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Comparing Responses of Ursolic Acid in Murine Macrophages Infected with *Mycobacterium Smegmatis* and *Mycobacterium avium*

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ABSTRACT

Mycobacterium smegmatis and *Mycobacterium avium* offer an advantage in examining tuberculosis-like effects and host immune defense. Therefore, the study aims to examine the effect of ursolic acid (UA) on the host immune system by analyzing cytokines concentration, such as TNF-α, IL-6, IL-1β, and nitrite oxide produced by murine macrophages infected with *Mycobacterium smegmatis* and *Mycobacterium avium*. Femurs of female C57BL/6 mice aged 6–8 weeks were used to culture the Bone marrow-derived macrophages (BMDM). On day 10, BMDM was infected with *Mycobacterium smegmatis* and *Mycobacterium avium* using a multiplicity of infection (MOI) amounting to 8:1, then TNF-α, IL-6, and IL-1β were analyzed using ELISA and nitrite oxide with Griess reagent. The results showed that UA decreased the production of three respective pro-inflammatory cytokines used in the study, both in BMDM infected by *Mycobacterium smegmatis* and *Mycobacterium avium*. For TNF-α, the reduction was nearly 65%-90% compared to the control. The decrease in the production of IL-6 occurred from 2700 pg/mL to 750 pg/mL for BMDM infected with *Mycobacterium smegmatis,* while the reduction was more significant in those infected using *Mycobacterium avium* with approximately 150 pg/mL compared to the control. Moreover, UA reduced by over 90% of IL-1β and this result was in line with the reduction of nitrite. UA decreases the production of pro-inflammatory cytokines such as TNF-α, IL-6, IL-1β, and nitrite. This result is preliminary but supports further study on the role of UA in immune defense from pathogenic and non-pathogenic mycobacterial infections.

Keywords: Ursolic acid, immune response, murine macrophage, mycobacteria infection

INTRODUCTION

Mycobacteria are well known for their acid-fastness and peculiar lipid-rich cell wall, involved in virulence (Mondino *et al*., 2013). The most studied pathogenic species include *Mycobacterium tuberculosis* (MTB), *Mycobacterium ulcerans, Mycobacterium leprae*, which are three human scourges among the top priorities of the World Health Organisation (WHO, 2021). *Mycobacterium avium* another mycobacteria species, is popular for opportunistic infections such as human immunodeficiency virus-positive compromised patients (Yone *et al*., 2012). Significant variability exists regarding their strategies to persist and multiply in the environment or host organism. For example, *M. avium* has developed strategies to evade the antimicrobial activities of macrophages by regulating the immune system through cytokines. The mycobacteria family also contains non-pathogenic species such as *Mycobacterium smegmatis* which have proved to be valuable hosts to express and study antituberculosis as well as genes-related pathogenic species (Sharbati *et al*., 2019). Both *M. avium* and *M. smegmatis* synthesize abundant glycopeptidolipids (GPLs), these surface-located GPLs exhibit pathogenicity by interfering with the host immune system (Biet *et al*., 2008). Although they both induce entirely different disease processes, the GPLs might confirm that *M. avium* and *M. smegmatis* are closely related and induce similar cytokine regulation.

Ursolic acid (3β-hydroxy-12-urs-2-en-28-oic acid) is a pentacyclic triterpenoid compound known to have pharmacological activities (Seo *et al*., 2018). Plant-isolated UA was found to have antituberculosis activity, both sensitive and resistant strains of MTB (Jyoti *et al*., 2015). It decreased mycolic acid concentration extracted from avirulent MTB H37Ra (Jyoti *et al*., 2015). This pentacyclic triterpenoid reportedly regulates the Mitogen-Activated Protein Kinase (MAPK) signaling pathway in leukemia cells involved in cytokine production (Gao *et al*., 2012). However, its regulation on infected macrophages with different mycobacteria virulence is unclear.

Therefore, this study aims to investigate comparatively the UA response in bone marrow-derived macrophages (BMDM) infected with the non-pathogenic strain of *M. smegmatis* and a pathogenic *M. avium*. It was hypothesized that the similar characteristics of these two mycobacteria can be explained by the induction of pro-inflammatory immune responses. To test this hypothesis, UA's impact was analyzed on the concentration of cytokines, such as TNF-α, IL-6, IL-1β, and nitrite oxide (NO) produced by macrophages infected with *M. smegmatis* and *M. avium*.

MATERIAL AND METHODS

This is an experimental laboratory study that compared the result of cytokines and nitrite concentration from BMDM infected with *M. smegmatis* and *M. avium* due to the administration of UA.

Isolation and culture of murine bone marrow-derived macrophages

Female C57BL/6 mice aged 6–8 weeks were obtained from the Preclinical Research Facility, University of Leicester, UK and were used as a source for BMDM. The preparation was based on a previous study (Pitaloka *et al*., 2020a). BMDM were cultured using complete Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), HEPES buffer, MEM amino acid, sodium pyruvate solution, and L-glutamine (Thermo Fisher Scientific, Waltham, MA, USA). Six-well tissue culture plates were initially seeded with 5.8×10^5 cells/well and incubated at 37°C in a 5% CO_2 humidified air chamber for 24h. The following day, the cells were supplemented with additional fresh complete DMEM containing 10 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) (Thermo Fisher Scientific, Waltham, MA, USA). The medium was changed every 3 days, and the cultures were incubated for 10 days. On the day before infection namely day 9, the medium was replaced with complete DMEM without GM-CSF, and the adherent cells were ready for infection on day 10.

Diff-Quik staining procedure

For the Diff-Quik staining procedure, one of 6 well-plate was initially added into the Diff-Quik stain solution I of xanthene dye five times for 1 s each and then added with solution II of thiazine dye twice for 1 s each time. The slides were rinsed by adding deionized water five times. The plate was air-dried and covered after the application of the mounting medium. All stains were microscopically analyzed at a magnification of 200X for the presence of different cells found in BMDM culture (Kellogg *et al*., 1996).

Cytotoxicity Assay

For the cytotoxicity assay, the adherent cells of BMDM were seeded at $1x10^6$ cells/mL. Seven concentrations namely 1, 5, 25, 50, 75, 100, and 200 µg/mL were tested with DMSO or stock diluent and untreated cells as controls. The toxicity of the UA was evaluated after 24 h incubation at 37°C in a 5% CO^2 humidified air chamber by applying the Alamar Blue Assay (Alfagene, Invitrogen, Carcavelos, Portugal). The samples were replaced by a 10% (v/v) Alamar blue solution, while the absorbance was read at 570 nm for reduced form; resorufin and 620 nm for oxidized form; resazurin. The data were analyzed by calculating the concentration of Alamar blue reduction and expressed as a percentage of the control (untreated cells).

In vitro infection of murine macrophages with *M. avium* and *M. smegmatis*

M. avium 724 and *M. smegmatis* with a density of 2.11×10^9 CFU/mL were used as the infectious agent. They were prepared from Cooper's Lab at the Department of Respiratory Sciences, College of Life Sciences, University of Leicester, UK. UA with purity >98% and endotoxin-free was isolated and obtained by Tokyo Chemical Industry (Tokyo, Japan). Dimethyl sulfoxide (DMSO) of cell culture grade was used as a diluent with the final concentration of 1%, while macrophages in 6-well plates were infected with a suspension of mycobacteria at a multiplicity of infection (MOI) of 8:1. Furthermore, controls and treatment groups of UA were incubated at 37°C in a 5% CO^2 humidified air chamber. After 18h, the supernatants were collected to analyze cytokines

and nitrite. The concentrations of UA used were 50μg/mL and 100 μg/mL based on the cytotoxic assay and previous study (Pitaloka *et al*., 2020a).

Measurement of pro-inflammatory cytokine production

TNF-α, IL-1β, and IL-6 produced by BMDM in the supernatants were measured using an Enzyme-linked immunosorbent assay (ELISA) kit (Biolegend, San Diego, USA). ELISAs were performed according to the protocol given by the manufacturer. Tetramethylbenzidine was used as a visualizing reagent and purchased from Sigma-Aldrich (St. Louis, MO, USA). ELISA plates were read for absorbance at 450 nm using an LT-4500 microplate reader and were analyzed using the LT-com data analysis software. Next, the concentration of cytokines was analyzed by interpolating from the sample absorbance to the standard curve.

Determination of nitrite

The nitrite (NO_2^-) concentration was determined using Griess reagent, composed of 1% sulfanilamide, 0.1% N-(1- naphthyl) ethylenediamine, and 2.5% phosphoric acid. In a flat-bottom 96-well microtiter plate, 100 μL of Griess reagent was added to 100 μL of each sample, followed by incubation for 10 min at room temperature, while the absorbance was read at 550 nm. The concentration of NO_2^- was analyzed by interpolating from the sample absorbance to the standard curve.

Statistical analysis

Intergroup differences in treatment effects were probed with one-way analysis of variance, using Tukey's post hoc test for multiple comparisons. The significance of intergroup differences for other parameters was determined using a two-way analysis of variance and Bonferroni's post hoc test. All analyses and graphs were carried out using Prism GraphPad software v.8.0. All experiments were performed at least three separate times, with data obtained in duplicate wells in each experiment.

RESULT AND DISCUSSION

Isolation and culture of bone marrow-derived macrophages

To culture macrophages, bone marrow cells were initially isolated from wild-type C57BL/6 mice using the established protocol previously described by (Pitaloka *et al*., 2020a). The quality of bone marrow depends on how quickly femurs and tibias are obtained as well as extracted (Zhang *et al*., 2008). Subsequently, the bone marrow cells with two bones per mice were aseptically harvested in less than 1 h on day 0 to ensure it was in good condition, while the non-adherent cells were collected and plated into 6 well plates on day 1. The bone marrow cells were cultured in the presence of GM-CSF 10 nm/mL for 10 days to induce proliferation and differentiation of myeloid progenitor *in vitro* culture.

Overnight stimulation of GM-CSF was considered to be 16 h, on day 3, the myeloid cells were found to have started differentiating (Figure 1b). The adherent cells were 90% confluent on day 9 and ready for infection (Figure 1f). GM-CSF bone marrow cell-derived macrophages (GM-BMMs) were the primary cells up to 70% of the mixed populations. It also comprised the bone marrow cell-derived DCs (Figure 1f).

Diff-Quik staining was used to determine differential cells found in the macrophages culture. The staining was carried out in one of 6 well-plates after all the experiments had been performed. Based on the results, most of the cell cultures were macrophages including eosinophils and lymphocytes (Figure 2).

Cytotoxicity Assay

The cytotoxicity analysis of UA on BMDM was evaluated by alamar blue as it is a quantitative measure of cell proliferation. After 24 h exposure, at concentrations up to 75 μg/mL, the UA did not produce significant changes on the cell viability compared to the control or untreated cells. However, UA was found to decrease the BMDM viability to approximately 75% at 100μg/mL and 20% at the highest concentration used in this study. Based on this result and the previous study by (Pitaloka *et al*., 2020), 50 μg/mL and 100 μg/mL of UA were used for further analysis.

Measurement of pro-inflammatory cytokines

An experiment was made to investigate the production of TNF-α, IL-6, IL-1β, and NO analyzed as nitrite due to the administration of UA to macrophages infected with *M. smegmatis* and *M. avium*. The interaction between mycobacteria and the host immune system began with phagocytosis by macrophages which modulate the immune response. Mycobacteria can activate macrophages through Toll-Like receptor 2, stimulate ERK1/2 phosphorylation, and increase the production of pro-inflammatory cytokines such as TNF-α, IL-6,

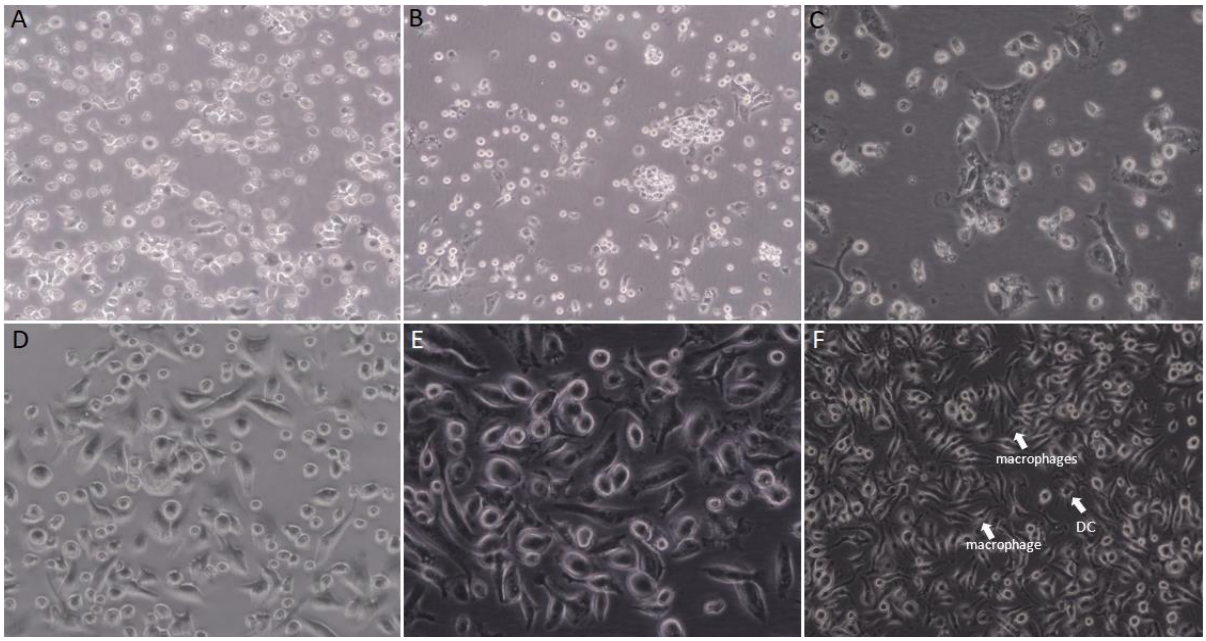

Figure 1. Culture of bone marrow-derived macrophages from the femur of C57BL/6 mice in day 1 (a), day 3 (b), day 5 (c), day 6 (d), day 7 (e), day 9 (f). DC; dendritic cell.

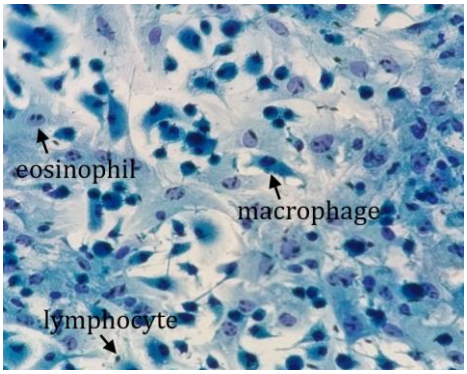

Figure 2. BMDM at day 11 stained with Diff-Quik

IL-1, and NO (Nathan & Shiloh, 2000; Wang *et al*., 2000).

UA was found to reduce the concentration of TNF-α, IL-6, and IL-1β with the presence of *M. smegmatis* and *M. avium* compared to control. This pentacyclic triterpene reduced the production of TNF-α by 65% from approximately 250 pg/mL to 90 pg/mL. The reduction was dose-dependent, hence, UA at 100 µg/mL reduced more than 90% or became less than 10 pg/mL of TNF-α concentration (figure 3a). BMDM treated with DMSO of 0.01 µg/mL also did not affect the concentrations of the cytokines and appeared to have a similar pattern to the control. Meanwhile, the single BMDM treatment without UA and bacteria did not produce the respected cytokines (Figure 3a). UA decreased the production of IL-6 from 2700 pg/mL to 750 pg/mL in BMDM infected with *M. smegmatis* compared to the control (figure 3b). Moreover, the reduction was more significant in BMDM infected with *M. avium* with approximately 150 pg/mL concentration than the control bacteria amounting to 1500 pg/mL. Similar to TNF-α, the reduction was also dose-dependent. Compared to control bacteria, the cytokine production was reduced by more than 90% for IL-1β compared to the control (Figure 3c). The reduced concentration of IL-6 during mycobacterial infection will increase the sensitivity of macrophages to interferon-gamma (IFN-γ) and accelerate the formation of vesicle

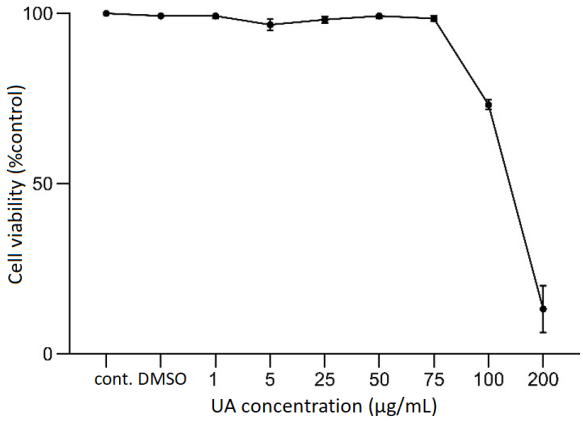

Figure 3. The effect of UA on BMDM exposed for 24 h. Statistically significant differences, $p < 0.05$ are denoted with * in relation to respective control at 24 h. The results are presented as mean of ± SD of n = 3.

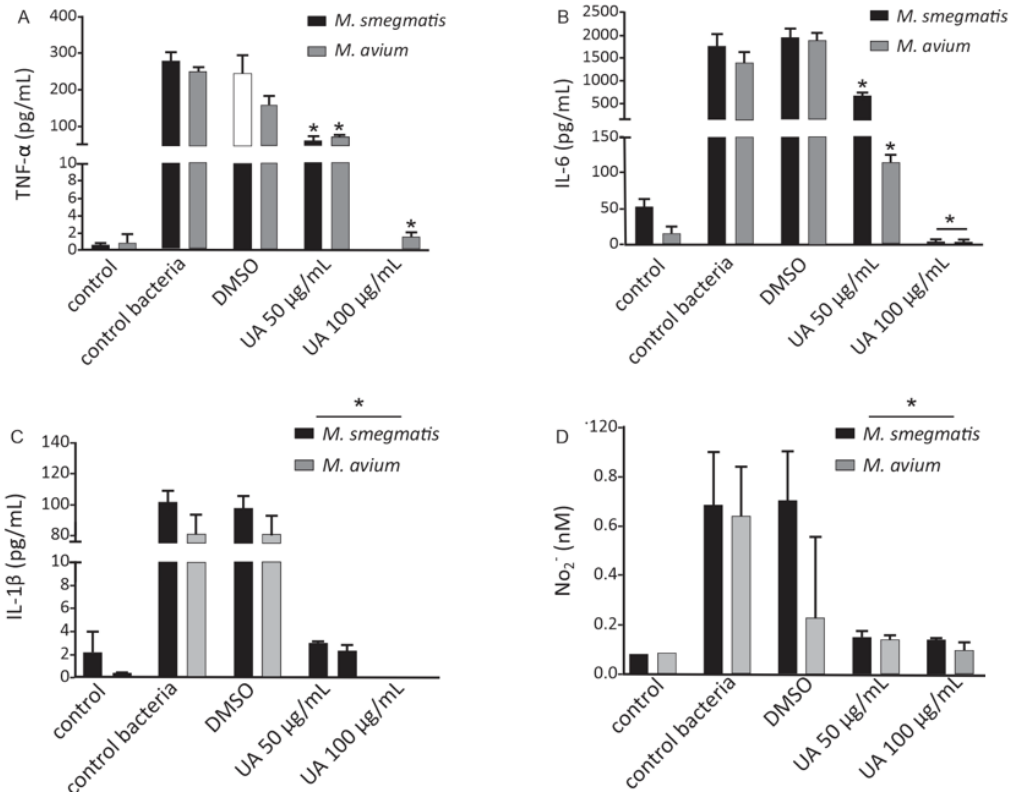

Figure 4. The concentrations of TNF-α (a), IL-6 (b), IL-1β (c), and NO2− (d) after UA treatment on BMDM infected with *M. smegmatis* (black bar) and *M. avium* (grey bar). The results are presented as the mean of ± SD, each performed in triplicate; *p<0.05

apoptosis, thereby increasing the clearance of pathogen from the host (Lee & Kornfeld, 2010). Furthermore, a previous study demonstrated that non-tuberculous mycobacteria induce the AIM2-inflammasome in an IFN-β dependent manner and reduce the production of IL-1β, while UA might decrease cellular perturbations (Briken *et al.*, 2013; Lugrin & Martinon, 2018).

Immune response towards different mycobacteria strains can vary, depending on the structure of proteins, concentration of mycolic acid, and the bacteria morphology. Host response to *M.*

avium mainly revolves around $CD4^+$ T-Cells action and IFN-γ production, highest in early infection than MTB (González-Pérez *et al*., 2013). Infected macrophage also expresses the MAV2054 protein due to the rise of Reactive Oxygen Species (ROS), which induce primary cytokines such as TNF, IL-6, and monocyte chemoattractant protein-1 (Lee *et al*., 2016).

Meanwhile, *M. smegmatis,* the rapid grower non-pathogenic mycobacteria, immediately induces TNF production upon infection in C57BL/6 BMDM accompanied by a strong apoptosis response from Cas-3 activation in BALB/C (Bohsali *et al*., 2010). These differences in mechanism presumably make cytokines production slightly higher in *M. smegmatis* than *M. avium.*

A previous study reported similar results in both mycobacteria, *M. smegmatis* is currently used widely as a control in tuberculosis and NTM studies (Andreu *et al*., 2004; Drapal *et al*., 2018). Some experiments suggested these mycobacteria as a model or surrogate host in drug studies for MTB (Lelovic *et al*., 2020; T *et al*., 2020). *M. smegmatis* also acts as a surrogate house for *M. avium* gene or protein studies (Danelishvili *et al*., 2006; Kannan *et al*., 2020; K.-I. Lee *et al*., 2016). These experiments raise the possibility of *M. smegmatis* as a potential model and safer investigation alternative to *M. avium*.

Measurement of nitrite production

The result in cytokines concentration was in line with that of nitrate, UA was found to reduce NO concentration in macrophages infected with *M. smegmatis* and *M. avium* to 0.2 nM compared to the control bacteria with approximately 0.7 nM. Meanwhile, DMSO reduced NO concentration in macrophages infected with *M. avium* to 0.25 nM but did not affect those infected with *M. smegmatis* with a concentration of 0.7 nM (figure 3d).

This result is due to UA cytokine-repressing ability during mycobacterial infection (López-García *et al*., 2015; Zerin *et al*., 2015; Zhao *et al*., 2019) which reduces post-infection cytokines and NO production in both species. A previous study reported that NO plays a vital role in regulating the size, quantity, and quality of granuloma formation in *M. avium*-infected mice. Furthermore, reduced NO levels were associated with an increased number of T and B cells, as well as granulocytes within the granulomatous lesions (Abukhalid *et al*., 2021). Another investigation showed that UA administration in macrophages infected by *M. tuberculosis* helps to reduce induced Nitrogen Oxide Synthase (iNOS) and Nitrogen Oxide (NO) production (López-García *et al*., 2015; Zerin *et al*., 2015). This result supports the use of UA as an anti-inflammatory drug in controlling body response against Non-tuberculous Mycobacteria (NTM) infection.

CONCLUSION

Based on the results, UA decreased the production of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β, as well as nitrite in both BMDM infected by *Mycobacterium smegmatis* and *Mycobacterium avium*. This study provides experimental evidence on the application of UA as a candidate to support immune defense against pathogenic and non-pathogenic mycobacteria infection in the future.

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