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SARS-CoV-2 induces inflammasome-dependent pyroptosis and downmodulation of HLA-DR in human monocytes

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Abstract

Infection by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has been associated with leukopenia and uncontrolled inflammatory response in critically ill patients. A better comprehension of SARS-CoV-2-induced monocyte death is essential for the identification of therapies capable to control the hyper-inflammation and reduce viral replication in patients with COVID-19. Here, we show that SARS-CoV-2 induces inflammasome activation and cell death by pyroptosis in human monocytes, experimentally infected and from patients under intensive care. Pyroptosis was dependent on caspase-1 engagement, prior to IL-1ß production and inflammatory cell death. Monocytes exposed to SARS-CoV-2 downregulate HLA-DR, suggesting a potential limitation to orchestrate the immune response. Our results originally describe mechanisms by which monocytes, a central cellular component recruited from peripheral blood to respiratory tract, succumb to control severe 2019 coronavirus disease (COVID-19).

Author summary

Since its emergence in China in late 2019, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused thousands of deaths worldwide. Currently, the number of individuals infected with SARS-CoV-2 and in need of antiviral, anti-inflammatory, anticoagulant and more invasive treatments has overwhelmed the health systems worldwide. In our study, we found that SARS-CoV-2 is capable of inducing inflammatory cell death in human monocytes, one of the main cell types responsible for anti-SARS-CoV-2 immune response. As a consequence of this intracellular inflammatory mechanism (inflammasome engagement), an exacerbated production of inflammatory mediators occurs. The infection also decreases the expression of HLA-DR in monocytes, a molecule related to the orchestration of the immune response in case of viral infections. We also demonstrated that the HIV-1 protease inhibitor, atazanavir (ATV), prevented the uncontrolled inflammatory response, cell death and reduction in HLA-DR expression in SARS-CoV-2-infected monocytes. Our study provides relevant information on the effects of SARS-CoV-2 infection on human monocytes, as well as on the effect of ATV in preventing these pathological effects on the host.

65

66 ## Introduction

67 Severe acute respiratory coronavirus 2 (SARS-CoV-2), the etiological agent of
68 the 2019 coronavirus disease (COVID-19), emerged in China, causing a major public
69 health burden in decades. Patients with COVID-19 may develop an asymptomatic or mild
70 disease or be affected by the life threatening acute respiratory distress syndrome (ARDS),
71 which is characterized by elevated serum levels of proinflammatory mediators, the
72 cytokine storm (1–3). In the respiratory tract of patients with severe COVID-19,
73 monocytes/macrophages may be the main source of uncontrolled levels of the pro-
74 inflammatory mediators TNF-α and IL-6 (4). Plasmatic levels of IL-6 have been
75 associated with mortality, intensive care admission and hospitalization, representing a
76 poor prognostic factor for COVID-19 (5). The uncontrolled inflammation promoted by
77 SARS-CoV-2 in severe COVID-19 is not acute, it negatively associates with viral loads
78 in nasopharyngeal swabs and represents an important event from 7 to 10 days after onset
79 of illness (6,7). The SARS-CoV-2-induced cytokine storm associates with intense cell
80 death (8,9).

81 There are various mechanisms involved in cell death, which are differently
82 engaged from development to responses to infection (10). For certain diseases, such as
83 COVID-19, in which the immunopathogenesis mechanisms associate with poor clinical
84 outcomes, controlling the way cells collapse to infection is vital for the host (10). For
85 example, cell death from necrosis, which can occur from necroptosis to pyroptosis, tends
86 to exacerbate inflammation due to the rupture of the cellular plasma membrane.
87 Pyroptosis, in particular, begins with the activation of the inflammasome, an intracellular
88 structure that involves several intracellular molecules such as caspase-1(10,11), leads to
89 leakage of the cytoplasmic content, favors inflammatory infiltrate (11) and amplifies of
90 the inflammatory response (12). These mechanisms of cell death contrasts with apoptosis,
91 a more controlled process that maintain the host's homeostasis.

92 In severe COVID-19, the cytokine storm associates with high levels of tissue
93 insult, judged by increased levels lactate dehydrogenase (LDH) and D-dimer in the
94 plasma (6–8). Moreover, high LDH levels and leukopenia in severe COVID-19 points
95 out that white cells loses the integrity of plasma membrane (6–8). Among these cells,

monocytes should orchestrate the equilibrium between innate and adaptative immune responses, which may be presumably affected during cytokine storm. Indeed, severe immune dysregulation with low antigen presenting capacity is evidenced by reduced expression of HLA-DR in monocytes during COVID-19, which is strongly associated with severe respiratory failure (4).

The leukopenia of patients with severe COVID-19 seem to precede the cytokine storm (13–18). Moreover, in other virus-induced cytokine storm episodes in the respiratory tract, such as induced by influenza A virus, monocytes and macrophages are severely affected (13). Thus, we hypothesized that the monocyte cell death induced by SARS-COV-2 exacerbates the production of inflammatory cytokines, as well as impairs the immune balance in the hosts. In fact, we found that SARS-CoV-2 triggers the activation of the inflammasome, leading to pyroptosis in human monocytes, by experimental or natural infection. Pyroptosis was dependent on caspase-1 engagement, prior to IL-1ß production and dysregulation of cytokine release. Monocytes that survive after SARS-CoV-2 challenge downregulate HLA-DR, being less likely to properly orchestrate the immune response. Finally, we show that the reproposed antiviral drug atazanavir (ATV) could block this deleterious loop in favor of the host.

Material and Methods

Reagents

Atazanavir (ATV) and Ribavirin were received as donations from Instituto de Tecnologia de Fármacos (Farmanguinhos, Fiocruz). The antiviral Lopinavir/ritonavir (4:1 proportion) was pruchased from AbbVie (Ludwingshafen, Germany). ELISA assays were purchased from R&D Bioscience. Lipopolysacchadides - LPS, adenosine triphosphate (ATP), the specific inhibitor of caspase-1 (AC-YVAD-CMK), pan-caspase inhibitor (ZVAD-FMK), RIPK1 (Necrostatin-1 – Nec-1) and IL-1 receptor (IL-1RA) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). All small molecule inhibitors were dissolved in 100 % dimethylsulfoxide (DMSO) and subsequently diluted at least 10^4-fold in culture or reaction medium before each assay. The final DMSO concentrations showed no cytotoxicity. The materials for cell culture were purchased from Thermo Scientific Life Sciences (Grand Island, NY), unless otherwise mentioned.

126

127 **Cells and Virus**

128 African green monkey kidney (Vero, subtype E6) cells were cultured in DMEM
129 high glucose supplemented with 10 % fetal bovine serum (FBS; HyClone, Logan, Utah)
130 and 100 U/mL penicillin, and 100 µg/mL streptomycin (P/S). Vero cells were incubated
131 at 37°C in 5 % CO_2 atmosphere.

132 Human primary monocytes were obtained through plastic adherence of peripheral
133 blood mononuclear cells (PBMCs), which were obtained from buffy coat preparations of
134 healthy donors by density gradient centrifugation (Ficoll-Paque, GE Healthcare). In brief,
135 PBMCs (2.0 x 10^6 cells) were plated onto 48-well plates (NalgeNunc) in RPMI-1640
136 without serum for 2 to 4 h; then, non-adherent cells were removed by washing and the
137 remaining monocytes were maintained in DMEM with 5% human serum (HS; Millipore)
138 and penicillin/streptomycin.

139 SARS-CoV-2 was isolated and expanded on Vero E6 cells from a nasopharyngeal
140 swab of a confirmed case from Rio de Janeiro, Brazil. Experiments were performed after
141 one passage in cell culture, when Vero E6 cells with DMEM plus 2% FBS in 150 cm^2
142 flasks were incubated at 37 °C in 5 % CO_2 atmosphere. Cytopathic effect was observed
143 daily and peaked 4 to 5 days after infection. All procedures related to virus culture were
144 handled at biosafety level 3 (BSL3) multiuser facility, according to WHO guidelines.
145 Virus titers were determined as the tissue culture infectious dose at 50% (TCID50/mL).
146 Virus stocks were kept in -80 °C ultralow freezers. The virus strain was sequenced to
147 confirm the virus identity and its complete genome is publicly deposited (GenBank
148 accession no. MT710714).

149 **Yield-reduction assay**

150 Human primary monocytes were infected with multiplicity of infection (MOI) of
151 0.01 at density of 2-8 x 10^5 cells/well in 48-well culture plates, depending on the total cell
152 number from each donor. After 1 h at 37 °C, cells were washed, and various
153 concentrations of compounds were added in DMEM with 2% FBS. After 48 h, the

supernatants were harvested and virus replication was quantified by real time RT- PCR and infectious titers by TCID50/mL.

Virus titration

Monolayers of Vero cells (2 x 10^4 cell/well) in 96-well culture plates were infected with log- based dilutions of the supernatants containing SARS-CoV-2 for 1 h at 37°C. The cells were washed and fresh medium with 2% FBS was added. After 3 to 5 days, the cytopathic effects were scored in at least 10 replicates per dilution by independent readers, who were blind with respect to source of the supernatant. Reed and Muench scoring method was employed to determine TCID50/mL.

Flow cytometer analysis

For flow cytometry analysis, monocytes were diluted in labelling buffer (10^6 cells/mL). Then, 100 µL of cell samples were marked with 5 µL of AnnexinV and PI for 15 minutes for cell death evaluation. Around 10,000 gated events were acquired using FACSCalibur and the analysis was performed using the CellQuest software. Monocytes were gated through cell size (foward light scatter, FSC) and granularity (side light scatter, SSC) analysis.

Human monocytes were stained for caspase-1 activity with FAM-YVAD-FMK (fluorescent-labeled inhibitor of caspase-1 [FLICA] and FAM-FLICA Caspase-3/7 activity or HLA-DR APC.H7 or IgG APC.H7. Caspase-1 and caspase-3/7 activity was determined via flow cytometry (FACSCalibur) by detecting FLICA fluorescence and expression of HLA-DR as mean fluorescence intensity (MFI) value for each sample. Acquisition of data was set to count a total of 10,000 events, and the FLOWJO software package was used to analyze the data.

Microscopic analysis

Human primary monocytes were plated on glass coverslips at density of 2-8 x 10^5 cells/well in 48-well plates. Infection was performed for 2 h at 37 °C and then fresh medium with 2% FBS was added. After 24 h, the cells were washed with Binding buffer and stained with PI (0.5 µg/mL) for 5 minutes. Next, the cells were fixed with 3.7% formaldehyde for 30 minutes at room temperature. The nuclei were stained with DAPI

(1µg/mL) for 5 min and the coverslips were mounted using an antifade mounting medium (VECTASHIELD). Fluorescence was analyzed by fluorescence microscopy with an x100 objective lens (Olympus, Tokyo, Japan).

Measurements Inflammatory Mediators and cell death marker

The levels of IL-1ß, TNF-α, IL-6, IL-8 and LDH were quantified in the culture supernatants from infected and uninfected monocytes using ELISA kits, according to the manufacturer's intructions (R&D System).

Extracellular lactate dehydrogenase (LDH) was quantified using Doles® kit according to manufacturer's instructions. In brief, cell culture supernatants were centrifuged at 5,000 rpm for 1 minute, to remove cellular debris, and then 25 µL were placed into 96-well plates and incubated with 5 µL of ferric alum and 100 µL of LDH substrate for 3 minutes at 37 °C. Nicotinamide adenine dinucleotide (NAD, oxidized form) was added followed by the addition of a stabilizing solution. After 10 minutes, the reaction was read in a spectrophotometer at 492 nm.

Western blot assay

Cellular extracts of $1x10^6$ cells were homogenized in the RIPA lysis buffer in the presence of proteinase inhibitor cocktail (Roche), and the protein levels were measured by BCA protein assay kit. A total of 20 µg of protein was loaded onto a 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) for separation by electrophoresis and the protein bands were then transferred to a polyvinylidene difluoride membranes (ImmobilonP-SQ, Millipore). The membranes were blocked with 5 % albumin diluted in Tris-buffered saline containing 0.05 % of Tween 20 for 2 hours at room temperature and incubated with the specific primary antibodies (Cell Signaling Technology), to detect pro-caspase-1 and cleaved-caspase-1, after overnight incubation at 4 °C. After washing, membranes were incubated with secondary antibodies (IRDye® 800CW Goat-anti-Mouse and IRDye® 680LT Goat anti-Rabbit IgG Antibody, LI-COR, Lincoln) for 30 minutes at RT. The protein bands were visualized by digital fluorescence (Odyssey® CLx Imaging System), and protein density was analyzed by the ImageJ software. All the data were normalized by β-actin expression quantification.

213 **Human subjects.**

214 We prospectively enrolled severe COVID19 RT-PCR-confirmed cases, as well as
215 SARS-CoV-2-negative healthy controls. Blood samples were obtained from 12 patients
216 with severe COVID-19 within 72 hours from intensive care unit (ICU) admission in two
217 reference centers (Instituto Estadual do Cérebro Paulo Niemeyer and Hospital Copa Star,
218 Rio de Janeiro, Brazil). Severe COVID-19 was defined as critically ill patients presenting
219 viral pneumonia on computed tomography scan and in mechanical ventilation. All
220 patients had SARSCoV-2 confirmed diagnostic through RT-PCR of nasal swab or
221 tracheal aspirates. Peripheral vein blood was also collected from 8 SARS-CoV-2-negative
222 healthy control participants as tested by RT-PCR on the day of blood sampling. The
223 characteristics of severe (n = 12), and control (n = 8) participants are presented in Table
224 1. Severe COVID-19 patients usually present older age and higher prevalence of
225 comorbidities as obesity, cardiovascular diseases and diabetes as in previously reported
226 patient cohorts. In the present study, the SARS-CoV-2-negative control group was
227 designed to include subjects of older age and chronic non-communicable diseases, so it
228 is matched with critically ill COVID-19 patients (Table 1). Patients with acute respiratory
229 distress syndrome (ARDS) were managed with neuromuscular blockade and a protective
230 ventilation strategy that included low tidal volume (6 mL/kg of predicted body weight)
231 and limited driving pressure (less than 16 cmH2O) as well as optimal PEEP calculated
232 based on the best lung compliance and PaO_2/FiO_2 ratio. In those with severe ARDS and
233 PaO_2/FiO_2 ratio below 150 despite optimal ventilatory settings, prone position was
234 initiated. Our management protocol included antithrombotic prophylaxis with enoxaparin
235 40 to 60 mg per day. Patients did not receive routine steroids, antivirals or other anti-
236 inflammatory or anti-platelet drugs. The SARS-CoV-2- negative control participants
237 were not under anti-inflammatory or anti-platelet drugs for at least two weeks. All clinical
238 information were prospectively collected using a standardized form - ISARIC/WHO
239 Clinical Characterization Protocol for Severe Emerging Infections (CCPBR).

240 **Ethics statement.**

241 Experimental procedures involving human cells from healthy donors were
242 performed with samples obtained after written informed consent and were approved by
243 the Institutional Review Board (IRB) of the Oswaldo Cruz Foundation/Fiocruz (Rio de
244 Janeiro, RJ, Brazil) under the number 397-07. The National Review Board approved the

study protocol (CONEP 30650420.4.1001.0008), and informed consent was obtained from all participants or patients' representatives.

Statistical analysis

The assays were performed in blinded way. They were performed by one professional, codified and read by another fellow. All experiments were carried out at least three independent times, including a minimum of two technical replicates in each assay. The equations to fit the best curve were generated based on R^2 values ≥ 0.9. Student's T-test was used to access statistically significant P values <0.05.

Results

SARS-CoV-2 promotes pyroptosis in human monocytes

To characterize the mechanism by which SARS-CoV-2 triggers monocyte death, human primary monocytes were infected with SARS-CoV-2 or treated with LPS+ATP. Next, cell death was analyzed by quantifying the LDH activity in the culture supernatant, and by AnnexinV/propidium iodide (PI) cell labeling through for flow cytometry and fluorescence microscopy. As shown in Figure 1A, SARS-CoV-2 increased LDH levels similarly to the positive control, LPS+ATP (Figure 1A). Flow cytometry and fluorescence microscopy images demonstrated higher percentages of PI^+ cells in the infected cultures, as well as a significant increase in mean fluorescence intensity (MFI). Both, LDH leak and PI labeling characterize cell membrane disruption in human monocytes after SARS-CoV-2 infection (Figure 1B-E). These data suggest that SARS-CoV-2 infection can induce cell death characterized by loss of plasma membrane integrity suggestive of pyroptosis cell death.

SARS-CoV-2 induces pyroptosis in monocytes through inflammasome activation

To gain insights on the mechanisms of monocyte cell death in SARS-CoV-2 infection, we accessed caspase-1 activation - a key events that require inflammasome's proteolytic activity. Cells stimulated with LPS+ATP were used as a positive control of inflammasome activation. We observed that SARS-CoV-2 induced pro-caspase-1 cleavage, similarly to positive control (Figure 2A and 2B). To confirm these results, cells were labeled with FAM-YVAD-FLICA (as an indicative of caspase-1 activation) and

analyzed by flow cytometry. We found that SARS-CoV-2 infection induced the activation of caspase-1 in monocytes in the same magnitude to the positive control group (Figure 2C and 2D). Moreover, SARS-CoV-2-induced caspase-1 activation was a specific event, since we did not observe activation of the apoptotic caspases-3 and -7 in infected monocytes (Figure 2E and 2F). As caspase-1 activity is critical for the production of IL-1β (19,20), we measured the levels of this cytokine in our experiments. Our data show that SARS-CoV-2 infection was able to increase IL-1β production, this phenomenon being prevented with pretreatment with AC-YVAD-CMK, and not altered with a necroptosis inhibitor. (Figure 2G). Importantly, inhibition of IL-1R engagement reduced SARS-CoV-2-mediated caspase-1 activation and LDH release (Figure S1), suggesting that inflammasome-dependent IL-1β secretion amplify caspase-1 activation and pyroptosis in SARS-CoV-2 infection.

Inflammasome activation amplify pro-inflammatory cytokines secretion in infected monocytes

To evaluate the effect of the activation of inflammasome and pyroptosis on the immune response, we quantified the production of key cytokines in the amplification of the inflammatory process observed clinically in a patient during the course of the infection with IL-6 and TNF-α. Remarkably, caspase-1 specific inhibition, but not pan-caspase or RIPK1 inhibitor, also led to lower levels of the pro-inflammatory cytokines IL-6 and TNF-α, highlighting the participation of the caspase-1-IL-1β axis in inflammatory amplification during SARS-CoV-2 infection. (Figure 3A and 3B). To confirm the effects in SARS-CoV-2 infection in the immune response, whether the observed effect is specifically related to inflammasome activation, we evaluated the production of IL-8, in which induction is independent of the inflammasomes pathway. SARS-CoV-2 infection induced a higher IL-8 production, which was not prevented by caspase-1 inhibition (Figure 3C). In addition, inhibition of IL-1R engagement reduced SARS-CoV-2-mediated was able to significantly decrease the production of IL-1 β, IL-6, TNF-α and did not alter the production of IL-8 (Figure S2).

Infection with SARS-CoV-2 decreases the expression of HLA class II in human monocytes independently of the activation of inflammasomes

To investigate the impact of inflammasome activation and cell death through pyroptosis on the orchestration of anti-SARS-CoV-2 immune response, cells were then marked with HLA-DR and analyzed by flow cytometry. We found that SARS-CoV-2 infection induced a decrease in HLA-DR expression in monocytes (Figure 4A and 4B). However, the inhibition of caspase-1 (Figure 4A and 4B) and IL-1R engagement (Figure S3) did not prevent the SARS-CoV-2-mediated decreasing of HLA expression. These results suggest that even in disassociation with the engagement of SARS-CoV-2-induced inflammasome activation, monocytes exposed to the new CoV will present limited ability to orchestrate the immune response.

Atazanavir prevented inflammasome-dependent pyroptosis

Given our findings that pyroptosis and decreased HLA-DR expression are triggered during SARS-CoV-2 replication, we tested whether atazanavir (ATV), an HIV protease inhibitor recently shown to possess antiviral activity against the new CoV in monocytes (17), could prevent these events. Flow cytometry analysis of SARS-CoV-2-infected monocytes treated with ATV demonstrated a significant reduction in caspase-1 activity, observed by the decreased FAM-YVAD-FLICA labeling (Figure 5A and 5B). Other orally available repurposed anti-SARS-CoV-2 drugs, such as lopinavir (LPV) and ribavirin (RBV), did not affect SARS-CoV-2-induced pyroptosis (Figure 5C). Moreover, treatments with ATV did not alter the activity of caspase-1, -3 and -7 in monocytes exposed to LPS and ATP, used as a positive control of pyroptosis (Figure S4), indicating a specific antiviral activity of this drug. Consistently, treatment with ATV also reduced the levels of IL-1β, IL-6 and TNF-α in SARS-CoV-2-infected monocytes, when compared to the untreated cells (Figure 5D-F). ATV did not interfere with the production of IL-8, which is independent of inflammasome engagement (Figure 5G).

We then confirmed whether ATV can prevent SARS-CoV-2-induced pyroptosis. Lower levels of LDH were detected in the supernatants of SARS-CoV-2-infected monocytes treated with ATV, when compared to infected and untreated cells (Figure 5H). The treatment with ATV was also able to prevent the decrease of HLA-DR expression in infected monocytes (Figure 5I and J). LPV and RBV did not alter the HLA-DR expression

in monocytes infected with SARS-Cov-2 (Figure S5). Since ATV inhibits the early proteolytic processing of viral antigens, an early event during SARS-CoV-2 replication, this drug represented the most upstream process pharmacologically inhibited in this investigation to prevent pyroptosis and HLA-DR knockdown.

Inflammasome activation and pyroptosis are risk factors in critically ill COVID-19 patients

To clinically validate our findings, we evaluated if monocytes obtained from critically ill patients with COVID-19 would also display signals of inflammasome engagement and pyroptosis. We observed that monocytes from COVID-19 patients had increased caspase-1 activation (Figure 6A and 6B) and significantly higher PI+ staining, when compared to monocytes from healthy donors (HD) (Figure 6C and 6D). Corroborating with our *in vitro* data, we also detected higher levels of IL-1β in the plasma of critically ill patients (Figure 6E). Therefore, the *in vitro* results from the previous sections stand on the shoulders of the clinical relevance of monocytes in patients with severe COVID-19.

SARS-CoV-2 infection induces inflammasome-dependent pyroptosis and downmodulation of HLA-DR monocytes, which can be prevented by atazanavir

A representative scheme to summarize the effect of SARS-CoV-2 infection upon in the increase of TNF-α and IL-6 levels, as part of an immunodysregulation promoted by pyroptosis. SARS-CoV-2-induced pyropotosis engages inflammasomes, to activate caspase-1 and IL-1ß. Release of IL-1ß amplify the activation of inflammasomes during the SARS-CoV-2 infection. In parallel, SARS-CoV-2 also induces the HLA-DR downregulation. ATV prevented SARS-CoV-2-induced deleterious effects on monocyte biology.

Discussion

COVID-19 has caused in less than 8 months over 800,000 deaths worldwide (21) and represent the major public health crisis of the beginning of 21st century, leading to an unpredictable impact in global economics (22,23). SARS-CoV-2 infection triggers an uncontrolled inflammatory response and marked leukopenia with consequent lung/respiratory dysfunction, which are the characteristics of the most severe

365 manifestations of the COVID-19 (24,25). Similarly, to other respiratory viruses (26-29),
366 SARS-CoV-2 induces a cytokine storm, characterized by an uncontrolled inflammatory
367 response mediated by monocytes/macrophages, when they should orchestrate the
368 antiviral immune response (30). In this work, we demonstrate that SARS-CoV-2 infection
369 triggers inflammasome activation, increases IL-1ß secretion by monocytes, resulting in
370 pyroptotic cell death, which could be a key event for SARS-CoV-2 pathogenesis in
371 critically ill patients (31). This deleterious immune dysregulation loop triggered by
372 SARS-CoV-2 may be impaired by ATV.

373 Our results demonstrate that SARS-CoV-2 leads to an intense cell death in human
374 monocytes, observed by the increase in LDH release in infected cultures, as well as by
375 the higher number of PI^+ cells when compared to uninfected controls. Even though the
376 pretreatment of infected monocytes with the pan-caspase inhibitor ZVAD-FMK
377 prevented IL-1β secretion, it was not able to prevent the release of LDH. Indeed, others
378 have demonstrated that the treatment with ZVAD-FMK induces necroptosis in diverse
379 cell types (32-34). Notably, as pyroptosis is an inflammatory and programmed cell death
380 mediated by inflammasome and inflammatory caspase-1 activation (10,11), we
381 investigated the engagement and activation of these structures in human monocytes
382 infected with SARS-CoV-2. Activation of caspase-1 and increased production of IL-1ß
383 were observed in infected monocytes. This is correlated to the activation of
384 inflammasomes, because the pretreatment of infected monocytes with YVAD prevented
385 caspase-1 activation. These *in vitro* results are in accordance with the literature and with
386 our finding described here that indicate the formation of inflammasomes in patients with
387 severe COVID-19 (35). We also showed that the release of IL-1ß could be promoted by
388 the activation of inflammasomes during the SARS-CoV-2 infection, because blockage of
389 IL-1ß receptors reduced caspase-1 activation and cell death. These results corroborate
390 with studies showing that the increase in IL-1ß production is associated with severe
391 COVID-19 (36-38). Under our experimental conditions, the increase in IL-1ß precedes
392 the unbalanced IL-6 release. These information should not be neglected when considering
393 biopharmaceuticals to tackle cytokine storm in severe COVID-19.

394 Monocytes that survived from SARS-CoV-2 infection displayed decreased HLA-
395 DR expression, which has also been described in monocytes isolated from COVID-19
396 patients (39,40). Our results suggest that this modulation of HLA expression is not

397 directly associated with the activation of inflammasomes, and may be involved by other
398 inflammatory pathways, since we and others have observed increased levels of IL-6 by
399 myeloid cells impairing HLA-DR membrane expression (41).

400 To stablish the clinical relevance of SARS-CoV-2-induced pyroptosis in
401 monocytes (41,41), we analyzed peripheral monocytes isolated from patients with severe
402 COVID-19. We found that the cells from the patients displayed higher caspase-1
403 activation, when compared with monocytes isolated from HD. Recent clinical data reveal
404 high levels of LDH and consistent leukopenia in critically ill COVID-19 patients
405 (6,7,35,42-44). Our data also demonstrate intense monocyte death in COVID-19 patients,
406 as detected through flow cytometry analyzes. Altogether, these data suggest that the
407 severity of COVID-19 may be associated with inflammasome activation in monocytes
408 that results in large amounts of IL-1ß and generates an excessive inflammatory response,
409 further characterized by high levels of IL-6 and TNF-α. Consistently, treatment with IL-
410 1RA has been associated with clinical and inflammatory improvements in critically ill
411 COVID-19 patients (45). These results are in line with clinical case reports that
412 demonstrate that monocytes/macrophages are key cells in the deleterious pro-
413 inflammatory events that characterize the most serious cases of COVID-19 (46-49).

414 To the best of our knowledge, effective therapies for COVID-19 should ideally
415 combine antiviral/anti-inflammatory drugs to reduce viral load and to mitigate the
416 cytokine storm. In the present study, we showed that ATV, an antiretroviral approved in
417 2003 for HIV-1 treatment and previously described by us as having anti-SARS-CoV-2
418 effects in different cell types (including monocytes)(50), to block new CoV-induced
419 pyroptosis and HLA-DR knockdown in monocytes. In addition, ATV inhibited the
420 release of LDH and the production of IL-1ß, f IL-6 and TNF-α from SARS-CoV-2-
421 infected monocytes, which are key players in the cytokine storm associated with COVID-
422 19 (51,52).

423 In this work, we originally describe that infection with SARS-CoV-2 can induce
424 pyroptotic cell death by inflammasome activation, which may be related to the intense
425 leukopenia and exacerbated inflammation seen in severe cases of the COVID-19. Since
426 there is no specific therapy for this disease, our results point out that ATV has a promising
427 therapeutic potential against SARS-CoV-2-induced cell death.

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FIGURE LEGENDS

Figure 1. SARS-Cov-2 induces monocyte cell death through pyroptosis. Human monocytes were infected with SARS-CoV-2 (MOI 0.1) for 24 h. As a positive control, monocytes were stimulated with LPS (500ng/mL) for 23 h and after this time, incubated with ATP (2 mM) for 1 h. **(A)** Cell viability was assessed through the measurement of LDH release in the supernatant of monocytes. **(B, C and D)** Monocytes` monolayers were stained with propidium iodide (PI) plus Annexin V and pyroptotic cell death were determine by flow cytometer analysis and **(E)** labeled with DAPI to visualize the nuclei fluorescence microscopy. Images and Graph data are representative of six independent experiments. Data are presented as the mean ± SEM * $P < 0.05$ *versus* control group (MOCK).

Figure 2. SARS-CoV-2 induce inflammasome activation in human monocytes. Human monocytes were pretreated with caspase-1 inhibitor (AC-YVAD-CMK – 1 μM), pan-caspase inhibitor (ZVAD-FMK - 10 μM) or necroptosis inhibitor (Nec-1 - 25 μM) for 1 h and infected with SARS-Cov-2 (MOI 0.1) for 24 h. Monocytes were stimulated with LPS (500ng/mL) for 23 h and after this time were stimulated with ATP (2 mM) for 1 h as a positive control group for the formation of inflammasomes and pyroptosis induction. **(A, B)** Monocytes` monolayers were used for determination of the levels of pro-caspase-1 and cleaved caspase-1 by western blot analysis. **(C - F)** Monocytes were stained with FAM-YVAD-FLICA and FAM-FLICA to determine the caspase-1 and caspase-3/7 activity, respectively, by flow cytometry. **(G)** Cell culture supernatants were collected for the measurement of the levels of IL-1β. Western blot, histogram and graph data are representative of six independent experiments. Data are presented as the mean ± SEM $^{\#}$ $P < 0.05$ *versus* infected and untreated group.

Figure 3. Inflammasome activation amplify pro-inflammatory cytokines secretion in infected monocytes. Human monocytes were pretreated with caspase-1 inhibitor (AC-YVAD-CMK – 1 μM), pan-caspase inhibitor (ZVAD-FMK - 10 μM), necroptosis inhibitor (Nec-1 - 25 μM) or with inhibitor of IL-1ß receptor (IL-1RA – 1 μM). Monocytes were stimulated with LPS (500ng/mL) for 23 h and after this time were stimulated with ATP (2 mM) for 1 h as a positive control group for the formation of inflammasomes and pyroptosis induction. Cell culture supernatants were collected for the measurement of the levels of **(A)** IL-6, **(B)** TNF-α and **(C)** IL-8. Graphs data are

representative of six independent experiments. Data are presented as the mean ± SEM $^{\#}$ $P < 0.05$ *versus* infected and untreated group.

Figure 4. Infection with SARS-CoV-2 decreases the expression of HLA in human monocytes independently of the activation of inflammasomes. Human monocytes were pretreated with caspase-1 inhibitor (AC-YVAD-CMK – 1 μM) for 1 h and infected with SARS-Cov-2 for 24 h. **(A, B)** Monocytes were stained with HLA-DR APC.H7 or Isotype control APC.H7 to determine the HLA-DR expression by flow cytometry. Mean fluorescence intensity (MFI) value for each sample was represented in graphics. Histogram and graphs datas are representative of six independent experiments. Data are presented as the mean ± SEM * $P < 0.05$ *versus* uninfected group (MOCK).

Figure 5. Atazanavir prevented inflammasome-dependent pyroptosis. Human monocytes were infected with SARS-Cov-2 and treated with atazanavir – ATV (10 μM), ribavirina (10 μM) or Lopinavir (10 μM) for 24 h. **(A, B and C)** Monocytes were stained with FAM-YVAD-FLICA or FAM-FLICA and HLA-DR APC.H7 or IgG APC.H7 to determine the caspase-1 and caspase-3/7 activity and HLA-DR expression, respectively and analyzed by flow cytometry. **(D – G)** Culture supernatants were collected and the levels of IL-1β, IL-6, TNF-α and IL-8 were determined by ELISA. **(H)** Assessment of cell viability through the measurement of LDH release in the supernatant of monocytes. **(I and J)** Monocytes were stained with HLA-DR APC.H7 to determine the HLA-DR expression by flow cytometry. Histogram and graphs datas are representative of six independent experiments. Data are presented as the mean ± SEM * $P < 0.05$ *versus* control group (MOCK); $^{\#}$ $P < 0.05$ *versus* only infected group.

Figure 6. Inflammasome activation and pyroptosis are risk factors in critically ill COVID-19 patients. Monocytes isolated from blood samples of critically ill patients with COVID-19 and healthy donors. **(A and B)** monocytes were stained with FAM-YVAD-FLICA or **(C and D)** propidium iodide (PI) and analyzed by flow cytometry. **(E)** plasma was separated and the levels of IL-1β were determined by ELISA. Western blot, histogram and graphs datas are representative of 9 critically ill patients and 8 healthy donors. Data are presented as the mean ± SEM * $P < 0.05$ *versus* healthy donors (HD).

652 **Figure 7.** Representative scheme of the SARS-CoV-2 infection in activation of
653 inflammasome and pyroptosis in monocyte with downregulation of HLA-DR expression
654 and effects of ATV treatment in this phenomenon.

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Table 1: Characteristics of COVID-19 patients and control subjects.

Characteristics[1]	Control (N=8)	Covid-19 (N=9)
Age, years	52.6 (47 – 60)	61 (33 – 79)
Sex, male	8 (100 %)	6 (66.7 %)
Respiratory support		
Oxygen supplementation	0 (0 %)	3 (33.3 %)
Mechanical ventilation	0 (0 %)	6 (66.7 %)
SAPS 3		59.25 (31 – 75)
PaO2/FiO2 ratio	-	210.75 (87 – 509.5)
Vasopressor	-	3 (33.3 %)
Time from symptom onset to blood sample, days	-	10 (6 – 18)
Status on Jun 30th		
Dead	-	5 (55.55 %)
Discharged	-	1 (11.1 %)
Hospitalized	-	3 (33.3 %)
Comorbidities		
Obesity	1 (11.1 %)	1 (11.1 %)
Hypertension	3 (33.3%)	3 (33.3 %)
Diabetes	0 (0%)	0 (0 %)
Cancer	0 (0%)	0 (0 %)
Chronic heart disease[2]	0 (0%)	0 (0 %)
Presenting symptoms		
Cough	0 (0 %)	3 (33,3 %)
Fever	0 (0 %)	3 (33,3 %)
Dyspnea	0 (0 %)	4 (44,4 %)
Headache	0 (0 %)	0 (0 %)
Anosmia	0 (0 %)	0 (0 %)
Laboratory findings on admission		
Lymphocyte count, cells/mm^3	-	1,355.8 (552 –2,564)
Platelet count, x 1000/mm^3	-	169.6 (92 – 278)
Leukocytes	-	8,036 (8.03-18,670)
C Reactive Protein, mg/L	-	14.78 (0.1 – 30.8)

FIGURE 1

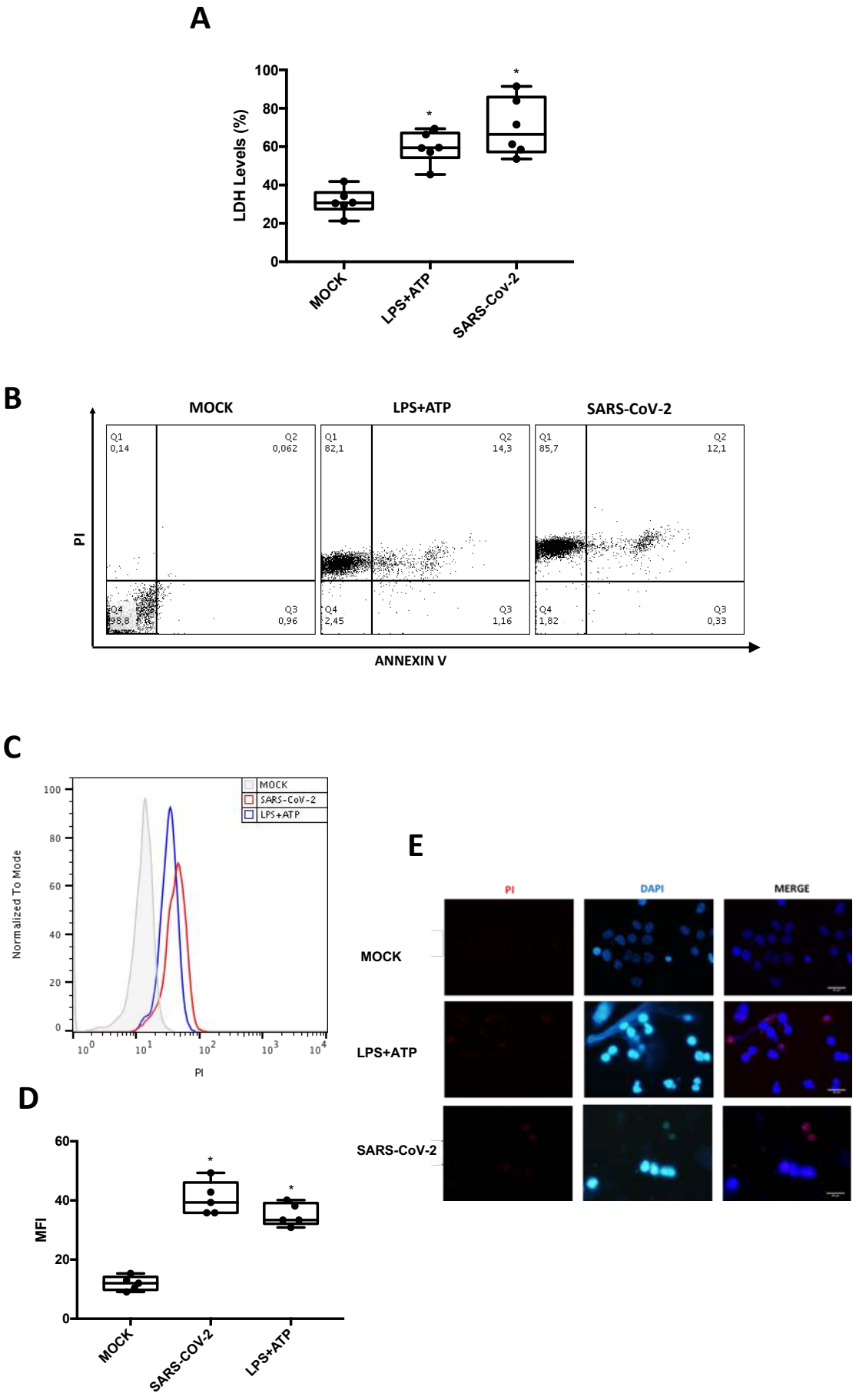


FIGURE 2

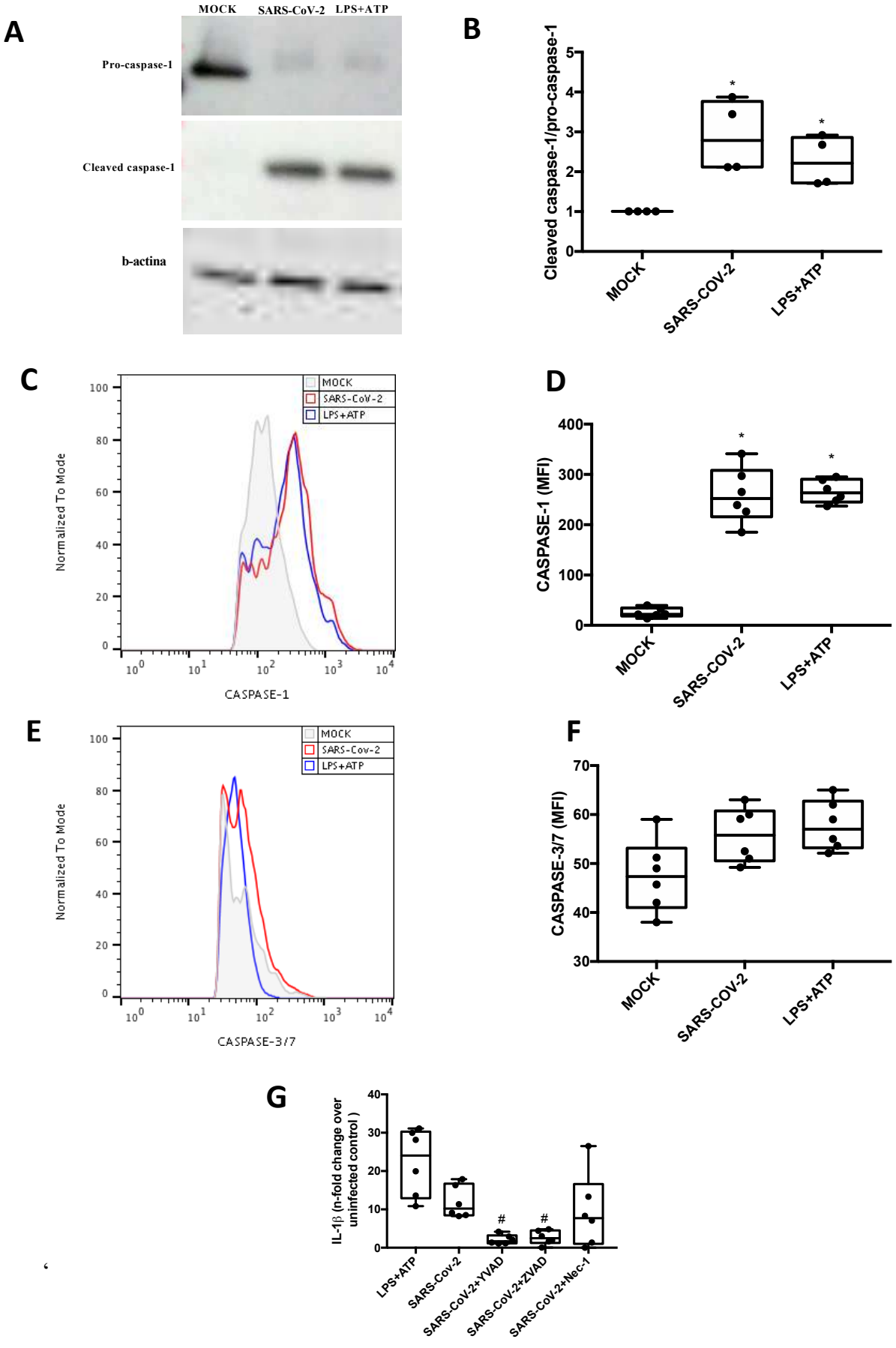


FIGURE 3

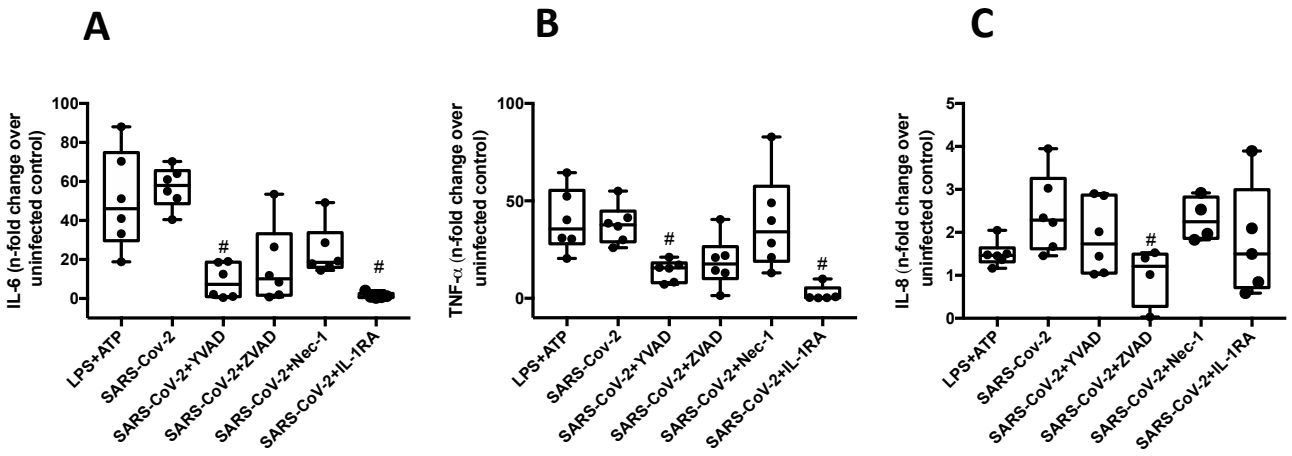


FIGURE 4

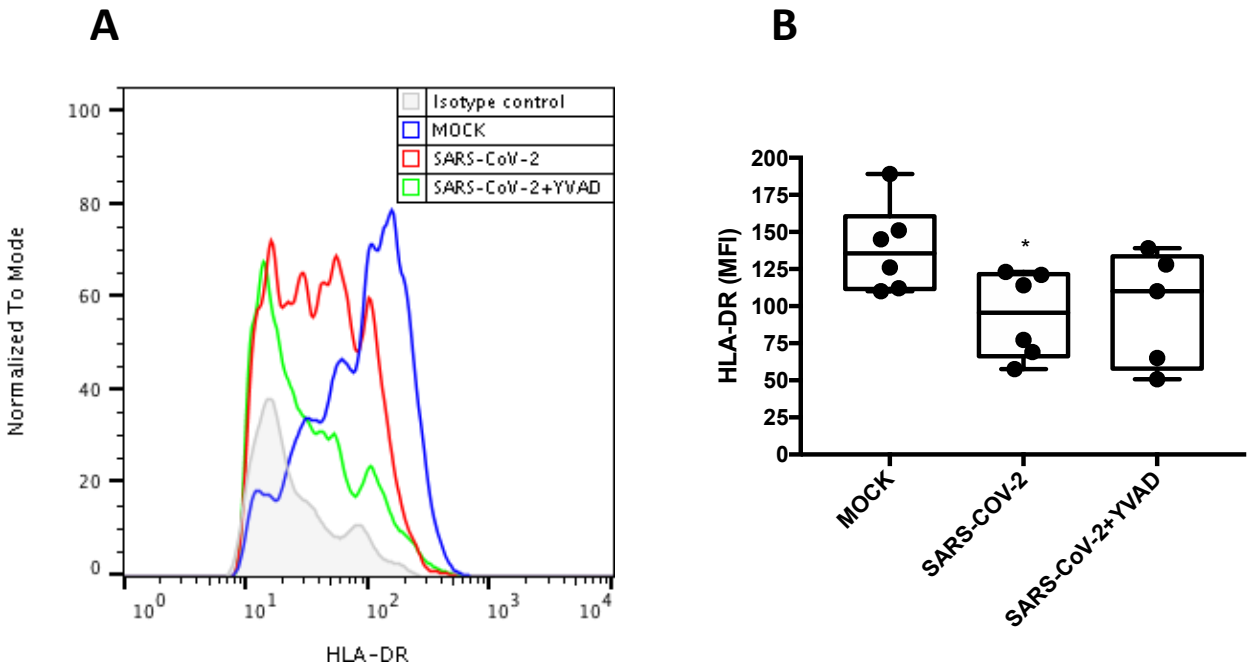
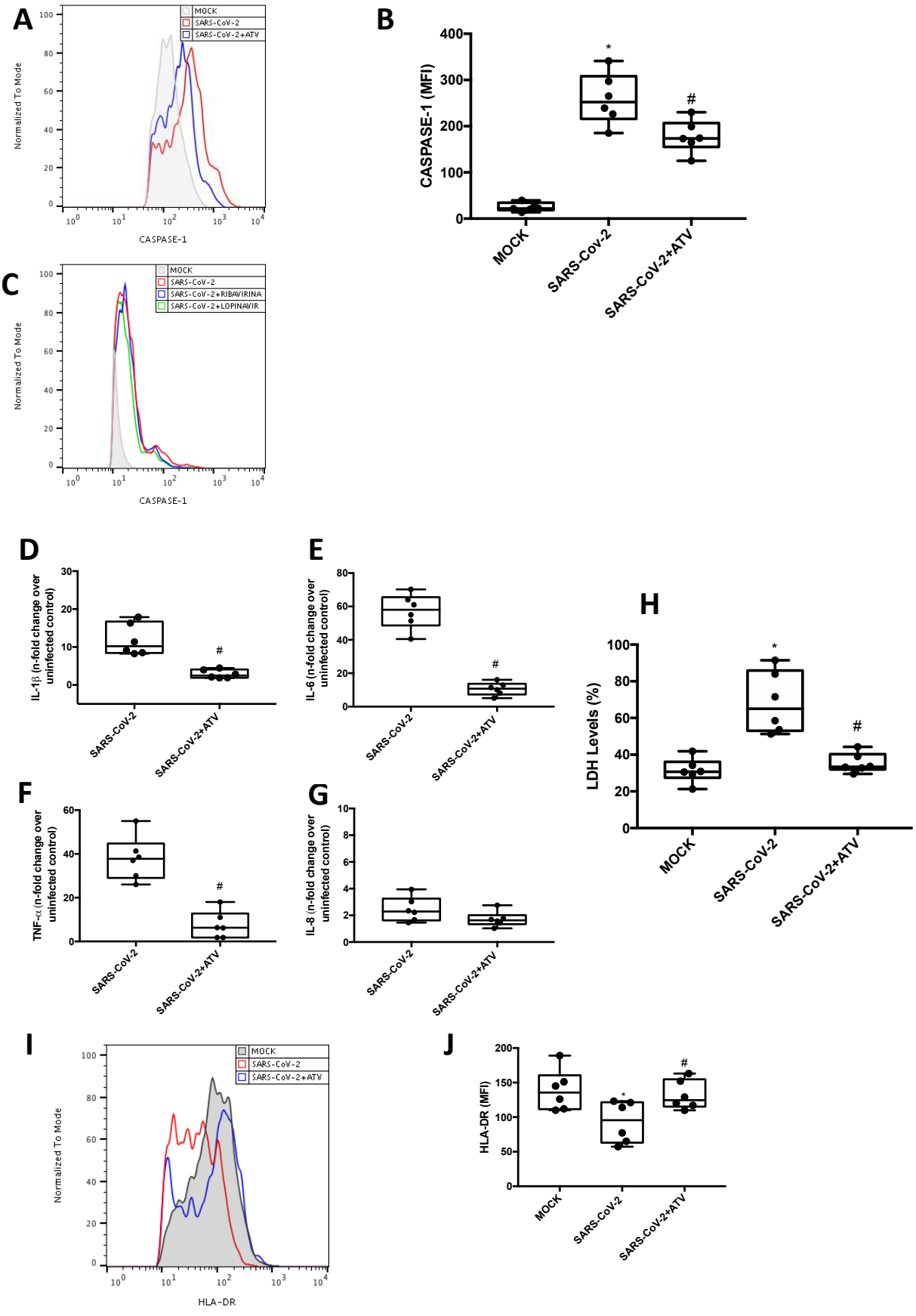


FIGURE 5



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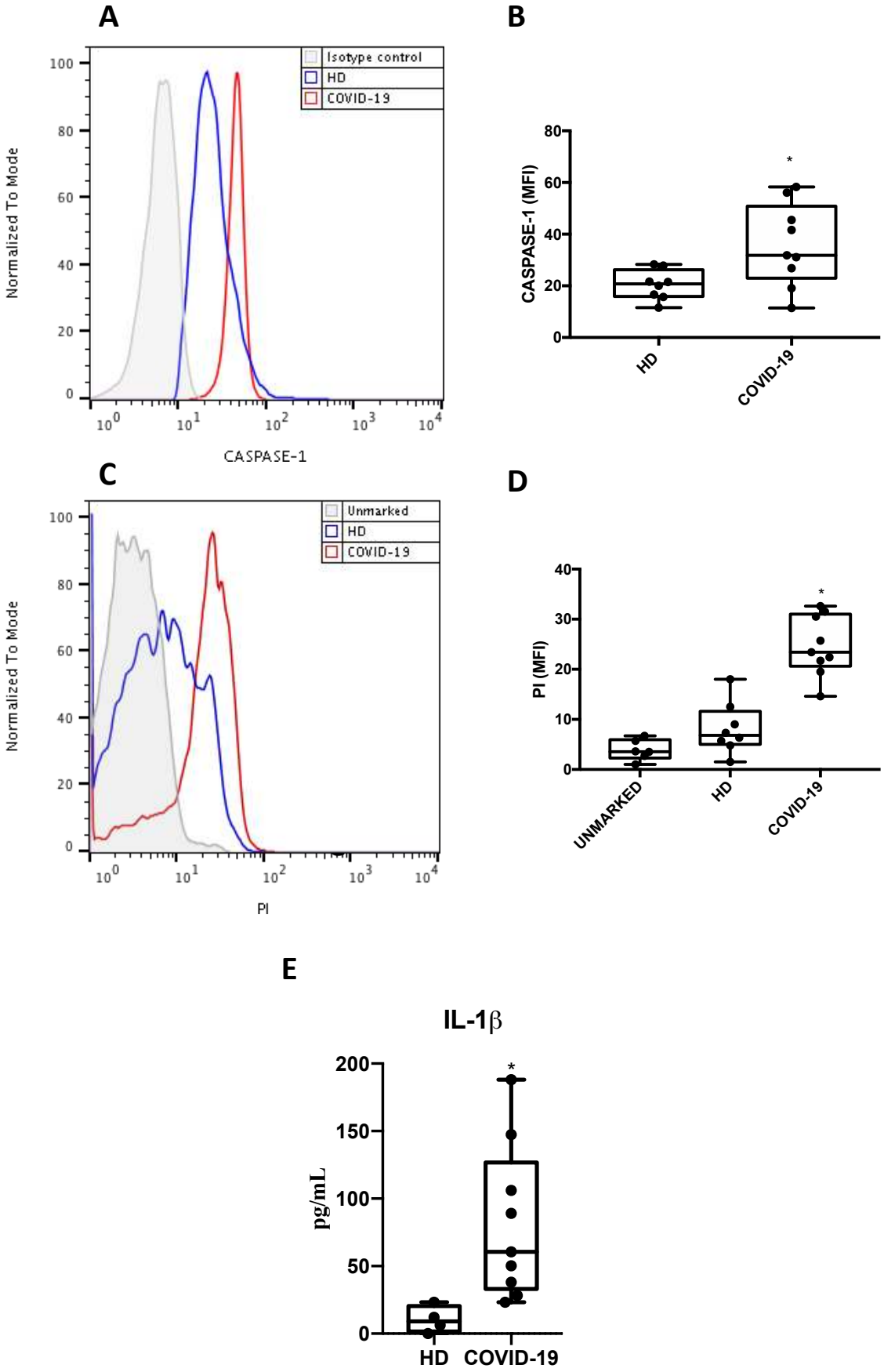


FIGURE 7

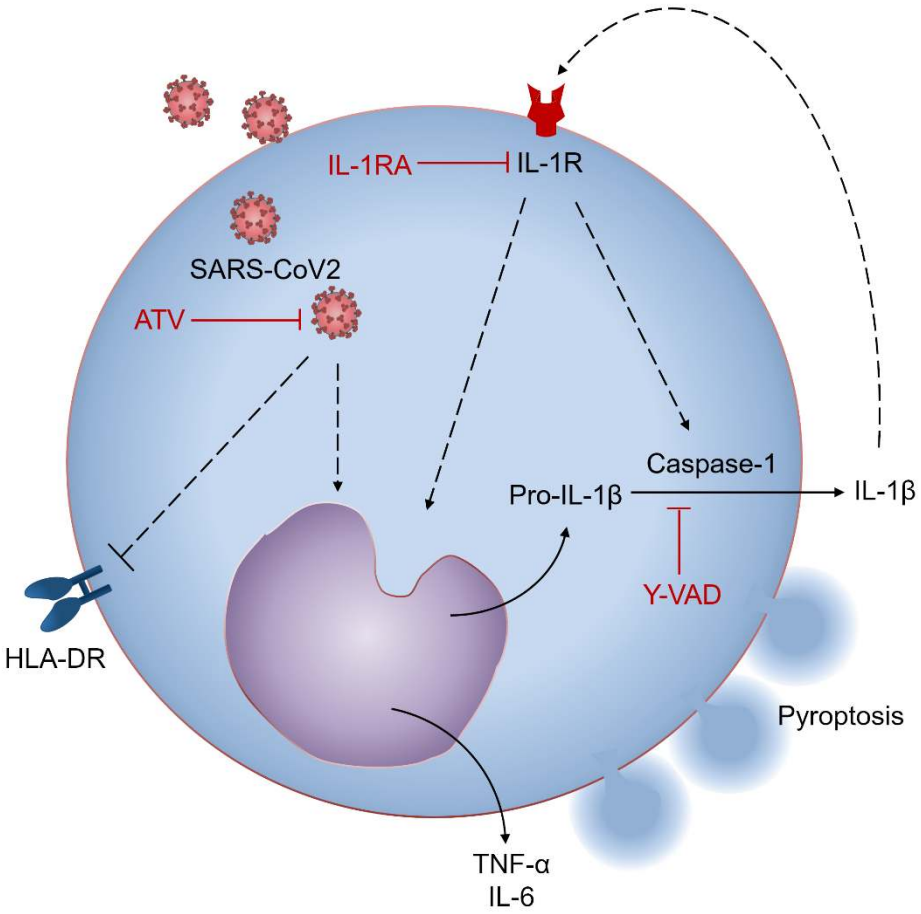
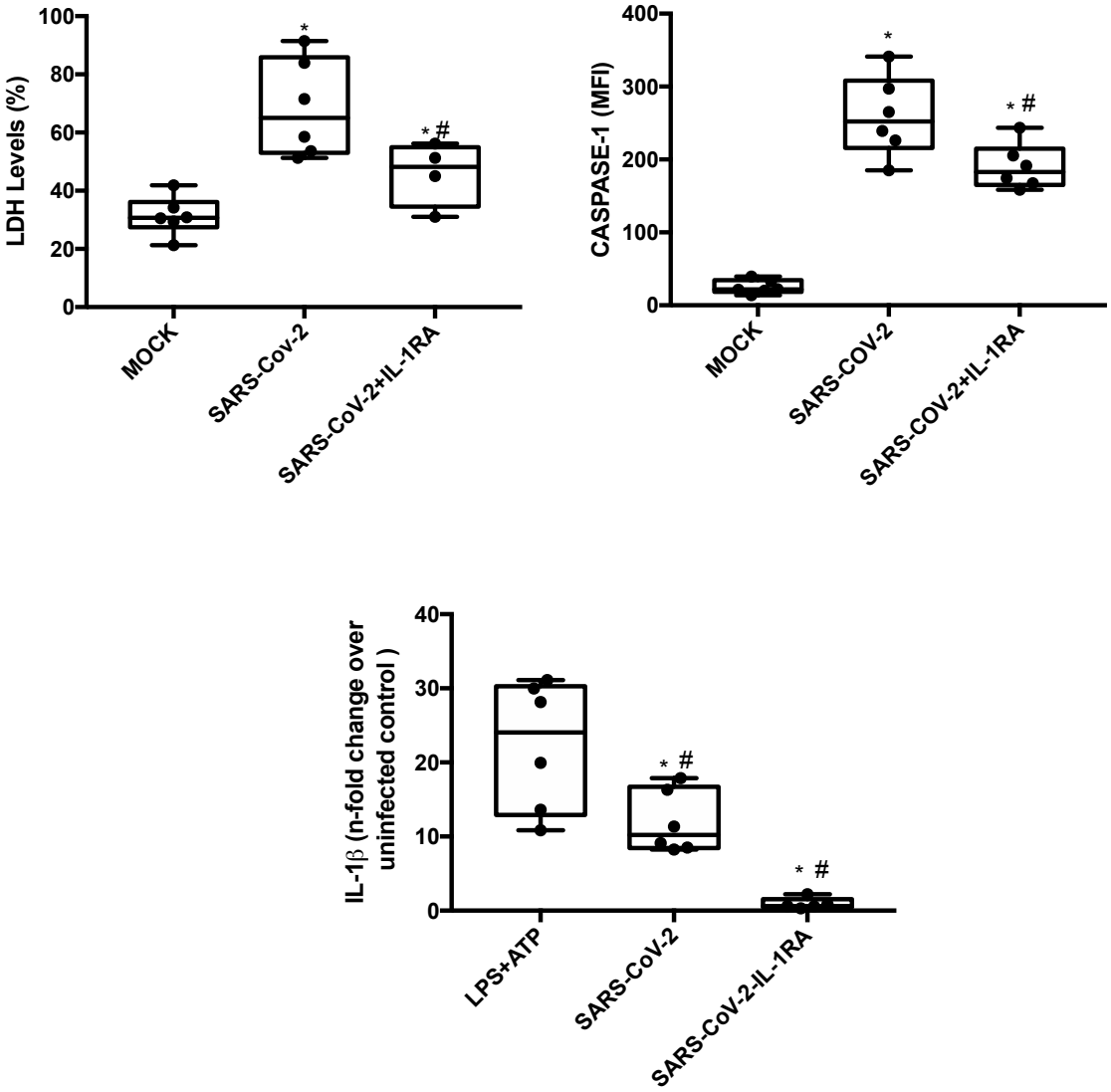
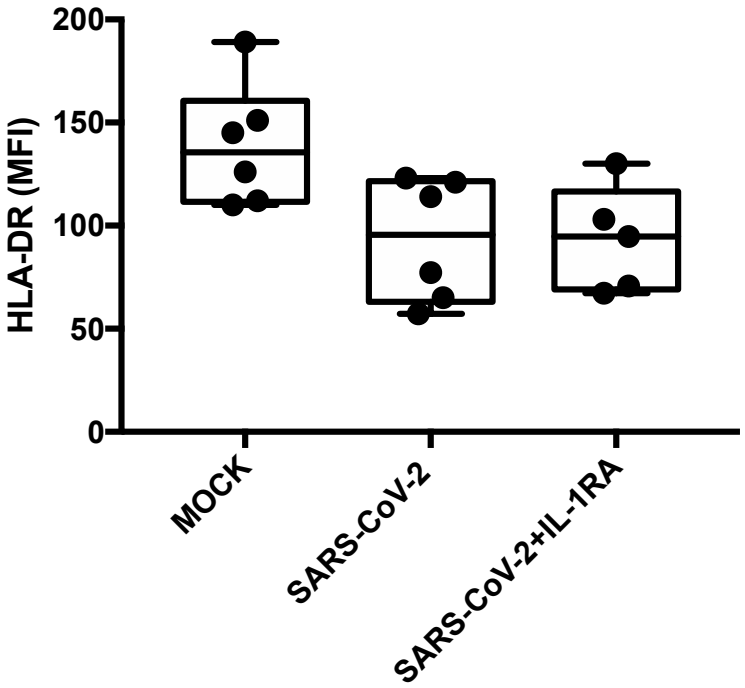


FIGURE S1



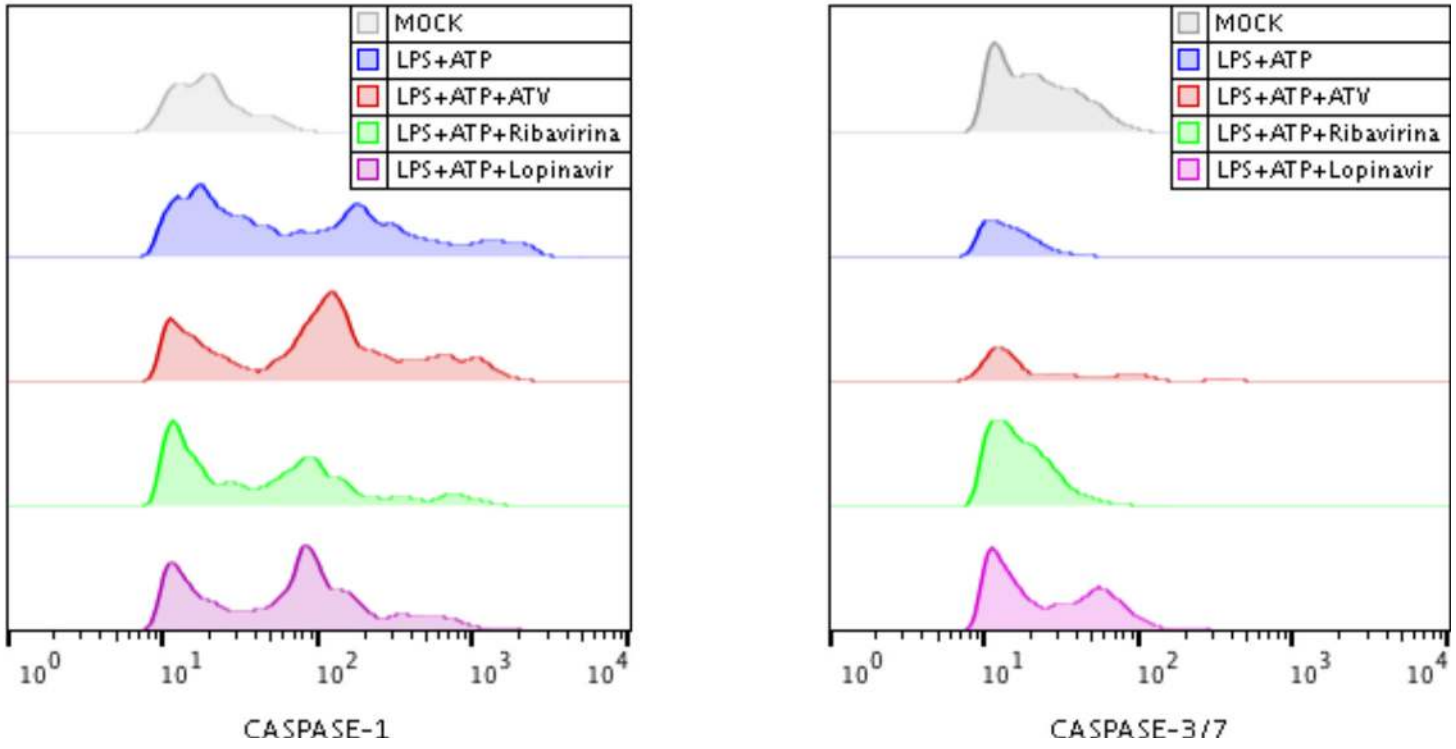
S1 Fig. SARS-CoV-2 induce inflammasome activation and pyroptosis in human monocytes by IL-1β amplification. Human monocytes were pretreated with IL-1β receptor (IL-1RA – 1 μM) for 1 h and infected with SARS-Cov-2 (MOI 0.1) for 24 h. Cell viability was assessed through the measurement of LDH release in the supernatant and monocytes were stained with FAM-YVAD-FLICA to determine the caspase-1 and caspase-3/7 activity, respectively, by flow cytometry. Cell culture supernatants also were collected for the measurement of the levels of IL-1β. Data are presented as the mean ± SEM $^{\#}$ $P < 0.05$ *versus* infected and untreated group; * $P < 0.05$ *versus* control group (MOCK). Graphs are representative of four independent experiments.

FIGURE S2



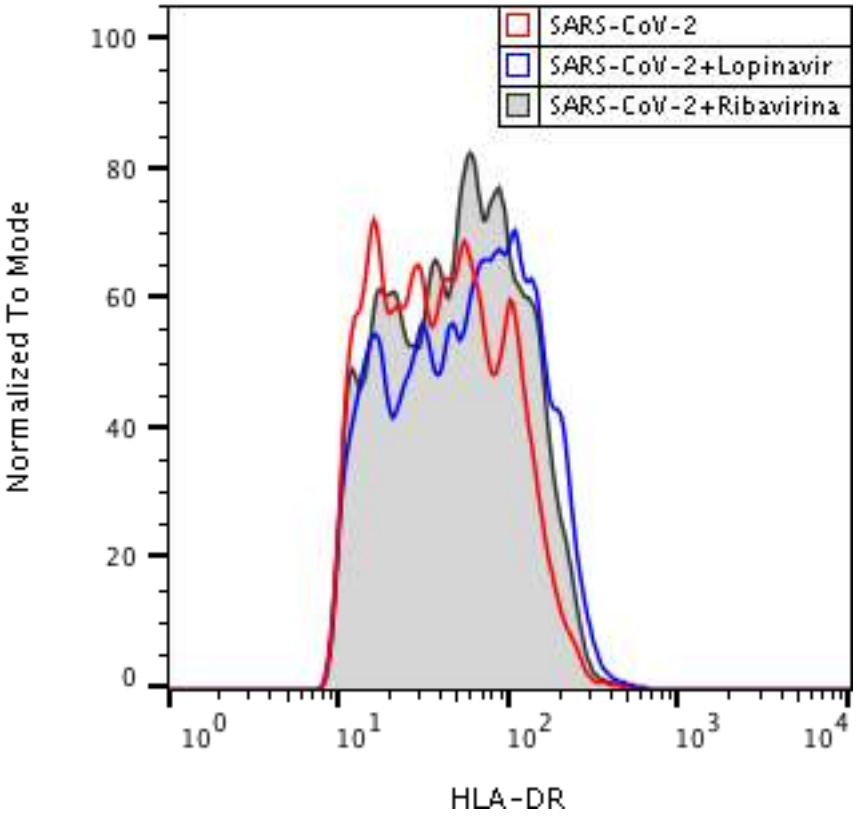
S2 Fig. Infection with SARS-CoV-2 decreases the expression of HLA in human monocytes independently of the IL-1ß activation. Human monocytes were pretreated with inhibitor of IL-1ß receptor (IL-1RA – 1 μM) for 1 h and infected with SARS-Cov-2 for 24 h. Monocytes were stained with HLA-DR APC.H7 or IgG APC.H7 to determine the HLA-DR expression by flow cytometry. Mean fluorescence intensity (MFI) value for each sample was represented in graphics. Graphs datas are representative of four independent experiments. Data are presented as the mean ± SEM $^{\#}$ $P < 0.05$ *versus* uninfected group (MOCK).

FIGURE S3



S3 Fig. Evaluation of anti-inflammatory activity of ATV, LPV and ribavirin in LPS-induced monocytes. Human monocytes were stimulated with LPS (500ng/mL) for 23 h and after this time were stimulated with ATP (2 mM) for 1 h as a positive control group for the formation of inflammasomes and pyroptosis induction. Simultaneously, cells were treated with atazanavir – ATV (10 μM), LPV (10 μM) or ribavirin (10 μM) for 24 h. Monocytes were stained with FAM-YVAD-FLICA (blue) or FAM-FLICA (green) to determine the caspase-1 and caspase-3/7 activity and HLA-DR expression, respectively, and analyzed by flow cytometry. Histograms are representative of six independent experiments.

FIGURE S4



S4 Fig. Effects of LPV and ribavirin in preventing the SARS-CoV-2-related reduced expression of HLA-DR in monocytes. Human monocytes were infected with SARS-Cov-2 and treated with LPV (10 μM) or ribavirina (10 μM) for 24 h. Monocytes were stained with HLA-DR APC.H7 or IgG APC.H7 to determine the HLA-DR expression by flow cytometry. Histogram data are representative of six independent experiments.