Original Article

Lidocaine reinforces the anti-inflammatory action of dexamethasone on myeloid and epithelial cells activated by inflammatory cytokines or SARS-CoV-2 infection

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A B S T R A C T

Background: Severe cases of Coronavirus Disease 2019 (COVID-19) that require admission to the Intensive Care Unit (ICU) and mechanical ventilation assistance show a high mortality rate with currently few therapeutic options available. Severe COVID-19 is characterized by a systemic inflammatory condition, also called "cytokine storm", which can lead to various multi-organ complications and ultimately death. Lidocaine, a safe local anesthetic that given intravenously is used to treat arrhythmias, has long been reported to have an anti-inflammatory and pro-homeostatic activity.

Methods: We studied the capacity of lidocaine to modulate cytokine secretion of mouse and human myeloid cell lines activated by different cytokines or Toll Like Receptor (TLR) ligands (flagellin (FliC), Lipopolysaccharide (LPS), Polyinosinic:polycytidylic acid (Poly I:C) and N-Palmitoyl-S- [2,3-bis(palmitoyloxy)-(2RS)-propyl]-(R)-cysteinyl-(S)-seryl-(S)-lysyl-(S)-lysyl-(S)-lysyl-(S)-lysine x 3HCl (Pam3Cys-SKKKK)) or by Severe acute respiratory syndromecoronavirus 2 (SARS-CoV-2) infection to epithelial cells. Reporter cell lines were used to study modulation of lidocaine of specific signaling pathways.

Results: Lidocaine used in combination with dexamethasone, had an additive effect in the modulation of cellular inflammatory response triggered by Tumoral Necrosis Factor alpha

(TNFα), Interleukin 1 beta (IL-1β) as well as different TLR ligands. We also found that lidocaine in combination with dexamethasone modulates the Nuclear factor kappa B (NF-κB) pathway, inflammasome activation as well as interferon gamma receptor (IFNγR) signaling without affecting the type I interferons (Type I IFNs) pathway. Furthermore, we showed that lidocaine and dexamethasone treatment of epithelial cells infected with SARS-CoV-2 modulated the expression of chemokines that contribute to pro-inflammatory effects in severe COVID.

Conclusions: We reported for the first time *in vitro* anti-inflammatory capacity of lidocaine on SARS-CoV-2 triggered immune pathways. These results indicated the potential of lidocaine to treat COVID-19 patients and add tools to the therapeutic options available for these concerning cases.

At a glance commentary

Scientific background on the subject

Although affecting a low percentage of cases, progression to severe COVID-19 is still associated to high mortality and therapeutic options are needed.

What this study adds to the field

Lidocaine combined with dexamethasone showed additive anti-inflammatory effects in vitro in myeloid and epithelial cells activated with different cytokines or SARS-CoV-2 infected epithelial cells, without compromising type I interferon signaling. This drug combination could be an option for treating severe COVID 19 cases.

Coronavirus Disease 2019 (COVID-19) was declared as a pandemic by the World Health Organization (WHO) in March 2020, few months after the new virus Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was identified. Since then, more than 300 million people were infected in spite of different measures taken to reduce the viral circulation [1,2]. Although the vast majority of infected persons developed mild or asymptomatic forms of the disease, less than 10% developed a severe form that could evolve from an upper respiratory illness to pneumonia (phase IIa of the disease) with eventual systemic compromise (phase IIb of the disease), requiring mechanical ventilatory assistance [3]. This situation could ultimately evolve towards an inflammatory phase of the disease with potential compromise of the vascular system with alterations in hemostasis (phase III of the disease), showing in this case a non-negligible mortality rate [4]. It has been reported that COVID-19 patients entering Intensive Care Unit (ICU)/requiring mechanical ventilation support presented a death rate between 30 and 60% depending on the country, local epidemiological situation, among other factors [1,3,5—8].

The main complication in severe cases is the respiratory failure secondary to severe acute respiratory distress syndrome (ARDS), becoming the main cause of mortality. Accumulating evidence suggests that patients with severe COVID-19 experience a hyperinflammatory syndrome characterized by fatal, fulminant hypercytokinemia associated with multiple organ failure, so called "cytokine storm" [4]. In addition to respiratory failure due to SARS-CoV-2, reports have shown a high incidence of acute heart failure, brain, kidney and liver damage; as well as thrombotic events including acute myocardial infarction, cerebrovascular accident, acute arterial ischemia of the extremities and intestine, and venous thromboembolic disease [9]. Increased risk of thrombosis is originated in an imbalance of endothelial cell, platelets and coagulant system function secondary to inflammatory process in a vicious circle generated by this two processes that are mutually reinforced, so called immuno-thrombosis [10,11]. Since the beginning of the SARS-CoV-2 pandemic, different treatments for severe disease have been evaluated in clinical trials around the world. However, so far there are few specific therapeutic alternatives for patients who develop severe disease, one of those recommended by the WHO are the biologic Tocilizumab that targets and blocks the Interleukin 6 receptor (IL-6R), a drug used to treat rheumatoid arthritis (RA), or Anakinra that blocks the Interleukin 1 beta (IL-1β) receptor. Also a combination of neutralizing antibodies against the SARS-CoV-2 virus has been recommended for severe COVID-19 patients by the WHO (https://www.who.int/publications/i/item/WHO-2019-nCoV-therapeutics-2021.3) or the recently FDA-authorized antiviral drugs Paxlovid. Although promissory [12], these type of drugs are difficult to be used at large-scale worldwide, especially in low-and middle income countries due to their costs. Therefore, steroids such as dexamethasone has being indicated as first-line treatment for patients with severe COVID-19, as it has been early shown that this intervention reduces the mortality rate of COVID-19 patients receiving invasive mechanical ventilation or oxygen [13].

An effective treatment for the management of patients with severe COVID-19 with respiratory compromise could help to prevent the high death burden of this disease and ICUs collapse, since in spite of vaccines' implementation, outbreaks remained as a matter of concern worldwide. Drug repurposing has been an important activity seeking for safe, affordable drugs that can be readily switched for clinical application. Under this approach, the use of lidocaine as an immunomodulatory and anti-inflammatory drug has been proposed. Lidocaine is a local anesthetic, also used as antiarrhythmic agent with an anti-inflammatory effect, which is currently approved for the treatment of cardiac arrhythmias. Its mechanism of action is exerted through its ability to inhibit

sodium entry through fast channels of the cell membrane. Besides the applications as local anesthetic or antiarrhythmic, different studies that evaluated the administration of intravenous lidocaine in a perioperative way showed that it exerts positive effects by attenuating the inflammatory response [14–16]. Interestingly, it has been shown that intravenous lidocaine administration attenuates inflammation of the lower respiratory tract in an ischemia-reperfusion injury model [17].

In this study, we evaluated the anti-inflammatory/immunomodulatory capacity of lidocaine in combination with dexamethasone, anticipating the possibility of this drug combination to improve the impact of corticosteroid treatment for severe COVID-19. We used different cell lines with different pro-inflammatory stimuli, showing that in all cases lidocaine is able to modulate the pro-inflammatory activation of epithelial and myeloid cells by triggers that are relevant in COVID-19 pathophysiology, including viral infection by itself.

Materials & methods

Cell lines and growing conditions

The cells were maintained at 37 °C in a 5% CO_2 atmosphere in Complete Roswell Park Memorial Institute (RPMI) (C-RPMI): RPMI + 10% Fetal Bovine Serum (FBS) medium (Gibco), 100 U/mL of penicillin, 100 μg/ml of streptomycin or Complete Dulbecco's Modified Eagle Medium (DMEM) (C-DMEM): DMEM+10% FBS medium (Gibco), 100 U/mL of penicillin, 100 μg/mL of streptomycin unless otherwise stated.

THP-1 human monocytic cell line. The cells were plated using C-RPMI at 2.5×10^5 cells/well in 48-well plates. They were stimulated overnight (ON) with stimuli as indicated. The culture supernatant was harvested and stored at −80 °C until use.

THP1-Apoptosis-associated speck-like protein containing a CARD (ASC)-Green Fluorescent Protein (GFP) human monocytic cell line (InvivoGen®) (ASC adapter protein reporter cell line). The cells were plated using C-RPMI at 3.6×10^5 cells/well in 96-well plates. As a positive control, they were stimulated with 1 μg/mL of ultra-pure Escherichia coli Lipopolysaccharide (EcLPS) (Sigma−Aldrich) for 3 h (prime) and 500 ng/mL of plasmid cDNA (pcDNA) were transfected with Lipofectamine™ LTX Reagent with PLUS™ Reagent (Invitrogen®) according to the manufacturer's instructions. They were stimulated with different stimuli detailed in the text and incubated ON. The supernatants were collected and stored at −80 °C until use, and the cells were prepared for microscopic analysis (see below).

THP1-XBlue™-defMyD human monocytic cell line (InvivoGen®) (Nuclear factor kappa B (NF-κB)/Activator Protein-1 (AP-1) secreted alkaline phosphatase (SEAP) reporter cell line). The cells were plated using C-RPMI at 1.8×10^5 cells/well in 48-well plates. As a positive control, they were stimulated with 50 ng/mL of hTNFα, according to manufacturer's recommendations. They were stimulated with different stimuli detailed in the text and incubated ON. The supernatants were collected and stored at −80 °C until their use to measure cytokines and SEAP activity.

J774 murine monocytic cell line. The cells maintained in C-DMEM were seeded in plates at a rate of 2.5×10^5 cells/well in 48-well plates. They were stimulated ON with stimuli as indicated. The culture supernatant was harvested and stored at −80 °C until use to measure cytokines.

RAW 264.7 murine macrophage cell line. The cells maintained in C-DMEM were seeded in 96-well plates at 2×10^5 cells/well. They were stimulated ON with stimuli as indicated. The culture supernatant was harvested and stored at −80 °C until use to measure cytokines.

B16 type I interferons (type I IFNs) reporter cell line (InvivoGen®). The cells maintained in C-DMEM were seeded in plates at 1×10^5 cells/well in plates 96-well. They were stimulated ON with stimuli as indicated. As a positive control, cells were stimulated with 50 μg/mL of Polyinosinic:polycytidylic acid (poly I:C) (Sigma Aldrich®). The culture supernatant was harvested and stored at −20 °C (to measure SEAP activity) or at −80 °C (to be used as a source of type I IFNs) until use.

B16 interferon gamma (IFNγ) reporter cell line (InvivoGen®). The cells maintained in C-DMEM were seeded in 96-well plates at 7.5×10^4 cells/well. They were stimulated ON with stimuli as indicated. As a positive control, they were stimulated with 5 ng/mL of mIFNγ (BD®). The culture supernatant was harvested and stored at −20 °C until use to measure SEAP activity.

HEK-hTLR4 reporter cell line (InvivoGen®). The cells maintained in C-DMEM were seeded at 3×10^4 cells/well in 96-well plates. The cells were incubated ON or to 70% confluence. Medium was fully replaced and cells were stimulated ON as indicated. The culture supernatant was harvested and stored at −20 °C until use to measure SEAP activity.

HeLa−NF-κB-luc reporter cell line. The cells maintained in C-DMEM were seeded in 96-well plates at 2.5×10^4 cells/well. The cells were incubated for 48 h, washed with Phosphate-Buffered Saline (PBS) and stimulated with stimuli as indicated. As a positive control, they were stimulated with 10 ng/mL hTNFα. The cells were then incubated for 5−6 h, medium discarded, washed with PBS and lysed with Cell Culture Lysis 5x Reagent lysis buffer (Promega®). The lysates were stored at −20 °C until use to measure luciferase activity.

Calu-3 cell line. The cells maintained in C-DMEM were seeded in 48-well plates at 2×10^5 cells/well. The cells were incubated for 48 h, washed with culture medium and stimulated with stimuli as indicated. The cells were then incubated for 18 h, medium discarded, washed with PBS and lysed with Lysis Buffer Pure Link RNA mini Kit (Invitrogen®). The lysates were stored at −80 °C until use.

VERO cell line (American Type Culture Collection (ATCC) CCL 81). The cells were grown in Minimum Essential Medium Eagle (MEM) (MEM, Lot # SLBS0116V, SIGMA) supplemented with 10% FBS at 37 °C in a 5% CO2 atmosphere and were seeded in 96-well plates at 1.5×10^6 cells/plate. At confluence, the cells were treated with different concentrations of lidocaine for a cytotoxic assay or infected with SARS-CoV-2 for a viral cytopathic inhibition assay (see below).

Stimuli/enzymes

Inflammasome activators: Ultra-pure EcLPS (InvitroGen®), transfected pcDNA (generated by PCR cloning of a cDNA

fragment of a random not related gene) with Lipofectamine™ LTX Reagent with PLUS™ Reagent (Invitrogen®) following the manufacturer instructions.

Toll Like Receptor (TLR) agonists: poly I:C (Sigma Aldrich®), EcLPS ultrapure (InvitroGen®), Flagellin (FliC) was produced in our laboratory as previously reported [18], N-Palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-(R)-cysteinyl-(S)-seryl-(S)-lysyl-(S)-lysyl-(S)-lysyl-(S)-lysine x 3HCl (Pam3Cys-SKKKK) (EMC microcollections®).

Proinflammatory cytokines: hTNF-α (PreproTech®), mIFNγ (BD®)

Enzymes: Recombinant Ribonuclease A (RNase A) (Qiagen®).

Observation of ASC-GFP specks by fluorescence microscopy

THP1-ASC-GFP cells were harvested after ON stimulation, washed with PBS, and centrifuged on the same plate. Nuclei were stained with 40,6-diamidino-2-phenylindole (DAPI) and fixation with 2% Paraformaldehyde (PFA) was performed. The total number of cells and ASC-GFP spots per field were determined using a Nikon Eclipse Ti fluorescence microscope and analyzed with ImageJ software. Each experimental condition was carried out in triplicate and three photographs were taken per well. The ratio between cells with condensed intense fluorescent ASC-GFP green dots over the total number of cells per field was calculated. The result shown in this work is the result of three independent experiments.

Quantification of cytokines in culture supernatants

The culture supernatants collected from the different stimulated cells were thawed on the day of the test and used for the determination of hIL-1β, hIL-8, mL-6 concentrations using OptEIA™ Enzyme Linked Immunosorbent Assay (ELISA) Set (BD®), according to the manufacturer's specifications.

SEAP activity assessment

Alkaline phosphatase enzyme activity in culture supernatant of THP1-XBlue™-defMyD (InvivoGen®) and HEK-hTLR4 cells was measured using p-Nitrophenyl Phosphate, Disodium Salt (PNPP) as substrate dissolved in alkaline phosphatase buffer, the reaction was stopped with 0.1 M Ethylenediaminetetraacetic acid (EDTA) and optic density (OD) was measured at 405 nm in a spectrophotometer plate reader.

Luciferase activity assessment

Luciferase enzyme activity was measured in cell lysates (lysates with Cell Culture Lysis 5x Reagent from Promega®) of HeLa/NFκB-luc cells using the Luciferase Assay System kit (Promega®), according to the manufacturer's instructions. Luminescence was measured on a Luminoskan TL Plus luminometer (Labsystems®).

In vitro modulation with lidocaine and dexamethasone

All modulation tests with lidocaine in combination or not with dexamethasone were carried out by incubating the cells at the indicated concentrations of modulators 30 min before adding the stimulus and for the entire indicated incubation time.

SARS-CoV-2 strain used

A SARS-CoV-2 strain isolated from a clinical sample positive for COVID-19 in Buenos Aires, Argentina was used in this study. This strain was *in vitro* characterized by staff of the *"Respiratory Viruses Service. National Reference Center of Viral Respiratory Diseases, National Institute of Infectious Diseases. ANLIS Dr. Carlos G. Malbran"*, receiving the identification hCoV-19/Argentina/C121/2020. The viral whole genome was also sequenced, being available at GISAID database (accession numbers EPI_ISL_420,600). This strain carries the mutation D614G that was frequent at the moment of its isolation. Viral stock was produced by infecting VERO cells and titrated following standard procedures, the Reed and Muench method and expressed in tissue culture infective dose 50% (TCID50)/ml.

Lidocaine cytotoxic effect on VERO cells

Different lidocaine concentrations were tested: 2 mM, 1 mM, 0.2 mM and 0.1 mM on Vero cells ATCC CCL 81 monolayers. The maximum concentration that did not affect cell viability and morphology was 1 mM. The development of cytotoxic effect was monitored during 96 h post − treatment.

Inhibition of viral cytopathic effect on VERO cells

This test consisted in adding lidocaine in the a) pre-incubation and b) incubation phases. a) It was carried out in triplicate adding 100μl/well of MEM supplemented with 2% FBS and 1% antibiotic − antimycotic mixture (100X, Gibco) and 1 mM of lidocaine. The incubation was for 1 h at 37 °C in a 5% CO2 atmosphere. After that, the inoculum was discarded, sterile 1× PBS pH 7.2 wash was carried out twice and the monolayers were infected with SARS-CoV-2 D614G virus at 10^3 TCID50/mL. After 1 h of adsorption, the inoculum was removed, 2 washes were carried out and 200μl/per well of MEM supplemented with 2% FBS and 1% antibiotic − antimycotic (100 ×, Gibco) was added. b) The medium was discarded and the monolayers were infected with SARS-CoV-2 D614G virus at 10^3 TCID50/mL plus 1 mM lidocaine (final concentration). After 1 h of adsorption at 37 °C in a 5% CO2 atmosphere, the inoculum was removed, sterile 1× PBS pH 7.2 wash was carried out twice and 200 μl/per well of MEM supplemented with 2% FBS and 1% antibiotic − antimycotic (100 ×, Gibco) plus 1 mM of lidocaine was added. Negative controls (cells incubated without lidocaine and cells incubated in presence of lidocaine 1 mM) and positive control (cells infected with SARS-CoV-2 D614G virus at 10^3 TCID50/ml) were included.

The development of cytopathic effect was monitored in both pre - incubation and incubation assays until 96 h post − infection.

Infection of Calu-3 cells with SARS-CoV-2 STRAIN Wuhan

Cells were maintained in C-DMEM at 37 °C in a 5% CO_2 atmosphere and were seeded in 48-well plates at 2×10^5 cells/

well. Cells were incubated for 48 h, washed with culture medium and incubated for 30 min with lidocaine in combination or not with dexamethasone and stimulated with stimuli as indicated. Then, cells were incubated for 18 h, medium discarded, washed with PBS and lysed with Lysis Buffer Pure Link RNA mini Kit (Invitrogen®). Lysates were stored at −80 °C until use.

RNA isolation from Calu-3 cells and quantitative real-time PCR

Total RNA from SARS-CoV-2 (Wuhan strain) infected Calu-3 cells was isolated with RNA Spin Mini kit (GE Healthcare). Reverse transcription was performed using 1 µg of the total RNA using MML-V polymerase and random primers from Molecular Probes Inc (Invitrogen, Carlsbad, CA, USA) were used. Quantitative real-time PCR was performed using SYBER Green Supermix (Invitrogen, cat 11,761–100, USA) on an IQ-Cycler (Bio-Rad). The specific primers to amplify fragments corresponding to specific gene targets were used: β-actin, forward, 5′-CCT GGC ACC CAG CAC AAT-3′, reverse, 5′-GCC GAT CCA CAC GGA GTA CT-3′; C-X-C motif chemokine ligand 10 (CXCL10), forward, 5′-TCC ACG TGT TCA GAT CAT TGC-3′, reverse, 5 TG A TGG CCT TCG ATT CTG G-3′; CeC Motif Chemokine Ligand 20 (CCL20), forward, 5′-CCA AGA GTT TGC TCC TGG CT-3′, reverse, 5′-TGC TTG CTG CTT CTG ATT CG-3′; Dynamin-like GTPase myxovirus resistance protein 1 (Mx-1), forward, 5′-GCC GGC TGT GGA TAT GCT-3′, reverse, 5′-TTT TTT ATC GAA ACA TCT GTG AAA GC-3′; C-X-C Motif Chemokine Ligand 2 (CXCL2), forward, 5′-AAG GTG AAG TCC CCC GGA C-3′, reverse, 5′-GCC CAT CT TGA GTG TGG CT-3′. All data are presented as relative expression units after normalization to the β-actin gene. Measurements were conducted in triplicate.

Statistic analysis

All experiments were repeated at least three times with similar results. ONE WAY ANOVA (Bonferroni post-hoc test) or Student's t-test (Tukey's post-hoc test) were used accordingly. All quantitative data are expressed as mean ± SEM. A $p < 0.05$ ($p \leq 0.05$) was considered statistically significant. Graphics and data analysis were performed using GraphPad Prism8 (GraphPad software).

Results

Lidocaine reinforces the immunomodulatory capacity of dexamethasone in vitro

Murine and human myeloid and epithelial cell lines were used to study the capacity of lidocaine to modulate pro-inflammatory activation pathways in vitro, with different pro-inflammatory stimuli like TLR-agonists and different pro-inflammatory cytokines of relevance in severe COVID-19 conditions. The murine monocytic-macrophage lines RAW 264.7 and J774 and the human monocytic cell line THP-1 were treated with poly I:C, a TLR3 agonist, used as surrogate stimuli to roughly emulate RNA virus infection. Cell lines were

pretreated for 30 min with different amounts of lidocaine and then stimulated with 40 ng/mL of poly I:C. Pro-inflammatory cytokines were measured in culture supernatants: mIL-6 in the case of RAW 264.7 and J774 cells, and hIL-8 in that of THP-1. In all three cases modulation of pro-inflammatory cytokine secretion can be observed compared to stimulated and unmodulated control, in a dose-dependent manner. For the case of RAW 264.7, mIL-6 secretion is significantly decreased for conditions stimulated with poly I:C and modulated with 0.1 and 0.4 mM lidocaine when compared to the condition stimulated with poly I:C alone [Fig. 1A]. For J774, a similar significant modulation of mIL-6 secretion is observed for the conditions treated with 0.4 and 2 mM lidocaine compared to the untreated control, but no significant difference is observed for the condition treated with 0.1 mM lidocaine [Fig. 1B]. In the case of THP-1, hIL-8 secretion is significantly inhibited for the cases treated with 0.4 and 2 mM lidocaine [Fig. 1C]. Bearing in mind the need for an effective and widely accessible therapeutic for the treatment of severe COVID-19 patients, we designed similar assays as presented in Fig. 1, but using a combination of lidocaine with the corticosteroid dexamethasone, a drug that is used in first line for COVID-19 patients admitted to ICU. RAW 264.7 cells were pretreated with different concentrations of lidocaine in combination or not with 0.5 µM dexamethasone, stimulated with poly I:C [Fig. 2A] or FliC [Fig. 2B] and mIL-6 was measured in culture supernatant after an ON incubation. Again, a significant decrease in mIL-6 secretion in all treated conditions compared to the unmodulated stimulated condition was observed. On the other hand, additive action of lidocaine was observed with dexamethasone in the case of cells stimulated with poly I:C and modulated with 0.2 mM lidocaine +0.5 µM dexamethasone compared to the action of only 0.2 mM lidocaine [Fig. 2A]. The same cells stimulated with FliC and modulated with 0.2 mM lidocaine +0.5 µM dexamethasone secreted significantly less mIL-6 compared to their counterparts modulated with only 0.2 mM lidocaine or 0.5 µM dexamethasone [Fig. 2B]. In EcLPS-stimulated human THP-1 cells, it was also observed that hIL-8 secretion decreases significantly in all modulated conditions, and the combination of 1 mM lidocaine with 1 µM dexamethasone induces significantly less hIL-8 secretion compared to both counterparts, modulated only with 1 mM lidocaine or 1 µM dexamethasone [Fig. 2C]. On the other hand, THP-1 cells modulated with lidocaine and/or dexamethasone and stimulated with the TLR2 agonist Pam3Cys-SKKKK also showed a significant decrease in hIL-8 secretion compared to the condition without any of the modulators. Furthermore, the additive action of dexamethasone to lidocaine showed significant differences at all concentrations compared to its counterparts modulated only with lidocaine (at the same concentration) [Fig. 2D]. It is noteworthy that the signaling via TLR2 and TLR3 pathways have been identified as pathways activated directly by viral infection in humans and that the interaction with the viral protein S enhances the TLR4 signaling capacity of Lipopolysaccharide (LPS), which may contribute to the inflammatory syndrome associated with severe forms of the disease [19–22]. It has also been identified that the E protein (Envelope) of the SARS-CoV-2 virus interacts with TLR2 (human and murine) and activates NF-κB via Myeloid differentiation primary response 88 (Myd88), stimulating pro-inflammatory cytokines such as Interleukin 6 (IL-6),
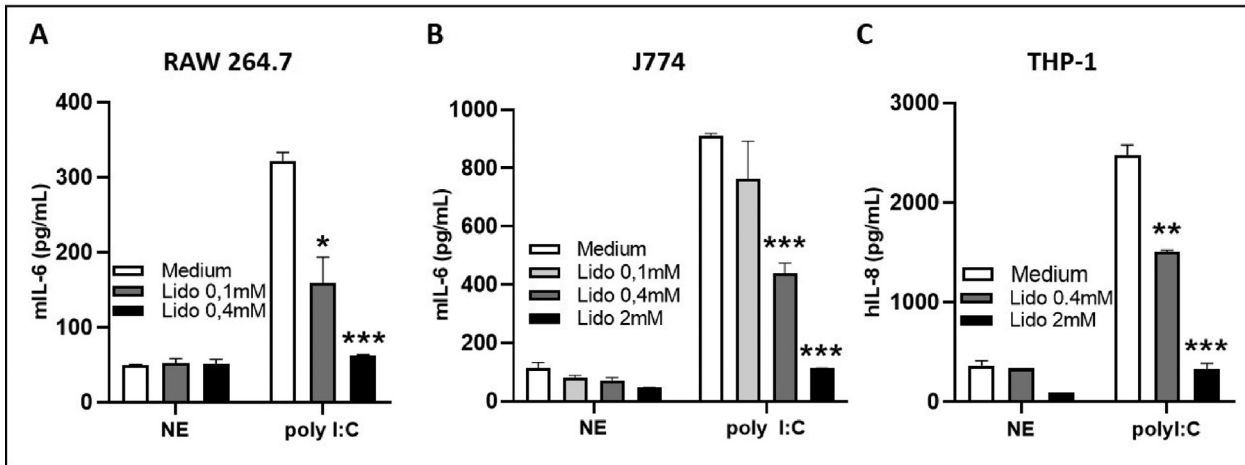
Fig. 1 **Dose-dependent immunomodulatory capacity of lidocaine on different monocytic-macrophage cell lines stimulated with a TLR3 agonist.** RAW 264.7 (A), J774 (B) and THP-1 (C) cells were stimulated ON with poly I:C in presence of different concentrations of lidocaine or not. Pro-inflammatory cytokines, mIL-6 and hIL-8, were measured in supernatants. *($p < 0.05$), **($p < 0.01$), ***($p < 0.001$), a result significantly different from the stimulated unmodulated condition. Data are represented as mean ± SEM of three replicates. Graphs are representative of at least two independent assays.
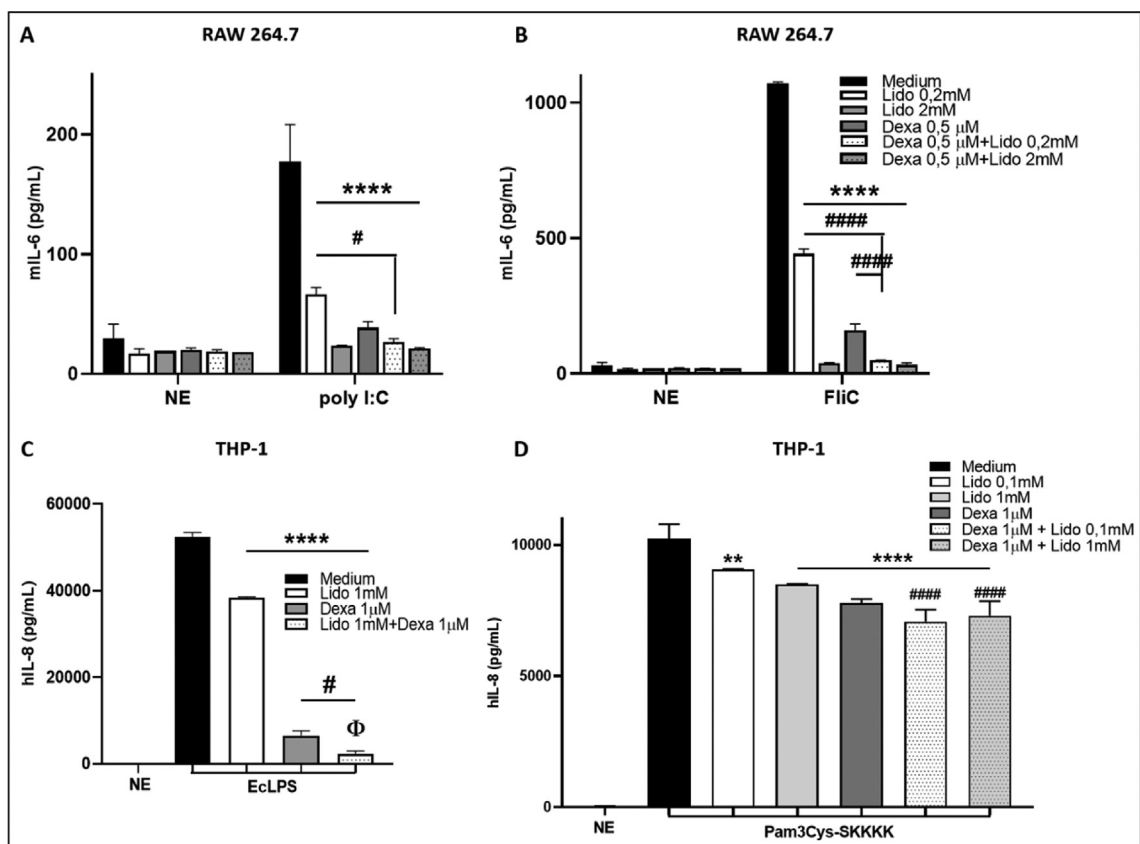

Fig. 2 **Lidocaine in combination with dexamethasone has an additive modulatory effect on TLR3, TLR5, TLR4 and TLR2 signaling pathways.** RAW 264.7 cells were stimulated ON with poly I:C (A) and FliC (B), modulated or not with lidocaine and/or dexamethasone, and mIL-6 was measured in culture supernatants. THP-1 cells were stimulated ON with EcLPS (C) and Pam3Cys-SKKKK (D) in presence or not of different concentrations of lidocaine and/or dexamethasone, and hIL-8 was measured in supernatants. * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), **** ($p < 0.0001$), a result significantly different from the stimulated unmodulated condition. # ($p < 0.05$), ## ($p < 0.01$), ### ($p < 0.001$), #### ($p < 0.0001$), a result significantly different from its counterpart stimulated and modulated with only lidocaine at the same concentration. Φ ($p < 0.05$) a result different from its counterpart stimulated and modulated with only dexamethasone at the same concentration. Data are represented as mean ± SEM of three replicates. Graphs are representative of at least three independent assays.

Tumoral Necrosis Factor alpha (TNFα), IFNγ, IL-1β; being this interaction independent of the viral infection [19].

Lidocaine modulates different signaling pathways: TNFR, interferon gamma receptor (IFNγR) and NF-κB without affecting type I interferon signaling

The inflammatory condition presented by COVID-19 patients admitted to the ICU is characterized by hypercytokinemia that can lead to multi-organ failure and death. Two key cytokine players reported to be at high levels in severe patients are IFNγ and TNFα [23,24]. For this reason, stimulations with hTNFα on reporter THP1-XBlue™-defMyD cells in the presence of lidocaine or in combination with dexamethasone were performed. As readout, hIL-8 secretion by ELISA in culture supernatant after ON incubation was measured. A significant decrease in hIL-8 secretion was observed in stimulated and modulated cells, either with lidocaine or lidocaine + dexamethasone [Fig. 3A]. Furthermore, a synergistic inhibitory effect was observed when stimulated cells were incubated with a combination of 1 μM dexamethasone +1 mM lidocaine. On the other hand, the murine B16 IFNγ reporter SEAP cell line was stimulated with mIFNγ and again, SEAP activity in the supernatants was suppressed in a dose−response manner with a wide range of lidocaine and dexamethasone concentrations and combinations [Fig. 3B]. Again, a synergistic effect of lidocaine and dexamethasone was observed in various combinations.

To get a further insight on the mechanism behind this inhibition, the NF-κB pathway was studied, as it is a master transcription factor regulator of pro-inflammatory genes in different conditions (stress, effect of cytokines, bacterial or viral antigens, etc). The uncontrolled secretion of many of the pro-inflammatory cytokines and chemokines in severe COVID-19 are under the control of this factor pathway [4]. The action of lidocaine and dexamethasone was studied in different human reporter cell lines for NF-κB: two epithelial cell lines HeLa (luciferase reporter) and HEK-hTLR4 (SEAP reporter) and a monocytic-macrophage cell line THP1-XBlue™-defMyD (SEAP reporter), which were stimulated with hTNFα, EcLPS and hTNFα respectively. In the case of HEK-hTLR4 stimulated with EcLPS, a significantly lower SEAP activity with any suppressor in comparison with medium treatment was observed. Although not significant, a tendency in additive capacity was observed when modulators were used in combination [Fig. 3C]. The same behavior is observed in THP1-XBlue™-defMyD and HeLa cells stimulated with hTNFα and modulated with different concentrations of lidocaine and/or dexamethasone. Again, these reporter cell lines showed no significant additive effect in inhibiting cell activation, but a tendency to it (data not shown).

The type I IFNs response is crucial to control viral infections, and that caused by SARS-CoV-2 is not an exception. The implications of a late or null type I IFNs response and poor prognosis for the development of the pathology have been reported [25,26]. To determine the effects of lidocaine on this pathway, we used the B16 type I IFNs SEAP reporter cell line. Stimulation with poly I:C induced an autocrine Interferons alpha and beta (IFNα/β) signal that results in a high production of SEAP, that was drastically diminished when we treated it with RNase prior to stimulation [Fig. 3D]. Treatment with lidocaine and dexamethasone separately or in combination significantly decreased SEAP activity compared to the unmodulated control. This modulation is via TLR3 and not through IFNAR, since in the same assay performed with B16 supernatant of previously stimulated cells with poly I:C (as a source of type I IFNs), no modulation was observed [Fig. 3E]. In this case, the pre-treatment of the supernatant with RNAse does not alter the enzyme activity signal, indicating that the stimulation is effectively given by type I IFNs and not by remnants of the previously used poly I:C.
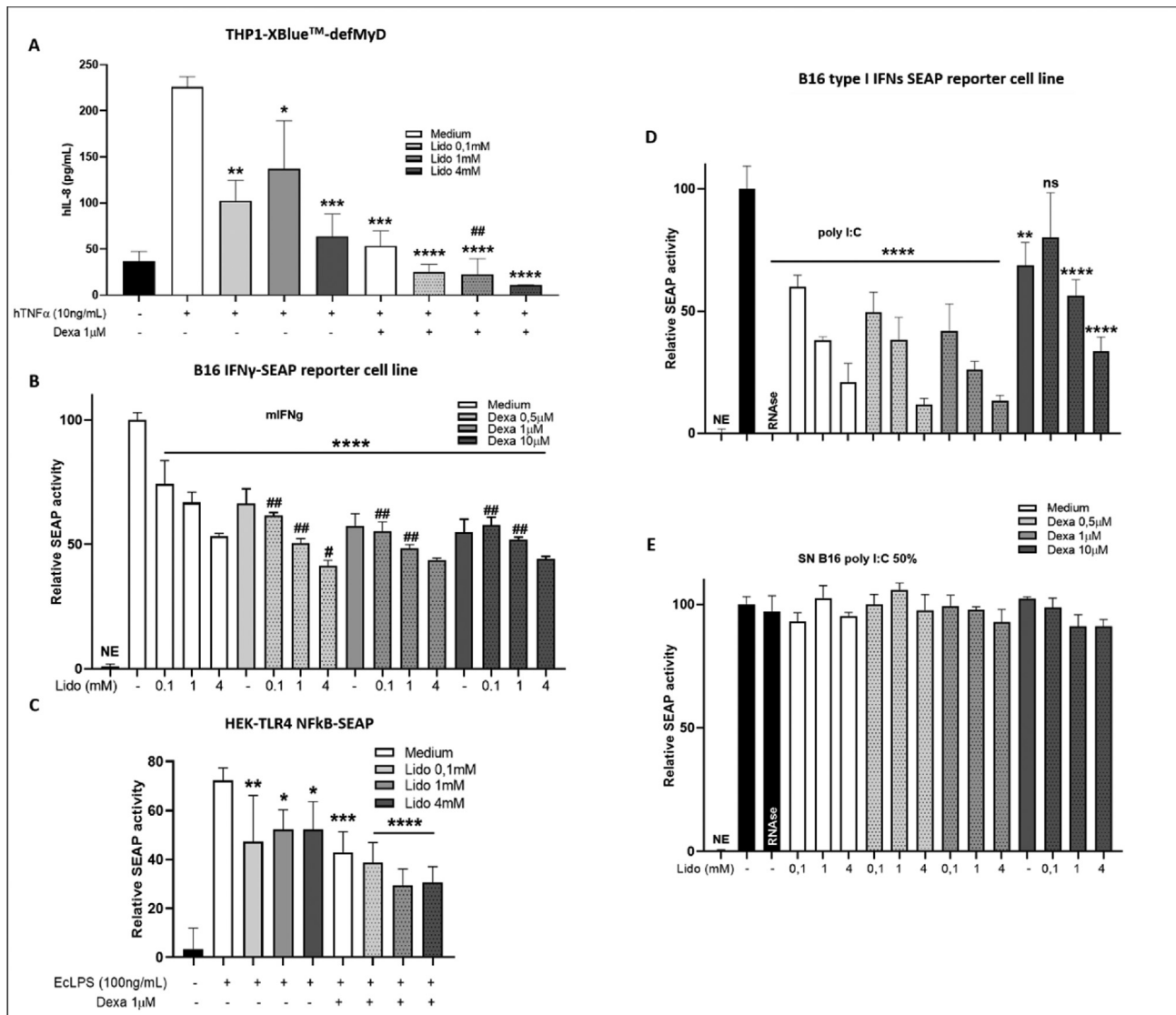
Lidocaine and dexamethasone additively modulate the activation of the inflammasome

The inflammasome is an innate pathway that has been reported to be involved in the dysregulation of the pro-inflammatory response in severe COVID-19 [27,28]. GFP reporter cells for ASC were stimulated with an EcLPS Prime for 3 h and then transfected with pcDNA in the presence of lidocaine and/or dexamethasone. Under the fluorescence microscope, a decrease in the amount of intense fluorescent green specks could be observed compared to the unstimulated condition [Fig. 4A]. Furthermore, the positive ASC-GFP cells/total cells ratio was significantly decreased in the 0.1 mM lidocaine +1 μM dexamethasone treated condition compared to the control without modulators [Fig. 4B]. These synergistic results were confirmed with the abrogation of hIL-1β secretion with 0.1 mM lidocaine +1 μM dexamethasone, whereas 0.1 mM lidocaine exerted no suppressive effect [Fig. 4C].

Lidocaine and dexamethasone additively modulate the exacerbated inflammatory response in the context of in vitro infection by SARS-CoV-2

In order to determine if there is any interaction between lidocaine and SARS-CoV-2 viral replication, a viral replication inhibitory assay was performed at first instance. A cytotoxic assay on Vero cells was performed using incremental doses of lidocaine from 0,1−3 mM was performed as preliminary dose−response experience. It was observed that the maximum dose that did not produce cytotoxic effect on Vero cells was 1 mM. Then, no inhibitory effect on viral replication was observed both on Vero cells monolayers pretreated with 1 mM lidocaine and then infected with SARS-CoV2 nor in the case of performing the infections in the presence of the drug at 1 mM (data not shown).

Afterwards, the effect of lidocaine pretreatment on pro-inflammatory activation upon viral infection on epithelial cells was evaluated. Calu-3 airways epithelial cells, which are sensitive to viral infection [29,30], were infected at Multiplicity of Infection (MOI) 1. Gene expression was assessed by RT-qPCR at 16 h post-infection. Different pro-inflammatory genes (CXCL10, CCL20, CXCL2) were significantly induced upon viral infection [Fig. 5]. CXCL10 and CCL20 showed a significant down-modulation by lidocaine or lidocaine + dexamethasone treatment. CXCL2 expression showed a non-significant downregulation in the modulated conditions with lidocaine and/or dexamethasone. On the other hand, anti-viral genes such as Mx-1 were induced by viral infection and were not modulated by lidocaine/

Fig. 3 **Lidocaine in combination with dexamethasone has an additive modulatory effect different signaling pathways. TNFR signaling (A):** THP1-XBlueTM-defMyD cells were stimulated ON with hTNFα and modulated or not with lidocaine and/or dexamethasone, and hIL-8 was measured in culture supernatants. **IFNγR signaling (B):** B16 IFNγ SEAP reporter cells were stimulated ON with mIFNγ in presence or not of different concentrations of lidocaine and/or dexamethasone, and SEAP activity was measured in supernatants. **NF-κB signaling (C):** HEK-hTLR4 SEAP reporter cells were stimulated ON with EcLPS and modulated or not with lidocaine and/or dexamethasone, and relative SEAP activity was measured in culture supernatants. **Lidocaine and dexamethasone additively modulate poly I:C TLR3 signaling, but do not affect IFNAR signaling (D−E).** B16 type I IFNs SEAP reporter cells were stimulated ON with poly I:C (D) or with supernatant from the same cell line previously stimulated with poly I:C (E) and modulated or not with lidocaine and/or dexamethasone. SEAP activity was measured in culture supernatants. * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), ****($p < 0.0001$), a result significantly different from the stimulated unmodulated condition. # ($p < 0.05$), ## ($p < 0.01$), a result significantly different from its counterpart stimulated and modulated with only lidocaine at the same concentration. Data are represented as mean ± SEM of three replicates. Graphs are representative of at least two independent assays.

dexamethasone, possibly reflecting differential signaling that controls the different type of genes.

Discussion

The multiple waves of COVID-19 cases that have affected different regions worldwide have shown a varied heterogeneity in clinical consequences with a low proportion of severe cases that were associated to increased age and presence of comorbidities [31]. Vaccination has impacted in the severity and mortality of the disease, but occasionally, even with the new variants, there are still severe cases in the vaccinated population, mostly associated to increased age and/or comorbidities, or to an impaired immune response to vaccines [32]. This situation added to a variable proportion of population in different
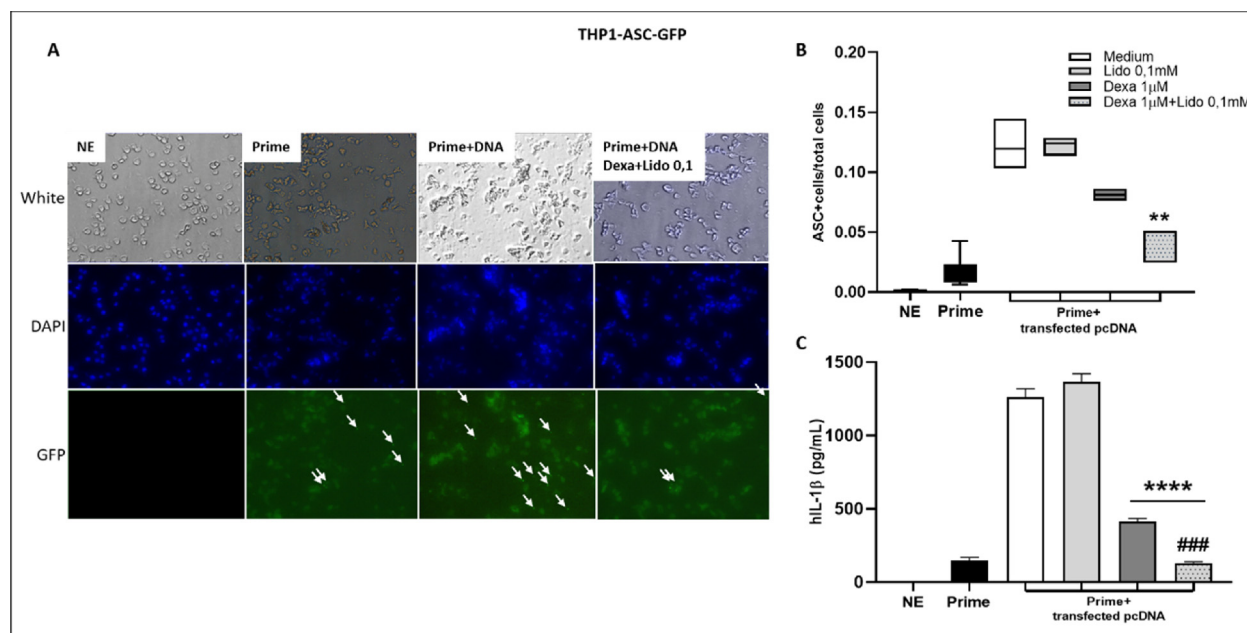
Fig. 4 Lidocaine in combination with dexamethasone has an additive modulatory effect on the innate inflammatory pathway of inflammasome activation. THP1-ASC-GFP cells were primed with EcLPS for 3 h, stimulated ON with transfected pcDNA and modulated or not with lidocaine and/or dexamethasone. Cells were prepared for fluorescent microscopy observation (A, 20×), intense green specks and cells were counted and ASC + cells/total cells ratio calculated (B) and hIL-1β was measured in culture supernatants (C). * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), **** ($p < 0.0001$), a result significantly different from the stimulated unmodulated condition. # ($p < 0.05$), ## ($p < 0.01$), ### ($p < 0.001$), a result significantly different from its counterpart stimulated and modulated with only lidocaine at the same concentration. Data are represented as mean ± SEM of three replicates. Graphs are representative of at least two independent assays.
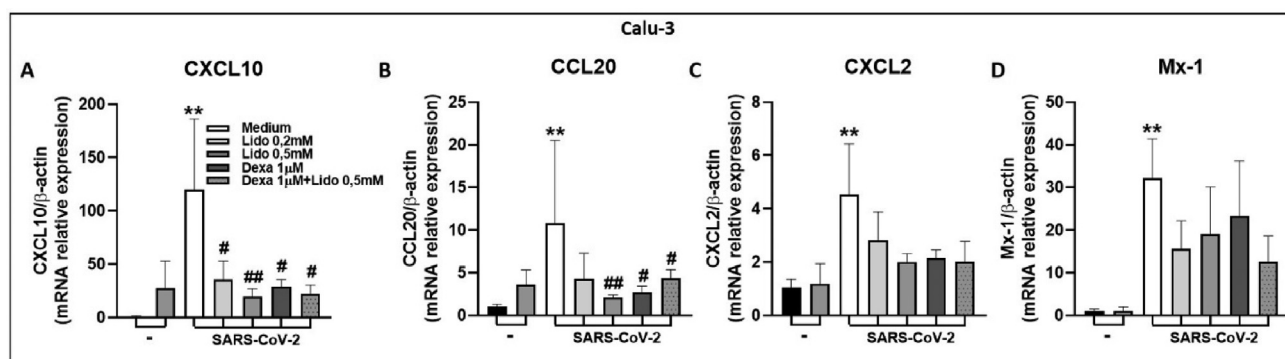
Fig. 5 Lidocaine and dexamethasone modulate pro-inflammatory gene expression in epithelial cells infected by SARS-CoV-2. Calu-3 cells were infected ON with the virus SARS-CoV-2 Wuhan type strain at a MOI of 1 and modulated or not with lidocaine and/or dexamethasone. Expression levels of CXCL10, CCL20, CXCL2 and Mx-1 mRNA were measured. *($p < 0.05$), **($p < 0.01$), ***($p < 0.001$), ****($p < 0.0001$), a result significantly different from the stimulated unmodulated condition. Data are represented as mean ± SEM of three replicates. Graphs are representative of at least two independent assays.

countries refusing voluntary vaccination, claim for improving the treatment options to manage severe COVID-19 cases. In particular, reported mortality in COVID-19 patients in ICU ranges from 20 to 60% depending on different factors with an average of around 30% [33]. Besides, treatment is centered in viral clearance, such as use of specific monoclonal antibodies or drugs that target the viral cycle at different steps such as remdesivir or molnupiravir, the use of anti-inflammatory drugs can help avoiding severe complications due to a dysregulated

immune response associated to phase IIb and III in COVID-19 severe patients [34]. In this line of treatment, the use of dexamethasone or other corticosteroids that ameliorated the systemic inflammatory immune response has been shown to reduce mortality and length of stay (LOS) in ICU [13] and has been adopted worldwide. Still, the optimal timing and dosing of this treatment has been matter of clinical investigation [35–37]. In spite of the benefits of using corticosteroids to treat hospitalized COVID-19 patients, high mortality is still observed in

ICUs and additional tools to manage these patients are long awaited, consequently, important efforts to improve anti-inflammatory treatments are ongoing [38]. Along this time, we learned that the so-called cytokine storm in COVID-19 patients consists in an auto-sustained loop triggered by viral infection but comprising several host actors such as endothelial cells, neutrophils, macrophages, platelets, lymphocytes and epithelial cells that contribute in different timings with several cytokines and inflammatory mediators [39]. This complex interplay creates a challenging scenario to disarm the network by targeting a single cytokine or receptor, since timing of the interventions become more critical when single targets are used [40]. In this context, the results we show here related to inhibition of inflammasome and TLR activation by lidocaine in a dose–response additive effect to dexamethasone [Figs. 1–3] indicate that this might be a promising combination. Enhanced TLR4 signaling by interaction with viral proteins has been reported during COVID and also TLR2, TLR3 and TLR7 signaling are triggered by viral derived ligands during infection [19,41]. Also coinfections or bacterial translocation can contribute to a TLR-mediated amplification of inflammatory pathways [42]. It has been described that lidocaine inhibits activation of NF-κB pathway [43], however, the interaction with dexamethasone has not been shown so far.

Furthermore, we have shown that lidocaine can modulate monocyte/macrophage activation stimulated with TNFα [Fig. 3] and inflammasome pathway [Fig. 4], both pathways that have been shown to be an important player in COVID-19 cytokine storm [39]. Moreover, lidocaine/dexamethasone combined had an additive effect in modulating IFNγR signaling. Although this cytokine is not elevated in patients that have not developed acute respiratory disease, in later phases it can contribute to tissue destruction being part of the immunopathological axis of severe COVID-19 [44]. Interestingly, lidocaine treatment did not affect type I IFN signaling in host cells [Fig. 3]. This pathway has been shown to be critical in viral infection contention [45,46], consequently interventions that may affect its function could increase the clinical risk especially if applied in early phases of the infection [47]. Our results indicate that this would not be the case of lidocaine treatment.

Lidocaine modulates the inflammatory response caused by viral infection with SARS-CoV-2 Wuhan like strain [Fig. 5]. Although in this experiment we could not detect an additional effect of lidocaine on dexamethasone, we speculate that this can be due to the differences in conditions and readouts used in this case compared with experiments performed using cytokines or TLR ligands as triggers of inflammation. As mentioned before, many pathologies including severe COVID-19, are driven by host dysregulated immune response more than by viral infection itself [39]. Thus, decreased pro-inflammatory hIL-8 secretion and downregulated gene expression of different pro-inflammatory genes are encouraging results in order to try to control the disproportionate immune response observed in severe COVID-19 ICU patients.

Although lidocaine usage to modulate inflammation in severe COVID-19 patients has been envisaged [48], there have been few concreted attempts so far. A case of a severe patient receiving i. v. lidocaine infusion in June 2020 was recently published [49] indicating a good response to treatment and lack of adverse events. Treatment used in this study was lidocaine infusion at a rate of 45 mg/h to a dose of 0.6 mg/kg/h in intervals of 12 h over a period of 5 days. Along this treatment, a drop in D dimer and CRP was observed. In November 2020 a controlled randomized trial to test the effect of i. v. administration of lidocaine in COVID-19 or not COVID respiratory distress syndrome and inflammation was initiated in France (NCT04609865). The dosing regimen to be tested is an infusion of lidocaine of 1 mg/kg (ideal weight), followed by 3 mg/kg/h for the first hour, 1.5 mg/kg/h for the second hour, 0.72 mg/kg/h for the next 22 h and then 0.6 mg/kg/h for 14 days at most or 24 h after extubation or ventilator-weaning [50]. Dosing regimen could be critical to determine clinical efficacy of the intervention, since lidocaine can modulate different signaling pathways related to inflammation at concentrations in the range of 10–200 μM, partly overlapping with the concentrations tested in our experiments. In particular, inhibition of purinergic signaling through P2X7R, which contributes to inflammatory activity is observed in this range [51]. Furthermore, NF-κB inhibition and immunometabolic changes in immune cells has been also described as mediators of lidocaine anti-inflammatory capacity [52–54]. One important concern regarding translational applications of lidocaine as systemic anti-inflammatory agent is associated to unwanted secondary effects observed when lidocaine blood concentrations rise higher than 20 μM [43]. An interesting approach has been proposed by Hasan et al. [51], that reported a series of COVID-19 cases treated with lidocaine administered by subdermal route at a continuous infusion rate of 1 mg/kg/h during 6 days. In this way, plasma concentrations remain below safety threshold and authors argue that higher concentrations in the draining lymphatic systems allow higher exposure of immune cells to the drug to increase its immunomodulatory effects. Authors report a series of 20 severe COVID-19 cases treated using this dosing regimen, 19 among them successfully recovered from disease. These alternative administration routes and the combinatory effect with corticosteroids reported here may contribute to improve and broaden the options to manage severe COVID-19 patients available so far.

Beyond COVID-19 treatment, the combination of lidocaine and dexamethasone analyzed in our experiments could be an interesting alternative for other pathological conditions that are driven by an exaggerated inflammatory response, such as ventilator-induced lung injury [55,56].

Overall, we have shown that lidocaine combined with dexamethasone can modulate cellular activation pathways triggered by endogenous or exogenous pro-inflammatory signaling. Circuits modulated by both drugs are critical to restrain cytokine storm in severe COVID-19. This novel approach using lidocaine as anti-inflammatory therapy, could enlarge the tools to manage severe COVID-19 patients and be useful in other inflammation-driven pathological situations.

Funding

Conflicts of interest

None.

Data availability

The data that support the findings of this study are available from the corresponding author, MR, upon reasonable request.

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