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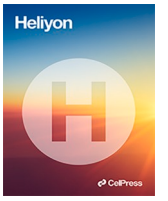

Pseudomonas aeruginosa quorum-sensing molecule N-(3-oxododecanoyl)-L-homoserine lactone mediates Ca^{+2} dysregulation, mitochondrial dysfunction, and apoptosis in human peripheral blood lymphocytes

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ABSTRACT

N-(3-oxododecanoyl)-L-homoserine lactone is a *Pseudomonas aeruginosa* secreted quorum-sensing molecule that mediates the secretion of virulence factors, biofilm formation and plays a pivotal role in proliferation and persistence in the host. Apart from regulating quorum-sensing, the autoinducer signal molecule N-(3-oxododecanoyl)-L-homoserine lactone (3O–C12-HSL or C12) of a LasI–LasR circuit exhibits immunomodulatory effects and induces apoptosis in various host cells. However, the precise pathophysiological impact of C12 on human peripheral blood lymphocytes and its involvement in mitochondrial dysfunction remained largely elusive. In this study, the results suggest that C12 (100 μM) induces upregulation of cytosolic and mitochondrial Ca^{+2} levels and triggers mitochondrial dysfunction through the generation of mitochondrial ROS (mROS), disruption of mitochondrial transmembrane potential (ΔΨm), and opening of the mitochondrial permeability transition pore (mPTP). Additionally, it was observed that C12 induces phosphatidylserine (PS) exposure and promotes apoptosis in human peripheral blood lymphocytes. However, apoptosis plays a critical role in the homeostasis and development of lymphocytes, whereas enhanced apoptosis can cause immunodeficiency through cell loss. These findings suggest that C12 exerts a detrimental effect on lymphocytes by mediating mitochondrial dysfunction and enhancing apoptosis, which might further impair the effective mounting of immune responses during *Pseudomonas aeruginosa*-associated infections.

1. Introduction

Pseudomonas aeruginosa is a ubiquitous opportunistic gram-negative pathogen, causing severe morbidity and mortality in immunocompromised individuals [1]. It causes chronic infections in both cystic fibrosis and non-cystic fibrosis patients [2]. Approximately 16.2 % of intensive care unit patients acquire *P. aeruginosa* infections due to nosocomial transmission [3,4], with mortality rates soaring from 18 % to 61 %, higher than other infections [5,6]. *P. aeruginosa* possesses a dynamic range of mechanisms for adaptation and survival, due to which it causes disease in both plants and animals [7]. Furthermore, it possesses multiple mechanisms for antibiotic resistance and virulence factors, making it resistant to several antibiotics and capable of causing life-threatening infections [8].

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Quorum sensing (QS) is a density-dependent communication mechanism in prokaryotes that regulates gene expression and secretion of virulence factors. *Pseudomonas aeruginosa* employs an arsenal of virulence factors driven by QS to thrive and persist in the host [9–11]. QS coordinates the expression of genes through the production of the auto-inducer signal molecules [12]. At threshold density, these molecules bind to their cognate receptor proteins, thereby controlling gene expression [13]. There are four different quorum-sensing systems in *P. aeruginosa*: LasI–LasR, RhlI–RhlR, PQS (Pseudomonas quinolone signal-dependent QS system), and the IQS (integrated quorum-sensing signal) system. The LasI–LasR QS circuit uses N-3-oxo-dodecanoyl-L-Homoserine lactone (C12) as an auto-inducer signal molecule [14], N-butyryl-L-homoserine lactone (C4) is an auto-inducer for RhlI–RhlR, 2-heptyl-3-hydroxy-4 (1H)-quinolone (PQS) for the PQS system [15], and 2-(2-hydroxylphenyl)-thiazole-4-carbaldehyde (IQS) for the IQS system [16,17].

C12 is a primary quorum-sensing auto-inducer signal molecule that mediates the secretion of *P. aeruginosa*-associated virulence factors and biofilm formation [18,19]. At the same time, C12 QS mutants are shown to have decreased pathogenicity in the host [7,8]. For host colonization, C12 regulates the secretion of virulence factors such as alkaline protease, elastases, lipases, neuraminidase, exotoxin A, and secretion of siderophore [20]. Among other virulence factors, C12 also regulates the T2SS (type II secretion system) [20]. The presence of C12 molecules in the sputum and infected lungs of chronic cystic fibrosis patients makes this QS signal molecule clinically relevant for a better understanding of the disease conditions [21].

C12 is also shown to exert an immunomodulatory effect in mammalian hosts; it has been demonstrated that C12 activates pathogen-associated molecular patterns (PAMPs) and inhibits protein translation through eukaryotic initiation factor 2 (eIF2α) phosphorylation [22]. Moreover, C12 activates mitogen-activated protein kinases (MAPK kinase) signaling by activating p38 mitogen-activated protein kinases (p38 MAP kinase) [22]. Furthermore, C12 downregulates immune function through its interaction with peroxisome proliferator-activated receptor (PPAR), and it can inhibit PPAR even at a low dose of 1 nM [23]. Activation of the mitochondrial intrinsic apoptotic pathway is also implicated in the detrimental effect of C12 [24–26]. Additionally, C12 also alters calcium signaling, activating Ca^{+2}-dependent pro-apoptotic events in various host cells [27–31]. Moreover, C12 is also shown to induce neutrophil migration through actin remodeling and calcium mobilization [32]. In immune cells, C12 induces apoptosis by inducing mitochondrial depolarization and phosphatidylserine (PS) exposure [27]. Furthermore, C12 downregulates inflammatory responses in macrophages and induces apoptosis [25,33]. Meanwhile, in dendritic cells, C12 mediates an anti-inflammatory response by impeding the expression of pro-inflammatory cytokines like Interleukin-12 (IL-12) and Interferon-γ (IFN-γ) and by increasing anti-inflammatory cytokine Interleukin-10 (IL-10) [34,35]. According to these studies, C12 may contribute to establishing chronic infection by suppressing adaptive immunity and inducing cell death in host cells.

Lymphocytes are a crucial component of the immune system, and their homeostasis is essential for proper immune function, and their dysregulation is associated with immune pathophysiology [36]. During infection, lymphocytes play a vital role in identifying foreign antigens, proliferating, and differentiating to develop effector functions that eliminate pathogens, this coordinated response by lymphocytes is instrumental in combating infections by maintaining immune function through B-cell mediated adaptive immune responses and T Cell-Mediated Immunity [37]. Pathogens can modulate lymphocyte function to subvert immune responses [38]. C12 has been shown to impair immune function; as it reduces PBMCs cell viability and induces apoptosis in human Jurkat T lymphocytes through the mitochondrial pathway [39,40]. However, the intricate molecular underpinning of human peripheral blood lymphocytes with C12 and its downstream cellular effects on mitochondria are still unknown.

Mitochondria play a pivotal role in regulating crucial cellular physiology and homeostasis [41]. They are responsible for energy metabolism, cellular respiration, and ATP generation [42]. In parallel, mitochondria coordinate signaling pathways such as innate immunity, stress response, and calcium signaling [43,44]. Energetic impairment and oxidative stress perturbations in mitochondria can be detrimental to cellular physiology and mediate pathophysiological events [45].

The role of cellular Ca^{+2} signaling in maintaining homeostasis is well-established, and it is tightly regulated by intracellular stores and channels present in the plasma membrane [46]. However, capacitive Ca^{+2} influx of extracellular Ca^{+2} through channels and intracellular Ca^{+2} release from the endoplasmic reticulum have been shown to be apoptogenic [47]. Aberrant cytosolic Ca^{+2} transfer from the cytosol to mitochondria leads to mitochondrial Ca^{+2} overload, which further mediates apoptosis signaling [48]. The decisive role of cell fate between life and death depends on the amount of Ca^{+2} taken in the mitochondrial matrix [49,50]. In contrast, it has been shown that the transient rise in mitochondrial Ca^{+2} at physiological levels regulates respiratory chain activity and ATP synthesis [49]. However, excessive mitochondrial Ca^{+2} overload is pathological and induces apoptosis by generating genotoxic mROS (mitochondrial ROS), causing mitochondrial transmembrane potential loss ($\Delta\Psi m$), and opening the mPTP (mitochondrial permeability transition pore). Prolonged mPTP opening induces the efflux of mitochondrial cytochrome *c*, intermembrane space proteins, and ions to the cytosol; furthermore, the release of cytochrome *c* leads to the induction of caspase-mediated apoptosis [51].

This study evaluated the role of C12 in the impairment of human peripheral blood lymphocytes and demonstrated that C12 induces cytosolic and mitochondrial Ca^{+2} imbalance, perturbations in mitochondrial functions, and subsequent induction of apoptosis. These results are helpful in unraveling the potential interplay between *P. aeruginosa*-associated QS signal molecules and human immune lymphocyte cells.

2. Materials and methods

2.1. Lymphocytes isolation

For lymphocyte isolation, 10 mL of blood was collected from healthy volunteers (n = 6, male = 3, female = 3, age = 22–38 years) into a K_2-EDTA vacutainer (BD Biosciences, San Jose, CA, USA) and inverted 4–6 times. Peripheral blood mononuclear cells (PBMCs) were isolated by carefully layering the blood on top of Polymorphprep (Axis-shield AG, UK) in a 1:1 ratio and centrifuged at *500×g* for

40 min at room temperature [27]. The Buffy coat was carefully taken out and suspended in Hank's Balanced Salt Solution (HBSS) (Himedia, India) and washed twice with 5 mL of HBSS at *300×g* for 5 min. PBMCs were then maintained at 1×10^6 cells/mL in an appropriate culture medium or buffer and seeded in 12-well plates for further experiments. PBMCs consist of lymphocytes (B-cells, T-cells, and NK cells), dendritic cells, and monocytes. The lymphocyte cell population was gated during the analysis using the flow cytometer BD Accuri C6 (BD Biosciences) and FlowJo analysis software (BD Biosciences).

2.2. MTT assay

Isolated peripheral blood mononuclear cells (PBMCs) were seeded in a tissue culture grade 96-well plate at a density of 10,000 cells per well in RPMI-1640 medium (Himedia) supplemented with 10 % FBS (Gibco, Thermo Scientific, USA) and 100 U/mL penicillin-streptomycin (Gibco). The cells were then treated with varying concentrations of C12 (10, 25, 50, and 100 μM, Sigma-Aldrich, USA) and then incubated for 2 h at 37 °C with 5 % CO_2 under humidified conditions. After the incubation, 50 μg/100 μL of MTT (Himedia) was added to each well and further incubated for 4 h; after the appearance of formazan crystals, the supernatant medium was discarded, and the formazan crystals were dissolved by adding 100 μL DMSO (Himedia) to each well and mixed properly; the absorbance at 570 nm was measured using a multimode plate reader (BioTEK, Synergy, USA). The results were expressed as percentage cell viability [52].

2.3. Calcium measurement

For the measurement of cytosolic Ca^{+2} and mitochondrial Ca^{+2}, cells were stained either with Fluo-4 AM (5 μM) (Molecular Probes, Invitrogen, USA) or Rhod-2 AM (5 μM) (Molecular Probes, Invitrogen) respectively for 45 min at 37 °C in HBSS without Ca^{+2} and Mg^{+2} (Himedia), following the manufacturer's instructions. Stained PBMCs were washed three times with HBSS (without Ca^{+2} and Mg^{+2}) and subsequently treated with C12 (100 μM) for 2 h; here, DMSO (0.1 % v/v) was used as the vehicle control. The flow cytometer BD Accuri C6 with lymphocyte gating was used to analyze PBMCs after C12 treatment using the FL-1 channel for cytosolic Ca^{+2} (Fluo-4 AM) or the FL-2 channel for mitochondrial Ca^{+2} (Rhod-2 AM). The data were analyzed using FlowJo software.

2.4. Mitochondrial ROS measurement

The generation of mitochondrial ROS was assessed using MitoSOX dye (Molecular Probes, Invitrogen). Isolated PBMCs were treated with C12 (100 μM) for 2 h, and DMSO (0.1 % v/v) was used as the vehicle control. After the treatment, the cells were stained with MitoSOX (5 μM) for 15 min at 37 °C [52]. Stained cells were subsequently washed thrice and resuspended in HBSS. The generation of mitochondrial ROS was measured using an FL-2 channel in flow cytometer BD Accuri C6, and data were analyzed using FlowJo software with lymphocyte gating.

2.5. Mitochondrial membrane potential measurement assay

The mitochondrial membrane potential (MMP) was assessed using the cationic probe 5,5 V, 6,6 V-tetrachloro-1,1 V 3,3 V-tetra ethylbenzimidazolcarbocyanine iodide (JC-1, Mitoscreen kit, BD Biosciences). Briefly, isolated cells were treated with C12 (100 μM, 2 h) and carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP, 10 μM, 30 min) (Sigma-Aldrich). After treatment, the cells were stained with JC-1 (5 μM) for 15 min at 37 °C, as per manufacturer's instructions. Stained and treated PBMCs were subsequently washed thrice with HBSS, and mitochondrial potential ($\Delta\psi m$) were analyzed using the BD Accuri C6 flow cytometer and FlowJo software with lymphocyte gating.

2.6. Mitochondrial permeability transition pore formation assay

mPTP (mitochondrial permeability transition pore) opening was assessed by the cobalt quenching assay of Calcein-AM fluorescence. Briefly, isolated cells were pre-stained with 1 μM Calcein-AM (Molecular Probes, Invitrogen) in the presence of 1 mM $CaCl_2$ at 37 °C in the dark for 30 min. After Calcein-AM loading, 1 mM of $CoCl_2$ was added, and cells were then incubated for another 10 min [27]. Quenched cells were then treated with C12 (100 μM, 2 h) and A23187 calcium ionophore (Sigma-Aldrich) (1 μM, 1 h). mPTP opening was analyzed using the flow cytometer BD Accuri C6 (BD Biosciences), and the data were analyzed using FlowJo software.

2.7. Apoptosis detection assay

An apoptosis detection assay based on Annexin V-FITC and PI was used to assess PS exposure and cell death [53]. Briefly, isolated PBMCs were treated with C12 (100 μM, 2 h). After treatment, cells were stained with Annexin V-FITC (2.5 μg/mL) (Santa Cruz Biotechnology, USA) and Propidium iodide (10 μg/mL) (Sigma-Aldrich) for 20 min at room temperature in Annexin V binding buffer as per manufacturer's protocol. Apoptosis induction was detected using the flow cytometer BD Accuri C6. Data were analyzed using FlowJo software with lymphocyte gating.

2.8. Statistical analysis

All experiments were performed independently and in triplicate (repeated at least three times). The data are presented as the mean value ± standard error of the mean (SEM) and were considered statistically significant at $p < 0.05$. Significance was calculated by One-way ANOVA, and statistical analysis was performed using GraphPad Prism (Version 8.0.1).

3. Results

3.1. C12 reduces PBMCs cell viability

The cytotoxic effect of C12 on isolated human PBMCs was initially investigated. Cells were treated for 2 h with varying concentrations of C12, and DMSO (0.1 % v/v) was used as the vehicle control. A dose-dependent decrease in cell viability due to C12 exposure was observed (Fig. 1a), with significant cell death observed from C12 concentrations of 50 μM onwards, whereas C12 (10–25 μM) does not show any effect on PBMCs cell viability. Additionally, PBMCs treated with C12 (100 μM, 2 h) exhibited a 50 % reduction in cell viability. These results indicate that C12 reduces PBMC viability within 2 h. To study the impact of C12 on lymphocytes, the lymphocyte population was gated from the whole PBMCs, as depicted in Fig. 1b, which shows a SC/FSC plot of whole PBMCs that are gated for the lymphocyte's population. Further, to understand the mechanistic insight into C12-induced detrimental effects on lymphocytes, all experiments were conducted at a cytotoxic dose of C12 (100 μM) within a 2-h time frame.

3.2. C12 induces Ca^{+2} dysregulation in lymphocytes

Ca^{+2} regulates diverse aspects of cellular physiology, and its dysregulation is linked with impaired homeostasis. Sustained Ca^{+2} overload is shown to be pro-apoptotic and mediates apoptosis in diverse cell types [54]. Several investigations have linked the role of cytosolic Ca^{+2} overload with mitochondrial dysfunction, which further aggravates cellular signaling to apoptosis or necrosis [55]. C12 is also shown to be a potent modulator of Ca^{+2} signaling in different cells and induces Ca^{+2}-dependent apoptosis [28,30]. So, it is intriguing to evaluate the impact of C12 on cytosolic Ca^{+2} in peripheral blood lymphocytes. Isolated PBMCs were stained with Fluo-4 AM and examined by flow cytometer with population gating on lymphocytes. The findings revealed that treatment with C12 (100 μM) for 2 h elevated cytosolic Ca^{+2} levels compared to the DMSO control (Fig. 2a and b).

Furthermore, mitochondria buffer the transient rise in cytosolic Ca^{+2} to evade cytosolic Ca^{+2} overload, but excessive and sustained Ca^{+2} overload induces apoptosis. To delineate the role of mitochondria during cytosolic Ca^{+2} release, mitochondrial Ca^{+2} levels were measured, and it was observed that C12 induces a rise in mitochondrial Ca^{+2} levels (Fig. 2c and d). These results suggest that C12 (100 μM) increases cytosolic Ca^{+2} and mitochondrial Ca^{+2} in peripheral blood lymphocytes, which might alter other Ca^{+2}-dependent physiological processes.

3.3. C12 induces mitochondrial ROS generation in lymphocytes

It has been shown that sustained and excessive cytosolic Ca^{+2} signal causes mitochondrial Ca^{+2} overload, leading to mitochondrial dysfunction and ROS generation [49,56]. Hence, we investigated whether C12 induces mitochondrial ROS generation in human lymphocytes. PBMCs treated with C12 were stained with the cell-permeable mitochondrial ROS-specific fluorescent dye MitoSOX, which emits enhanced fluorescence upon reaction with mitochondrial ROS. It was observed that C12 (100 μM) induces significant mitochondrial ROS generation (Fig. 3a and b) at a 2 h time frame, in comparison to DMSO control (Fig. 3a and b).

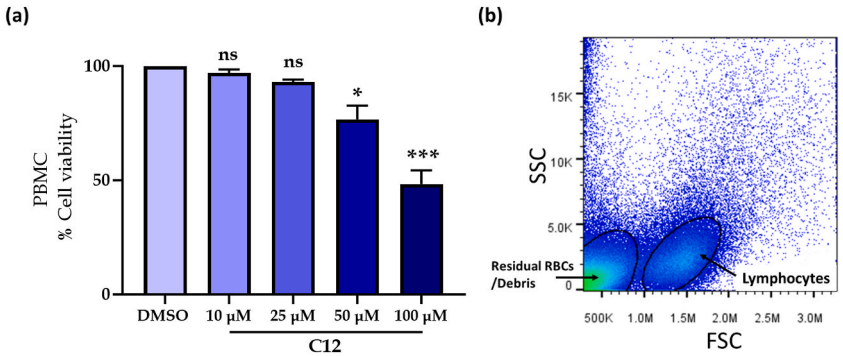

Fig. 1. C12 decreases PBMCs' cell viability. (a) The percentage cell viability of PBMCs was determined by treating PBMCs with varying concentrations of C12 (10–100 μM) for 2 h, followed by an MTT assay. (b) The lymphocyte population was gated from total PBMCs for further analysis of lymphocytes. The results are presented as mean ± SEM and represent significant data, ns = non-significant, *$p < 0.05$ and ***$p < 0.001$.

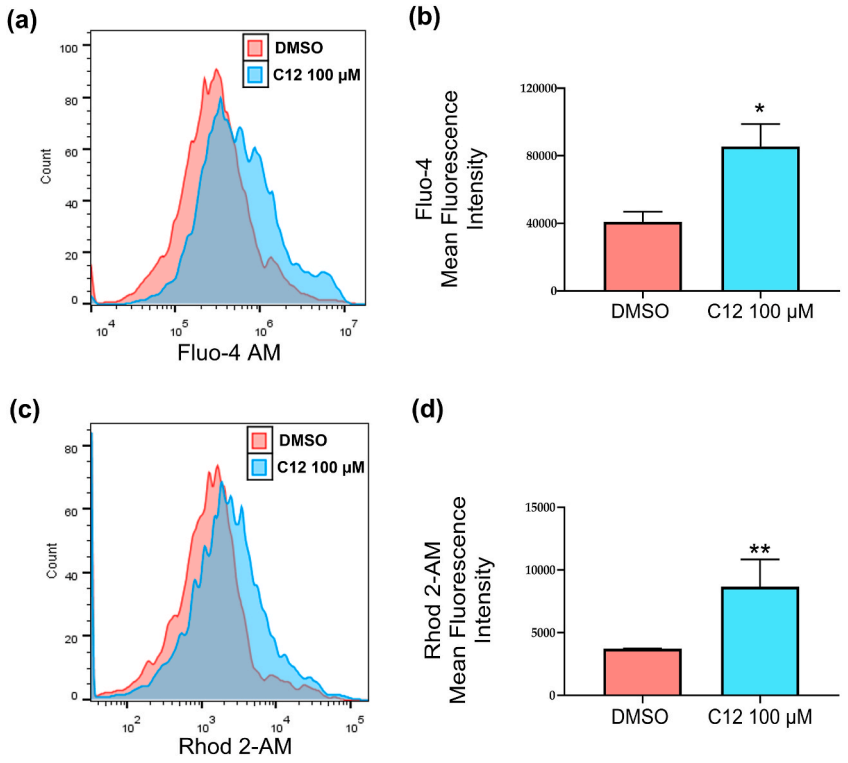

Fig. 2. C12 induces calcium dysregulation in peripheral blood lymphocytes. (a) PBMCs were stained with Fluo-4 AM (5 μM) for 45 min, followed by C12 (100 μM) treatment for 2 h. Cytosolic calcium levels in lymphocytes were analyzed by flow cytometer after gating the lymphocyte population. (b) Bar graphs represent the mean fluorescence intensity (MFI) of FL-1 (Fluo-4 AM) in Fig. 2a. (c) PBMCs were stained with Rhod-2 AM (5 μM) for 45 min. Stained cells were treated with C12 (100 μM) for 2 h, and mitochondrial calcium levels in lymphocytes were analyzed by flow cytometer after gating the lymphocytes population. (d) Bar graphs represent the mean fluorescence intensity (MFI) of FL-2 (Rhod-2 AM) in Fig. 2c. The results are presented as mean ± SEM and represent significant data, *$p < 0.05$, **$p < 0.01$.

3.4. C12 induces mitochondrial membrane potential drop in lymphocytes

Excessive mitochondrial ROS generation is linked to MMP loss, so we then analyzed MMP with JC-1 dye; a decrease in FL-2 (JC-1 aggregates-polarised mitochondria) and an increase in FL-1 (JC-1 monomers-depolarised mitochondria) shows a loss in MMP and mitochondrial dysfunction [57,58]. For MMP estimation, PBMCs were treated with C12 (100 μM, 2 h) and then stained with JC-1 dye for 15 min, washed thrice in HBSS, and resuspended in HBSS, and acquired using a flow cytometer. It was observed that C12 causes

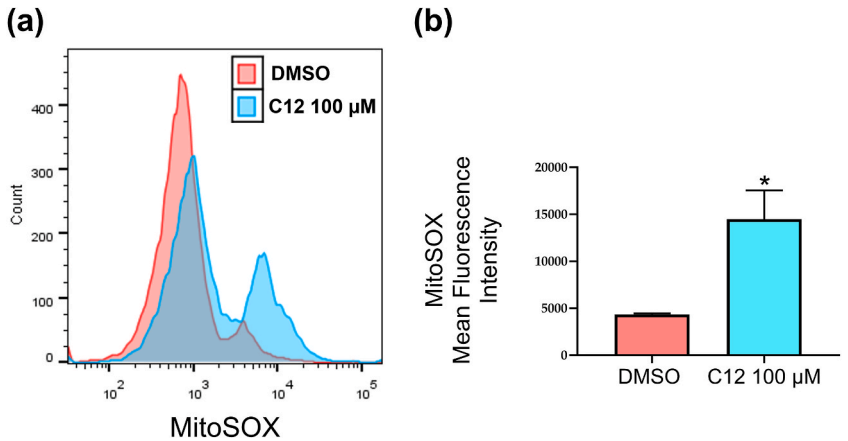

Fig. 3. C12 induces mitochondrial ROS generation in peripheral blood lymphocytes. (a) PBMCs were treated with C12 (100 μM) for 2 h, and the generation of mitochondrial ROS was analyzed by flow cytometry after staining with MitoSOX (5 μM) for 15 min. (b) Bar graphs represent the mean fluorescence intensity (MFI) of FL-2 (MitoSOX) in Fig. 3a. The results are presented as mean ± SEM and represent significant data, *$p < 0.05$.

MMP loss in 58.6 % of lymphocytes as compared to DMSO control (25.6 %) (Fig. 4a and b); here, CCCP (mitochondrial depolarizing agent) was used as a positive control. Based on these results, it was concluded that C12 induces mitochondrial depolarization in human peripheral blood lymphocytes.

3.5. C12 induces mitochondrial permeability transition pore (mPTP) opening in lymphocytes

As mPTP opening promotes cell death due to increased mitochondrial Ca^{+2} that mediates mitochondrial dysfunction [59], we investigated the impact of C12 on mPTP opening in human lymphocytes using the Calcein-AM/$CoCl_2$ assay. Calcein-AM is a cell-permeable fluorophore that is trapped in all subcellular compartments, including mitochondria. $CoCl_2$ quenches all the Calcein-AM except in the mitochondrial matrix when the mPTP pore is closed. As mPTP opens, $CoCl_2$ quenches the Calcein-AM in the mitochondrial matrix, resulting in decreased fluorescent intensity. This assay allows differentiation between open and closed mPTP status of mitochondria. Upon treatment with C12 (100 μM, 2 h), lymphocytes decreased Calcein-AM fluorescence, suggesting that C12 induces mPTP opening of mitochondria in lymphocytes as compared to DMSO control (Fig. 5a and b). Additionally, A23187 calcium ionophore, a known inducer of mPTP opening, was used as a positive control. These results suggest that C12 induces mPTP pore opening in human peripheral blood lymphocytes.

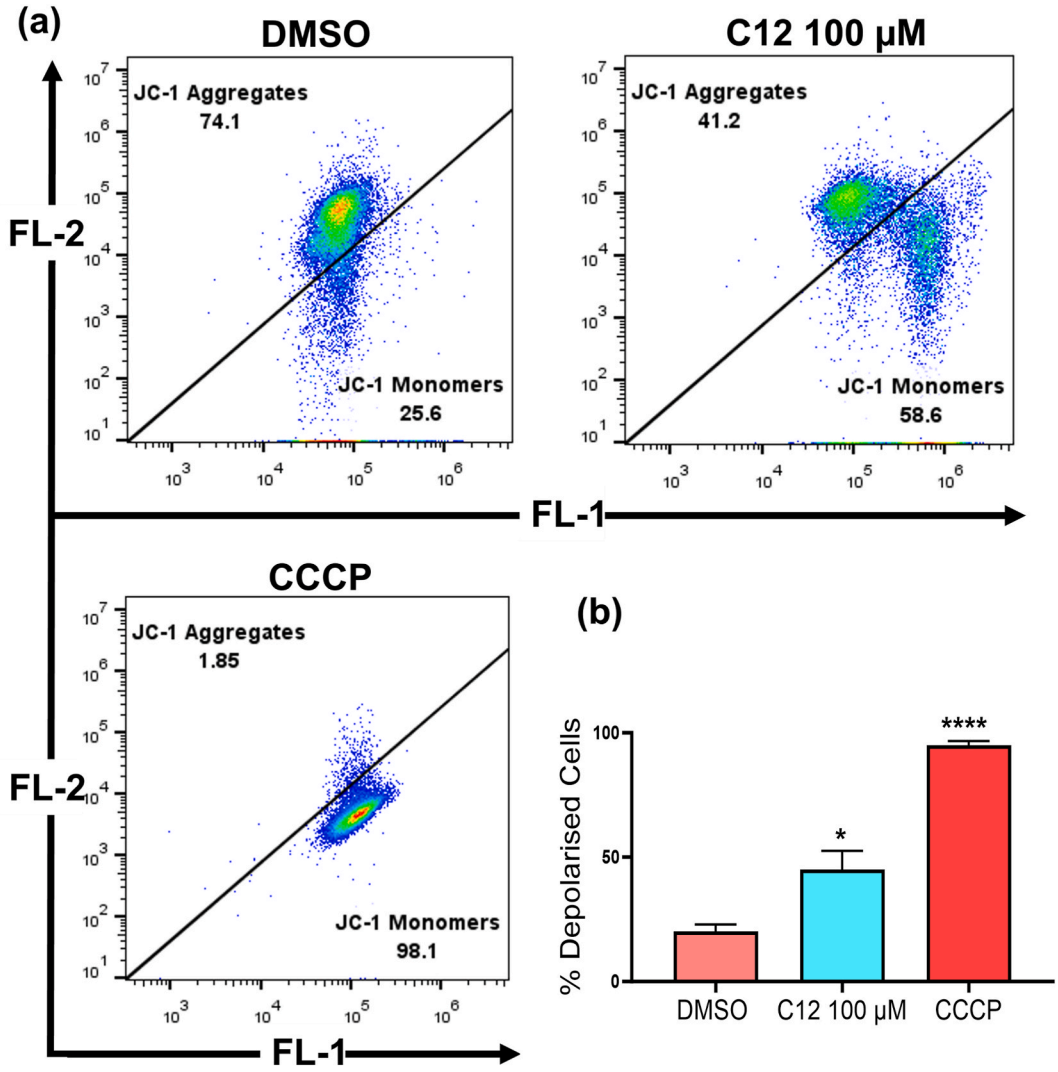

Fig. 4. C12 induces mitochondrial potential loss in peripheral blood lymphocytes. (a) PBMCs were treated with C12 (100 μM, 2 h), and mitochondrial membrane potential (MMP) was analyzed by flow cytometry after staining with JC-1 (5 μM) for 15 min. MMP was assessed by red fluorescence (FL-2, JC-1 aggregates) and green fluorescence (FL-1, JC-1 monomers) fluorescence intensity. (b) Bar graphs represent the percentage of cells with low membrane potential from Fig. 4(a). The results are presented as mean ± SEM and represent significant data, **p < 0.01, ****p < 0.0001.

3.6. C12 induces apoptosis in lymphocytes

Since mitochondrial dysfunction is a mediator of the mitochondrial-driven intrinsic apoptosis pathway, the effect of C12 on the induction of apoptosis in lymphocytes was investigated. C12-induced cell apoptosis was assessed by using the Annexin V-FITC/PI assay. The Q4 quadrant marks control cells, which are negative for both Annexin V-FITC and PI staining. The Q3 quadrant marks cells undergoing early apoptosis, which are positive for Annexin V-FITC due to higher PS exposure and negative for PI staining. The Q2 quadrant marks cells at the late stage of apoptosis, which are positive for both Annexin V-FITC and PI staining, as during the late stage of apoptosis, cells lose their membrane permeability and stain positive for PI. While the Q1 quadrant marks cells undergoing necrotic cell death, which is only positive for PI staining [60]. Based on these analyses, it was found that C12 (100 μM) exposure markedly increased apoptosis in lymphocytes cells, with 53.2 % of cells undergoing apoptosis (early apoptosis Q3-22.9 % + late apoptosis Q2-30.3 %) (Fig. 6a and b) as compared to the DMSO control. According to these results, it was concluded that C12 induces apoptosis in peripheral blood lymphocytes.

4. Discussion

In this study, we examined the detrimental effect of C12 on human peripheral blood lymphocytes, and found that C12 induces cytosolic and mitochondrial Ca^{+2} dysregulation, leading to mitochondrial dysfunction and apoptosis in lymphocytes. These results imply that C12 can modulate innate and adaptive immunity by enhancing apoptosis in human peripheral blood lymphocytes. To accurately simulate the impact of C12 on lymphocytes, it is crucial to consider its concentrations in relevant clinical and in-vitro settings. C12 has been detected in the sputum of CF patients at a concentration of up to 1–22 nM [61]. However, in the murine infection model, it was found to be present at concentrations of 1–2 μM [62]. Interestingly, under in-vitro biofilm conditions, C12 can reach up to 300–600 μM [63]. The low levels of C12 detected in CF patients' sputum samples may be attributed to various factors, such as the inefficiency of organic solvent extraction and C12 degradation [61]. Additionally, the presence of bacteria in the form of

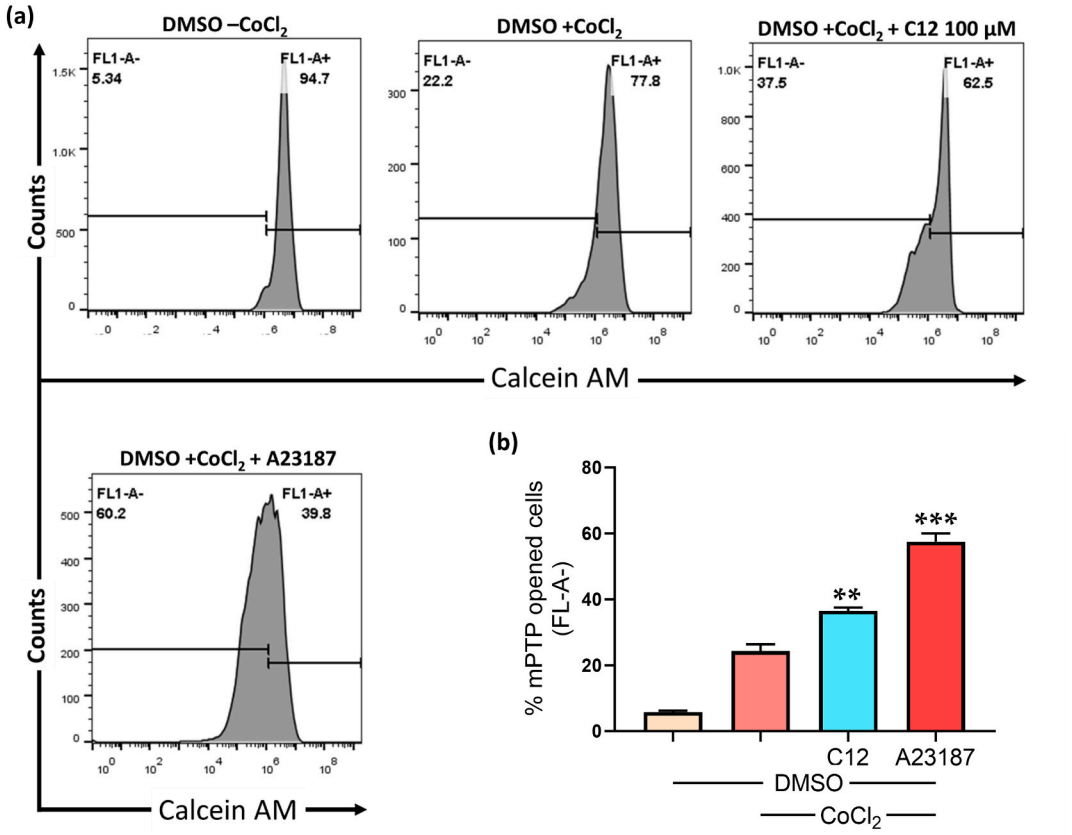

Fig. 5. C12 induces mitochondrial permeability transition pore opening in human lymphocyte cells. (a) PBMCs were treated with C12 (100 μM) for 2 h, and mitochondrial permeability transition pore openings were analyzed via flow cytometry using the Calcein-AM + $CoCl_2$ assay. Cells were stained with Calcein-AM (1 μM) for 15 min, and then cytosolic Calcein-AM was quenched by incubating with 1 mM $CoCl_2$. (b) Bar graphs represent the percentage of mPTP opened cells from the FL-1 channel (Calcein-AM fluorescence) from Fig. 5a. The results are presented as mean ± SEM and represent significant data, $^{**}p < 0.01$, $^{***}p < 0.001$.

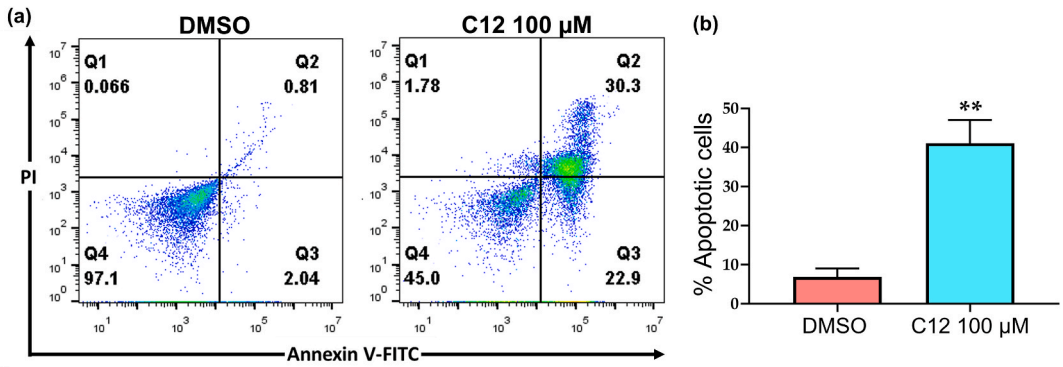

Fig. 6. C12 induces apoptosis in peripheral blood lymphocytes. (a) The Annexin V-FITC/PI assay was used to measure the induction of apoptosis in PBMCs treated with 100 μM C12 for 2 h. (b) Bar graph representing the percentage of apoptotic cells from Fig. 6a. The results are presented as mean ± SEM and represent significant data, $^{**}p < 0.01$.

microcolonies or biofilms in small patches in the lungs, where high levels of autoinducer accumulate at high cell densities, may lead to further dilution of C12 in the sputum [61]. Furthermore, the host may be exposed to a higher concentration of C12 at the interphase of biofilm.

Therefore, in this study, a dose of 100 μM C12 was used to ensure comparability with previous studies that investigated C12-induced cell death in other cell types within the range of 1 μM–100 μM and a time frame of 1–2 h [27,52]. C12 has been found to induce apoptosis in various host immune cells, including platelets, Jurkat T-lymphocytes, macrophages, and neutrophils [27,28,33,39,52]. Additionally, beyond immune cells, C12 has been observed to cause cell death in intestinal goblet cells, airway epithelial cells, and endothelial cells [64–66]. Recent research has also demonstrated that C12 can induce plasma membrane disorders and trimerize tumor necrosis factor receptor 1 (TNFR1), leading to apoptosis in macrophages independently of its ligand, tumor necrosis factor α (TNF-α) [67]. Moreover, it has been shown that the toxicity of C12 depends on the carbon chain length, as C4 does not exhibit any toxicity in host cells. Furthermore, studies have indicated that host Paraoxonase 2 mediates C12-dependent toxicity in human intestinal epithelial cells (LS174T) [68], human embryonic kidney cells (HEK293T) [69], and mouse embryo fibroblast cells (MEFs) [69], suggesting that toxicity due to the free fatty acid chain may be the underlying mechanism.

Intrigued by these studies, we investigated the detrimental role of C12 on human peripheral blood lymphocytes, and it was found that C12 induces a loss of cell viability in human PBMCs, as demonstrated by the MTT assay. As C12 induces apoptosis by primarily triggering the mitochondrial-mediated apoptotic pathway and involves the dysregulation of cytosolic Ca^{+2} homeostasis to induce apoptosis, we further examined the role of C12 in Ca^{+2} dysregulation and mitochondrial dysfunction in human peripheral blood lymphocyte cells. Ca^{+2} signaling plays an important role in maintaining physiological homeostasis, and it is tightly regulated by intracellular stores and plasma membrane channels [70]. Conversely, under genotoxic stress, capacitive Ca^{+2} influx from extracellular Ca^{+2} through channels and intracellular Ca^{+2} release from the endoplasmic reticulum causes cytosolic Ca^{+2} overload, which has been shown to be apoptogenic [70]. Moreover, previous research has demonstrated that C12 induces IP3R-dependent release of ER Ca^{+2} to induce cytosolic Ca^{+2} overload [27]. Based on our results, it was found that C12 induces cytosolic Ca^{+2} overload in human lymphocyte cells, which is consistent with previous studies showing that C12 mediates cytosolic Ca^{+2} overload in human blood immune cells like neutrophils and platelets [27,29].

Mitochondria transiently buffer the cytosolic Ca^{+2} insult by taking up ER Ca^{+2} through the IP3R-VDAC-MCU channel [71]. Abnormal Ca^{+2} transfer through the IP3R-VDAC-MCU axis causes mitochondrial Ca^{+2} overload, further contributing to the transmission of apoptosis signals [71]. The amount of Ca^{+2} taken up in the mitochondrial matrix plays a decisive role in a cell's fate between life and death [31]. Physiologically, an increase in mitochondrial Ca^{+2} enhances ATP synthesis and the activity of the respiratory chain in the mitochondria. However, sustained pathological Ca^{+2} overload leads to apoptosis by generating ROS in mitochondria, depolarizing mitochondria, and opening the mitochondrial permeability transition pore (mPTP). Consequently, prolonged mPTP opening releases intermembrane space proteins and cytochrome *c* from the mitochondria into the cytosol, leading to caspase-mediated apoptosis [72]. Earlier, C12 was shown to induce MCU-dependent mitochondrial Ca^{+2} overload, as the MCU-specific inhibitor Ru360 abrogated C12-dependent mitochondrial Ca^{+2} overload in human neutrophils [27]. Therefore, we further investigated the role of C12 in mediating mitochondrial Ca^{+2} dynamics using the mitochondrial Ca^{+2} specific dye Rhod-2 AM. These investigations revealed that C12 (100 μM) causes a rise in mitochondrial Ca^{+2} levels, suggesting that C12 dysregulates calcium dynamics in human peripheral blood lymphocytes. Mitochondrial Ca^{+2} overload is known to mediate mitochondrial redox impairment [73], and there is a bidirectional interaction between ROS and calcium signaling, where ROS affects calcium signaling, and calcium signaling leads to ROS production. In comparison, it has been shown that free radicals are formed by activating ROS-generating enzymes through Ca^{+2} signaling [74]. Therefore, we investigated the mitochondrial redox balance in lymphocytes using the mitochondrial ROS-sensitive dye MitoSOX, and it was found that C12 (100 μM) causes mitochondrial ROS generation. Excessive mitochondrial ROS has a detrimental effect on mitochondrial physiology as it mediates oxidative damage to mitochondrial DNA, energetic impairment, and subsequent mitochondrial depolarization [75]. Intrigued by these findings, mitochondrial depolarization was assessed using JC-1 dye in the presence of C12, and the results suggest that C12 (100 μM) causes a drop in mitochondrial membrane potential in lymphocytes.

Moreover, the obtained results align with earlier reports of C12-associated mitochondrial depolarization events in mouse embryonic fibroblasts [76], neutrophils [27], platelets [28], and macrophages [52].

mPTP is the large nonspecific channel in the inner mitochondrial membrane [77,78]. Under physiological conditions, mPTP allows the free flow of low molecular weight ions and solutes across the mitochondrial membrane [79]. However, under pathological conditions, mPTP opening becomes unregulated, and sustained opening results in the loss of mitochondrial solutes, cytochrome *c*, and apoptotic proteins to the cytosol [80]. Two significant causes of mPTP opening are sustained oxidative stress in mitochondria and mitochondrial calcium overload [81,82]. During apoptosis, mPTP opening is regarded as an irreversible point in cell death [83]. Therefore, we further investigated the impact of C12 on lymphocytes' mPTP opening, and it was found that C12 induces mitochondrial pore opening. Based on these results, it was concluded that C12 has a detrimental impact on mitochondrial physiology by aberrantly opening the mitochondrial channels that play a pivotal role in mitochondrial-dependent apoptosis. Aberrant mPTP opening-dependent mitochondrial dysfunction is known to impair cellular energetics, leading cells to progress to apoptosis [84]. So, we then investigated the C12-dependent apoptosis on human lymphocytes by measuring the levels of PS exposure. The results suggest that C12 (100 μM) induces a marked increase in PS exposure, as shown with Annexin V-FITC/PI assay, indicating that C12 induces apoptosis in human peripheral blood lymphocytes. Based on these findings, it was concluded that C12 impairs Ca^{+2} signaling and mitochondrial function, and induces apoptosis in human peripheral blood lymphocytes.

5. Conclusion

In conclusion, the findings of this study revealed a novel perspective with implications for the involvement of C12-driven mitochondrial dysfunction in human peripheral blood lymphocytes. That can potentially lead to impaired immune responses against *P. aeruginosa*-associated infections. The study revealed that *P. aeruginosa* secreted QS signal molecule C12 (100 μM) triggers various events associated with calcium dysregulation, induction of mitochondrial dysfunction (increased mROS generation, mitochondrial depolarization, mPTP opening), and increased apoptosis in human peripheral blood lymphocytes. Therefore, these results imply that novel therapeutic strategies that restore mitochondrial dysfunction may improve immune responses and prevent pathogenic *P. aeruginosa* infections. Moreover, other strategies, such as quorum quenching therapy [85,86], have recently emerged as a promising approaches to mitigate the negative impact on host immune responses by either degrading or inhibiting the synthesis of C12, which can potentially restore the balance between apoptosis and immune function in lymphocytes and might facilitate more efficient immune responses against the pathogen. Additionally, conducting detailed studies to understand C12's stability under pathological conditions and how the host-lactonase initiates the release of cytotoxic long-chain free fatty acids is necessary.

Overall, this study provides insights into the pathophysiological consequences of C12 on human peripheral blood lymphocytes and their involvement in mitochondrial dysfunction. Consequently, further research is warranted to identify mitochondrial targets that can be developed as a combinatorial therapeutic strategy with quorum quenching therapy to effectively mitigate the detrimental effects of C12 on host immune responses.

Compliance with ethical standards

All the volunteers who participated in the study were duly informed, and they provided their consent by signing consent forms; these forms had received prior approval from the institutional ethics committee at Motilal Nehru National Institute of Technology (MNNIT) Allahabad, Prayagraj, India (Ref. No. IEC/19–20/09). Blood samples from healthy volunteers were collected at the Pathology Laboratory of the Institute Health Centre at MNNIT Allahabad.

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Author contribution statement

Ankit Kushwaha: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper. Vishnu Agarwal: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Data availability statement

Data will be made available on request.

Additional information

No additional information is available for this paper.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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