold increase $p < 0.0001$), TNF-α (TAI $= 6.62 \pm 0.48$, 5.6 fold increase $p < 0.00001$) as compared to the untreated control (TAI $= 1.0 \pm 0.08$). Statistical comparison were made for gene expression levels of IL-8, IL-1β and TNF-α between SARS-COV 2 Spike protein and the HI SARS-COV2 treated microglia, and only IL-8 gene expression was significantly higher in the HI SARS-COV2 treated microglia. No significant differences were obtained in the IL-1β and TNF-α gene expression levels between SARS-COV 2 Spike protein and the HI SARS-COV2 treated microglia.

Effect of SARS-COV2 on Levels of Secreted Cytokines in Microglial Cells

We examined the levels of key cytokines in the culture supernatants of the microglial cultures treated with 0.5 µg/ml of SARS-COV 2 recombinant spike protein using the

Fig. 1 A; Effect of SARS-COV2 spike protein/Heat inactivated SARS-COV2 virus on Cell Viability of microglia. Results of the CCK-8 cell viability experiments, showing no significant difference in cell viability at 24–48 h post treatment with SARS-COV2 spike protein/Heat inactivated SARS-COV2. Data (Mean $\pm$ SD) represent % cell viability calculated with respect to the untreated control from 3 separate experiments done in triplicate. **B**; Effect of SARS-COV2 spike protein /heat inactivated SARS-COV2 virus on IL-8, IL1β and TNF-α gene expression in microglia as quantitated by QPCR. Results are expressed as the mean $\pm$ SD from separate experiments (n $=3$) done in triplicate. A p value of < 0.05 is considered a statistically significant difference

Table 1 Effect of SARS-COV2 spike protein on levels of pro-inflammatory cytokines

Microglia	IL-1β	TNF-α	IL-6	IL-10	IL-12	IL-17A	IL-23	IL-33
Conc units	pg/ml	pg/ml	pg/ml	pg/ml	pg/ml	pg/ml	pg/ml	pg/ml
UT	11.98 ± 0.71	3.15 ± 0.70	1622.7 ± 360.4	7.12 ± 0.88	4.29 ± 0.86	0.89 ± 0.17	2.89 ± 0.46	26.04 ± 5.9
SARS-COV2 spike	14.12 ± 0.53	12.91 ± 0.89	$58,880.65 \pm 1177.6$	16.41 ± 1.78	9.29 ± 1.20	0.41 ± 0.069	7.83 ± 1.25	33.95 ± 7.80
p value	*0.04*	*0.0001*	*0.000001*	*0.002*	*0.01*	*0.01*	*0.001*	*0.05*

LEGENDplex™ bead-based cytokine immunoassay. Table 1 shows that the levels of cytokines IL1β, TNF-α, IL-6, IL-10, IL-12, IL-17A, IL-23 and IL-33 in the culture supernatants of the microglial cultures treated with 0.5 μg/ml of SARS-COV 2 recombinant spike protein were significantly higher than the levels of these cytokine in the untreated control cultures.

Effect of SARS-COV2 on ACE2 Expression in Microglial Cells

Our immunofluorescent staining data (Fig. 2; panel a) showed that when compared to the untreated control,

treatment of microglia with recombinant SARS-COV2 spike protein lead to a 50% increase ($p < 0.05$), in the ACE2 expression [Fig 2; Panel a (i-iii); Panel b (i-iii); Panel c (i-iii); Fig. 3; Panel a(i-iii)].

Effect of SARS-COV2 on HIF-1α Expression in Microglial Cells

Our immunofluorescent staining data (Fig. 2; panel b) showed that when compared to the untreated control, treatment of microglia with recombinant SARS-COV2 spike protein leads to a 57% increase ($p < 0.05$) in HIF-1α expression [Fig. 2; Panel b(i-iii)].
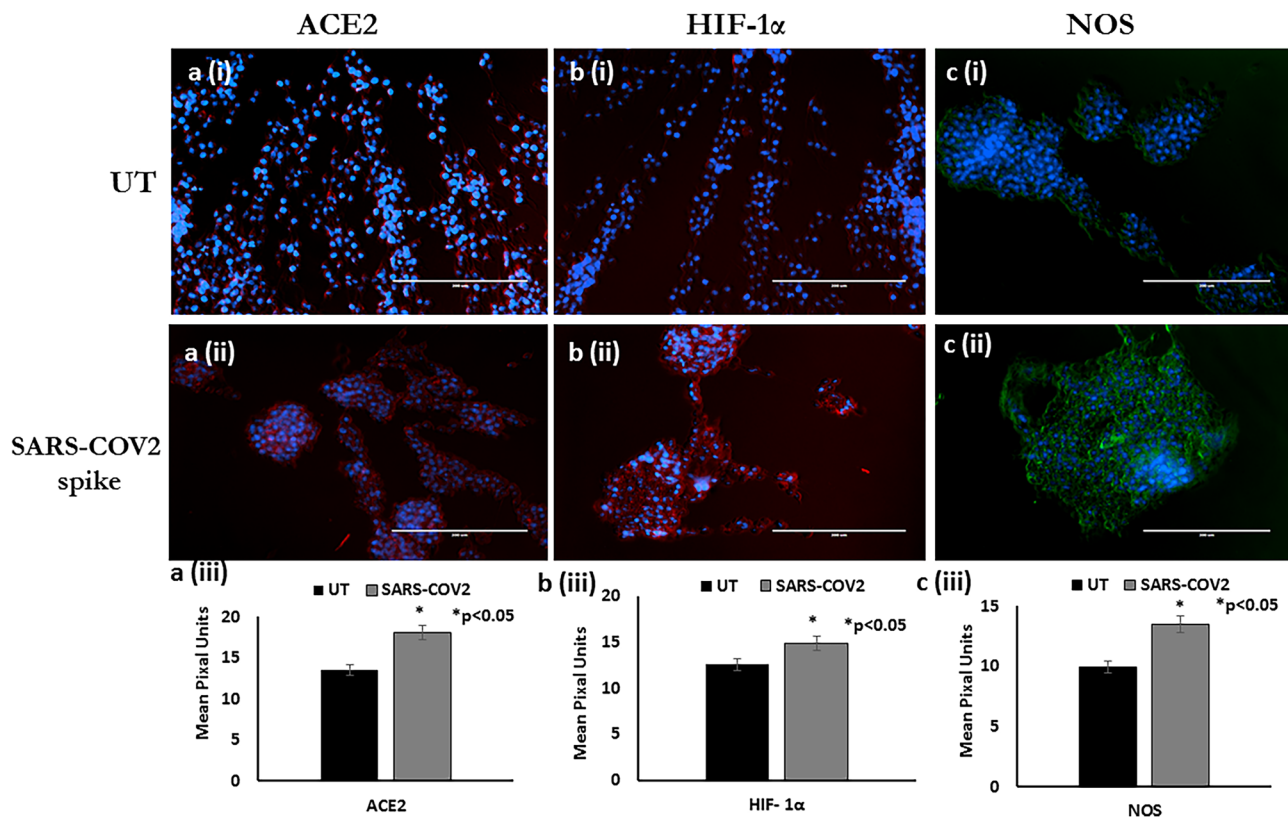

Fig. 2 Effect of SARS-COV2 spike protein on ACE2, HIF-1α and NOS expression in microglia. Results from the Immunofluorescence staining experiments showing representative images: Panel **a**: ACE2 expression, Panel **b**: HIF-1α and Panel **c**: NOS that includes the following (**i**) Untreated control; (**ii**) Recombinant SARS-COV2 spike protein treated (**iii**) Quantitative histogram. Red stain in Panel **A** and **B** is Alexa Fluor 647 dye and Green staining in Panel **C** is the Alexa Fluor 488 dye. Expression was quantitated using ImageJ software and statistical comparisons were done based on a comparison with the respective untreated control. A *p* value of < 0.05 is considered a statistically significant difference. Standard immunostaining protocols were followed. Data are representative images from 3 separate experiments done in triplicate

Effect of SARS-COV2 on NOS Expression in Microglial Cells

Our immunofluorescent staining data (Fig. 2; Panel C) showed that when compared to the untreated control, treatment of microglia with recombinant SARS-COV2 spike protein leads to a 62% increase ($p < 0.05$) in the NOS expression [Fig. 2; Panel c(i-iii)].

Effect of SARS-COV2 on Caspase 3/7 Expression in Microglial Cells

We observed that microglial cells treated with 0.5 µg/ml of SARS-COV 2 recombinant spike protein for 24 h, showed increased apoptosis (1.4-fold increase, $p < 0.01$) as evident by increased Caspase 3/7 expression as compared to the untreated control [Fig. 4; Panel a(i-iii)].

Effect of SARS-COV2 on Mitochondrial Activation in Microglial Cells

We observed increased mitochondrial activation (1.4-fold increase, $p < 0.01$) as quantitated by the fluorescent intensity of the MitoTracker™ Red CMXRos dye in microglial cells

treated with 0.5 µg/ml of SARS-COV 2 recombinant spike protein for 24 h as compared to the untreated control [Fig. 4; Panel b(i-iii)].

Effect of SARS-COV2 on Reactive Oxygen Species (ROS) Production in Microglial Cells

We quantitated the fluorescent intensity of the ROS using the CM-H2DCFDA reagent in microglial cells treated with 0.5 µg/ml of SARS-COV 2 recombinant spike protein, our data shows a significant increase in ROS production (1.4-fold increase, $p < 0.01$) in SARS-COV2 protein treated cells as compared to the untreated control [Fig. 4; Panel c(i-iii)].

Effect of SARS-COV2 on Activation of the Inflammasome in Microglial Cells

Our immunofluorescent staining data (Fig. 3; Panel A-B) showed that compared to the untreated control, treatment of microglia with recombinant SARS-COV2 spike protein lead to a 64% increase ($p < 0.05$) in the expression of NLRP3 [Fig. 3; Panel A(i-iii)] which suggests inflammasome activation which further amplifies pro-inflammatory cytokines secretion as indicated by a

Fig. 3 Effect of SARS-COV2 spike protein on NLRP3 and TNF-α expression in microglia. Results from the Immunofluorescence staining experiments showing representative images: Panel **a**: NLRP3 expression, Panel **b**: TNF-α expression that includes the following (**i**) Untreated control; (**ii**) Recombinant SARS-COV2 spike protein treated (**iii**) Quantitative histogram. Red stain in Panel **A** and **B** is Alexa Fluor 647 dye. Expression was quantitated using ImageJ software and statistical comparisons were done based on a comparison with the respective untreated control. A *p* value of < 0.05 is considered a statistically significant difference. Standard immunostaining protocols were followed. Data are representative images from 3 separate experiments done in triplicate
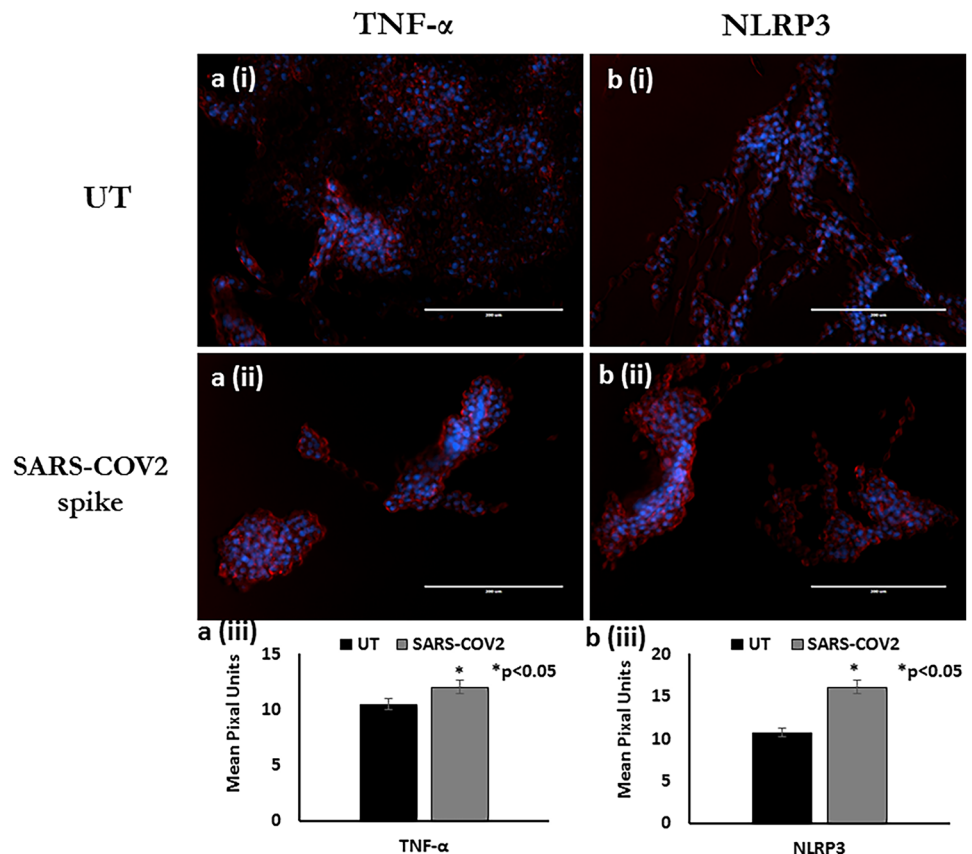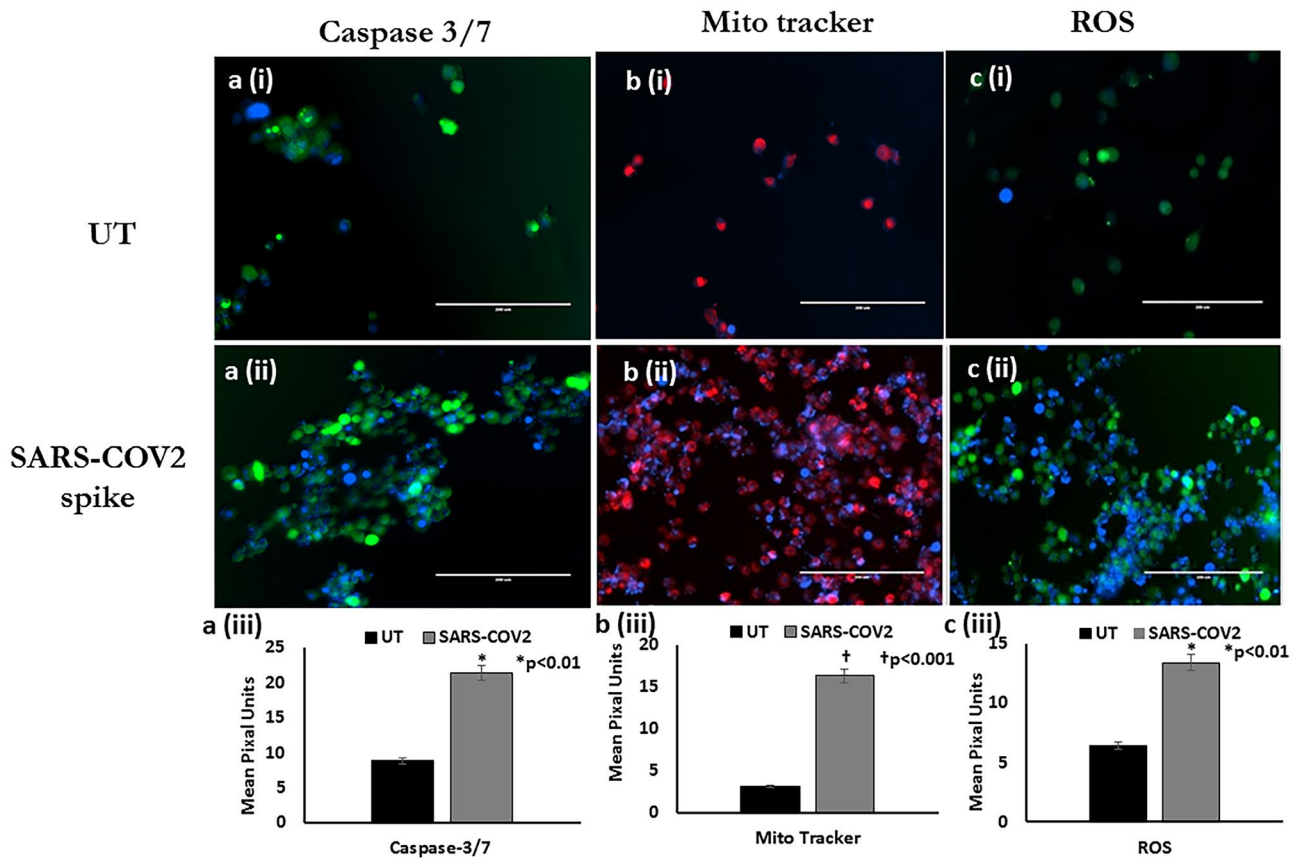
Fig. 4 Effect of SARS-COV2 spike protein on Caspase 3/7, MitotrackerTM and ROS expression in microglia. Results from the fluorescence staining experiments showing representative images: Panel **a (i-iii)**: Caspase 3/7 expression, Panel **b (i-iii)**: Mitotracker and Panel **c (i-iii)**: ROS that includes the following (**i**) Untreated control; (**ii**) Recombinant SARS-COV2 spike protein treated (**iii**) Quantitative histogram. Green stain in Panel **A** and **C** is a green fluores- cence protein dye and Red staining in Panel **B** is the Red CMXRos dye. Expression was quantitated using ImageJ software and statistical comparisons were done based on a comparison with the respective untreated control. A p value of < 0.05 is considered a statistically significant difference. Standard manufacture provided protocols were followed. Data are representative images from 3 separate experiments done in triplicate

30% increase, ($p < 0.05$) in expression of TNFα [Fig. 3; Panel B(i-iii)].

Effect of SARS-COV2 on Mitochondrial Bioenergetics in Microglial Cells

Figure 5 shows the oxygen consumption rate (OCR) is measured before and after the addition of different inhibitors to derive several parameters of mitochondrial respiration in microglial treated with 0.5 μg/ml of SARS-COV 2 recombinant spike protein or heat inactivated SARS-COV2 and comparators were the untreated microglial cells. Our data shows that treatment with both SARS-COV 2 recombinant spike protein and HI SARS-COV2 resulted in a significant increase in mitochondrial respiration. Figure 6 shows a significant increase in both basal (67% increase, $p < 0.01$) and maximal (149% increase, $p < 0.001$) OCR in response to 0.5 μg/ml of SARS-COV 2 recombinant spike protein

in microglial cells as compared to the respective untreated controls. Treatment of microglial cells with HI SARS-COV2 also resulted in significant increase in both basal (73% increase, $p < 0.01$) and maximal (140% increase, $p < 0.001$) OCR as compared to the respective untreated controls.

Effect of SARS-COV2 on Microglial Morphology

Figure 6A, shows a representative image of morphological features of an untreated microglial cell consistent with healthy immortalized microglia, with some ruffling which was predominantly restricted to cell boarder and presence of long filamentous mitochondria and/or endoplasmic reticulum. Figure 6B, shows a representative image of a microglial cell treated with recombinant -SARS-COV 2 spike protein showed increase in the apoptotic cells and fragmentation of organelles, including mitochondria.

Fig. 5 Measurement of oxygen consumption rate (OCR), in microglia treated with recombinant SARS-COV2 spike protein/ heat inactivated SARS-COV2. OCR was measured in real time, under basal condition, and in response to mitochondria inhibitors: oligomycin (Oligo, 1 µM), cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP, 1.5 µM), and antimycin A plus rotenone (Rot/AA, 0.5 µM). Data represent mean and standard deviation from 3 separate experiments with 5 replicates / sample. A "t- test" was used for statistical analysis. P values are reported in the figure
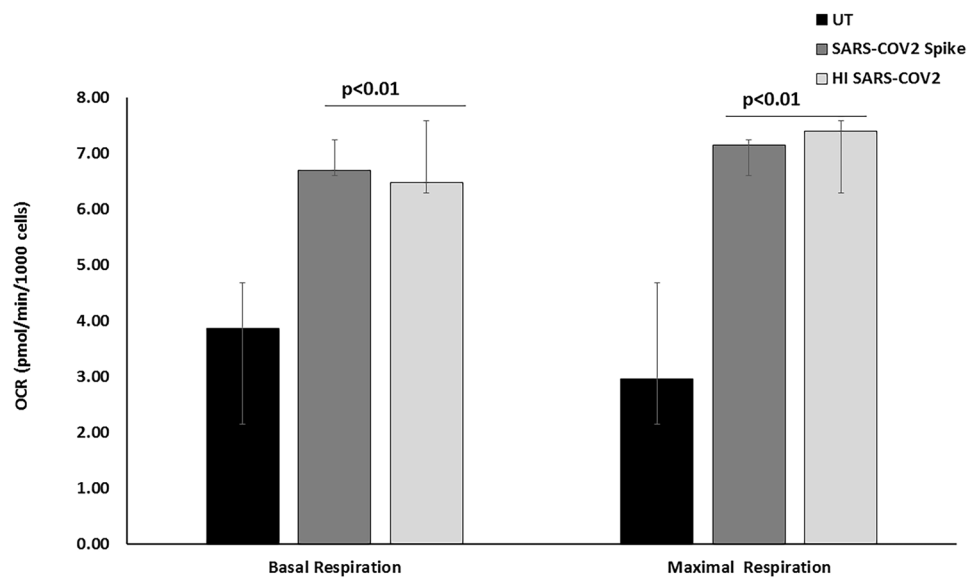

Figure 6C shows representative images of a microglial cell treated with HI SARS-COV2 and showed increased vertical ruffling, consistent with cells reacting to foreign particles and increased vacuoles, consistent with increase in membrane ruffling, micropinocytosis and mitochondrial fragmentation.

Fig. 6 **A–C**; Representative Nanolive images of microglia treated with SARS-COV2 spike protein /Heat inactivated SARS-COV2 virus showing SARSCOV2 induced changes in Microglial morphology. The yellow box inlet highlight region of interest showing increased vacuoles, increase in membrane ruffling, micropinocytosis and mitochondrial fragmentation. The Nanolive scope used 60×microscopy objective and class 1 low power laser with wavelength of 520 nm. Data are representative images from 3 separate experiments done in triplicate. **6D:** Representative Raman spectra of microglia treated with SARS-COV2 spike protein /heat inactivated SARS-COV2
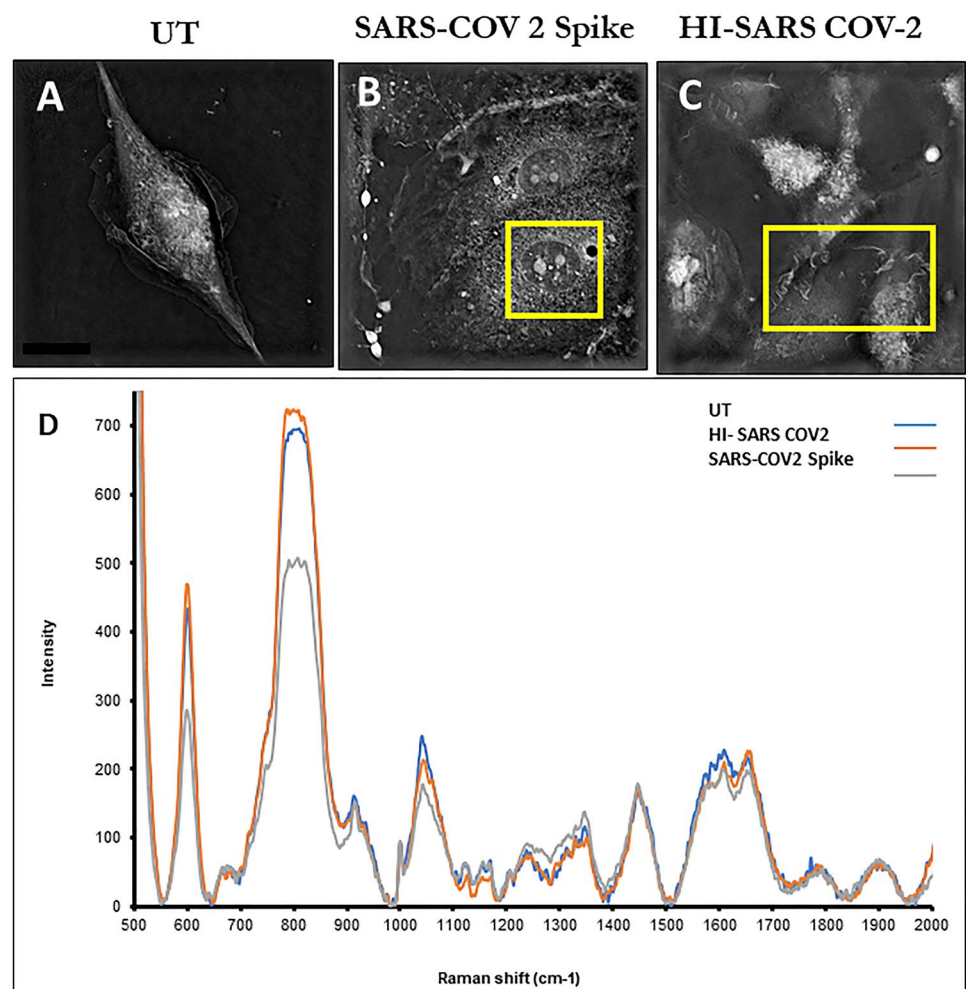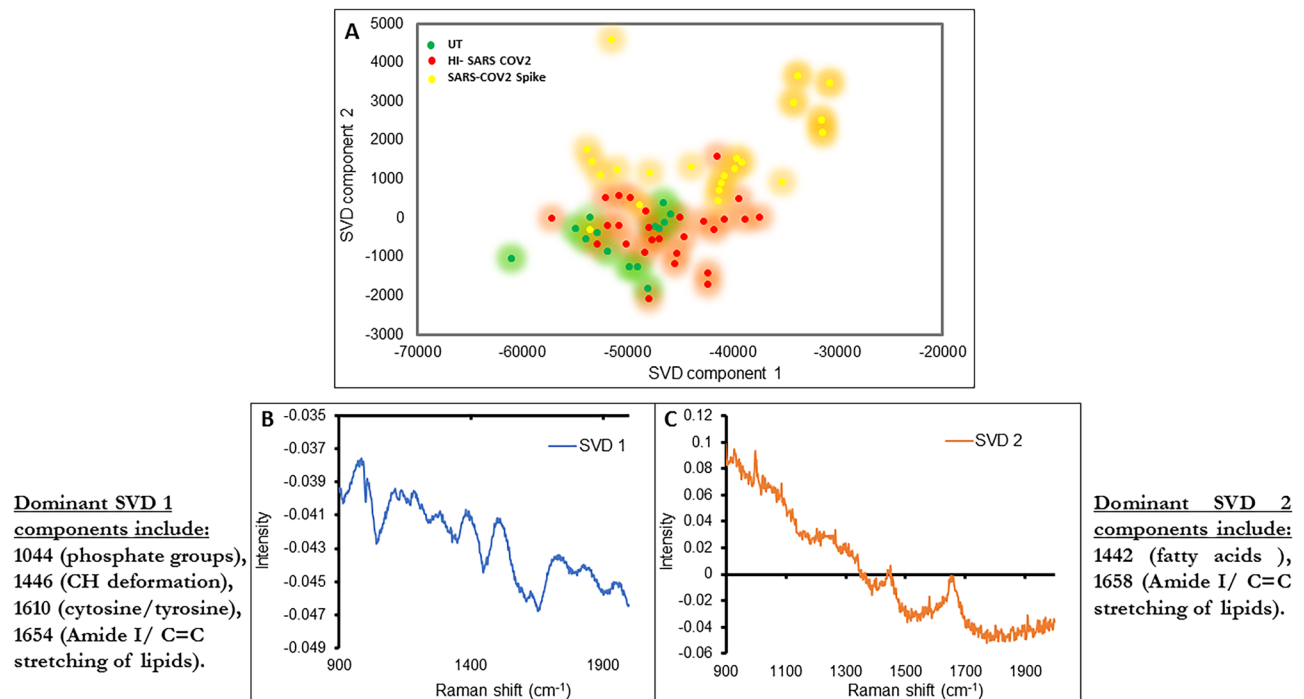
Fig. 7 Single Value Decomposition (SVD) analysis of Raman spectra of microglia treated with SARS-COV2 spike protein /heat inactivated SARS-COV2. Spectra was restricted to values above 900 cm -1. SVD analysis decomposes a matrix (stacked Raman spectra) into matrix U, $\sum$ and VT where U, V are real/complex unitary matrix and $\sum$ was a positive real diagonal matrix. Matrix U provides the critical spectra information and matrix V given the coordinate of SVD cluster for each spectrum base on matrix U. SVD scatter plot are plotted with matrix V where each data point represents one Raman spectrum. The spectra with similar feature form a cluster but spectra with different feature get separated in SVD scatter plot. The feature pickup for separation depends on the matrix U and is plotted as SVD1 and SVD2. SVD1 correspond to the x-axis separation and SVD 2 correspond to y-axis separation in SVD scatter plot

Use of Raman Spectroscopy for Prognostic Evaluation of Mitochondrial Function in SARS-COV2 Treated Microglial Cells

Figure 6D shows a representative Raman spectral image of microglia treated with 0.5 µg/ml of SARS-COV 2 recombinant spike protein or the heat inactivated SARS-COV2 using the untreated microglial cells as comparators. Figure 6D represents an average of all spectra which had a visible phenylalanine peak at ~ 1004 cm-1. Notable differences are seen in the peak centered around 800 cm-1, 1044 cm-1 and 1650 cm-1. Peak 800 cm-1 is a set of peaks related to pyrimidine bases, DNA, RNA and Peak 1044 cm-1 correspond to phosphate groups and Peak 1650 cm-1 corresponds to Amide I.

Figure 7A-C, shows spectra which is restricted to values above 900 cm-1 and SVD analysis conducted. Panel A shows SVD analysis and shows the differences between the untreated control and the recombinant SARS-COV2spike protein /HI SARS-COV2 treated microglia. Panel B shows dominant SVD 1 component includes: 1044 cm-1 (phosphate groups), 1446 cm-1 (CH deformation) and 1650 cm-1 (Amide I/ C = C stretching of lipids) and Panel C shows the

dominant SVD 2 component includes: 1442 cm-1 (fatty acids) and 1650 cm-1 (Amide I/ C = C stretching of lipids). SVD analysis shows that the control cluster is well separated from recombinant SARS-COV2 spike protein treated cluster, but slightly closer to the HI SARS-COV2 treated cluster in SVD scatter plot, which suggest that the microglia treated with SARS-COV2 spike protein have bigger chemical composition changes as compare to microglia treated with HI SARS-COV2

Discussion

The involvement of the CNS manifestation in COVID-19 patients is becoming increasing clear with continued neurological symptoms in long haulers of COVID-19 disease, implying an urgent need to examine the mechanisms that underlie the neuropathogenesis process (Solomon et al. 2020; Baig 2020; Graham et al. 2021). Given, the neurological manifestations of SARS-COV2, it is more evident that SARS-COV2 is potentially neurotropic in humans and neuroinvasion may occur by transsynaptic transfer across infected neurons, entry via the olfactory nerve, via the infection of

brain microvascular vascular endothelial cells or leukocyte transmigration across the blood–brain barrier (BBB) (Arbour et al. 2000; Li et al. 2013; Bohmwald et al. 2018; Paniz-Mondolfi et al. 2020; Baig et al. 2020). Microglia are central players of the CNS homeostasis maintenance and inflammatory response; therefore, our goal was to examine the SARS-COV2 induced changes in microglial morphology, the mechanisms that underlie the neuroinflammatory response and molecular signatures resulting in modulation of microglial function.

Figure 1A shows that the dose of SARS-COV2 spike protein and HI SARS-COV2 at which the microglia were treated no significant toxicity at 24 h, but a small decrease in cell viability with increase in time exposure to 48 h, thus all of our subsequent assays are conducted between the 24–48-h time points. Figure 1B shows a significant increase in the gene expression levels of proinflammatory cytokines IL-8, IL-1β and TNF-α. Table 1 shows a significant increase in levels of pro-inflammatory cytokines in SARS-COV2 treated microglial cultures. Additional immunofluorescence data shows a SARS-COV2 induced increase in TNF-α expression [Fig. 3, Panel a(i-iii)]. Several studies have reported significant increase in the systemic levels of pro-inflammatory cytokines (Ruan et al. 2020; Marton et al. 2021; Rowaiye et al. 2021). Hyper-inflammatory response induced by SARS-COV2 is a major cause of disease severity and death and studies have reported significant increase in the systemic levels of pro-inflammatory cytokines such as IL-2, IL-10, IP-10, monocyte chemoattractant protein-1 (MCP-1), and tumor necrosis factor-α (TNFα) (Merad and Martin 2020; Ruan et al. 2020; Marton et al. 2021; Rowaiye et al. 2021). The COVID-19-related cytokine response was quite distinct from the traditional cytokine storm associated with sepsis with sustained elevated cytokine levels over days and weeks, and relative absence of coordination between cytokines. [Liu et al. 2020]. IL-8 plays a key role in the recruitment and activation of neutrophils, commonly elevated in patients with COVID-19. Elevated TNF-α, contributes to organ damage and had significant prognostic value. IL-8, is associated with survival time and IL-1β is believed to have limited predictive value for disease outcome.

In the CNS, basal levels of IL1β, TNFα, and IL6 are necessary for optimal neurons and glial function to maintain CNS homeostatis (Camacho-Arroyo et al. 2009; Borsini et al. 2015). Viral infection results in cytokine induced stimulation of neuroendocrine responses via the activation of the hypothalamus–pituitary–adrenal (HPA) axis and the elevation of the core body temperature, ultimately promoting disease-symptoms and elevated cytokine levels in the brain parenchyma which serve as mediators of neurotoxic and neurodegenerative pathology (Camacho-Arroyo et al. 2009; Borsini et al. 2015). In the context of

COVID-19, pathological excess of cytokines may lead to vascular remodeling and BBB leakage, increasing the entry of danger-associated molecular pattern (DAMPs) and pathogen-associated molecular pattern (PAMPs) associated with the peripheral viral infection. Cytokine storm is a distinct immunological character of COVID-19 infection resulting in significant neuro-inflammation that is attributed to an increase in pro-inflammatory cytokines. In the CNS during SARS-COV 2 infection, the elevated level of cytokines IL1β, TNF, and IL6 serve as a mediator of neurotoxic and neurodegenerative pathology (Gonçalves de Andrade et al. 2021). Cytokine storm is the hyper-induction of pro-inflammatory cytokines, which is major hallmark of SARS-COV2 infection. Activation of the innate and adaptive immune system results in producing pro-inflammatory cytokines and a feedback loop of inflammation. Recent studies have shown that the cytokine response induced by SARS-COV2 is different from the cytokine storm caused by other respiratory viruses, as SARS-COV2 does not always induce significant amounts of cytokines such as IL-2, IL-10, IL-4 but causes dysregulation of the Type-I interferon (IFN) response and its associated cytokine activation (Olbei et al. 2021; Channappanavar and Perlman 2017)). The main effectors of the Type-I IFN signaling are IFN-α and IFN-β, which activate cytokines, such as IL-12 and IFN-γ (Kang et al. 2018; Bhardwaj et al. 1996). We did not observe significant change in IL-2, IL-4, but observed a significant increase in IL-6, IL-10, IL-12, IL-17A, IL-23 and IL-33 in SARS-COV2 treated microglia as compared to the untreated control. Our data suggests that the SARS-COV2 neuropathogenesis process may be driven by different inflammatory response patterns than other respiratory viruses and that the cytokine response during viral infection may be a dynamic process, that may results in modulation of levels of different members of the cytokine family during the course of the infection, in order to maintain a homeostatic balance in the CNS, however, should the cytokine dysregulation continue and the excessive cytokine production contributes to brain tissue inflammation and the neuropathogenesis of COVID-19.

We observed increased gene expression of IL-8, IL-1β and TNF-α in both SARS-COV2 spike protein and heat inactivated SARS-COV2 treated microglial cells, as compared to the untreated control, however only significantly higher IL-8 cytokine expression was seen in the heat inactivated SARS-COV 2 treated microglia when compared to the microglia treated with SARS-COV2 spike protein. Although, we did not measure cytokine levels from the heat inactivated SARS-COV 2 treated microglia, the increased IL-8 gene expression levels quantitated by QPCR suggests that we would also observe an increase in other cytokine levels concurrent with what we observed in SARS-COV2 treated microglial cells. Although both SARS-COV2 spike protein and heat inactivated

SARS-COV2 treatment of microglia may result in a robust cytokine response, modulation of cytokine levels may be variable given that the cytokine release is a dynamic process. Predictive biomarkers of pathogenic inflammation may help guide targetable immunotherapy that may have significant benefit in controlling neuro-inflammation and the consequent SARS-COV2 associated neuropathology.

Inflammatory response is associated with the assembly/ activation of a cell-intrinsic defense platform known as the inflammasome. Several reports suggest that the main role of NLRP3 inflammasome in the pathogenesis of SARS-COV2 is its overactivation contributing to cytokine storm (Freeman and Swartz 2020; Ratajczak and Kucia 2020; Fu et al. 2020). We observed a significant increase in the expression of NLRP3 expression on treatment with SARS-COV2 (Fig. 3b) indicating that SARS-COV2 induces inflammasome activation in microglia. Several studies have shown that NLRP3 inflammasome is activated in response to SARS-COV2 infection (Xu et al. 2020; Irrera et al. 2020; Freeman and Swartz 2020; van den Berg and Te Velde 2020). The NLRP3 inflammasome promotes inflammation via cleavage and activation of key inflammatory molecules including active caspase-1 (Casp1p20), IL-1β, and IL-18 (Rodrigues et al. 2021). Our data shows that NLRP3 inflammasome is activated in response to SARS-COV2 treatment in human microglia.

ACE-2 is an entry receptor for SARS-CoV2. Efficient replication of SARS-CoV2 depends on the expression level of ACE-2 (Hoffmann et al. 2020). Basal level of ACE2 expression in CNS cells is sufficient for viral entry (Puelles et al. 2020). We observed a significant increase in the ACE2 expression [Fig. 2, Panel a(i-iii)] in microglia when treated with SARS-COV2. In neuronal cell cultures, ACE2 is expressed both on the surface membrane and in the cytoplasm and its prevalent expression in the brain has the potential to infect neurons and glial cells throughout the CNS (Hoffmann et al. 2020; Reynolds and Mahajan 2021). We and others have reported that SARSCOV2 induces increased ACE2 expression by brain microvascular endothelial cells (Reynolds and Mahajan 2021; Buzhdygan et al. 2020) and increases BBB permeability to SARS-COV2.

We observed a significant increase in the HIF-1α expression [Fig. 2, Panel b(i-iii)] in microglia when treated with SARS-COV2 that indicates that an accumulation of HIF-1α occurs in microglia, this is believed to be due to increased HIF-1α expression as well as inhibited proteasome degradation (Vassilaki and Frakolaki 2017). Hypoxia and HIF-1α stabilization may trigger or enhance the cytokine storm. Hypoxia plays important roles at the inflammation site by inducing pro-inflammatory cytokines production and induces HIF-1α expression (Serebrovska et al. 2020). Hypoxia and HIF-1α can either stimulate or inhibit cytokine-mediated inflammatory response. HIF-1α regulates important cellular processes such as cell proliferation, metabolism and angiogenesis. Hypoxia may trigger mitochondrial dysregulation, acidosis, altered mitochondrial membrane permeability, and eventually insufficiency of ATP biosynthesis inducing cell death. Modulation of the mitochondrial respiratory chain in hypoxia occurs via the generation of H_2O_2, and other reactive oxygen /nitrogen species (Zorov et al. 2014).

Mitochondrial dysfunction and associated oxidative stress drive the production of pro-inflammatory cytokines that, in turn, play an important role in the immune response. (Arnoult et al. 2011). Our data shows that SARS-COV2 treatment increases mitochondrial activation [Fig. 5, Panel b(i-iii)], ROS [Fig. 4, Panel c(i-iii)] and NOS [Fig. 2, Panel c(i-iii)] activation. Mitochondrial dysfunction occurs when oxidative modification of the respiratory chain complexes occurs and this event amplifies and promotes further oxidative damage. Our data suggest that SARS-COV2 treatment of microglial cells results in increased mitochondrial respiration which affects mitochondrial function, and could influences SARS-COV2 intracellular survival or enable evasion of host immune system. Mitochondrial manipulation by SARS-CoV-2 results in increased oxidative stress and increased cytokine production. A recent report indicate that mitochondrial dysfunction contributes to a systemic immune response in COVID-19 pathogenesis (Ajaz et al. 2021), thus given that microglia are the immune cells of the CNS, the SARS-COV2 induced mitochondrial dysfunction in microglia observed in our study could potentially drive Neuro-COVID progression.

Elevated ROS production, upregulates nitric oxide synthase expression, indicating development of oxidative and nitrosative stress impacting immune function, apoptosis, inflammatory response, as well as organ and tissue dysfunction in COVID-19 infection (Chernyak et al. 2020; Chang et al. 2021). H_2O_2 triggers the expression of many genes that upregulate pro-inflammatory cytokines, such as IL-1β and TNFα, and inducible nitric oxide synthase (iNOS) via activation of the NF-κB pathway (Nanduri 2008). These pro-inflammatory cytokines activate macrophages, neutrophils, endothelium cells via NADPH oxidase (NOx) to produce more superoxide and H_2O_2 and these superoxide radicals and NO are toxic to mitochondria (Tan et al. 2016). In COVID-19 patients it is likely that the inability of the mitochondria to utilize oxygen despite normal tissue oxygen saturation may be exacerbated (Cecchini and Cecchini 2020). SARS-COV2 induced excessive ROS production results in the development of oxidative stress followed by cell and tissue damage (Chernyak et al. 2020). The interaction between ROS and cytokines generates a self-sustaining cycle between cytokine storm and oxidative stress production that eventually leads to multiorgan failure in patients with COVID-19. Alternately, NO also inhibits the replication cycle of SARS-COV2 suggesting that the production of NO by inducible nitric oxide synthase (iNOS) results may produce an antiviral effect (Fang et al. 2021) and could be used as a therapeutic to kill the virus.

Evidence suggests that there is a direct relationship between apoptosis and COVID-19 pathogenicity and that the induction of apoptosis is a hallmark of SARS-COV2 infection. (Donia and Bokhari 2021). Our data shows that SARS-COV2 treatment increased the expression of Caspase3/7 potentially initiate microglial cell apoptosis [Fig. 4, Panel a(i-iii)], Apoptosis triggered by SARS-COV2 has a complex role in host antiviral immunity, and might facilitate the viral clearance or act as a mechanism for virus-induced tissue injury and disease progression [Kaminskyy and Zhivotovsky (2010); Ampomah and Lim (2020)]. SARS-COV2 induces apoptosis via the extrinsic apoptotic pathway Ren et al. (2020).

Mitochondria play a central role in the COVID neuropathogenesis process by virtue of the fact that it is a major reactive oxygen species (ROS) producer, and a target of ROS. The proximity of mtDNA to the ROS-generating electron transport chain makes mtDNA susceptible to oxidative damage. Our data shows that treatment with both SARS-COV 2 spike protein and HI SARS-COV2 resulted in a significant increase in mitochondrial respiration. Figure 5 shows a significant increase in both basal and maximal OCR in response to both SARS-COV 2 recombinant spike protein and HI SARS-COV2. Our results suggest that SARS-COV2 increased mitochondrial respiration in microglia which could lead to bioenergetic dysfunction and production of ROS and increased oxidative stress. Host responses against SARSCOV2 depend on mitochondrial functions and mitochondrial DNA itself acts as a danger-associated molecular pattern (DAMP) and mitochondrial dysfunction drives a systemic immune response in COVID-19 pathogenesis. (Singh et al. 2020). Since mtDNA encodes vital components of the OXPHOS and protein synthesis machinery, oxidative damage-induced mtDNA mutations that impair either the assembly or the function of the respiratory chain will, in turn, further accumulation of ROS, which results in a vicious cycle leading to energy depletion in the cell and ultimately cell death. Our results show that SARS-COV2 increased mitochondrial respiration in microglia which could lead to bioenergetic dysfunction and production of ROS and increased oxidative stress.

Figure 6 (Panel A-C) shows recombinant SARS-COV 2 spike protein and HI SARS-COV 2 induced changes to microglial morphology and Nanolive images show fragmentation of cell organelles such endoplasmic reticulum, increased vacuoles, increase in membrane ruffling, micropinocytosis and mitochondrial fragmentation, all of which are indicative of cellular stress and apoptosis initiation. Figure 6D shows Raman spectra for microglial cells treated with recombinant SARS-COV 2 spike protein and HI SARS-COV 2. We used single value decomposition (SVD) to analyze the Raman spectra. (Fig. 7A-C) The SVD allow us to differentiate the Raman spectra and provided important differences between the spectra. Our data indicate that chemical changes in proteins, observed by Raman measurements, precede morphological changes.

Thus, our data suggests that SARS-COV2 induces a significant inflammatory response, increased oxidative stress, inflammasome activation and mitochondrial dysfunction in microglial cells, all of which contribute to COVID associated neuropathology. This study provides important mechanistic insights into SARS-COV2 induced mitochondrial dysfunction which underlies COVID-19 associated neuropathology.

Declarations

Ethical Approval (X) Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors. This is an in-vitro cell culture study.

Conflict of Interest None of the Authors have any conflicts of interests.

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