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Guineensine increases the production of pro-inflammatory cytokines in activated M1 macrophages and shows inhibitory effects on resting and M2 macrophages

La guineensina aumenta la producción de citocinas proinflamatorias en macrófagos M1 activados y exhibe efectos inhibidores sobre los macrófagos en reposo y los macrófagos M2

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ABSTRACT

Guineensine, a natural compound from pepper plants, is an endocannabinoid uptake inhibitor and a potential indirect CB1 receptor (CB1R) agonist. While its cannabimimetic effects are established through inhibition of AEA (N-arachidonoylethanolamide) and 2-AG (2-arachidonoylglycerol) cellular uptake, its role in inflammation and macrophage activation remains unclear. Using the human THP-1 macrophage-like cell line, we investigated the effects of guineensine. Our results showed that when cells are stimulated with lipopolysaccharide (LPS) and IFN-γ (M1 activation), guineensine increased the production of IL-1β, TNF-α, and monocyte chemoattractant protein (MCP-1), with the latter two increasing up to 2.5-fold. Conversely, guineensine significantly decreased MCP-1 and IL-8 levels in both resting macrophages and those stimulated with IL-4 and IL-13 (M2 activation). Notably, guineensine did not significantly affect phagocytosis or respiratory burst (ROS production) in activated macrophages. These findings suggest that guineensine differentially modulates the immune response in M1, M2, and resting macrophages.

KEY WORDS: Guineensine, Inflammation, Cytokines, Macrophages.

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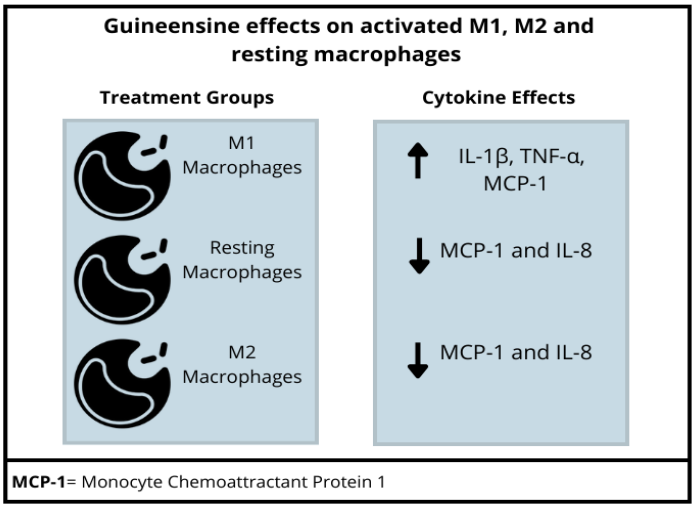
ABSTRACT

La guineensina, un compuesto natural de la planta del pimiento, es un inhibidor de la recaptación de endocannabinoides y un posible agonista indirecto del receptor CB1 (CB1R). Si bien sus efectos cannabimiméticos se basan en la inhibición de la recaptación celular de AEA (N-araquidonoiletanolamida) y 2-AG (2-araquidonoilglicerol), su papel en la inflamación y la activación de macrófagos aún no se comprende completamente. Utilizando la línea celular humana similar a macrófagos THP-1, investigamos el efecto de la guineensina. Nuestros resultados muestran que cuando las células se estimulan con lipopolisacárido (LPS) e IFN-γ (activación de M1), la guineensina aumenta la producción de IL-1β, TNF-α y proteína quimioatrayente de monocitos (MCP-1), aumentando estas dos últimas hasta 2.5 veces. Por el contrario, la guineensina redujo significativamente los niveles de MCP-1 e IL-8 tanto en macrófagos en reposo como en aquellos estimulados con IL-4 e IL-13 (activación de M2). Cabe destacar que la guineensina no afectó significativamente la fagocitosis ni la producción de ROS en macrófagos activados. Estos hallazgos sugieren que la guineensina modula de forma diferencial la respuesta inmunitaria en macrófagos M1, M2 y en reposo.

KEY WORDS: Guineensina, Inflamación, Citocinas, Macrófagos.

GRAPHICAL ABSTRACT



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Introduction

Guineensine is a fatty acid-derived isobutylamide conjugated to an aromatic ring through a six-carbon chain (Okogun & Ekong,1974). This compound has been isolated from several species of the plant genus *Piper*, such as *P. guineense* Schumach, *P. longum*, and *P. nigrum* (black pepper), which is considered the king of the spices worldwide. Guineensine is the fourth most abundant component of the acetone extracted from black pepper seeds (Singh, G *et al*., 2004). In addition to the use of the dry form of the spice, the aqueous extract of black pepper is widely used in Ayurvedic medicine due to the extensive biological activity attributed to the different components of the fruit.

Guineensine inhibits the activity of the alpha-glucosidase-I enzymes (Pullela *et al*., 2006) and cholesterol acyltransferase (Lee *et al*.,2004). A recent *in vivo* study has reported that guineensine induces dose-dependent cannabimimetic effects (hypomotility, hypothermia, antinociception, and catalepsy) (Nicolussi *et al*.,2014). The authors of this study suggest that guineensine could act as an indirect CB1 receptor (CB1R) agonist. They showed that guineensine is an inhibitor of endocannabinoid cellular uptake independent of fatty acid amide hydrolase (FAAH) or monoacylglycerol lipase (MAGL). This effect was found to be more evident in anandamide uptake (AEA) in U937 cells (EC_{50}= 290 nM) and HMC-1 cells (EC_{50}= 617 nM) (Nicolussi *et al*.,2014). Furthermore, this study also showed that guineensine administration reduces pro-inflammatory cytokine production during LPS-induced endotoxemia in a mouse model of acute inflammation. Together, these findings suggest that guineensine may be a promising therapeutic agent for controlling inflammatory effects and pain. However, little is known about the role of guineensine on the primary cells involved in inflammation and immune responses.

The mononuclear phagocyte system (MPS) is key to the development of innate and adaptive immune responses. The MPS comprises monocytes, macrophages, and dendritic cells (DC). In particular, monocytes and macrophages are crucial for initiating and resolving pathogen- or tissue-damage-induced inflammation. Monocytes are peripheral blood leukocytes that, in addition to serving as a reservoir for macrophages and DC, exhibit phagocytic activity and produce reactive oxygen species and inflammatory cytokines in response to various stimuli, such as exogenous bacterial products, lipopolysaccharide (LPS), and endogenous cytokines (Silva *et al*.,2018). Furthermore, it has been observed that the binding of the hematopoietic factors GM-CSF and IL-3 to their heterodimeric receptors formed by the common beta subunit and specific alpha (CD116 or CD123, respectively) is a potent factor for monocyte differentiation in macrophages (Tarique *et al*.,2015) and enhances LPS-induced TNF-α production in monocytes (Borriello *et al*., 2017). A greater response to GM-CSF has been observed, as CD116 is constitutively expressed, whereas CD123 is not. However, basal expression of CD123 has been observed to increase by co-culture with IL-4 (Borriello *et al*., 2015). This cytokine activation makes clear the role that monocytes can play in initiating the inflammatory response to various aggressor stimuli.

Macrophages are tissue-resident cells derived from embryonic precursors or peripheral blood monocytes. These cells perform a wide range of functions, including eliminating pathogens

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and apoptotic cells, maintaining homeostasis, promoting angiogenesis and tissue repair, and controlling tumor progression, all in response to various environmental signals (Silva *et al*.,2018). There are two main types of activated macrophages, M1 and M2. M1 macrophages, also known as classically activated macrophages, are activated by an inflammatory microenvironment, typically LPS and interferon (IFN)-γ, but also including GM-CSF and TNF-α; these cells are pro-inflammatory, tumoricidal, bactericidal, and associated with antigen presentation and clearance of intracellular pathogens. M2 macrophages, including tumor-associated immune-regulatory macrophages, in contrast, are activated by IL-4 and IL-13 (or TGF-β, IL-10, or other tumor-associated factors) and are associated with tumor promotion, immunoregulation, tissue remodeling, and anti-inflammatory responses (Tarique *et al*.,2015).

Regarding cytokine production, activated M1 cells produce pro-inflammatory cytokines, including TNF-α, IL-1β, IL-6, IL-12, Type I IFN, MCP-1, and IL-8. Whereas IL-10, IL-4, IL-1RA, and TGF-β are mainly produced by activated M2 cells. Many studies have shown the role of these two subtypes in inflammatory pathways, and their emergence appears to determine the fate of inflammatory signaling and disease progression (Saqib *et al*.,2018). Hence, this article focuses on the importance of analyzing the effect of guineensine on cytokine synthesis and the function of resting or activated macrophages.

Material and Methods

Preparation of Guineensine Extract

Guineensine (≥ 95 % pure in HPLC) was isolated from dry fruits of *P. nigrum* L. (obtained from Delaca AG, Switzerland). Powdered fruits (1.05 kg) were extracted with n-hexane, $CHCl_3$, and CH_3OH for guineensine isolation. Guineensine was characterized and identified by multidimensional NMR spectroscopy and mass spectrometry as previously described by Nicolussi S *et al*. Dimethyl sulfoxide (DMSO) was used to dissolve guineensine (Nicolussi *et al*.,2014).

Cell culture and THP-1 cell line differentiation

Human THP-1 monocytes were procured from the American Type Culture Collection (Cat. TIB-202, organism: *Homo sapiens*; disease: Acute monocytic leukemia; cell type: monocyte). Cells were maintained in RPMI 1640 medium containing 10 % heat-inactivated fetal bovine serum, 2 mM L-glutamine, 20 μM β-mercaptoethanol, 100 U/mL penicillin, and 100 U/mL streptomycin (complete RPMI medium). Cells were cultured in a humidified incubator at 37 °C with 5 % CO_2. Cultures were passaged every 3 days by resuspension to a density of 2 × 10^5 viable cells/mL in fresh complete medium. THP-1 cells were seeded at 1 × 10^6 cells/mL in 12-well culture plates and treated with 200 nM phorbol myristate acetate (PMA; Sigma-Aldrich) for 72 hours to induce differentiation into macrophages. After the PMA treatment, cells were gently washed twice with sterile phosphate-buffered saline (PBS, pH 7.2) to remove any residual PMA, and then rested in a fresh complete medium for 24 hours before the experiment.

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Cytokine production by THP1 cells

Resting THP-1 macrophages were pre-treated with guineensine at 290 nM or 1000 nM concentrations for 30 min. Following pre-treatment, the cells were stimulated for 24 hours to induce polarization: M1 Macrophages: Stimulated with 100 ng/mL LPS and 20 ng/mL IFN-γ; M2 Macrophages: Stimulated with 20 ng/mL IL-4 and 20 ng/mL IL-13; Unstimulated Control: No additional treatment. This polarization protocol is a classical technique for inducing macrophage differentiation, as previously described [11]. After the 24-hour stimulation period, cell supernatants were collected and clarified by centrifugation at 635 x g for 10 minutes. IL-1β, TNF-α, IL-12, MCP-1/CCL-2, and IL-8 concentrations were quantified using the LEGENDplex™ system (BioLegend, San Diego, CA, USA). Data acquisition was performed on an Attune Acoustic Focusing Cytometer (Life Technologies), and the analysis was conducted with the LEGENDplex™ Data Analysis Software (BioLegend). A total of 300 events for each cytokine bead population were acquired, as per the manufacturer's instructions. Results are expressed as concentration in pg/mL.

Phagocytosis assay

Resting THP-1 macrophages were treated with guineensine at 290 nM or 1000 nM concentrations for 24 hours. Following the treatment, cells were harvested using Accutase for 15 minutes at room temperature and washed twice with PBS. Subsequently, cells were incubated with 0.5 mg/mL of a pHrodo Green *E. coli* Bioparticle conjugate suspension (Life Technologies, Carlsbad, CA, USA) for 2 hours at 37 °C. The following control conditions were included: Blank (Unstained): Cells without bacteria; Control Baseline (CB): Cells incubated with bacteria but without guineensine treatment; Negative Control (NC): Cells pre-treated with 4 % paraformaldehyde and incubated at 4 °C to inhibit phagocytosis.

Bacterial internalization was quantified by measuring mean fluorescence intensity (MFI) using an Attune Acoustic Focusing Cytometer (Life Technologies). A total of 20,000 events within the cell region were acquired. We use the geometric mean to calculate MFI (mean fluorescence intensity). Data were analyzed using FlowJo v10.0.7 software. Relative phagocytosis was calculated as (MFI of experimental cells-MFI of B cells)/ (MFI of CB cells-MFI of B cells) X 100 %.

Reactive oxygen species determination

Intracellular ROS generation was quantified using the CellROX Green fluorogenic probe (Life Technologies, Carlsbad, CA, USA). THP-1 macrophages were treated, harvested, and washed as described for the phagocytosis assay. Following treatment, cells were incubated with 200 µM tert-butyl hydroperoxide (TBHP) for 1 hour to induce oxidative stress (positive control). Subsequently, cells were incubated with 0.5 µM CellROX Green reagent for an additional hour at 37 °C. SYTOX Red Stain (1 nM) was used to determine cell viability. Intracellular ROS production was measured by flow cytometry, and data were acquired on an Attune Acoustic Focusing Cytometer (Life Technologies). A total of 20,000 events within the cell region were collected per sample. Mean fluorescence intensity (MFI) was calculated as the geometric mean, and data were analyzed with FlowJo v10.0.7.

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Statistical analysis

Data are presented as the mean ± standard deviation (SD) of four independent experiments. Statistical significance was determined using one-way ANOVA with either Tukey's post-hoc test for multiple comparisons or Dunnett's post-hoc test for comparisons with the basal condition. A p-value of <0.05 was considered statistically significant.

Results and Discussion

Guineensine modulated cytokine levels in unstimulated THP-1 macrophage culture supernatants.

The effect of guineensine (290 nM and 1000 nM) on cytokine synthesis was evaluated in THP-1 macrophages. Cells were stimulated with LPS + IFN-γ or IL-4 + IL-13, with unstimulated cells used as a control group (Figure 1).

Guineensine treatment significantly decreased the secretion of both MCP-1 and IL-8 from THP-1 macrophages. MCP-1 levels were reduced by 80 % with 290 nM guineensine (2711.36 ± 274 pg/mL) and by 68 % with 1000 nM guineensine (4376.54 ± 19.19 pg/mL), when compared to the untreated control (13665.3 ± 163.9 pg/mL). Similarly, IL-8 secretion decreased by 78 % and 57 % at 290 nM (907.98 ± 12.59 pg/mL) and 1000 nM (1794.94 ± 80.43 pg/mL), respectively, relative to the untreated control (4122.87 ± 182.6 pg/mL).

When THP-1 cells were stimulated with LPS + IFN-γ, guineensine treatment at 290 nM enhanced the release of pro-inflammatory cytokines. IL-1β and TNF-α levels increased by 54 % and 78.6 %, respectively, compared to stimulated but untreated cells. The most pronounced effect was observed with MCP-1, which showed a >2.5-fold increase. In contrast, IL-8 secretion was not significantly affected by 290 nM guineensine, but decreased by 45.6 % at 1000 nM.

When the cells were stimulated with IL-4 + IL-13, guineensine synergized to inhibit the release of MCP-1 and IL-8 further. Treatment with 290 nM guineensine inhibited MCP-1 release by 69 % and IL-8 by 51 % ($p < 0.005$), relative to the stimulated but untreated control. This inhibitory effect was dose-dependent, with 1000 nM guineensine further decreasing MCP-1 and IL-8 levels by 80 % and 84 %, respectively.

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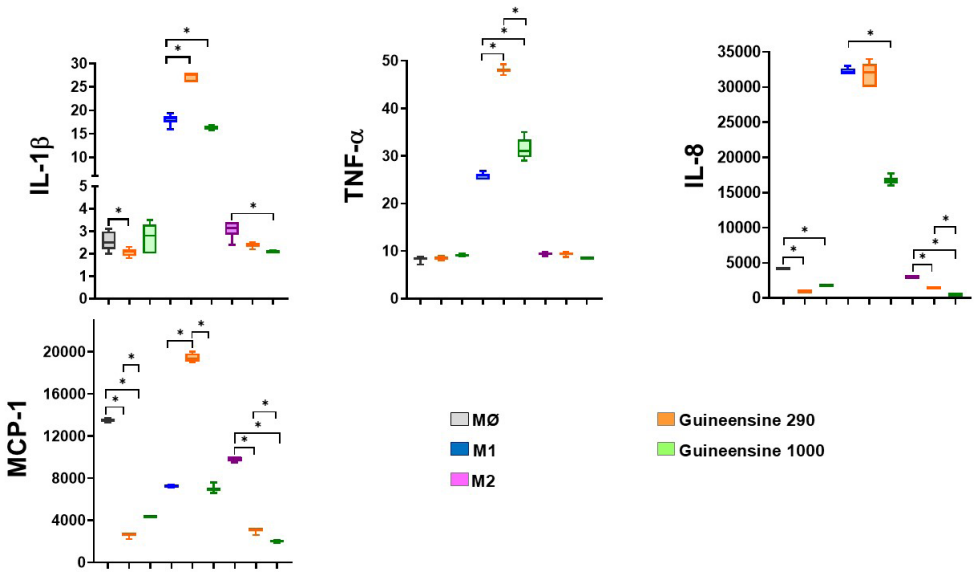

Figure 1. Secretion of pro-inflammatory cytokines by THP1 cells differentiated to macrophages, treated or not with guineensine and unstimulated (MØ), or stimulated with LPS plus IFN-γ (M1), or IL-4 plus IL-13 (M2).

LPS: lipopolysaccharide; IFN-γ: IL: interleukin; TNF-α: tumor necrosis factor alpha; MCP-1: monocyte chemoattractant protein-1. *p < 0.005. Data are presented as the mean ± standard deviation (SD) of four independent experiments.

Guineensine does not affect macrophage phagocytic activity.

To determine how guineensine influences macrophage phagocytic activity, we used pHRodo Green *E. coli* Bioparticles. When exposed to paraformaldehyde on ice, the cells' phagocytic activity was reduced by half compared to the control group. However, the phagocytic activity of cells treated with guineensine at 290 (51.07 %) or 1000 nM (46.5 %) was similar to that presented by THP-1 macrophages without guineensine (42.07 %) (Figure 2).

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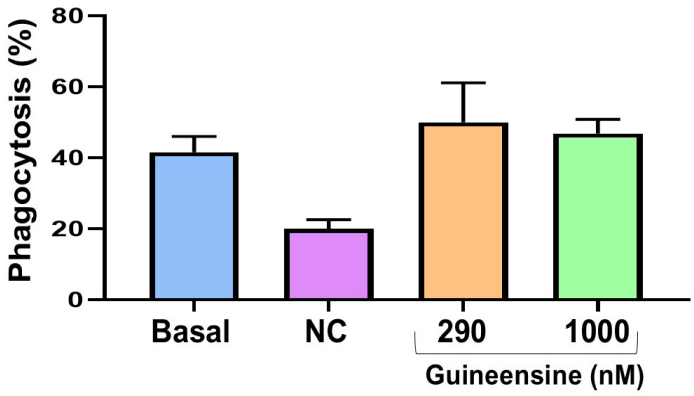

Figure 2. Effect of guineensine on the phagocytic activity of macrophage-differentiated THP-1 cells.

Paraformaldehyde on ice serves as a control of phagocytosis inhibition; a negative control (NC) was used. Data are representative of four independent assays. *$p < 0.005$.

Guineensine does not affect macrophage respiratory burst.

Then, the effect of guineensine on reactive oxygen species (ROS) production was analyzed in THP-1 macrophages using the CellROX Green fluorogenic probe. The respiratory burst was induced with tert-butyl hydroperoxide (TBHP). Treatment with guineensine at either 290 nM or 1000 nM did not significantly affect ROS production nor alter the oxidative burst induced by TBHP (Figure 3).

In the present study, we found that guineensine modulates the secretion of several cytokines (MCP-1, IL-8, IL-1β, and TNF-α) by resting or stimulated human macrophages, but this action does not affect cell functionality. This is important because macrophages are fundamental cells in the elimination of pathogenic agents.

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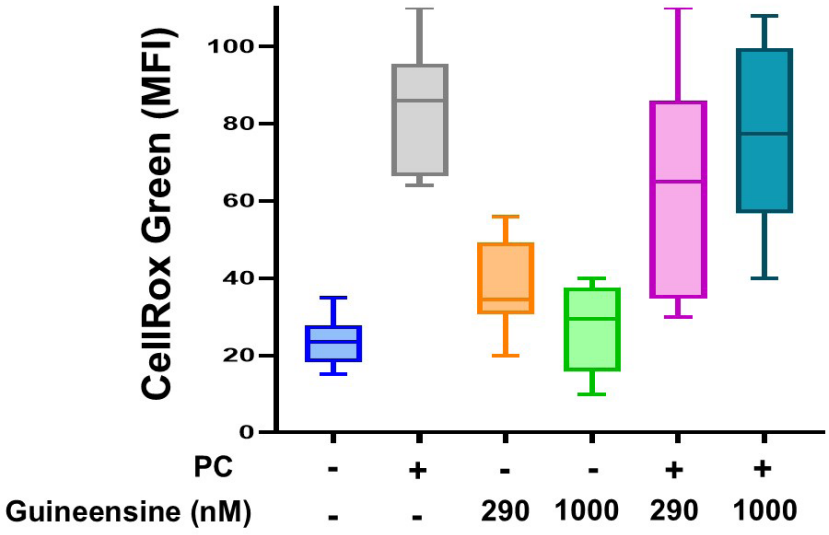

Figure 3. Effect of guineensine on the ROS production by macrophage-differentiated THP-1 cells.

As a positive control, we used (PC) TBHP. Data are representative of four independent assays. *$p < 0.05$.

A study showed that guineensine is a potent inhibitor of AEA cellular uptake; its effect was observed in U937 cells with an EC_{50} of 290 nM (95 % CI = 190-437 nM) and in HMC-1 with an EC_{50} of 617 nM (95 % CI = 451-844 nM) (Nicolussi *et al*.,2014). Furthermore, at 10 μM, it has been reported to interact with the δ receptor, the 5-HT2A and 5-HT2B serotonin receptors, and the dopamine transporter (Reynoso-Moreno *et al*., 2017). In our study, we evaluated the effects of N-isobutylamide at 290 nM and 1000 nM on THP-1 cells differentiated into macrophages. With the second condition, we intend to work outside the concentration range where it has been shown that guineensine inhibits the reuptake of endocannabinoids. Our results showed that guineensine modulates the synthesis of pro-inflammatory cytokines. In resting macrophages, guineensine reduced the expression of IL-8 and MCP-1, both chemokines important in initiating the inflammatory response. PMA, a potent inducer of differentiation, has been reported to upregulate CB1 and increase the CB1:CB2 transcript ratio from 1:17.5 to 1:3 over 5 days of culture (Han *et al*., 2009). However, our cells were cultured for only 24 hours after differentiation in RPMI-S, a medium enriched with 10 % FBS. It is known that FBS contains 2-AG at biologically active concentrations (250 to 700 nM) (Marazzi *et al*., 2011). This could be a limitation of the present study, as 2-AG is a full agonist at human CB2 and an inhibitor of cAMP accumulation, thereby attenuating cytokine

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production (Gonsiorek *et al*.,2000; Sugiura *et al*.,2000), an effect that could be enhanced by inhibiting cellular uptake of endocannabinoids. Interestingly, we observed a higher increase in MCP-1 in MØ than in those stimulated with LPS + IFN-γ. It is important to note that this chemokine is secreted by monocytes, macrophages, and dendritic cells, not by M1 macrophages. In this regard, some studies showed that THP1, U937, and primary macrophages stimulated with IFN-γ + LPS induce an M1 phenotype, with high production of IL-1, IL-18, RANTES, G-CSF, IFN-γ, GM-CSF, and IL-2, without significant changes in MCP-1 (Chimal-Ramírez *et al*.,2016). In particular, in our study, we observed increased IL-1β and IL-8 levels in macrophages stimulated with LPS + IFN-γ compared with macrophage differentiation with PMA alone, consistent with previous studies examining IL-1β and IL-8 production by M1 macrophages (Murray *et al*., 2014).

Moreover, when the cells were stimulated with IL-4 + IL-13, guineensine at 290 or 1000 nM maximized inhibition of IL-8 and MCP-1 production. Recently, it has been reported that M2-like human lung macrophages produce more 2-AG than AEA under baseline conditions; additionally, CB2 expression is higher than CB1 (Staiano *et al*., 2016). Thus, as in resting macrophages, it is likely that CB2 activation by 2-AG inhibits the synthesis of these cytokines.

Conversely, the release of IL-1β, TNF-α, and MCP-1 by the activation with LPS/IFN-γ is potentiated by the addition of guineensine at 290 nM. However, at 1000 nM, IL-8 release was decreased. This is probably because guineensine has greater activity in inhibiting cellular uptake of AEA. AEA production was evaluated in murine macrophages (J774) and in rat circulating macrophages, and was higher than that of 2-AG. Stimulation of J774 macrophages with either ionomycin or LPS was reported to induce 2- and 7.8-fold increases in 2-AG and AEA levels, respectively (Di Marzo *et al*.,1999). Likewise, *in vitro* LPS stimulation, with or without IFN-γ, decreases CB2 expression in macrophages (Carlisle *et al*.,2002). This could improve AEA binding to CB1. CB1 activation enhances macrophage pro-inflammatory activities through p38-MAPK activation, increasing TNF-α and MCP-1 production (Han *et al*., 2009). Additionally, 2-AG signaling via CB2 has been reported to induce the enhanced production of IL-8 and MCP-1 in HL60 cells (Kishimoto *et al*.,2004).

Our results agree with those of other studies evaluating the effects of CBD on the macrophage inflammatory response (Muthumalage & Rahman,2019; Yeisley *et al*., 2021). In one study, macrophages were exposed to different concentrations of CBD, which showed an anti-inflammatory profile by reducing LPS-induced IL-8 levels, similar to the findings of this study using guineensine at 1000 nM (Muthumalage & Rahman,2019). Similarly, another study showed reduced levels of IL-1β, TNF-α, and IL-6 after CBD treatment (Yeisley *et al*., 2021).

Interestingly, despite the modulation of cytokine synthesis by THP-1 cells, no significant effects on their functionality were observed. CB1 activation via AEA has been reported to directly induce intracellular ROS generation (Han *et al*., 2009). Moreover, activation of the CBD receptors has been shown to decrease the levels of phosphorylated p-mTOR S2448 and NOS-3, with an increase in COX-2, indicating induction of oxidative stress and autophagy (Yeisley *et al*., 2021). On the other hand, 2-AG signaling through CB2 has been shown to increase dectin-1-mediated phagocytosis of zymosan but not of *E. coli*, *S. aureus*, apoptotic cells, or latex beads (Shiratsuchi

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et al.,2008). A more recent work also indicates that AEA enhances phagocytosis through CB1 via the $G_{(\alpha)i/o/}$RhoA/ROCK signal axis, although this effect is abolished at high concentrations (Mai *et al*., 2015).

The results from these experiments demonstrate that guineensine modulates the monocyte/macrophage inflammatory response in a state-dependent manner, with differential effects observed in M1, M2, and resting macrophages. Given these findings, future studies should investigate the effect of guineensine on other immune cells involved in inflammation and immune responses, such as dendritic cells. A significant limitation of the present study is its *in vitro* nature; therefore, further research should evaluate the effects of guineensine in animal models. Such *in vivo* studies could focus on its potential therapeutic role in conditions like sepsis, bacterial translocation, and cannabinoid receptor-mediated pain.

Conclusions

Our study demonstrates that N-isobutylamide guineensine modulates cytokine production in THP-1 macrophages without affecting their functional capacity for phagocytosis or respiratory burst in the presence of bacterial antigens. We found that an intermediate dose of guineensine enhanced the production of pro-inflammatory cytokines, specifically IL-1β and TNF-α, in M1-activated macrophages. In contrast, guineensine inhibited IL-8 and MCP-1 secretion in both resting and M2-polarized macrophages.

Author Contribution

MJ. - Conceptualization, methodology, formal analysis, writing-original draft preparation. JMVP. - Conceptualization, validation, writing, review, and editing. JRVU. - methodology, writing-review, and editing. FSI. -formal analysis, writing, review, and editing. MCTB. - formal analysis, writing, review, and editing. JH. - formal analysis, writing, review, and editing. PCOL. - Conceptualization, methodology, writing—original draft preparation, project administration.

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Conflicts of Interest

There are no conflicts of interest.

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