The Inhibitory Effect of CBD on Proinflammatory Cytokine, IL-6, in LPS-Mediated Inflamed THP-1 Differentiated Macrophage Cells

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About 1.5 million Americans suffer from Rheumatoid Arthritis (RA), an autoimmune, chronic inflammatory disease with no cure. This is a condition in which the immune system mistakenly attacks healthy cells, leaving the body in a constant state of inflammation. A major characteristic of RA is unresolved inflammation specifically in the joints of the hands, wrists, and knees. People who have RA suffer from a lot of pain, experience bone, and joint deformity, and have a loss of function in the targeted areas. In patients with RA, the immune system is not working properly, and the immune responses are unregulated. Due to being an autoimmune disorder, our B cells are presenting healthy cells with autoantigens that trigger multiple signaling pathways that lead to the release of cytokines. A primary cytokine, IL-6, plays a role in the pathogenesis of RA due to its pro-inflammatory effects. IL-6 causes the release of acute phase proteins which cause fevers, iron deficiency, fatigue, fat and muscle loss, anorexia, and weakness. One way to treat RA is to target the inflammation directly by interrupting cytokine release. In this work, the effect of CBD was evaluated to determine if its application has the potential to decrease IL-6 expression/release by inflamed cells. Macrophages derived from THP-1 Cells cultured with PMA were treated with LPS to model inflammation in vitro and the expression of IL-6 was determined by ELISA to correlate in a dose-dependent manner to LPS.

Cytokines are any of a number of substances secreted by immune cells that have specific effects on the interactions and signaling of themselves or other immune cells.[1] These cytokines may be pleiotropic, meaning that they can have different effects on different cell types, and in fact, IL-6 was independently identified by several groups due to its varied activity on numerous cell types.[2] Amongst immune cells, IL-6 is a proinflammatory cytokine that is produced at the site of an infection or injury and plays a major role in host defense. IL-6 stimulates many immune responses including the signaling of acute phase proteins and hematopoiesis.[3] IL-6 also stimulates the differentiation of T and B cells.[4] During inflammation, IL-6 is elevated in order to defend against harmful stimuli then, when inflammation is resolved, IL-6 returns to normal levels.[5] IL-6 is beneficial to our bodies during the acute inflammatory response and helps in host defense.[5] During chronic inflammation, IL-6 remains elevated and is dysregulated, causing inflammation to persist. For example, in individuals with rheumatoid arthritis, IL-6 is sustained at higher levels than in normal healthy individuals and is associated with joint inflammation and destruction related to this disease.[6] Due to elevated IL-6 levels, inflammation persists long after the triggering event is passed and the inflammation itself leads to damage.

Rheumatoid arthritis (RA) is an autoimmune and chronic inflammatory disease. A major characteristic of RA is chronic inflammation, specifically in the joints of your hands, wrists, and knees which eventually leads to joint destruction and deformity.[7] IL-6 is elevated in RA patients and its production can lead to localized increases in autoantibody production in areas of inflammation.[8] These autoantibodies attack healthy cells and tissues, which is another characteristic of RA. This damage results in more IL-6 being produced and the cycle of a positive feedback loop of continuous IL-6 production and chronic inflammation. Currently, RA has no cure, but some medications are used to help treat the pain and keep the disease in remission.

IL-6 antagonists are used for a number of indications, specifically those dealing with autoimmunity and inflammation.[9] IL-6 antagonists work to decrease inflammation which helps reduce pain and improve mobility functions. The function of IL-6 antagonists is to stop the binding of the IL-6 protein to its membrane-bound and soluble receptor targets thereby reducing IL-6 signaling altogether.[8] Therapeutic antibodies, such as the anti-IL-6 receptor monoclonal antibody, Tocilizumab, were developed to treat rheumatoid arthritis and are still used today. Although Tocilizumab is well established as a viable therapeutic modality for RA, other alternatives have recently emerged to target IL-6 directly. The present study aims to target IL-6 release from pro-inflammatory cells.

Cannabidiol (CBD) is a non-intoxicating cannabinoid found in the cannabis Sativa plant.[10] CBD has many purported medical applications, including its use as an anti-inflammatory drug.[11] A recent study has shown that CBD reduced IL-6 levels in inflamed lung epithelial cells grown in culture.[12] Since this study showed CBD can affect IL-6, the present study looked at the effects of treating activated macrophages because these cells are significant producers of IL-6 and have been implicated in

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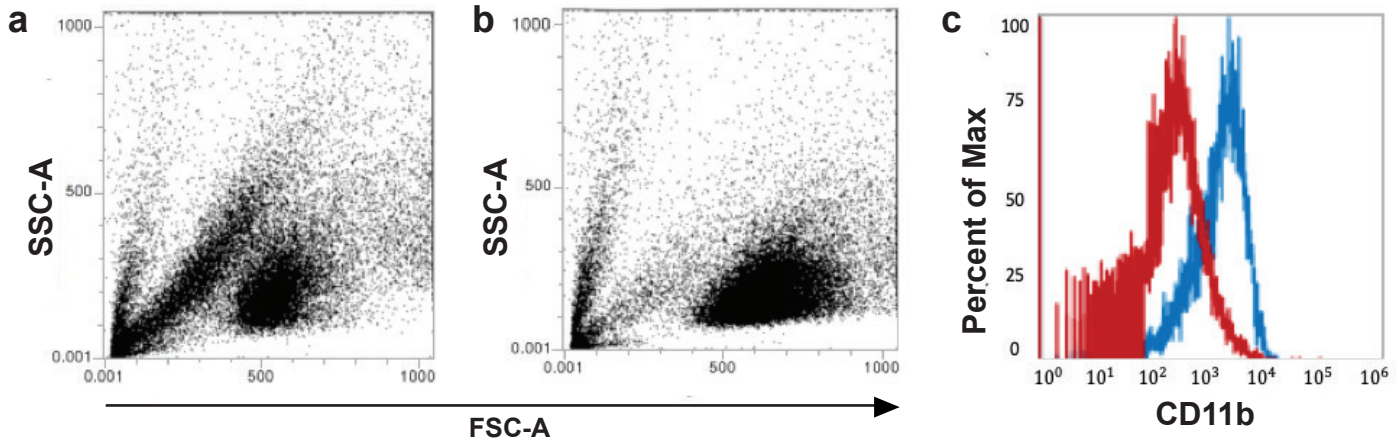

Figure 1 | Flow cytometric analysis of monocyte-like, undifferentiated (untreated) THP-1 cells and macrophage-like, differentiated (PMA-treated) THP-1 cells. Size and granularity of (**a**) Undifferentiated and (**b**) Differentiated THP-1 cells. (**c**) CD11b expression on undifferentiated (red) and Differentiated (blue) THP-1 cells.

perpetuating inflammatory states. Further, macrophages also express the receptors for CBD to enter the cell. Normally during immune cell signaling, a cytokine transcription factor known as NF-κB will translocate to the nucleus where it will facilitate the transcription of IL-6.[13] Prior to activation, NF-κB is prevented from entering the nucleus by its association with IκB, an inhibitor protein. It has been shown CBD can inhibit NF-κB from entering the nucleus by stabilizing the interaction between NF-κB and IκB.[14] This will, in turn, stop the production of IL-6 and reduce inflammation and some disease symptoms.[15]

In this study, an in vitro model was developed to explore the potential anti-inflammatory effect of CBD on IL-6 production by THP-1 macrophages. Briefly, lipopolysaccharide (LPS) was used to stimulate inflammation and cause the release of IL-6.[16] Before stimulating the cells with LPS, macrophages were generated from the THP-1 monocyte cell line. Phorbol 12-myristate 13-acetate (PMA) was used to differentiate cells of the human THP-1 monocyte cell line into macrophages.[17] Macrophages are a primary producer of IL-6 and contain the receptors needed for CBD to enter the cell.[18] The cells were stimulated with LPS following PMA exposure to assess its effect on modulating IL-6 release. This model will be used in future work to establish the potential of CBD to block the release of IL-6 protein and escape the chronic inflammatory feedback loop.

Materials and Methods

Cell culture and treatment

THP-1 monocytes were obtained through a generous gift by Dr. Ann Wozniak at the KU Medical Center. THP-1 monocytes were cultured and maintained in RPMI 1640 medium containing 10% FBS (HyClone, Cat. No. SH30541.03) and 1% penicillin- streptomycin (Gibco, Cat. No. 15140-122). Cells were incubated at 37˚C in a humidified environment, 5% CO_2 incubator. For PMA treatment, cells were plated in a 6-well plate and seeded at 300,000 cells/well. Cells were differentiated into macrophages at a final concentration of 100 ng/mL PMA (Sigma, Cat. No. 16561-29-8) in media for about 4 hours or until the cells became adherent. Media containing PMA was aspirated and replaced with fresh media. Cells were incubated for an additional 24 hours, allowing the cells to rest and recover. Cells were then stimulated with 0, 50, and 100 ng/mL LPS (eBioscience, Cat. No. 00-4976-93) for 24 hours. Following LPS stimulation, IL-6 levels were assessed by ELISA.

Flow cytometry analysis of THP-1 differentiation

THP-1 cells were plated at 300,000 cells/well in 6-well plates and treated with 100 ng/mL PMA for 4 hours, as mentioned above. Cells were harvested using a cell scraper, stained with PerCP/Cy5.5 anti-human CD11b antibody (Biolegend Cat. No. 101228), incubated for 15 minutes in the dark, and analyzed using flow cytometry.

Cell viability assay using MTT

Cell toxicity was analyzed using an MTT assay. Differentiated THP-1 cells were seeded at 80,000 cells/well in a 96-well plate. Cells were incubated for 24 hours after seeding to allow the cells to attach to the plate. CBD (0-50 μM) and LPS (0-100 ng/mL) were added to the wells and incubated for another 24 hours. After drug exposure, the media in the wells was removed and the MTT solution (5 mg/mL, MTT powder (Biosynth, Cat. No. 298-93-1) in media) was added. After incubating for 4 hours with the MTT solution, DMSO (Fisher Chemical, Cat. No. 67-68-5) was added to each well, the plate was placed on a shaker for 15 minutes, and read on the spectrophotometer at 570 nm.

Analysis of IL-6 by ELISA

Supernatants were collected from unstimulated and LPS-stimulated macrophages. Monocytes were differentiated into macrophages and stimulated with 0, 50, and 100 ng/mL of LPS for 24 hours. IL-6 production was measured by Enzyme-Linked Immunosorbent Assay (ELISA). Purified anti-human IL-6 antibody (Biolegend Cat. No. 501115) was used in the ELISA as the capture antibody. Captured IL-6 was detected using Biotin anti-human IL-6 (Biolegend Cat. No. 501201), Ultra Streptavidin-HRP (Thermo Fisher, Cat. No. N504), and TurboTMB (Thermo Fisher, Cat. No. 34022).

Results

THP-1 differentiation was confirmed by flow cytometry

Cells of the human monocyte (THP-1) cell line were differentiated into macrophages by a 37˚C incubation with Phorbol 12-myristate 13-acetate (PMA) for 4 hours at a final concentration of 100 ng/mL. Following the 4-hour incubation, the media was renewed, and the cells were incubated for 24 hours

to let the macrophages rest and recover. Cells were then analyzed by immunofluorescence staining using flow cytometry and undifferentiated (monocyte-like) and differentiated (macrophage-like) cells were stained with anti-CD11b antibody. **Figure 1** shows the increase in fluorescence of the macrophages (blue) compared to the monocytes (red). These results confirmed the monocyte-like CD11b- THP-1 cells did differentiate into CD11b+ macrophage-like THP-1s. This protocol was used throughout the rest of the experiments.

Cell viability assay on CBD and LPS

The toxicity of CBD and LPS were assessed by a cell viability assay using MTT. Cells were seeded at a density of 80,000 cells/mL and treated with PMA to differentiate into macrophages, as described above. Cells were then treated with CBD and LPS at different concentrations for 24 hours. After adding the MTT solution and DMSO, the plate was read on the spectrophotometer. Figure 2 illustrates the viability results after cells were co-cultured with various concentrations of CBD or LPS. These data indicated no evidence of any cell death as all conditions had statistically equal viability, with cells metabolizing the MTT powder into purple formazan crystals. Figures 2a and 2b represent the absorbance of the different concentrations of CBD and LPS, respectively.

IL-6 analysis by ELISA

To determine the effect of LPS stimulation on IL-6 secretion by macrophage-like THP-1 cells, the cells were differentiated with PMA as described above. The cells were then stimulated with LPS for 24 hours. Supernatants were collected and the expression of IL-6 was evaluated by ELISA. **Figure 3** shows the relative expression of IL-6 on the vertical axis by absorbance at 570 nm. The non-stimulated 'macrophages' (0 ng/mL) showed minimal absorbance, indicating little to no expression of IL-6. The expression of IL-6 in the 50 ng/mL LPS stimulated macrophages showed an increase in absorbance and even an even larger increase when cells were stimulated with 100 ng/mL LPS. The dose-dependent increase in absorbance is consistent with what was expected, suggesting that LPS is, indeed, triggering the production of IL-6 in response to LPS.

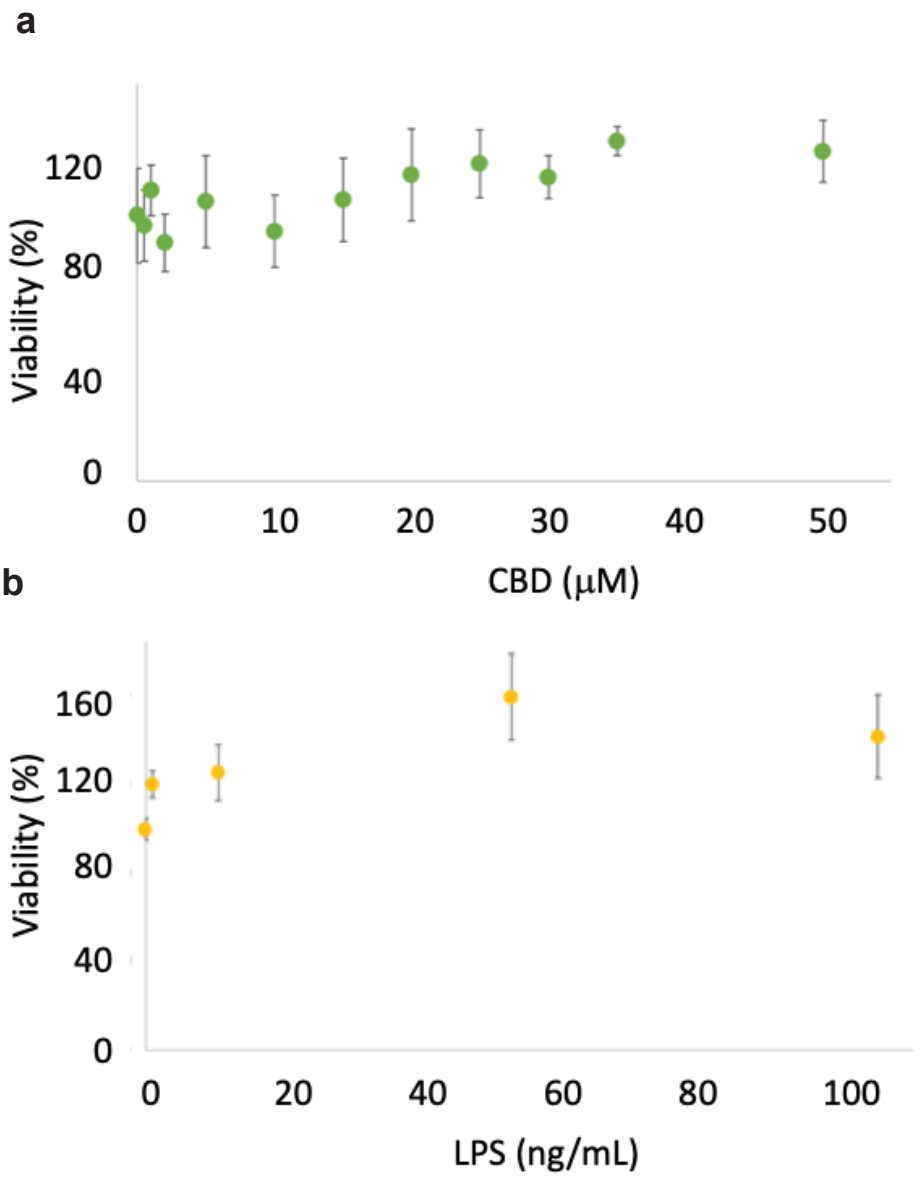

Figure 2 | MTT Cell viability assay of macrophage-like THP-1 Cells cultured with various concentrations of CBD or LPS. (**a**) Viability of macrophage-like THP-1 Cells co-cultured with concentrations of CBD from 0 mM to 50 mM. (**b**) Viability of macrophage-like THP-1 Cells co-cultured with various concentrations of LPS from 0 ng/mL to 100 ng/mL.

Discussion

The work described in this paper suggests that THP-1-derived macrophages are a viable model for studying LPS-mediated IL-6 production in vitro. We showed that PMA was effective in differentiating CD11b-ve monocytic THP-1 cells into macrophage-like cells expressing CD11b. We further demonstrated that the concentrations of LPS and CBD used were non-toxic to cells, allowing us to move forward with evaluating IL-6 expression. At non-toxic concentrations, LPS was capable of stimulating these cells to secrete IL-6 in a dose-dependent manner. This approach will enable the next steps of this research to be done on CBD's effects on inflammation by further investigating its effects on these cells, and ultimately, on primary cells.

This study proposes that LPS acts as an initial inflammatory signal sufficient to induce IL-6 production in macrophages. To determine if CBD could attenuate this expression, LPS stimulation will be performed as described above after a preliminary culturing with or without CBD. IL-6 production will be measured in the supernatants with the expectation that if CBD treatment specifically interrupts IL-6 secretion, IL-6 levels would be decreased from those observed with LPS stimulation alone.

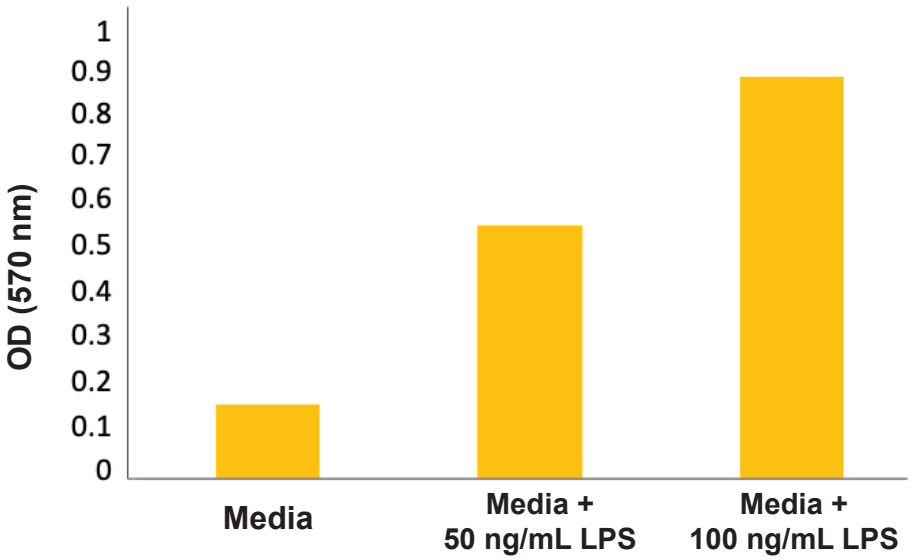

Figure 3 | Relative IL-6 production induced by LPS co-culture as determined by ELISA. Macrophage-like THP-1 cells were incubated with either media alone, 50, or 100 ng/mL of LPS for 24 hours. The supernatants were collected, and an ELISA was performed to detect any secreted IL-6 protein. All responses are relative and non-quantitated.

Future Directions

This work establishes a model system for evaluating the efficacy of CBD in modulating IL-6 production after the co-culture of macrophage-like cells with LPS. Further investigation into the mechanism of CBD-mediated modulation of IL-6 production would focus on the postulated stabilizing effect of CBD on IκB and whether this was sufficient to directly calm inflammation. Another future objective would be to evaluate the effects of LPS and CBD on freshly isolated macrophages instead of macrophages derived from a monocytic cell line.

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Author Biography

Lucy Johnson is a graduate of the KU Biotechnology Program at the University of Kansas Edwards campus. A native of Kansas City, she intends to stay in the area after graduation, hopefully relocating after a few years. Her plans after college include working in the Biotechnology industry to gain experience in the laboratory as well as business and development. Lucy has always wanted to help others with the work she does, so she wants to continue working toward exploring new scientific innovations and research. She would like to take on a role where she could be a mediator between the science and business worlds and improve the transparency of communication between the two.

Author Contributions

L.A.J. contributed to the experimental work, design, and writing of this work; J.F.T. contributed to the editing and design of this work; R.L.L. contributed to the editing and design of this work.

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