Niclosamide and ivermectin modulate caspase-1 activity and proinflammatory cytokine secretion in a monocytic cell line

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Abstract

The COVID-19 pandemic has led to an unprecedented demand for new and repurposed therapeutics to ameliorate the morbidity and mortality associated with SARS-CoV-2 infection. However, there is still a paucity of information relating to successful antiviral compounds. The repurposing of immune modulators, such as dexamethasone and tocilizumab, has shown significant improvement in survival rates. Repurposing of small molecules that may have antiviral and immunomodulatory potential may have significant impact on the pandemic. Niclosamide and ivermectin are being investigated for repurposing as potential treatments for COVID-19 patients. Both niclosamide and ivermectin have been proposed and studied based upon possible immunomodulatory and antiviral activity. To improve their posology, there are also ongoing efforts to nano-formulate these drugs, but a much greater understanding of their mechanisms of action is required to rationalise their plausibility as candidates. We have previously shown that niclosamide can affect responses to immune stimulation in *ex vivo* cells from healthy rats exposed via a long-acting injectable formulation. The current study aimed to further understand the effects of niclosamide and ivermectin on inflammasome activity in human cells due to the involvement of inflammasomes in the hyperinflammation and coagulation observed in severely ill COVID-19 patients. Caspase-1 activity and proinflammatory cytokine secretion in THP1 cells exposed to physiologically-relevant concentrations of niclosamide and ivermectin were measured as markers of inflammasome activity. Exposure to both niclosamide and ivermectin led to lower caspase-1 activity compared to untreated cells as well as resulting in lower secretion of IL-1β, IL-18, and TNF-α if treated prior to LPS induction. These data in their own right should not be interpreted as being a conclusive indicator of the utility of these drugs in COVID-19. However, the data presented suggests a putative mechanism for the proposed immune modulation. Substantive further work is still needed to determine the precise mechanism(s) that underpin these findings and whether the observations are relevant *in vivo*.

Abbreviations: Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), Coronavirus Disease 2019 (COVID-19), Lipopolysaccharide (LPS), nano-Silicon Dioxide (SiO2), Interleukin-1 beta and 18 (IL-1β and IL-18, respectively), and Tumor Necrosis Factor-alpha (TNF-α), Dimethyl Sulfoxide (DMSO)

Introduction

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), the virus responsible for the coronavirus disease 2019 (COVID-19) pandemic, has afflicted over 100 million people in the past year—three million of whom died [1,2]. To ameliorate this, extensive funding and collaboration across the globe have resulted in an unprecedented rate of vaccines being developed [3,4]. Despite the unprecedented progress with vaccines, a portfolio of interventions is needed that includes treatments for those who either cannot or will not be protected by the vaccines.

Several therapeutics are currently being repurposed for SARS-CoV-2 interventions—some of which has already been approved in countries like the UK, US, or Japan [5,6]. In spite of this, it is still important to consider pharmacokinetic and immunomodulatory information when repurposing therapeutics for SARS-CoV-2 [5,7,8]. Immune modulators like dexamethasone and tocilizumab have portrayed significant improvement in survival rates in COVID-19 patients [9,10].However, many interventions that have been studied were assessed without a strong preclinical case which raises significant concerns around benefit versus risk. A robust preclinical understanding should be a prerequisite for clinical trials and will considerably improve the success rate. Hydroxychloroquine was initially lauded for its antiviral activity, but was not supported on the basis of its pharmacokinetics relative to its antiviral activity (REF). Subsequently, the alternative immunomodulatory mechanism of action was purported by subsequently the drug failed clinical studies [11–14]. Thus, an assiduous assessment of antiviral and/or immunomodulatory plausibility will be critical to delivering SARS-CoV-2 therapeutic interventions.

As pharmacokinetic studies for potential treatments are underway, several have reported an immune system portraying hyperinflammation in patients with severe COVID-19 [16]. Increased amounts of inflammatory cytokines in the blood of COVID-19 patients, such as Interleukin-6 (IL-6) and Tumor Necrosis Factor alpha (TNF-α) (aptly dubbed a "cytokine storm"), have been highly associated with disease severity [17]. In conjunction with this, patients have exhibited decreased lymphocyte and monocyte counts pivotal for a substantial immune response to defend against SARS-CoV-2 [18]. Junqueira et al. has proposed that SARS-CoV-2 infection of monocytes may play an important role in the inflammatory response seen in patients [19]. Thus, treatments capable of restoring the immunological imbalance associated with the disease may have a role in regulating pathological inflammation.

One potential agent is niclosamide (NIC), an anti-parasitic which has shown *in vitro* antiviral effects and potential as an immunomodulator. *In vitro,* NIC inhibited SARS-CoV-2 entry by modifying endocytic pH—corroborating several findings claiming that low-pH endosomal proteases cleave the SARS-CoV-2 spike protein enabling the virus to enter its host cell [20]. Braga et al. posit NIC as an effective inhibitor of TMEM16F activity, a calcium-activated ion channel responsible for phosphatidylserine exposure, a protein involved in SARS-CoV-2's spike protein maturation. In addition to these antiviral effects, NIC has been implicated with the activation of the nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain containing 3 (NLRP3) inflammasome in THP-1 monocytes [22]. Interestingly, NIC also reduced the secretion of a number of proinflammatory cytokines—including IL-1β, IL-8, IL-6, and TNF-α in human synoviocytes [5,23]. Aside from the immunomodulatory effects of NIC, an evaluation of the pharmacokinetics at approved doses indicated that systemic concentrations achieving the in vitro antiviral concentrations were achievable in humans, albeit not for the entire dosing

interval [7]. Additionally, in an effort to improve the pharmacodynamics and clinical application of NIC, Hobson et al. engineered a nanoparticle formulation of NIC for long-acting drug delivery releasing drug systemically in rats for up to 28 days following a single intramuscular administration [5]. In this same study, we also demonstrate that NIC may modulate cytokine levels *ex vivo*.

Another anti-parasitic compound, ivermectin (IVM), has also been postulated as an antiviral, but systemic exposures at higher than approved doses are highly unlikely to achieve antiviral concentrations. IVM has also been proposed for its immunomodulatory activity based upon a mechanism of action involving allosteric modulation of the α7 nicotinic acetylcholine receptor (α7 AChR)(REF). Like NIC, IVM has also been implicated with inflammation. IVM increased the survival rate of mice treated with a lethal dose of lipopolysaccharide (LPS) and reduced production of IL-1β, IL-6, TNF-α from macrophages isolated from mice [24]. However, the target concentrations required for allosteric modulation of α7 AChR have not been robustly characterised in order to derive an in vivo target. It is critical that downstream studies incorporate an understanding of the concentration-response relationship in order that a robust understanding of the pharmacokinetic-pharmacodynamic relationship can start to be developed.

Here, the ability of NIC and IVMto modulate the immune system is explored further at clinically relevant concentrations. To accomplish this, we explored the interactions of these drugs with the inflammasome, NLRP3, and proinflammatory cytokine production in THP-1 monocytes.

Results

Determination of cytotoxic concentrations for NIC and IVM

Since cell death may confound any inquiries into the immunogenic potential of NIC and IVM, THP1 cells were treated with a range of concentrations of NIC or IVM to determine what concentrations result in significant cell death (**Fig. 1)**. For NIC, a 24-hour treatment period resulted in no significant increase of dead cells compared to control for all concentrations of NIC. As expected, cells treated with the proprietary lysis buffer showed a significant increase in cell death (**Fig. 1A)**. Upon 48 hours of NIC exposure, a significant reduction in MTT breakdown suggested increased cell death for cells treated with 125 or 250 ng/ml of NIC **(Fig. 1B)**. Similar results can be said of IVM, except IVM exhibited no cytotoxicity at 125 or 250 ng/ml of treatment for 48 hours **(Fig. 1C-D)**. NIC and IVM concentrations were guided by reported Cmax values found in pharmacokinetic studies of the compounds [25,26] Given these results and the Cmax values from other studies for reference (as discussed further in the "Methods" section), 35 and 60 ng/ml were selected for NIC and 45 and 250 ng/ml for IVM. Therefore, experiments following this use these concentrations when determining NIC and IVM's immunomodulatory effects.

NIC and IVM capabilities for NLRP3 modulation

In an effort to characterize the relationship between NIC or IVM and the NLRP3 inflammasome, a caspase-1 activity assay was employed **(Fig. 2)**. Formation of the NLRP3

inflammasome results in procaspase-1 self-cleavage followed by the subsequent secretion of mature proinflammatory cytokines IL-1β and IL-18 [27,28]. We therefore looked for NLRP3 activation via determining caspase-1 activity levels after a variety of treatments with NIC and IVM.

To determine whether NIC and IVM function as a treatment or prophylactic, we developed a "pre"-stimulated and "post"-stimulated sample, respectively. The "pre"-stimulated sample was first stimulated with LPS and SiO_2 followed by NIC or IVM treatment. Therefore, if caspase-1 activity levels in these "pre"-stimulated samples are lower than samples treated
with just LPS and SiO_2, NIC or IVM function as a treatment. It's obverse, "post"-stimulated samples, was first treated with NIC or IVM then stimulated with LPS and SiO_2. Therefore, a reduction in caspase-1 activity levels in these samples compared to the LPS and SiO_2 positive control posit NIC and IVM as a prophylactic. More information on this can be found in the "Methods" section.

As expected, THP-1 cells treated with LPS and SiO_2 raised caspase-1 activity ($P = 0.0142$)—confirming that cells treated with these compounds will result in an increase in caspase-1 activity. When THP-1 cells are exposed to only DMSO, 60 ng/ml of NIC, 45 ng/ml, or 250 ng/ml of IVM, caspase-1 activity levels decreased (P = 0.0053, 0.0013, 0.0053, and 0.0031, respectively). NIC in particular seems to decrease caspase-1 activity when introduced to THP-1 cells either after (black bars) or before (orange) LPS/ SiO_2 induction ($P = 5.086 \times 10^{-6}$ and 0.04267)—though this effect seems to depend upon the concentration of NIC introduced. IVM on the other hand does not seem to alter caspase-1 activity levels in conjunction with LPS/ SiO_2 induction **(Fig. 2B)**.

Proinflammatory cytokine modulation

Given that NIC and IVM seemed to interact with caspase-1 activation, we looked for proinflammatory cytokines associated with NLRP3 activation, namely IL-1β and IL-18, as well as other potent proinflammatory cytokines TNF-α, interferon-gamma (IFN-γ), and IL-6. Both IFN-γand IL-6 appeared in levels below the limit of detection by the Bio-plex-200 and therefore related data was not presented in this study.

LPS and SiO_2 induction resulted in a significant increase in IL-1β and IL-18 secretion, even more so than LPS alone, as expected (P = 0.0265 and 0.0002) **(Fig. 3A and C).** This is consistent with the notion that LPS and SiO_2 activate the NLRP3 complex resulting in the release of matured IL-1β and IL-18 [27,28]. However, mature TNF-α secretion is not dependent on SiO_2 and LPS inducing an increase in TNF-α secretion also confirms the positive control ($P = 2.670 \times 10^{-6}$).

Pre-stimulating THP-1 cells followed by treatment with DMSO significantly increased IL-1β, TNF-α, and IL-18 release (P = 0.0263, 0.0494, and 0.0188). As shown by the black bars, treating THP-1 cells with NIC and IVM after LPS and SiO_2 induction potentially reduced cytokine secretion relative to the DMSO samples for all three cytokines **(Fig. 3A-C)**. Even more glaringly, stimulating THP-1 cells with LPS and SiO_2 after NIC and IVM exposure, as made evident by the orange bars, resulted in a significant reduction in proinflammatory cytokine release ($P < 0.001$ for all samples except for the "Post-IVM" treatments for TNF-α, which resulted in P = 0.0028 and 0.03548 for 45 and 250 ng/ml, respectively) **(Fig. 3A-C)**. Interestingly, 45 ng/ml of IVM significantly reduced IL-18 levels at baseline (P = 0.0372) **(Fig. 3C and D)**. Besides 45 ng/ml of IVM, THP-1 cells treated with other concentrations of NIC

and IVM did not exhibit an increase or decrease in any of the secreted cytokines measured at baseline **(Fig. 3D and E)**.

Discussion

These results provide insight into how NIC and IVM may modulate the activity of NLRP3-activated monocytes at clinically relevant concentrations but cannot in their own right be used to justify the agents as interventions for COVID-19. Hitherto the interactions between NIC, IVM and these proinflammatory markers in THP-1 cells have been largely unidentified. The results of this study purport that NIC and IVM demonstrate an ability to decrease caspase-1 activity in a monocytic cell model at baseline. This suggests the compound's ability to potentially inhibit NLRP3 inflammasome activation. Additionally, NIC and IVM themselves do not stimulate the release of IL-1β and TNF-α.

These results also corroborate what others and our lab have seen previously for NIC, which reduced secretion of proinflammatory cytokines like IL-1β, IL-8, IL-6, and TNF-α in human synoviocytes [23]. Our lab has shown that inducing inflammation (via LPS) in blood collected from rats exposed to NIC resulted in less of an IL-1β secretion relative to control rats [5]. Specifically, inflammatory-stimulated rat blood accompanied by a vehicle control resulted in a fold difference of 2.6, whereas NIC treated groups demonstrated a fold difference of only 1.6. This idea that NIC may counterbalance inflammatory stimulation was shown in the "pre"-stimulated samples for both caspase-1 activity and proinflammatory cytokine secretion by NIC treated groups showing lower levels compared to groups treated with DMSO. Similarly, IVM has been demonstrated to reduce proinflammatory cytokine release in macrophages [24]. Though our investigation demonstrates neither an increase nor decrease in IL-1β and TNF-α release in IVM treated samples at baseline, 45 ng/ml of IVM reduced IL-18 secretion compared to controls. Unexpectedly however, 250 ng/ml of IVM did not reduce IL-18 secretion relative to controls suggesting that IL-18 secretion may only be modulated by lower concentrations of IVM.

Our findings do conflict with data from some other studies. Tran and team reported that NIC activates the NLRP3 inflammasome and this does not concur with NIC-treated THP-1 cells exhibiting decreased caspase-1 activity [22]. Tran's findings, however, were generated with THP-1 cells differentiated into macrophage-like cells potentially highlighting a different activation mechanism related to NIC treatment in monocytes relative to their mature obverse, macrophages. Our previous work also shown that blood collected from NIC treated rats exhibited a marked increase in proinflammatory cytokine concentrations, like IL-1β and TNF-α, when compared to control rats. The THP-1 cells in this study did not exhibit an increase in IL-1β or TNF-α at baseline. This ultimately suggests that the monocytes in the blood may not be responsible for this elevation in IL-1β and TNF-α seen in our previous rat study highlighting the multifactorial nature of this investigation. Several components within the rat blood may have contributed to this increase in proinflammatory cytokines, underscoring the importance of conducting similar experiments on not just monocyte models, but other types of immune cells in the blood.

In conclusion, NIC and IVM exhibit potential immunomodulatory effects in monocytes—reducing proinflammatory markers and NLRP3 formation and thus suppressed subsequent caspase-1 activation. Ultimate utility of any drug, including NIC, can only be empirically determined in sufficiently powered, randomised control trials, which themselves should only be initiated for candidates with strongly supportive preclinical data. Authors of

this paper do not suggest NIC as a treatment for SARS-CoV-2 until such trials are completed and advise so.

Methods

THP-1 cell culture

THP-1 cells were grown in Roswell Park Memorial Institute (RPMI) 1640 media supplemented with fetal bovine serum (FBS) yielding a 10% FBS solution both purchased from Gibco, UK. All culture flasks were incubated at 37°C in 5% CO_2. Cells were passaged every 3 to 4 days if confluent.

Niclosamide (NIC) and ivermectin (IVM) preparation

NIC and IVM were purchased from Tocris Biochemicals, UK, and Sigma-Aldrich (product no. I8898) respectively. Both NIC and IVM were solubilized in DMSO yielding stocks of 2 mg/ml of NIC and 10 mg/ml of IVM—both as guided by Tocris' solubility data [29,30].

Lactate dehydrogenase (LDH) cytotoxicity assay

THP-1 cells were plated in a 96-well flat-bottom plate at a density of 100,000 cells per well in 100 ul of media supplemented with FBS. Cells were exposed to concentrations ranging between 1.95 and 250 ng/ml for both NIC and IVM, which included the reported Cmax values of 82 and 78.04 ng/ml, respectively [25,31]. Three negative controls were also prepared: i) cells treated with 0.01% DMSO vehicle, ii) cells untreated with drug nor vehicle, and iii) culture media only. Cells were then incubated at 37°C in 5% CO_2 for 24 h. Following incubation, positive controls were constructed by treating cells with 5 ul lysis buffer for 15 min at room temperature. The LDH detection solution was made, administered, then measured as suggested by Sigma Aldrich's Cytotoxicity Detection Kit (LDH) protocol (catalogue # 11644793001). The absorbance of each well was analyzed with a BMG Labtech CLARIOstar microplate reader with a filter set to 492 nm.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay

THP-1 cells were plated in a 96-well round-bottom plate at a density of 100,000 cells per well in 100 ul of media supplemented with FBS. For similar reasons as in the LDH assay, cells were exposed to concentrations ranging between 1.95 and 250 ng/ml. As in the LDH assay, three negative controls were prepared: i) cells treated with 0.01% DMSO vehicle, ii) cells untreated with drug nor vehicle, and iii) culture media only. Cells were then incubated at 37°C in 5% CO_2 for 24 or 48 h. Following incubation, positive controls were treated with 5 ul of lysis buffer for 15 min at room temperature. Cells were centrifuged at 2000 rpm for 5 min and supernatant was removed keeping cell pellets intact. Then 50 ul of 0.0067M MTT was added to each well (MTT was purchased from Sigma Aldrich, product no. M5655) and diluted in phosphate-buffered saline (purchased from HyClone). Plates were then incubated at 37°C in 5% CO_2 for 2 h then centrifuged at 2000 rpm for 5 min with subsequent supernatant removal. Following the addition of 100 ul of DMSO, the absorbance of each well was analyzed with a BMG Labtech CLARIOstar microplate reader with a filter set to 570 nm.

Caspase-1/Inflammasome assay

THP-1 cells were plated in an opaque, 96-well flat-bottom plate at a density of 100,000 cells per well in 100 ul of media supplemented with FBS. Six controls were prepared: control wells contained either i) culture media only, ii) cells with culture media, iii) cells treated with lipopolysaccharide (LPS), iv) cells treated with nano-silica dioxide (SiO_2), v) cells treated with LPS and SiO_2, or vi) cells treated with 0.003% DMSO. LPS and SiO_2 were both purchased from Invivogen. As suggested by their purveyor, samples receiving LPS, SiO_2, or both, received either, 20 ng/ml LPS, 100 ug/ml SiO_2, or an initial 2 h treatment of 20 ng/ml LPS followed by an overnight treatment of 100 ug/ml SiO_2 resulting in a 24 h treatment time beginning from LPS delivery.

Treatment groups were partitioned into three categories: cells treated with the drug only, cells exposed to LPS/SiO_2 for 24 h followed by exposure to drug for 24 h (denoted as "Pre" for "Pre-LPS/SiO_2 activated" in **Figs. 2 and 3**) and cells exposed to drug for 24 h followed by LPS/SiO_2 for another 24 h (denoted as "Post" for "Post-LPS/SiO_2 activated" in **Figs. 2 and 3**). Two non-cytotoxic concentrations of each drug were selected, namely 35 and 60 ng/ml for NIC, and 45 and 250 ng/ml for IVM. These concentrations were selected based off the LDH and MTT cytotoxicity assays (**Fig. 1**), and studies assessing the pharmacokinetic characteristics of both NIC and IVM [25,26]. Following treatments, the protocol for measuring caspase-1 activity via Promega's Caspase-Glo 1 Inflammasome Assay (catalogue no. G9951) was executed. Luminescence from each sample was measured via Labtech CLARIOstar microplate reader.

Proinflammatory cytokine assay

THP-1 cells were plated in 24-well flat-bottom plates at a density of 1,000,000 cells per well in 1 mL of media supplemented with FBS. Control and treatment groups were mimicked from the caspase-1 experiment resulting in the same concentrations of reagent(s) added to each well. Following treatment, plates were spun at 2000 rpm for 5 min and cell supernatants were collected for subsequent storage at -80°C for future use.

Following 1 freeze/thaw cycle, supernatant samples were analyzed for proinflammatory cytokines IL-1b, TNF-a, IL-18, IFN-γ, and IL-6 as advised by the protocol given by R&D systems Human Premixed Multi-Analyte Kit (catalogue no. LXSAHM-05). Luminescence from each sample was measured using Bio-Rad's Bio-plex-200.

Statistical Analysis

All samples were tested for normality using the Shapiro-Wilk test. For normally distributed data, an unpaired Student's t-test was used to determine significant differences between two independent samples. For non-normal data, a Wilcoxon-Mann-Whitney test was used to discern significant differences. The significance level for all tests was defined as 0.05, and thus if $p < 0.05$, differences were deemed significant (all calculated using R). All data are presented as a mean with error measurements being standard deviation values.

Figures

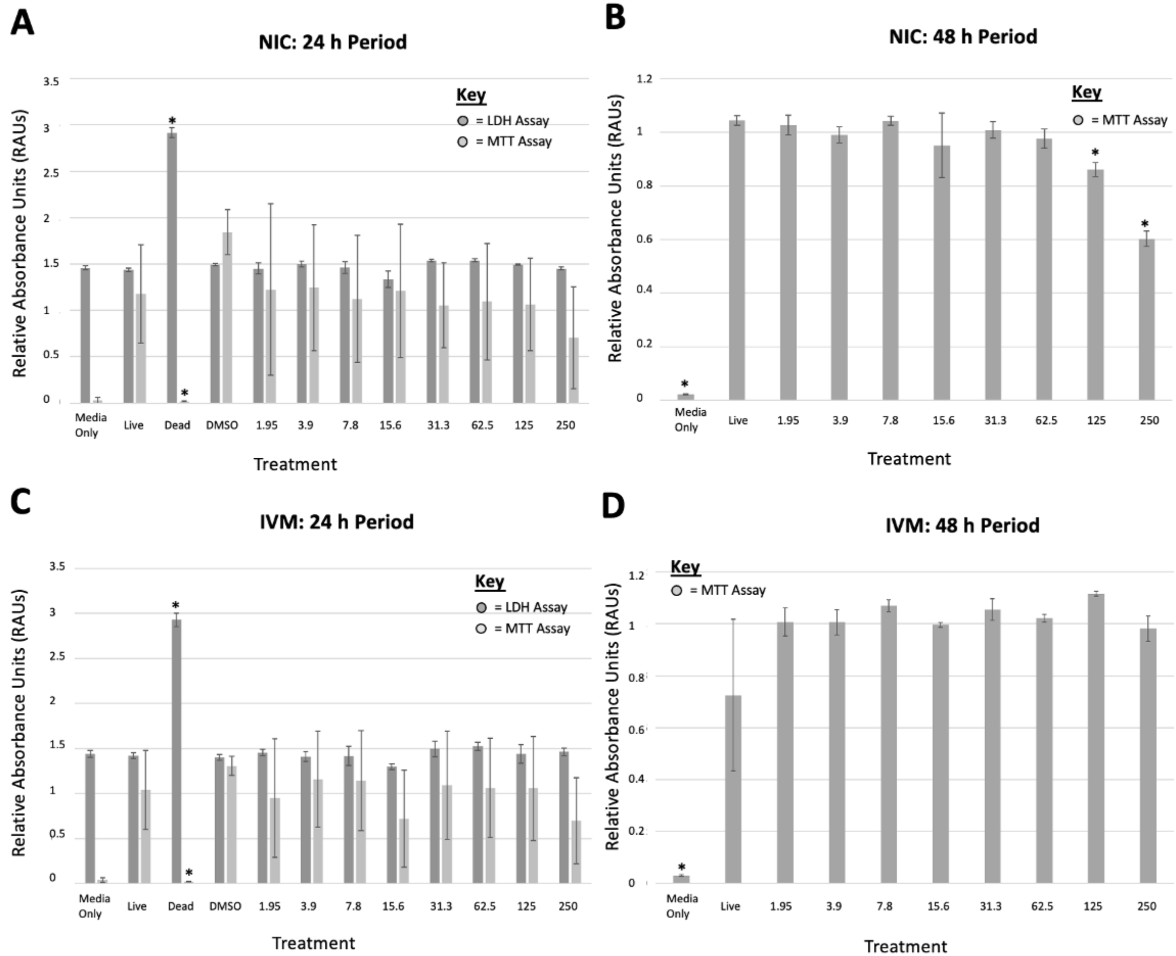
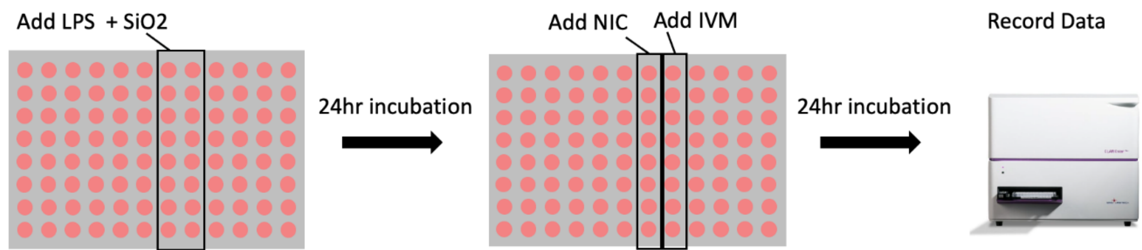
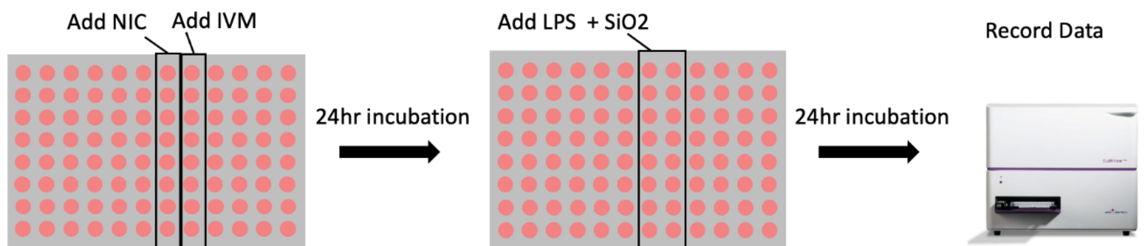


Fig. 1 Cytotoxicity of NIC and IVM in THP-1 cells. Bar plots depicting the relative absorbance units of THP-1 cells treated with control agents or varying concentrations of drug (units are in ng/ml) treated with either NIC (A and B) or IVM (C and D) for 24 or 48 hours. The LDH assay is denoted by the dark bar and the MTT the light bar. Asterisks, "*", indicate a significant difference between the indicated sample and the "Live" control. Significance level is equal to 0.05 and therefore a $p < 0.05$ results in a significant difference. Bars indicate mean ± SD.

A **Pre-Stimulated**



Post-Stimulated



B

Caspase-1 Activity

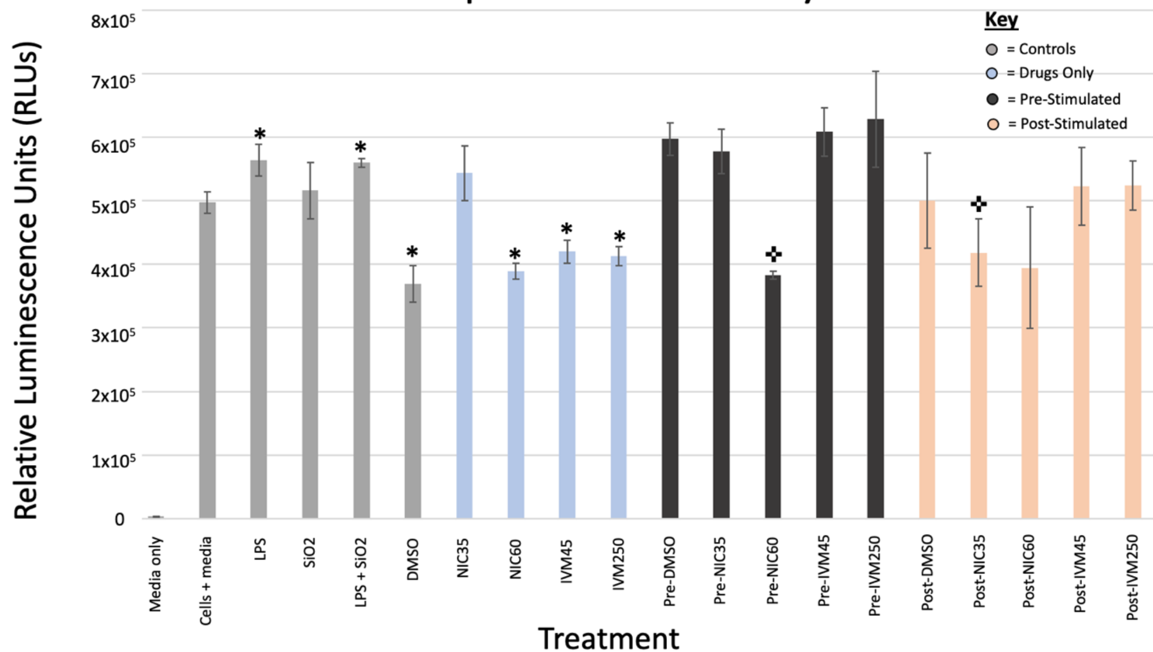


Fig. 2 Caspase-1 Activity of pre- and post-stimulated NIC or IVM treated THP-1 cells. (A) Experimental picture depicting how pre- versus post-stimulated samples were made. (B) Bar plot depicting relative luminescence units (RLUs) of active caspase-1 in THP-1 cells treated with controls (grey), NIC or IVM only (blue), LPS/SiO_2 first then NIC or IVM (black), or NIC or IVM first followed by LPS/ SiO_2 (orange). Asterisks, "*", indicate a significant difference between the indicated sample and the "Cells + media" control. Plusses, "✜", indicate a significant difference between the indicated sample and the "LPS + SiO_2" treated sample. Significance level is equal to 0.05 and therefore a $p < 0.05$ results in a significant difference. Bars indicate mean ± SD.

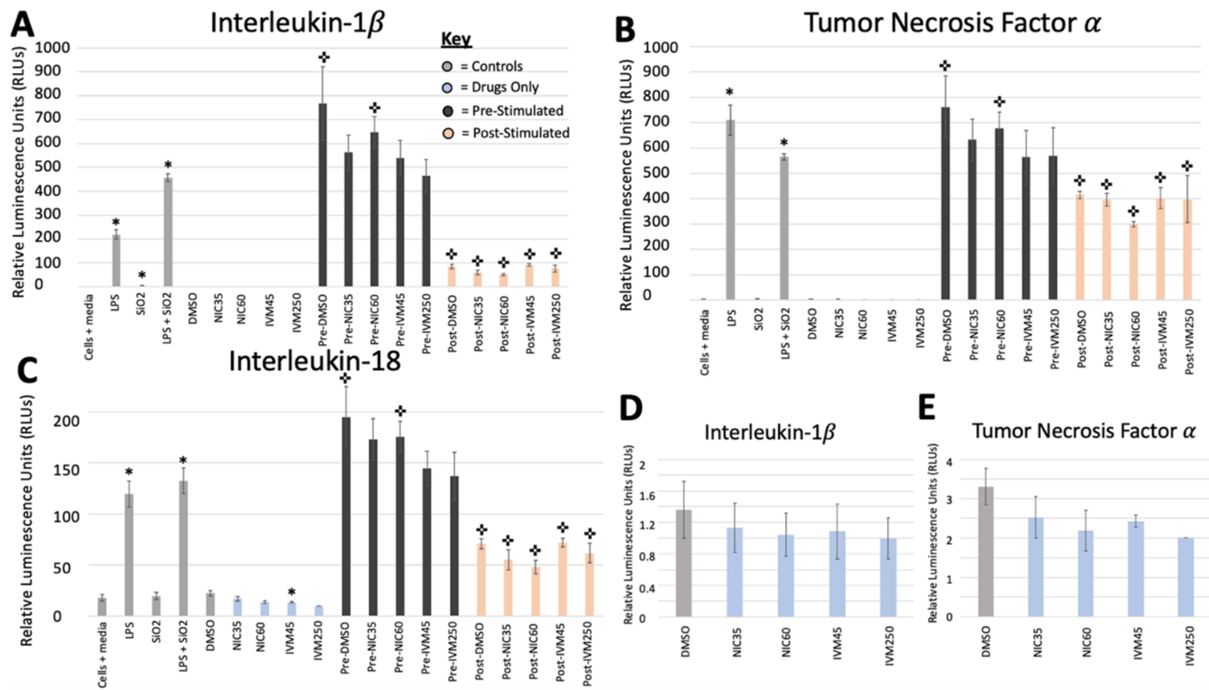


Fig. 3 Proinflammatory cytokine release of pre- and post-stimulated NIC or IVM treated THP-1 cells. Bar plot depicting relative luminescence units (RLUs) of (A) IL-1β, (B) TNF-α, and (C) IL-18. (D) and (E) depict a magnified version of the DMSO, NIC, or IVM treated THP-1 cells in parts (A) and (B). THP-1 cells treated with controls (grey), NIC or IVM only (blue), LPS/ SiO_2 first then NIC or IVM (black), or NIC or IVM first followed by LPS/ SiO_2 (orange). Asterisks, "*", indicate a significant difference between the indicated sample and the "Cells + media" control. Plusses, "✜", indicate a significant difference between the indicated sample and the "LPS + SiO_2" treated sample. Significance level is equal to 0.05 and therefore a $p < 0.05$ results in a significant difference. Bars indicate mean ± SD.

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