A cannabinoid receptor agonist shows anti-inflammatory and survival properties in human SARS-CoV-2-infected iPSC-derived cardiomyocytes

Luiz Guilherme H. S. Aragão[a¶], Júlia T. Oliveira[a¶], Jairo R. Temerozo[b,c], Mayara A. Mendes[a], José Alexandre Salerno[a,j], Carolina da S. G. Pedrosa[a], Teresa Puig-Pijuan[a,h], Carla Veríssimo[j], Isis M. Ornelas[a], Thayana Torquato[a], Gabriela Vitória, Carolina Q. Sacramento[d,e], Natalia Fintelman-Rodrigues[d,e], Suelen da Silva Gomes Dias[d], Vinicius Cardoso Soares[d,f], Leticia R. Q. Souza[a], Karina Karmirian[a,j], Livia Goto-Silva[a], Diogo Biagi[g], Estela M. Cruvinel[g], Rafael Dariolli[g,i], Daniel R. Furtado[a], Patrícia T. Bozza[d], Helena L. Borges[j], Thiago Moreno L. Souza[d,e], Marília Zaluar P. Guimarães[a,j,&*], Stevens Rehen[a,j,&*]

[a] D'Or Institute for Research and Education (IDOR), Rio de Janeiro, RJ, Brazil.

[b] National Institute for Science and Technology on Neuroimmunomodulation (INCT/NIM), Oswaldo Cruz Institute (IOC), Oswaldo Cruz Foundation (Fiocruz), Rio de Janeiro, RJ, Brazil.

[c] Laboratory on Thymus Research, Oswaldo Cruz Institute (IOC), Oswaldo Cruz Foundation (Fiocruz), Rio de Janeiro, RJ, Brazil.

[d] Laboratory of Immunopharmacology, Oswaldo Cruz Institute (IOC), Oswaldo Cruz Foundation (Fiocruz), Rio de Janeiro, RJ, Brazil.

[e] National Institute for Science and Technology on Innovation in Diseases of Neglected Populations (INCT/IDPN), Center for Technological Development in Health (CDTS), Oswaldo Cruz Foundation (Fiocruz), Rio de Janeiro, RJ, Brazil.

[f] Program of Immunology and Inflammation, Federal University of Rio de Janeiro (UFRJ), Rio de Janeiro, RJ, Brazil.

[g] Pluricell Biotech, São Paulo, SP, Brazil.

[h] Carlos Chagas Filho Institute of Biophysics, Federal University of Rio de Janeiro (UFRJ), Rio de Janeiro, RJ, Brazil.

[i] Department of Pharmacological Sciences, Icahn School of Medicine at Mount Sinai, New York, NY, United States.

[j] Institute of Biomedical Sciences, Federal University of Rio de Janeiro (UFRJ), Rio de Janeiro, RJ, Brazil.

¶ These authors contributed equally to this work

& These authors contributed equally to this work

* Corresponding authors

E-mail: marilia@lance-ufrj.org (MZPG) and srehen@lance-ufrj.org (SR)

Highlights

- Human iPSC-derived cardiomyocytes (hiPSC-CMs) express CB1 receptor.
- The cannabinoid receptor agonist, WIN 55,212-2 (WIN), does not influence SARS-CoV-2 infection in hiPSC-CMs.
- WIN reduces inflammation and death in SARS-CoV-2-infected hiPSC-CMs.

Abstract

Coronavirus disease 2019 (COVID-19) is caused by acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which can infect several organs and lead to loss of vital organ function, especially impacting respiratory capacity. Among the extrapulmonary manifestations of COVID-19 is myocardial injury, caused both directly and indirectly by SARS-CoV-2, and which is associated with a high risk of mortality. One of the hallmarks of severe COVID-19 is the “cytokine storm”, at which point the immune system malfunctions, leading to possible organ failure and death. Cannabinoids are known to have anti-inflammatory properties by negatively modulating the release of pro-inflammatory cytokines. Herein, we investigated the effects of the cannabinoid agonist WIN 55,212-2 (WIN) on SARS-CoV-2-infected human iPSC-derived cardiomyocytes (hiPSC-CMs). Although WIN did not modulate angiotensin-converting enzyme II, nor reduced SARS-CoV-2 infection and replication in hiPSC-CMs at the conditions tested, it had anti-inflammatory and protective effects by reducing the levels of interleukins 6, 8,18 and tumor necrosis factor-alpha (TNF-α) and lactate dehydrogenase (LDH) activity in these cells without causing hypertrophic cardiac damage. These findings suggest that cannabinoids should be further investigated as an alternative therapeutic tool for the treatment of COVID-19.

Keywords: WIN 55,212-2, Cannabinoids, SARS-CoV-2, COVID-19, human iPSC-derived cardiomyocytes

1. Introduction

Coronavirus disease 2019 (COVID-19) is caused by the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Most individuals with COVID-19 have classical respiratory symptoms, however, this disease affects multiple biological systems and organs, such as the nervous system (Carod-Artal, 2020), the digestive system (X. Wang et al., 2020), the urinary system (Puelles et al., 2020), the skin (Diaz-Guimaraens et al., 2020; Mahé et al., 2020) and the cardiovascular system (Maisch, 2020; Varga et al., 2020; Zheng et al., 2020).

The receptor angiotensin-converting enzyme II (ACE2) is a membrane receptor that acts as the major gateway to SARS-CoV-2 in host cells (Scialo et al., 2020).

Cardiomyocytes express ACE2, with high levels found in patients with pre-existing cardiac conditions, and SARS-CoV-2 infects iPSC-derived cardiomyocytes (hiPSC-CMs) through ACE2 (Dariolli et al., 2021; Sharma et al., 2020; Thum, 2020). Indeed, a *post-mortem* study of a child with COVID-19 revealed diffuse myocardial interstitial inflammation with immune cells infiltration and necrosis (Dolhnikoff, 2020). Recently, our group showed cardiac damage, such as microthrombi in small arteries and focal mild lymphocytic infiltrate in the ventricles, from *post-mortem* samples of an infant who died of COVID-19 (Gomes et al., 2020). Another study showed SARS-CoV-2 in the myocardial tissue, which was also positive for inflammatory markers such as $CD3^+$, $CD45^+$, and $CD68^+$ cells, and expressed substantial levels of tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), chemokine ligand 5, as well as interleukin (IL) -6, -8, and -18 (Lindner et al., 2020). Studies based on hiPSC-CM have shown that SARS-CoV-2 infection leads to upregulation of inflammation-related genes, including IL-6, IL-8, and TNF-α (Kwon et al., 2020; Wong et al., 2020). The increase of proinflammatory cytokines can cause several adverse effects in cardiomyocytes including arrhythmia (Keck et al., 2019), cellular hypertrophy (Smeets et al., 2008), cell death (Wang et al., 2016), conversion of fibroblasts into myofibroblasts (Wang et al., 2016) and alteration of action potential duration (Aromolaran et al., 2018). Additionally, patients with COVID-19 presented elevated levels of creatine kinase and lactate dehydrogenase activity (LDH), which are injury biomarkers (Chen et al., 2020; Zhou et al., 2020). Thus, there is an urgency to find drugs with the potential to mitigate SARS-CoV-2 infection and to treat severely affected patients, which often present cardiovascular system damage.

Cannabis sp. has been used for medicinal purposes for many centuries, with well-described effects on cardiovascular diseases (Mendizábal and Adler-

Graschinsky, 2007). *Cannabis* has several known compounds, named phytocannabinoids, including delta-9-Tetrahydrocannabinol (THC), which is the main psychoactive agent. Besides phytocannabinoids, there is intensive research on endocannabinoids, such as anandamide and 2-arachidonoylglycerol, and synthetic cannabinoids, such as WIN 55,212–2 (WIN). Studies have been investigating the potential of *Cannabis sativa* to modulate ACE2 expression in an attempt to decrease SARS-CoV-2 infection, but also to decrease viral replication and inflammation that are directly related to COVID-19 severity. Cannabinoids, including WIN, have been previously reported to affect human immunodeficiency virus 1 (HIV-1) expression *in vitro* (Peterson et al., 2004). In addition, *Cannabis* extracts decreased ACE2 expression in oral, intestinal, and airway epithelia *in vitro* (B. Wang et al., 2020). Noteworthy, cannabinoids have anti-inflammatory properties and exert their biological effect by interaction with the cannabinoid receptors type 1 (CB1R) and/or type 2 (CB2R), to both which WIN has high affinity (Devane et al., 1988; Munro et al., 1993). WIN reduced the number of LPS-activated microglia in an animal model of chronic brain inflammation (Marchalant et al., 2007). Another work showed that WIN decreased TNF-α and IL-6 plasma levels, and myeloperoxidase activity in mice with experimental colitis (Feng et al., 2016). Recently, an *in vitro* study showed that an extract fraction from *Cannabis sativa* Arbel strain and its formulation using phytocannabinoid standards presented anti-inflammatory activity in lung epithelial cells treated with TNF-α but proinflammatory activity in macrophages (Anil et al., 2021). Also, high-CBD cannabis sativa extracts presented anti-inflammatory properties in the epithelia pre-treated with TNF-α and IFN-γ (Lei et al., 2020).

Investigating the anti-inflammatory potential of *Cannabis sativa* in cardiomyocytes is important because the “cytokine storm” is a hallmark of COVID-19

and the cardiovascular system is mostly affected in severe cases. To date, there is no evidence of the effects of cannabinoids on hiPSC-CMs SARS-CoV-2 infection.

In this work, we aimed to investigate the effects of WIN, a synthetic cannabinoid that acts as a mixed CB1/CB2 receptors agonist, on hiPSC-CMs infected by SARS-CoV-2. WIN did not modulate ACE2 nor reduced SARS-CoV-2 infection and replication, but presented anti-inflammatory and protective properties by reducing the levels of proinflammatory cytokines and cell death in hiPSC-CMs.

2. Methods

2.1. Chemicals

WIN 55,212-2 mesylate was purchased from TargetMol (T4458). Stock and work solutions were prepared using 100% dimethyl sulfoxide sterile-filtered (DMSO; D2650 - Sigma-Aldrich).

2.2. iPS-Cardiomyocyte differentiation and purification

hiPSC-CMs were purchased from Pluricell (São Paulo, Brazil). hiPSC-CMs were used between day 25 and day 35 of differentiation. Our hiPSC-CMs were generated and previously characterized *in vitro* by Cruvinel et al. (2020) (Cruvinel et al., 2020). In this work, the purity of the cardiomyocyte population by TNNT2 flow cytometry and immunofluorescence assessment was average of 88.4% TNNT2+ cells +/- 8.4% (Fig S3). hiPSC-CMs were handled in four different groups: MOCK and SARS-CoV-2 (SARS-CoV-2 infection without WIN), which were also analyzed as controls in Salerno et al., 2021 (in preparation), MOCK WIN (no SARS-CoV-2 infection + WIN), and SARS-CoV-2 WIN (SARS-Cov-2 infection + WIN). All WIN-treated hiPSC-

CMs were pretreated with 1 µM WIN. After 24 hours, 1 µM WIN was re-added combined (or not) with SARS-CoV-2 infection.

2. 3. SARS-CoV-2 propagation

SARS-CoV-2 was expanded in Vero E6 cells from an isolate contained on a nasopharyngeal swab obtained from a confirmed case in Rio de Janeiro, Brazil (GenBank accession no. MT710714). Viral isolation was performed after a single passage in a cell culture in a 150 cm^2 flasks with high glucose DMEM plus 2% FBS. Observations for cytopathic effects were performed daily and peaked 4 to 5 days after infection. All procedures related to virus culture were handled in biosafety level 3 (BSL3) multiuser facilities according to WHO guidelines. Virus titers were determined as plaque-forming units (PFU/mL), and virus stocks were kept in -80°C ultralow freezers.

2.4. SARS-CoV-2 titration

For virus titration, monolayers of Vero E6 cells (2 x 10^4 cell/well) in 96-well plates were infected with serial dilutions of supernatants containing SARS-CoV-2 for 1 hour at 37°C. A semi-solid high glucose DMEM medium containing 2% FSB and 2.4% carboxymethylcellulose was added and cultures were incubated for 3 days at 37 °C. Then, the cells were fixed with 10% formalin for 2 hours at room temperature. The cell monolayer was stained with 0.04% solution of crystal violet in 20% ethanol for 1 hour. Plaque numbers were scored in at least 3 replicates per dilution by independent readers blinded to the experimental group and the virus titers were determined by plaque-forming units (PFU) per milliliter.

2.5. SARS-CoV-2 infection

hiPSC-CMs were infected with SARS-CoV-2 at an MOI of 0.1 in high glucose DMEM without serum. After 1 hour, cells were washed and incubated with complete medium with treatments or not for 48-72h. After, the supernatant was collected and cells were fixed with 4% paraformaldehyde (PFA) solution for posterior analysis.

2.6. Cytokine multiplex assay and LDH cytotoxicity assay

A multiplex biometric immunoassay containing fluorescent dyed microbeads was used for plasma cytokine measurement (Bio-Rad Laboratories, Hercules, CA, USA). The following cytokines were quantified: IL-6, IL-7, IL-8, and TNF-α; cytokine levels were calculated by Luminex technology (Bio-Plex Workstation; Bio-Rad Laboratories, USA). The analysis of data was performed using software provided by the manufacturer (Bio-Rad Laboratories, USA). A range of 0.51–8,000 pg/mL recombinant cytokines was used to establish standard curves and the sensitivity of the assay. Cell death was determined according to the activity of lactate dehydrogenase (LDH) in the culture supernatants using a CytoTox® Kit (Promega, USA) according to the manufacturer's instructions.

2.7. Gene expression analysis

Qualitative endpoint PCR reactions were executed with the following primer sequences: CB1R (forward 5'-ATGTGGACCATAGCCATTGTG-3'; reverse: 5'-CCGATCCAGAACATCAGGTAGG-3') and CB2R (forward 5'-GCTATCCACCTTCCTACAAAGC-3'; reverse: 5'- CTCAGCAGGTAGTCATTGGGG-3'). Glyceraldehyde-3-phosphate Dehydrogenase (GAPDH; forward: 5'-TTCGACAGTCAGCCGCATC-3'; reverse: 5'-GACTCCACGACGTACTCAGC-3') was

used as the endogenous housekeeping control gene. Each PCR reaction was performed in a 10 µL mixture containing 0.25 U GoTaq G2 Hot Start Polymerase (Promega), 1x GoTaq G2 Buffer, 1.5 mM MgCl2 (Invitrogen), 200 nM of each primer (forward and reverse), 200 µM dNTP mixture containing the four deoxyribonucleotides (dATP, dCTP, dTTP, and dGTP), and 10 ng of cDNA template. Appropriate negative controls and genomic DNA positive controls were incorporated into each experiment. Amplification thermal program included an initial denaturation step of 95°C for 3 min and 40 cycles of 95°C for 15 s, 60°C for 15 s and 72°C for 15 s using the ProFlexTM PCR System Thermal Cycler (Applied Biosystems). Subsequently, the total amount of PCR product was separated by electrophoresis at 110 V for 40 min in 1.8% agarose gel diluted in 1× Tris-acetate EDTA buffer (w/v) and stained with 0.01% of SYBR Safe (Thermo Fisher).

For real-time quantitative PCR, the reactions were carried out in triplicates in a reaction mixture containing 1x GoTaq qPCR MasterMix (Promega Corporation), 300 nM CXR Reference Dye, a final concentration of 200 nM of each (forward and reverse) SYBR green-designed primers (Thermo Fisher Scientific), and 10 ng of cDNA template per reaction. Appropriate negative controls were added in each run. The relative expression of the genes of interest: ACE2 (forward: 5'-CGAAGCCGAAGACCTGTTCTA-3'; reverse: 5'-GGGCAAGTGTGGACTGTTCC-3'), MYH6 (forward: 5'- GCCCTTTGACATTCGCACTG-3'; reverse: 5'-GGTTTCAGCAATGACCTTGCC-3'), MYH7 (forward: 5'-TCACCAACAACCCCTACGATT-3'; reverse: 5'-CTCCTCAGCGTCATCAATGGA-3') was normalized by human reference genes: Glyceraldehyde-3-phosphate Dehydrogenase (GAPDH; forward: 5'-GCCCTCAACGACCACTTTG-3'; reverse: 5'-CCACCACCCTGTTGCTGTAG-3') and Hypoxanthine Phosphoribosyltransferase 1

(HPRT-1; forward 5'-CGTCGTGATTAGTGATGATGAACC-3'; reverse: 5'-AGAGGGCTACAATGTGATGGC-3'). The reactions were performed on a StepOnePlusTM Real-Time PCR System thermocycler (Applied Biosystems). Thermal cycling program comprised of a denaturing step at 95°C for 3 min, followed by 40 cycling stages at 95°C for 15 sec, 57°C for 15 sec, 72°C for 15 sec and melt curve stage 95 °C, 15 sec; 60 °C, 1 min; 95 °C, 15 sec. qPCR data analysis was performed with the N0 method implemented in LinRegPCR v. 2020.0, which considers qPCR mean efficiencies estimated by the window-of-linearity method (Ramakers et al., 2003; Ruijter et al., 2009).

2.8. Immunofluorescence staining

Infected and mock-treated hiPSC-CMs were fixed using 4% paraformaldehyde solution (Sigma-Aldrich, EUA) for 1h and stored in 4ºC. After, cells were washed with PBS and then incubated with permeabilization/blocking solution (0.3% Triton X-100 / 1% bovine serum albumin + 3% normal goat serum) for 1h. Cardiomyocytes were incubated in primary antibodies diluted in a blocking buffer at 4º overnight (anti-SARS-CoV-2 convalescent serum from a positive COVID-19 patient (1:1000) and anti-cardiac troponin T (TNNT2) (1:500, MA5-12960 - Invitrogen). Next, cardiomyocytes were incubated with the secondary antibody diluted in a blocking buffer: goat anti-Human Alexa Fluor 647 (1:400; A-21445 - Invitrogen) and goat anti-Mouse 594 (1:400; A-11032 - Invitrogen) for 1h. Actin filaments were stained with Alexa Fluor 568 phalloidin (1:10; A-12380 - Life Technologies) for 1h. Nuclei were stained with 300 nM 4'-6-diamino-2-phenylindole (DAPI) for 5 minutes and each well was mounted with two drops of 50% PBS-Glycerol. Images (at least 10 fields per well) of hiPSC-CMs were acquired using Operetta® High-Content Imaging System (Perkin Elmer) with a 20x long working distance (WD) objective lens. To acquire images of hiPSC-CMs

immunostained for TNNT2 and F-actin (Fig S3), we used a Leica TCS-SP8 confocal microscope with the 63x objective.

2.9. Neutral red uptake assay

Briefly, hiPSC-CMs were seeded in 96-well plates. After reaching 80–90% confluency, the cells were exposed to eight concentrations of WIN 55,212-2 for 72h. Next, the medium was replaced, cells were washed with PBS 1x and 200 µL of neutral red dye diluted in the hiPSC-CMs medium was added to each well at a final concentration of 0.05 mg/mL. After 3h of incubation at 37ºC, the medium was removed and the cells washed again. Then, 100 µL of the developer solution was added (1% acetic acid-49% ethanol). To measure absorbance with a 540 nm filter, a spectrophotometer Tecan Infinite® 200 PRO (Life Sciences, Switzerland) was used.

2.10. Protein expression

After 24h treatment of hiPSC-CMs in 24-well plates, 100 µL of sample buffer without bromophenol blue (62.5 mM Tris-HCl, pH 6.8, containing 10% glycerol, 2% SDS, and 5% 2-mercaptoethanol) was added in each well, the samples were then frozen at -80ºC. Using a disposable pestle, the samples were broken down and cell extracts were boiled at 95ºC for 5 minutes and centrifuged at 4ºC 16,000x g for 15 min to collect the supernatant. Protein content was estimated using the Bio-Rad Protein Assay (# 5000006, Biorad). Next, bromophenol blue (0.02%) was added and extract samples (40ug/lane) were separated by electrophoresis 8% SDS polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked in 5% non-fat milk in Tris Buffered Saline with 0.1% Tween-20 (TBS-T) for 1 hour at room temperature. Membranes were incubated overnight at 4ºC with primary antibodies anti-ACE2 (1: 1000; MA5-32307 - Thermo Fisher), anti-ACTIN (1:

2000; MAB1501, Millipore) diluted in TBS-T with 5% non-fat milk and anti-CB1 (1:500; SC-10066, Santa Cruz). Membranes were washed to be incubated with peroxidase-conjugated antibodies IgG (H + L), HRP-conjugate: goat anti-mouse (1: 10.000, G21040, Molecular Probes) goat anti-rabbit (1: 10.000, G21234, Molecular Probes) and rabbit anti-goat (1: 2.000, 61-1620, Invitrogen). Using an ECL Prime Western Blotting System (# GERPN2232, Sigma) for 5 minutes, the signals were developed and chemiluminescence was detected with an Odyssey-FC Imaging System® (LI-COR Biosciences, EUA). The stripping protocol was used on the membranes after incubating them for three cycles of 10 minutes in the stripping buffer (pH 2.2, 200 mM glycine, 0.1% SDS, and 1% Tween-20). Next, the buffer was discarded and the membranes were washed three times with PBS and three times for 5 minutes with 0.1% TBS-T. Next, membranes were blocked again and proceeded with the above-described steps.

2.11. Statistics

Statistical analyses were performed using GraphPadPrism software version 8.0 (GraphPad, EUA). Results were expressed as the mean plus standard error of the mean (SEM). For the comparison between the two groups, unpaired two-tailed Student's t-test was performed. Two-way analysis of variance (ANOVA) followed by Tukey's test was used to compare three or more groups. A confidence interval of 95% was accepted.

3. Results

3.1. hiPSC-CMs express CB1R but WIN does not modulate ACE2

Since WIN is a mixed agonist of CB1/CB2 receptors, which are the main known cannabinoid receptors, we investigated if hiPSC-CMs also expressed them. We found that hiPSC-CMs express CB1 but not CB2 receptors (Fig S1).

Next, we investigated which WIN concentration was harmless for the hiPSC-CMs or could cause transitory or permanent cardiac hypertrophy (Albakri, 2019; Rahmatollahi et al., 2016; Wenzel, 1967). We performed a cell viability assay and found that WIN was harmless in concentrations up to 1 µM (Fig S2A). Also, compared with control, 1 µM WIN did not increase MYH6 and MYH7 mRNA levels (Fig S2B), which are genes that, when upregulated, may indicate hypertrophy, confirming the feasibility of using the chosen concentration. Therefore, we set 1 µM WIN as the usage concentration for further assays.

After confirming that hiPSC-CMs express both CB1R and ACE2, we asked whether WIN could modulate ACE2 expression and, subsequently, influence SARS-CoV-2 infection and replication within hiPSC-CMs. hiPSC-CMs were pre-treated with 1 µM WIN for 24 hours and analyzed for both mRNA and protein levels of ACE2. We observed that WIN-treated and untreated hiPSC-CMs were comparable regarding ACE2 mRNA expression as well as protein levels (Fig 1A and B).

3.2. WIN does not influence SARS-CoV-2 infection and replication in hiPSC-CMs

Next, we asked if WIN could reduce hiPSC-CMs SARS-CoV-2 infection by mechanisms other than through ACE2 modulation. For this, we pre-treated cells with 1 µM WIN for 24 hours and infected them with SARS-CoV-2 at a multiplicity of infection (MOI) of 0.1 for 48 hours. In this study, we defined the use of MOI 0.1 for all experiments because this MOI had already been set in hiPSC-CMs infected by SARS-CoV-2 (Sharma et al., 2020). Additionally, MOIs above 0.1 may not be physiologically

plausible viral doses to be found *in vivo*. Following the 48 hours of infection, we quantified convalescent serum (CS)-immunostained densities. As expected, we found that MOCK-infected cells presented no CS immunoreactivity. Of the cells infected (SARS-CoV-2), those pre-treated with WIN (WIN and SARS-CoV-2 WIN) presented CS immunoreactivity comparable with SARS-CoV-2 alone (Fig 2A and B).

Since viral infection and replication are directly related but orchestrated by different mechanisms, we asked whether WIN could decrease SARS-CoV-2 replication in hiPSC-CMs. We observed that despite SARS-CoV-2 WIN provoking a decrease in average viral titer, the difference from SARS-CoV-2 was not statistically significant (Fig 2C).

3.3. WIN reduces the secretion of IL-6, IL-8, TNF-α by hiPSC-CMs

The “cytokine storm” is a hallmark of severe COVID-19 cases and cannabinoids have well-known anti-inflammatory properties. We asked whether WIN could reduce the release of IL-6, IL-8, TNF-α *in vitro*. Cells pre-treated with 1 µM WIN were infected for 24, 48, and 72 hours, and the media harvested at each time point for the analysis of cytokines. We found that, at all time points post-infection, hiPSC-CMs released basal levels of cytokines. However, cells infected with SARS-CoV-2 released higher levels of cytokines when compared with control and SARS-CoV-2 WIN groups (Figure 3A, B and C).

3.4. WIN reduces LDH activity in hiPSC-CMs

SARS-CoV-2 infection causes apoptosis in hiPSC-CMs (Sharma et al., 2020). Therefore, we investigated whether WIN protects hiPSC-CMs from cell death by reducing the leakage levels of LDH, which is released when the plasma membrane is

damaged. Cells were pre-treated with 1 µM WIN for 24 hours, infected for 24, 48, and 72 hours, and the media analyzed at each time point for LDH activity. We observed that at 48 and 72 hours post-infection, LDH activity was higher in SARS-CoV-2 compared with MOCK, but it was lower in SARS-CoV-2 WIN compared with SARS-CoV-2 only (Figure 3D).

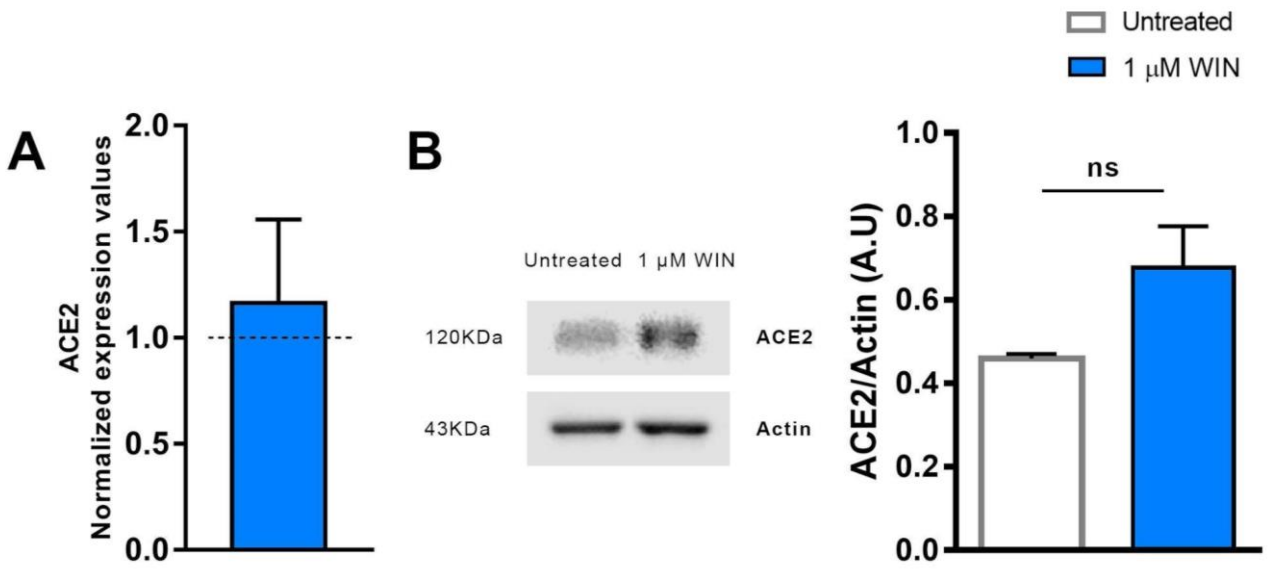

Fig 1. WIN does not modulate ACE2 in hiPSC-CMs. (A) Relative mRNA expression levels of ACE2 in hiPSC-CMs expressed as fold change relative to untreated condition (1.165±0.3929). (B) Quantification of western blots by densitometry normalized by actin expression. ACE2 mRNA and protein levels were statistically non-significant between treated (1.151±0.07 A.U.) and untreated (0.98±0.11 A.U.) hiPSC-CMs. Error bars represent standard errors of the means (SEMs) from three (A) and four (B) independent experiments.

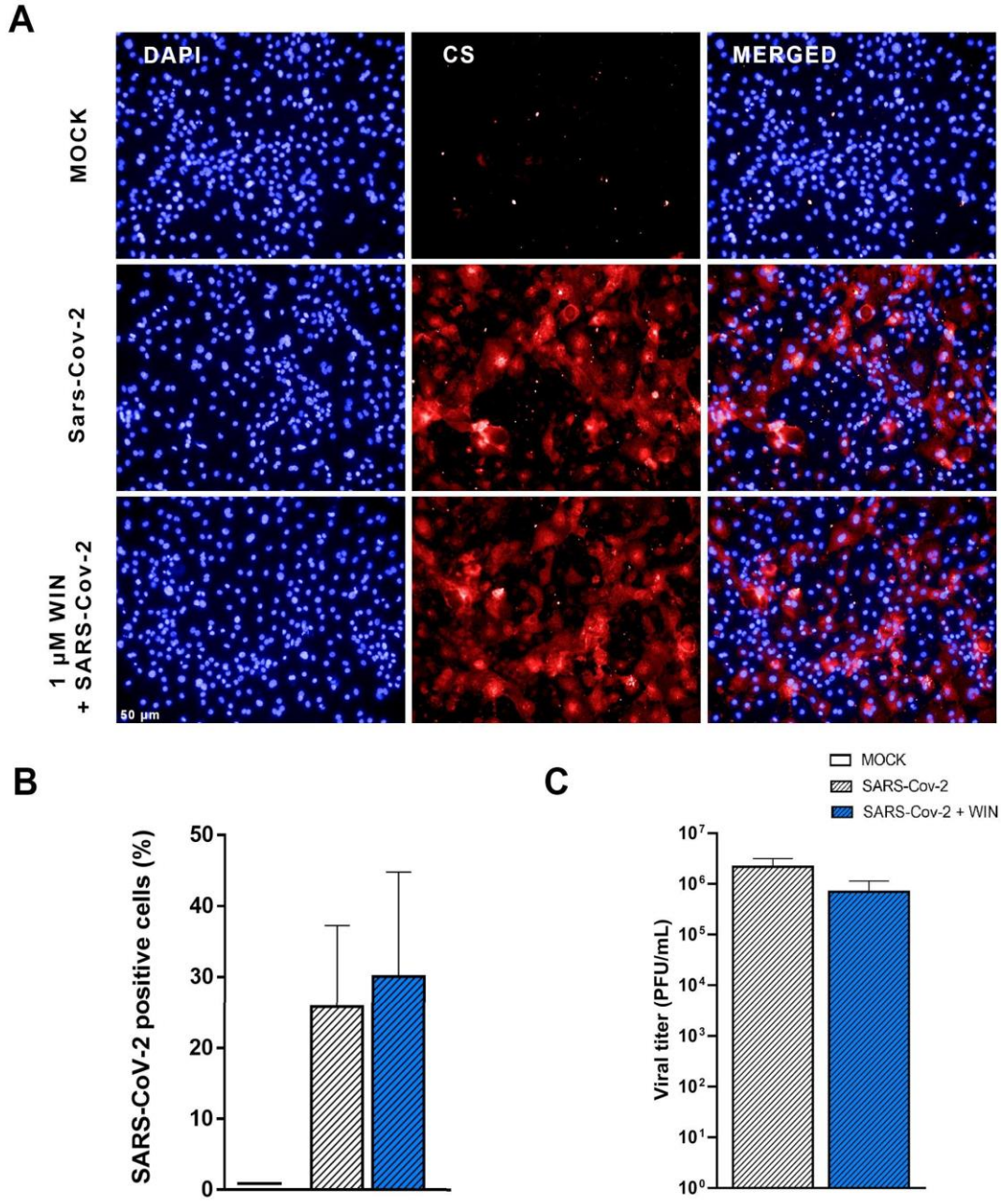

Fig 2. WIN does not reduce SARS-CoV-2 infection and replication in hiPSC-CMs. (A) Immunomicrographs of MOCK and SARS-CoV-2-infected hiPSC-CM pre-treated or not with 1 µM WIN for 24 hours. hiPSC-CM were immunostained for SARS-CoV-2 convalescent serum (CS) (red) and counterstained with DAPI (blue) at 48h post-infection. Scale bar: 50 µm. (B) Percentage of CS positive cells. CS immunoreactivity was comparable between treated (30±15%) and untreated (26±12%) hiPSC-CM. (C) Viral titer quantification by plaque forming units assay using the supernatants of the SARS-CoV-2 infected hiPSC-CMs. Viral titer was comparable between treated ($6.99 \times 10^5 \pm 4.39 \times 10^5$ PFU/mL) and untreated ($2.18 \times 10^6 \pm 9.96 \times 10^5$ PFU/mL) hiPSC-CM. Error bars represent standard errors of the means (SEMs) from three independent experiments.

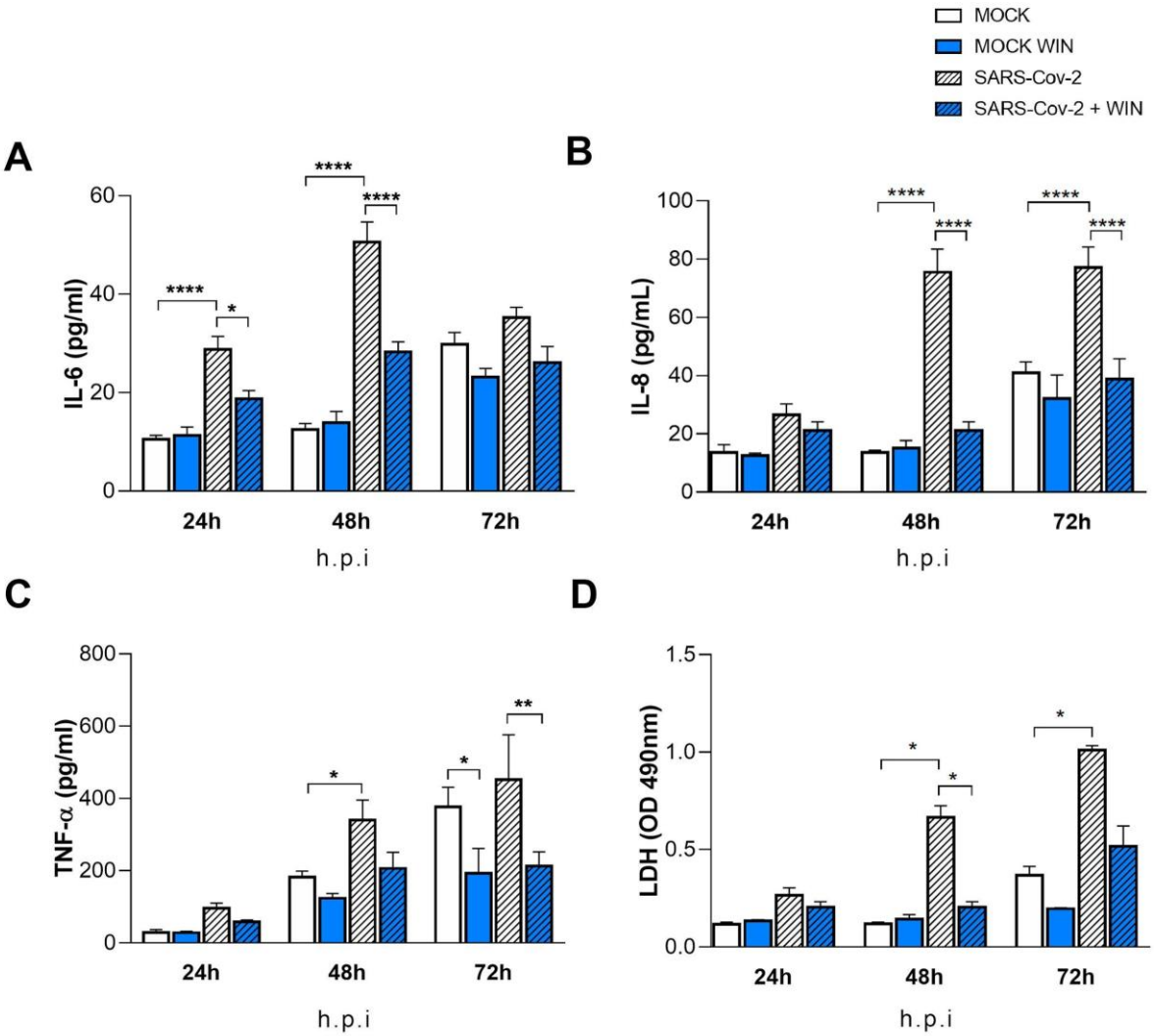

Fig 3. WIN reduces inflammatory markers and viral toxicity in hiPSC-CMs. Levels of IL-6 (A), IL-8 (B), TNF-α (C), and (D) activity of lactate dehydrogenase (LDH) from MOCK and SARS-CoV-2-infected hiPSC-CM, treated or not with 1 μM WIN for 24 hours, were analyzed at 24, 48 and 72 hours post-infection (h.p.i.). Cytokines levels and LDH activity were higher in SARS-CoV-2 compared with control (MOCK), and lower in SARS-CoV-2 WIN when compared with SARS-CoV-2. Data represent means and standard errors of the means (SEMs) from three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, **** $p < 0.0001$.

Discussion

Cannabinoids have been proposed as potential prevention and treatment of COVID-19, due to antiviral, cytoprotective and anti-inflammatory properties. In this study, we showed that the cannabinoid receptor agonist WIN reduced cell damage in SARS-CoV-2-infected hiPSC-CMs. Additionally, even though cardiomyocytes are not known for evoking robust inflammatory responses, WIN reduced the release of cytokines by these cells following a SARS-CoV-2 infection. To our knowledge, this is the first study showing anti-inflammatory and protective properties of a cannabinoid agonist in hiPSC-CMs infected with SARS-CoV-2.

We hypothesized that WIN could reduce the levels of ACE2 in hiPSC-CMs, consequently reducing SARS-CoV-2 infection and viral load in these cells. However, despite hiPSC-CMs expressing ACE2, it was not modulated by WIN in the conditions used by this study. ACE2 is downregulated in SARS-CoV-2 infected tissues (Gheblawi et al., 2020; Yan et al., 2020), which is harmful to the heart since ACE2 has a protective role in the cardiovascular system (Huentelman et al., 2005; Zhong et al., 2010). One possibility for the maintained levels of ACE2 in hiPSC-CMs is that WIN exerted a protective role through preventing receptor downregulation. Although it has been previously shown (B. Wang et al., 2020) that CBD-rich extracts reduced ACE2 mRNA and protein levels in some epithelia *in vitro* following TNF-α insult, this modulation had not been investigated in SARS-CoV-2 infected cardiomyocytes until now.

The modulation of cannabinoid receptors in cardiomyocytes has also not yet been explored, but the CB1 receptor, which is widely expressed in the central nervous system, can disrupt the ability of a virus to infect nervous tissue (Herrera et al., 2008). Our results showed that WIN did not reduce the infection nor the viral titer in hiPSC-

CMs. Several studies have examined the effect of cannabinoids on viral infections, especially regarding the role of CB1 and CB2 receptor activation (Reiss, 2010). The CB2 receptor agonist JWH-133 reduced CXCR4-tropic HIV-1 infection of primary CD4+ T cells, whereas the CB1 receptor agonist arachidonyl-29-chloroethylamide had no effect. In another study with HIV-1-infected primary human monocytes, agonists of CB2 receptors limited viral replication (Ramirez et al., 2013). There is still no consensus on the antiviral mechanisms of cannabinoids, however, it is well-known that the specific activation of the CB2 receptor plays a crucial role in the course of viral infection (Rossi et al., 2020). The fact that hiPSC-CMs do not express the CB2 receptor may indicate WIN's ineffectiveness in reducing SARS-CoV-2 infection and replication via CB1R in these cells *in vitro*.

Although immune cells and cardiac fibroblasts are typically the major players in cytokine production under cardiac stress conditions (Zhong et al., 2010), cardiomyocytes are also a local source of proinflammatory cytokines (Ancey et al., 2002; Atefi et al., 2011; Bozzi et al., 2019; Kleinbongard et al., 2011; Yamauchi-Takihara et al., 1995). In this work, hiPSC-CMs released IL-6, IL-8, and TNF-α at baseline levels and SARS-CoV-2 infection increased all cytokines. Indeed, infection for 24 or 48 hours by the *Trypanosoma cruzi* pathogen in hiPSC-CMs prompted these cells to produce proinflammatory cytokines that caused autocrine cardiomyocyte dysfunction (Bozzi et al., 2019). Cardiac damage in COVID-19 patients can be attributable to hypoxaemia due to respiratory dysfunction (Guo et al., 2020) but also to the “cytokine storm”, which is the uncontrolled systemic inflammatory response likely caused by an imbalance between regulatory and cytotoxic T cells (Meckiff et al., 2020). Although the “cytokine storm” is one of the hallmarks of SARS-CoV-2 infection (Coperchini et al., 2020), one can not rule out that cytokines locally released contribute

to tissue damage, as seen, for example, in *Trypanosoma cruzi* cardiac infection (Bozzi et al., 2019). Here we found that WIN decreased the levels of IL-6, IL-8, and TNF-α released by SARS-CoV-2-infected hiPSC-CMs. An *in vitro* study of cortical astrocytes treated with Amyloid-$\beta_{1–42}$, which is a toxic protein, showed that WIN reduced TNF-α and IL-1β levels, as well as prevented cell death (Aguirre-Rueda et al., 2015). In another study, WIN decreased the activity of peroxisome proliferator-activated receptor alpha and TNF-α levels in the heart tissue of mice with cardiac dysfunction (Rahmatollahi et al., 2016), reinforcing its anti-inflammatory and protective property in cardiac tissue.

THC and WIN are structurally different but they produce similar effects, and both are CB1 and CB2 receptor agonists (Compton et al., 1992). THC presented a protective role against hypoxia in neonatal murine cardiomyocytes by reducing the levels of LDH (Shmist et al., 2006). The authors found that neonatal murine cardiomyocytes expressed CB2R, but not CB1R, and that the cardioprotection by THC was via the CB2 receptor. Conversely, we found that hiPSC-CMs expressed only CB1 receptor but, in line with this study, WIN decreased LDH activity levels, suggesting that this effect was mediated by CB1R and/or other candidates, such as those of TRP (transient receptor potential channel) family (Freichel et al., 2017). However, given WIN's great affinity for CB1 receptors, we believe that the reduction in LDH activity could be attributed to CB1R activation (Shmist et al., 2006). Additionally, the modulation of TRP receptors by WIN depends on the use of high concentrations, such as above 10 µM (Jeske et al., 2006; Koch et al., 2011), which are 10 times the concentration used in this study.

Conclusion

Our investigation showed that pre-treatment with a cannabinoid receptor agonist reduced cytotoxicity and proinflammatory cytokines in SARS-CoV-2-infected hiPSC-CMs. These results suggest an anti-inflammatory and protective effect of cannabinoids, probably via CB1 receptor, in an *in vitro* model of cardiac SARS-CoV-2 infection. Further studies are needed to shed light on the role of cannabinoids in protecting the heart against SARS-CoV-2 infection as well as on their clinical use.

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Declaration of Competing interests

The authors declare no competing interests.

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