

FUNCTIONAL IMPACT OF ETHYL- β -D-GLUCURONIDE ON
MYCOBACTERIUM TUBERCULOSIS STIMULATED LUNG MACROPHAGES

by

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FUNCTIONAL IMPACT OF ETHYL- β -D-GLUCURONIDE ON GAMMA-IRRADIATED *MYCOBACTERIUM TUBERCULOSIS* STIMULATED LUNG MACROPHAGES

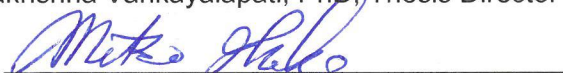
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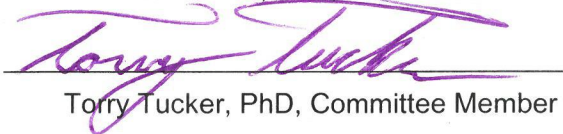
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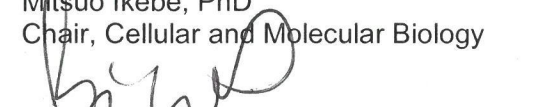
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ABSTRACT

Chronic alcohol abuse has been shown to alter immune defense mechanisms in humans and mice which makes the host susceptible to infections, including *Mycobacterium tuberculosis* (*Mtb*) infection. However, limited information is available on the mechanisms involved in alcohol-mediated host immune system dysfunction.

In this study, we determined the effects of ethyl- β -d-glucuronide (EtG), an alcohol-derived metabolite, on immune response of mice lung macrophages. We measured cytokine and chemokine production by gamma-irradiated *mtb* (γ -*mtb*) stimulated mice lung macrophages in the presence or absence of EtG. We also determined the effect of EtG on the metabolic state of γ -*mtb* stimulated mice lung macrophages.

We found that EtG inhibited secretion of IL-10 and enhanced production of macrophage inhibitory protein (MIP-1 β). We also found that EtG significantly inhibits oxidative phosphorylation of lung macrophages stimulated with γ -*mtb*.

PREFACE

Tuberculosis is an infectious disease caused by *Mtb*. It is the second leading infectious killer responsible for the death of over 1.6 million people in 2021. It is estimated that over 2 billion people are infected by latent tuberculosis infection and about 5-10 percent will develop active tuberculosis in their lifetime.

Previous studies have demonstrated that chronic alcohol disorder is an important risk factor in the reactivation of latent tuberculosis infection, and it is responsible for the disruption of host immune functions in response to bacterial and viral infections. However, limited knowledge is available on the mechanisms by which alcohol mediates these effects.

A previous metabolomic study in Dr. Ramakrishna Vankalayapati's laboratory has shown that there was an accumulation of EtG – an alcohol derived metabolite – in the serum and lungs of alcohol fed mice infected with *Mycobacterium tuberculosis*.

This study was carried out to observe the effects of EtG on lung macrophages – an important immune cell that first responds to tuberculosis infection. We carried out various techniques to observe the effects of the metabolite on the mitochondrial bioenergetics and cytokine production in lung macrophages. Findings from this study will be important to develop better methods to treat alcoholic individuals suffering from tuberculosis infection.

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LIST OF ABBREVIATIONS

EtG: Etyl- β -D-Glucuronide

EDTA: Ethylenediaminetetraacetic acid

ELISA: Enzyme-linked Immunosorbent Assay

FBS: Fetal Bovine Serum

γ -Mtb: Gamma-irradiated *Mycobacterium tuberculosis*

Glyco: Glycolysis

LPS: Lipopolysaccharide

MACS: Magnetic-activated cell sorter

PBS: Phosphate Buffered Saline

TNF- α : Tumor Necrosis Factor Alpha

MIP-1 β : Macrophage Inflammatory Protein – 1 beta

Oxphos: Oxidative Phosphorylation

HBSS: Hank's balanced salt solution

CRPMI: Complete Roswell Park Memorial Institute media

INTRODUCTION

In 2021, an estimated 10.6 million people suffered from tuberculosis infection, which claimed the lives of over 1.6 million people, making it the leading cause of death due to an infectious disease globally, only after Covid [1]. Immunocompromised conditions such as malnutrition, aging, diabetes, smoking, and alcohol abuse, as well as coinfections with HIV, can increase the risk of developing active TB in individuals with latent tuberculosis infection [2, 3]

Substantial work has described alcohol as a risk factor for activation of *Mycobacterium tuberculosis* (*Mtb*) infection [4, 5]. Published studies demonstrated that alcohol consumption of more than 40 g per day or a diagnosis of an alcohol use disorder resulted in a nearly three-fold increase in the risk of tuberculosis infection [6]. Based on this meta-analysis, alcohol consumption was estimated to be responsible for approximately 10% of all incident cases and deaths due to tuberculosis highlighting that heavy alcohol use impairs the host defense and increases the risk of *Mtb* infection by dysregulation of host defense mechanisms [7]. However, the mechanism by which alcohol jeopardizes immune integrity is poorly understood.

Alcohol abuse suppresses various components of the immune response, leading to an increased risk of infections [8]. The course and resolution of both bacterial and viral infections is severely impaired in alcohol-abusing patients,

resulting in greater patient morbidity and mortality [9-11]. Alcohol consumption has been shown to impair the phagocytic and antigen-presenting capacities of innate immune cells and suppresses production of cytokines such as IL-23, in response to *Mtb* infection [8, 12]. Alcohol-exposed dendritic cells produce more IL-10 and less IL-12, suggesting an inhibitory effect on dendritic cell function [13]. In humans and experimental mice, chronic alcohol consumption makes neutrophils hypo-responsive to bacterial infection [14]. Prolonged alcohol consumption induces interferon α (IFN- α) and tumor necrosis factor alpha (TNF- α) production [15]. Alcohol impairs natural killer cell trafficking, inhibits NK cell cytotoxicity and impairs adaptive immune responses mediated by B and T-cells [16, 17]. However, the mechanisms by which alcohol inhibit immune responses during *Mtb* infection are unknown.

Lung macrophages including alveolar macrophages are tissue-resident immune cells that respond first to *Mtb* infection. Previous studies have shown that these cells are major targets of *Mtb* infection, critical during the progression of active tuberculosis [18, 19]. The immunopathology of tuberculosis derives from a complex microenvironment in the lung, which shapes immune responses by lung macrophages [20, 21]. Lung macrophages carry out anti-microbial functions via different mechanisms including phagocytosis and the secretion of cytokines and chemokine [22, 23]. These proteins are also responsible for the regulation of immune functions [24]. Activated lung macrophages are polarized into two

phenotypically distinct states known as M1 and M2 macrophages [25]. These two activated macrophage states are characterized by their cytokine and chemokine production profiles as well as their bioenergetic profiles [26]. M1 macrophages also known as the classically activated macrophages are characterized by increased production of pro-inflammatory cytokines and utilizes glycolysis in energy metabolism. This phenotype is induced by LPS or IFN- γ [26, 27]. On the other hand, M2 known as alternatively activated macrophages induced by IL-4 or IL-13 and exhibit anti-inflammatory cytokine profile and derive energy by oxidative phosphorylation [28]. Studies have shown that chronic alcohol use and exposure decrease the phagocytic function of macrophages and leads to an increase in pro-inflammatory cytokines, there by promoting inflammation which may aid bacteria growth [29-31].

In a previous metabolomic study carried out in our laboratory, mice were fed with control and alcohol diets and infected with ~100 CFU of aerosolized *Mtb* H37Rv. Control and alcohol diet feeding was continued after infection. At three months post infection (p.i), serum and lung homogenates were collected, and metabolomics analysis were performed by Liquid chromatography–mass spectrometry (LC-MS). A list of altered metabolites in the lung and blood of alcohol diet fed *Mtb* infected mice compared to other groups was analyzed. It was observed that alcohol-fed mice infected with *Mtb* showed an accumulation of Ethyl-

β -D-glucuronide (EtG) in the lung homogenate and serum of these mice compared to control mice.

EtG is a direct non-oxidative metabolite of ethanol formed by the enzymatic conjugation of ethanol with glucuronic acid in the liver. This phase II metabolic reaction is catalyzed by mitochondrial membrane-bound Uridine 5'-diphosphoglucuronosyltransferase [32]. It is a known biomarker to test for ethanol use [33]. In addition, EtG can be used for monitoring the amount of alcohol use over time because it can be detected in hair and nails [34].

RESEARCH HYPOTHESIS

In this current study, we hypothesized that EtG exposure alters mitochondrial bioenergetics which can cause metabolic reprogramming and dysregulate antimicrobial function of lung macrophages by disrupting the cytokine and chemokine production during *Mtb* infection. To test this hypothesis, we determined the effect on EtG on cytokine and chemokine production by lung macrophages upon γ -*Mtb* stimulation. We also determined whether EtG affects the metabolic state of γ -*Mtb* cultured lung macrophages.

MATERIALS AND METHODS

Animals

Specific pathogen-free 4- to 6-week-old female C57BL/6 mice were purchased from the Jackson Laboratory and housed at the animal facility at the University of Texas at Tyler Health Science Center (UTHSC). All animal studies were approved by the Institutional Animal Care and Use Committee of UTHSC (Protocol #669). The animal procedures involving the care and use of mice were carried out in accordance with the guidelines of the NIH/OLAW (Office of Laboratory Animal Welfare). Gamma-irradiated *Mtb* (γ -*Mtb*) was utilized throughout this study to stimulate macrophages in vitro.

Isolation of lung macrophages

Mice were euthanized by CO₂ and lungs were dissected with scissors and forceps in a 40 μ m petri dish. They were further incubated at 37°C for 30 minutes in 500 μ l lysis buffer (containing 1U/ml dispase, 0.25% collagenase A, 2000U/ml DNase 1 and 80% RPMI) per 10⁷ cells. Afterwards, samples were filtered and smashed through a 40 μ m cell strainer and were suspended in Hank's Balanced Salt

Solution (HBSS) to a volume of 10 ml. Samples were centrifuged at 400 xg. Cell pellets were washed once more with HBSS after which they were suspended in 180 µl MACS buffer per 10⁷ cells. Samples were stained with 20 µl of anti-F4/80 microbeads ultrapure, mouse (Miltenyi Biotec) and incubated in the dark at 4°C for 15 minutes. Cells were washed by adding 2 ml of buffer per 10⁷ cells and centrifuged at 300xg for 10 minutes. Supernatant was aspirated completely and resuspended in 500 µl of buffer. Large separating (LS) columns were placed in the magnetic field of a suitable MACS separator. Columns were prepared by rinsing with 3 ml MACS buffer. Cell suspension was applied onto the columns and flow-through was collected containing unlabeled cells. Columns were washed thrice with 3 ml of MACS buffer and unlabeled cells were collected. Columns were removed from the separator and placed on a suitable collection tube. Five ml of MACS buffer was pipetted onto the column and was immediately flushed to release the magnetically labeled cells by firmly pushing the plunger into the column. The cells were centrifuged at 300 xg for 10 minutes and then resuspended in complete RPMI media (containing 10% fetal bovine serum and 1% penicillin and streptavidin).

Flow cytometry analysis

For surface staining, approximately 5×10^5 cultured lung macrophages were stained for 30 minutes at 4°C in 15 ml tubes with directly conjugated antibodies (below) diluted in 200 μ l of staining buffer (PBS containing 2% heat inactivated FBS). After staining, cells were washed twice, and fixed in 1% paraformaldehyde in PBS (MilliporeSigma) before acquisition using an Attune NxT acoustic flow cytometer (Invitrogen). The following antibody clones were used for this study: CD45 (Brilliant Violet 605), CD11b (PE/Cyanine 7), F4/80 (Brilliant Violet 421), Siglec F (PE/Dazzle), and Fixable Aqua.

Cytotoxicity assay

EtG was evaluated for its cytotoxicity against lung macrophages using cyQUANT LDH cytotoxicity assay kit (Invitrogen). Cell culture supernatants (50 μ l) were collected and transferred to a 96-well flat bottom plate after 48 h incubation of cultured cells with EtG at 2.25, 45, 90, and 375 μ M and stored at -70 °C. Reaction mixture (50 μ l) was transferred to well containing sample and incubated at room temperature for 30 mins. Stop solution (50 μ l) was added to each sample well to stop the reaction. Absorbance was measured at 490 nm and 680 nm using a spectrophotometer (Molecular Devices). The level of lactate dehydrogenase (LDH)

activity was determined by subtracting the 680 nm absorbance value from the 490 nm absorbance value. EtG concentrations were selected based on their cytotoxicity. Graph was plotted using GraphPad prism software.

Measurement of cytokine production

Lung macrophages were treated with 90 μ M of EtG per 50,000 cells and were stimulated with γ -mtb at 1 μ g/ml per 50,000 cells for 48 h. Cell culture supernatant (50 μ l) was collected for each sample. The Bio-Plex® MAGPIX™ Multiplex Reader was utilized to analyze samples. In culture supernatants, the following cytokines and chemokines were measured using a multiplex ELISA kit per the manufacturer's instructions. (Eotaxin/CCL11; GM-CSF; GRO- α /CXCL1; IFN- α ; IL-1 β ; IL-1 α ; IL-1RA; IL-6; IL-8/CXCL8; IL-10; IL-12 p70; IL-18; IL-23; IL-27; IP-10/CXCL10; MCP-1/CCL2; MIP-1 α /CCL3; MIP-1 β /CCL4; RANTES/CCL5; SDF1- α /CXCL12; and TNF- α). The manufacturer's protocol was followed for the multiplex bead assays [35]. Briefly, 25 μ l of standard and 25 μ l of sample along with 25 μ l of mixed beads was added to the appropriate wells; 25 μ l of matrix solution and 25 μ l of assay buffer was added separately into the standards wells and samples wells. After 2 h of incubation at room temperature with shaking followed by

washing, detection antibodies were added to the wells and incubated for 1 h at room temperature, followed by washing. Finally, 25 μ l of streptavidin-phycoerythrin per well was added and incubated for 30 min at room temperature. Then, the well contents were removed and washed with wash buffer. The mixed beads were re-suspended with 150 μ l of sheath fluid. Data was collected and analyzed using the Luminex xPONENT software (Luminex Corporation, USA).

Mitochondrial stress test

Lung macrophages treated with 90 μ M EtG per 250,000 cells and stimulated with γ -*Mtb* at 2 μ g/ml for 48 h were plated in XF96 plates. The mito-stress test (Agilent Technologies; Cat: 103015-100) and glycolysis stress test (Agilent Technologies; Cat: 103020-100) were performed using the Seahorse Xfe96 analyzer (Agilent, Santa Clara, CA). The cells were washed with XF base glyco buffer (1% Glutamine + DMEM medium) and oxphos buffer (1% glucose, 1% glutamine, 1% pyruvate + DMEM medium) respectively.

Measurement of OCR (oxygen consumption rate) was performed with subsequent injections of 1.5 μ M oligomycin, 1 μ M FCCP (carbonyl cyanide-4 trifluoromethoxy phenylhydrazone) and 0.5 μ M rotenone/antimycin A. Spare respiratory capacity was measured as the maximum OCR after FCCP injection subtracted from the

basal OCR under steady state conditions. While ECAR (extracellular acidification rate) measurement was carried out by injections of 10 mM glucose, 1 μ M oligomycin and 50 mM 2-DG (2-deoxy-d-glucose). GlycoATP and MitoATP production were calculated considering the proton efflux rate (PER), as extracellular acidification was a combined effect of both CO₂ produced by OXPHOS and H⁺ ions produced by lactate formed due to glycolysis. The following formula was used by the Wave Analysis Software to calculate the following: glycoATP Production Rate (pmol ATP/min) = glycoPER (pmol H⁺/min) mito ATP production Rate (pmol ATP/min) = OCRATP (pmol O₂/min)*2 (pmol O/pmol O₂)*P/O(pmol ATP/pmol O). Wave Desktop 2.6 software (Agilent) was used for the data analysis.

Statistical Analysis

All data analysis was performed using GraphPad Prism 8.0 (GraphPad software Inc.), and all results were expressed as the mean \pm standard error of the mean (SEM). For normally distributed data, an unpaired t-test was performed to compare between groups. One-way ANOVA followed by Tukey's test for multiple comparison was performed.

RESULTS

Purity of F4/80 positive cells

Lung macrophages were isolated using the magnetic bead–conjugated antibody cell isolation technique. Lung cells were incubated with anti-mouse F4/80 antibody mAb conjugated magnetic beads and passed through LS column. F4/80 positive cells were collected via flow-through from column. To confirm the purity of isolated lung macrophages, flow cytometry analysis was performed. The purity of isolated macrophages was around 85.1% (Fig.1) This analysis is a standard procedure in the laboratory for the characterization of lung macrophages and was performed once.

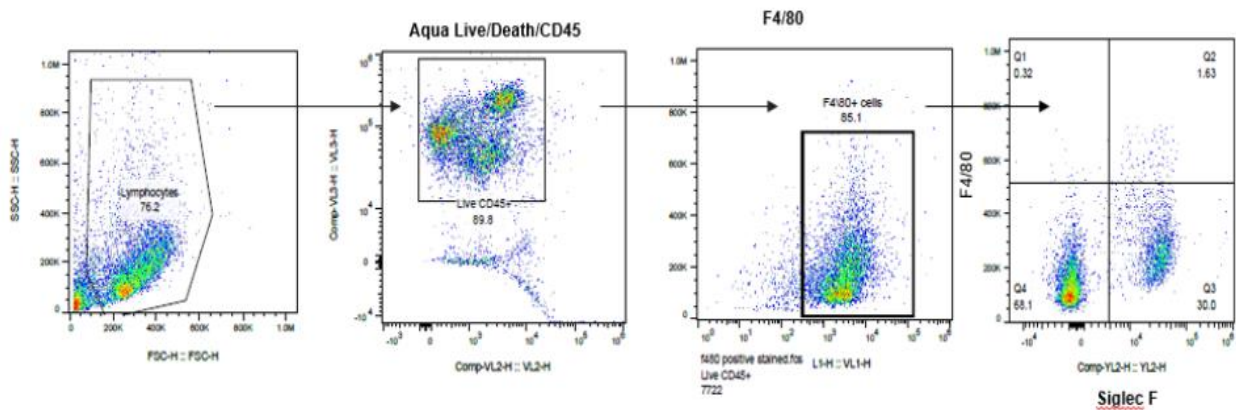


Figure 1: Characterization of isolated Lung Macrophages. Approximately 5×10^5 cultured cells were stained with 5 μ L each of anti-CD45, CD11b, F4/80 and Siglec F antibodies conjugated with fluorophores for 30 minutes at 4°C in 15 mL tubes. Samples were diluted in 200 μ L of staining buffer (PBS containing 2% heat inactivated FBS). After staining, the cells were washed twice, and fixed in 1% paraformaldehyde before acquisition using an Attune NxT acoustic flow cytometer. Experimental results were analyzed using FlowJo software. Data are representative of a single experiment. N = 1.

Ethyl-β-D-glucuronide is not cytotoxic to murine lung macrophages.

We next determined the viability of lung macrophages in the presence of various concentrations of EtG using lactose dehydrogenase (LDH) cytotoxicity assay. LDH is an enzyme located in the cytosol of many different cell types and released into culture medium upon cell damage or lysis. LDH concentrations in the culture medium can thus be used as an indicator of cell membrane integrity and cytotoxicity [36]. This experiment helped to determine the optimal concentration of EtG for subsequent cell culture studies. A previous study showed that the concentration of EtG in the blood of heavy drinkers after termination of heavy alcohol intake was 1.7 mg/L (7.65 μM) [37]. We utilize higher concentration for this experiment as cells are exposed to the metabolite in an acute condition within a limited time of 48 h. The purpose is to initiate a condition almost similar to a continuous exposure of a low concentration of the metabolite to immune cells in a physiological condition. We carried out LDH cytotoxicity assay to ensure that these high concentrations are not toxic to the cells. In this study, the quantity of LDH released after treating the cells with various concentrations of EtG for 48 hr was determined following the CyQUANT LDH cytotoxicity assay kit protocol. Cells without treatment and cells treated with 2.25, 45, 90 and 375 μM of EtG had low cytotoxicity levels of 16.26 ± 6.020 %, 9.66 ± 1.803 %, 11.36 ± 1.234 %, $10.35 \pm$

0.463 % and 18.55 ± 1.379 % as compared to cells treated with lysis buffer (Max LDH sample) which had a cytotoxicity level of 100.00 ± 1.439 % (Fig. 2).

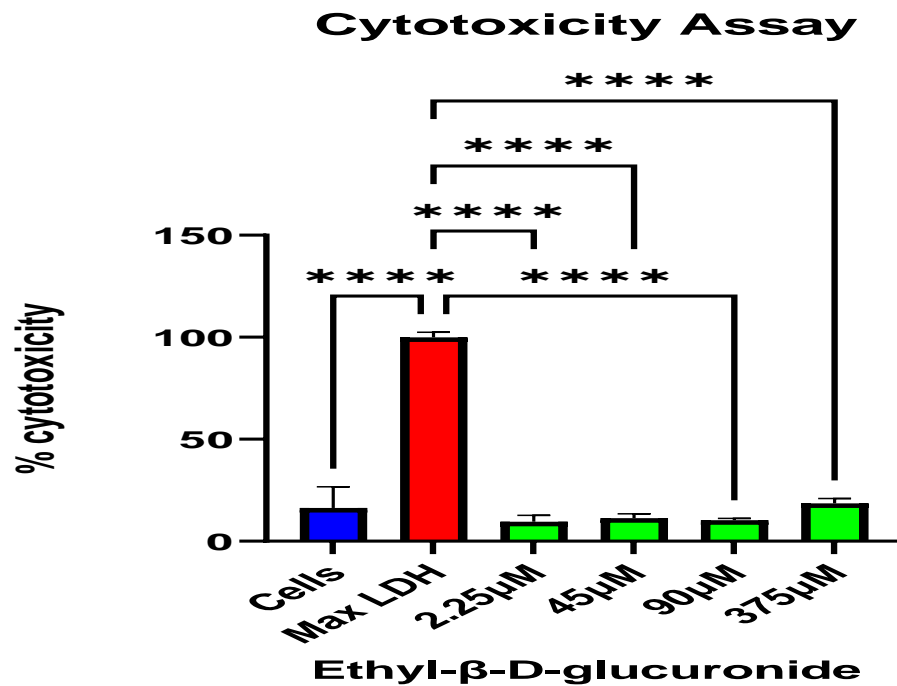


Figure 2: Cytotoxicity of Ethyl-β-D-Glucuronide on lung macrophages. Cell culture supernatants were collected after 48 h incubation of cultured cells with various concentrations of EtG and from cells without any EtG (Cells) and stored at -70 °C. The level of lactate dehydrogenase (LDH) was measured by aliquoting 50 μ L to each sample and measuring absorbance at 490 nm and 680 nm on a spectrophotometer. Maximum LDH activity was achieved by aliquoting 10 μ L of 10 X lysis buffer to the cells without any treatment. Data was analyzed on GraphPad prism software. The mean values, SEM values of three independent experiments and P values are shown. (****, $p < 0.0001$), $N = 3$.

Ethyl-β-D-Glucuronide inhibits anti-inflammatory cytokine production

Cytokines and chemokines are secreted by immune cells to fight infections, recruit other members of the immune system and regulate homeostasis [38]. We determined the effect of EtG on lung macrophages to produce cytokines and chemokines upon γ -*Mtb* stimulation. Isolated lung macrophages were treated with 90 μ M EtG and stimulated with γ -*Mtb*. After 48 hr, cytokines and chemokines in the cell culture supernatant were determined by multiplex ELISA analysis. The assay measured levels of cytokines and chemokines secreted by lung macrophages. Among 34 various cytokines and chemokines tested, EtG inhibited IL-10 secretion by γ -*Mtb* stimulated lung macrophages (from 114.09 ± 25.32 pg/ml to 58.93 ± 9.145 pg/ml, $p = 0.0486$, $N = 3$) (Fig. 3). In contrast, EtG enhanced macrophage inflammatory protein (MIP)-1 β secretion by γ -*Mtb* stimulated cells (from 31.99 ± 1.409 pg/ml to 38.46 ± 1.235 pg/ml, $p = 0.0053$, $N = 3$). There were also notable effects on other cytokines and chemokines production including IL-17A, IL-22, RANTES, GM-CSF. However, there was no statistical significance in these results (Fig 3).

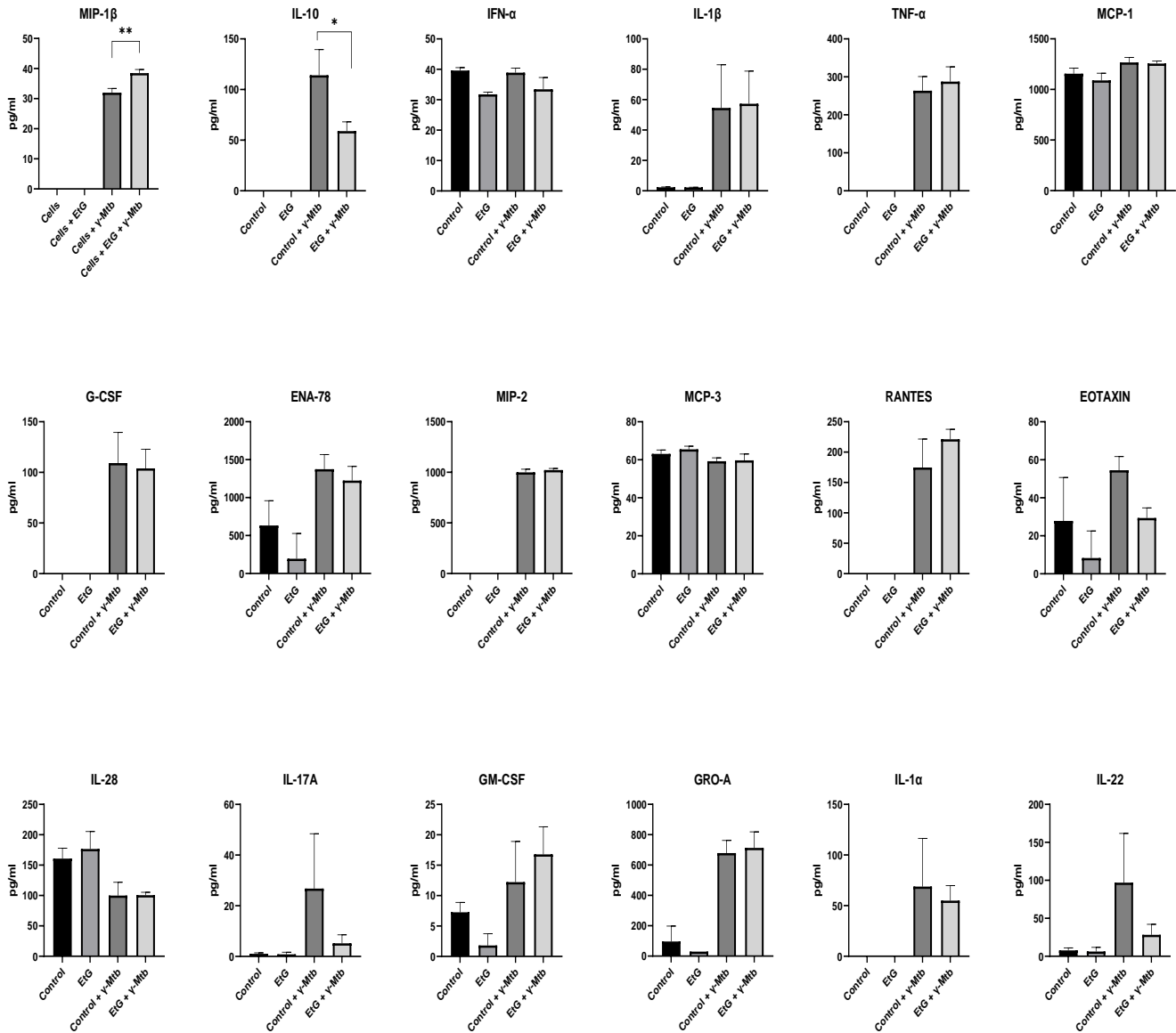


Figure 3: Effect of Ethyl- β -D-Glucuronide on cytokines and chemokines secretion by γ -Mtb stimulated lung macrophages. Lung macrophages were treated with EtG before stimulated with γ -Mtb. The cell free culture supernatants from control and γ -Mtb stimulated samples were collected, and cytokine and chemokine levels were determined by multiplex ELISA. The mean values and SEM values are shown for three independent experiments. (*, p < 0.05; **, p < 0.01) N = 3.

Ethyl- β -d-glucuronide inhibits oxidative phosphorylation of γ -*Mtb*
stimulated lung macrophages.

We further investigated the effect of EtG on the metabolic state of γ -*Mtb* –cultured lung macrophages. We used mito stress test to study oxidative phosphorylation rate and glycolysis stress test to determine glycolysis in the cells. There was a reduction of oxidative phosphorylation by γ -*Mtb* stimulated lung macrophages, although the results lacked statistical significance. This reduction is characterized by a decrease in basal respiration (from 86.774 ± 20.30 pmol/min to 59.185 ± 18.60 pmol/min, $P = 0.72$, $N = 3$), maximum respiration (from 271.016 ± 72.84 pmol/min to 168.038 ± 54.30 pmol/min, $P = 0.64$, $N = 3$), ATP production (from 67.746 ± 16.00 pmol/min to 48.047 ± 14.95 pmol/min, $P = 0.79$, $N = 3$), and sparse respiratory capacity levels (from 184.246 ± 55.54 pmol/min to 98.851 ± 37.06 pmol/min, $P = 0.61$, $N = 3$) (Fig. 4). On the other hand, there was no obvious effect of EtG on glycolytic parameters in γ -*Mtb* stimulated lung macrophages including, the basal glycolysis (from 47.698 ± 11.14 mpH/min to 47.476 ± 11.62 mpH/min, $P > 0.99$, $N = 3$), glycolytic capacity (from 60.984 ± 13.93 mpH/min to 58.8 ± 17.30 mpH/min, $P = 0.99$, $N = 3$), and glycolytic reserve (from 13.284 ± 2.813 mpH/min to 11.321 ± 2.919 mpH/min, $P = 0.98$, $N = 3$) (Fig. 5). These results demonstrate that EtG inhibits oxidative phosphorylation of γ -*Mtb* –cultured lung macrophages.

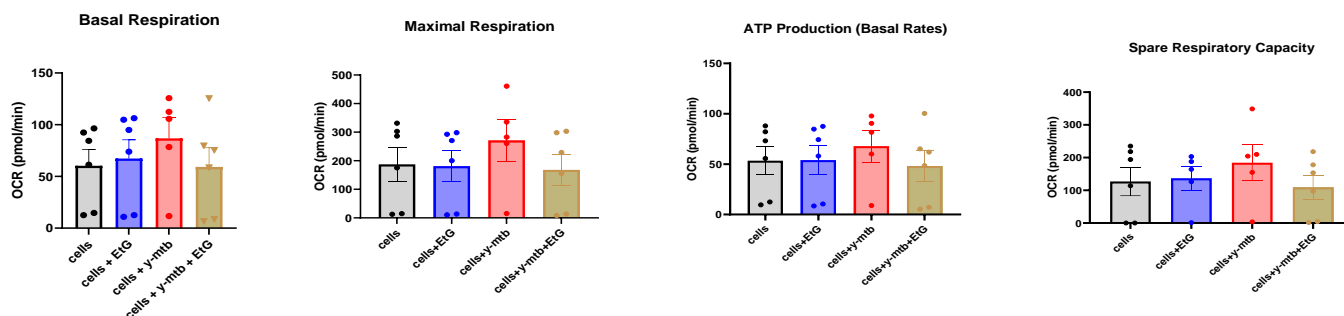
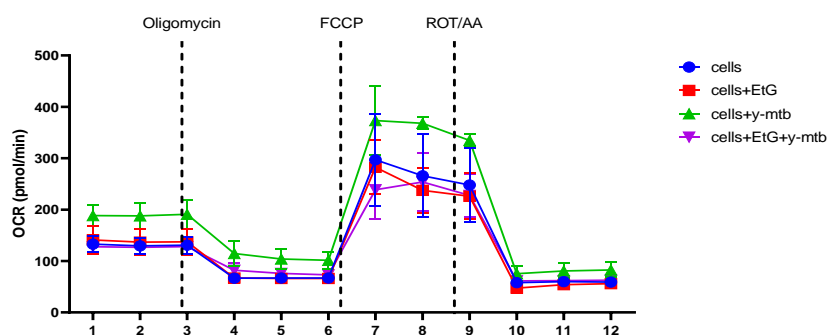
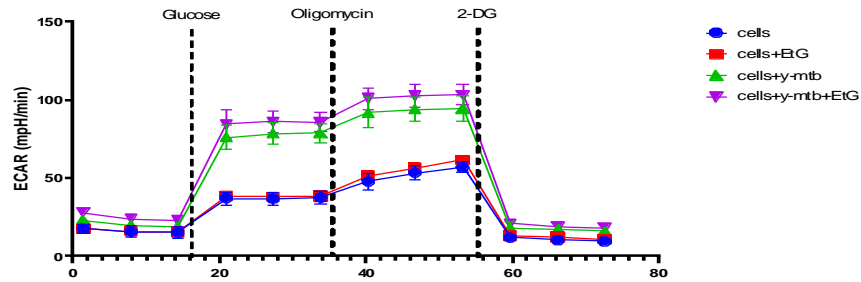
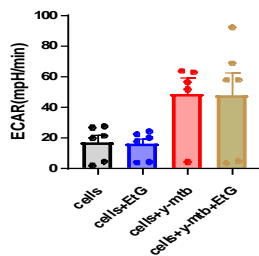


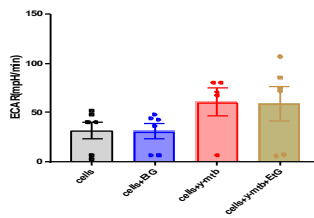
Figure 4: Effects of Ethyl- β -D-Glucuronide on energy metabolic profile of γ -Mtb stimulated Lung Macrophages. Lung macrophages were treated with EtG then, they were stimulated with γ -Mtb. Control and γ -Mtb stimulated cells were treated with Oligomycin, FCCP, and ROT/A drugs for measure of OCR per manufacturer's instructions. The mean values and SEM are shown for three independent experiments. N = 3.



Basal Glycolysis



Glycolytic Capacity



Glycolytic Reserve

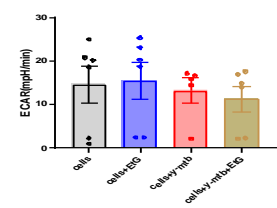


Figure 5: Effects of Ethyl- β -D-Glucuronide on energy metabolic profile of γ -mtb stimulated Lung Macrophages. Lung macrophages were treated with EtG then, they were stimulated with γ -Mtb. Control and γ -Mtb stimulated cells were treated with glucose, oligomycin and 2-DG drugs for measure of ECAR per manufacturer's instructions. The mean values and SEM are shown for three independent experiments. N = 3.

DISCUSSION

Chronic alcohol consumption impairs host immune defense and increases susceptibility to various infections, including *Mtb* [39-41]. However, there is a dearth in information regarding the mechanisms involved in alcohol-mediated host immune impairment during *Mtb* infection and other intracellular infections. In this study, we determined the effects of the alcohol derived metabolite EtG on cytokine and chemokine production and the metabolic state of γ -*Mtb* stimulated lung macrophages from mice.

We found that EtG inhibits IL-10 production by γ -*Mtb* stimulated mice lung macrophages. IL-10 is a potent anti-inflammatory cytokine produced by various hematopoietic cells including B cells, neutrophils, macrophages, and some dendritic cells subsets [42, 43]. IL-10 has a suppressive effect on IL-12 and TNF- α production [44, 45]. Studies have shown that chronic alcohol abuse causes an increase in TNF- α production by sensitization to LPS which can lead to lung injury, inflammation, and mortality [31, 46]. In the current study, we noticed an increase in TNF- α secretion as well as some other pro-inflammatory cytokines. There was an increase in the production of granulocyte-macrophages colony-stimulating factor (GM-CSF), a cytokine responsible for the expansion and differentiation of myeloid cells. RANTES, a CC-chemokine that promotes infiltration of most immune cells to the site of infections, also showed increased secretion in EtG-treated lung

macrophages. Studies have shown GM-CSF to be implicated in the induction of sepsis, a condition characterized by an uncontrolled inflammatory response. Although there was no statistical significance in these results, which may be due to limitation in the number of experiments performed, a trend in the effects of the metabolite on pro-inflammatory cytokines was observed. We also noticed a reduction in IL-17A production by EtG. IL-17A is a part of the IL-17 family known to be a signature cytokine of activated CD4⁺ T helper subset, T helper 17 (Th17) cells. IL-17A has also been identified to be produced by neutrophils at certain conditions [47, 48]. The secretion of IL-17A may have been as a result of the presence of a small population of activated T helper cells and or neutrophils as isolated cells were not entirely macrophages as confirmed in the flow cytometry analysis. EtG may mediate a reduction in IL-22 production by lung macrophages stimulated by γ -*Mtb*. IL-22 is a potent immune-regulator and a part of the IL-10 family responsible for repair and regeneration of epithelial cells following injury [49]. This finding further suggests that EtG may mediate inhibition of anti-inflammatory cytokines production. More experiments are required to confirm these findings with statistical significance. We found that EtG enhances production of MIP-1 β by γ -*Mtb* stimulated lung macrophages. MIP-1 β is an important chemokine that acts on various immune cells [50]. It drives co-stimulation of purified human T cell and human T cell clone proliferation and IL-2 production [51]. They stimulate the release of proinflammatory cytokines in malignancy [52]

Previous studies have shown that the levels of MIP-1 β involved in monocyte recruitment were increased in the liver of patients with alcoholic liver disease (ALD) [53] Our findings suggest that an increase in MIP-1 β can further enhance inflammatory responses during *Mtb* infection in alcoholic host.

The classically activated macrophages or M1 and alternatively activated macrophages or M2 are distinct by their cytokine production profiles and pathways in bioenergy generation. M1 is characterized by the production of pro-inflammatory cytokine and dependence on glycolysis for energy generation [54]. M2 utilizes oxidative phosphorylation to generate energy and is associated with anti-inflammatory cytokine production [55]. To determine the effect of EtG on the bioenergetic profile of lung macrophages stimulated with γ -*Mtb*, we compared glycolysis and oxidative phosphorylation levels in the macrophages. We carried out the mito stress test to measure important parameters of mitochondrial function by directly measuring the oxygen consumption rate (OCR) of cells. Cells were injected with oligomycin to inhibit ATP synthase which causes a decrease in electron flow through the electron transport chain (ETC), thereby decreasing mitochondrial respiration. This effect is closely related to ATP production which is needed to meet energy demand. EtG may have caused a reduction in ATP production in treated cells suggesting that it limits the cell's ability to meet energy demand via oxidative phosphorylation during stressful conditions. Carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) was the next drug injected

into cells to collapse the proton gradient and disrupt the mitochondrial membrane potential. This results in uninhibited flow of electrons through the ETC. This effect gives insight into the maximum respiration rate the cells can achieve during metabolic challenges that require energy demand. This parameter is also used in defining the spare respiratory capacity of the cells which measures the cell's capacity to respond to increased energy demand. Our results suggest that EtG may limit maximum respiration rate and spare respiratory capacity of lung macrophages in *Mtb* infected alcoholic mice. However, the mito stress assay results yielded no significance. This may have been caused by the limited number of experiments and uneven numbers of cells used for each experiment. More experiments may be carried out to confirm the inhibitory effects of EtG on oxidative phosphorylation in lung macrophages during *Mtb* infection.

Our findings suggest that EtG may prevent polarization of macrophages into M2 phenotype by inhibiting oxidative phosphorylation thereby limiting regulation of pro-inflammatory response by reduction of potent anti-inflammatory cytokines such as IL-10. The glucose stress test was carried out to show the effect of EtG on glycolysis in lung macrophages. There was no evidence of the effect of EtG on glycolysis in the stimulated macrophages. Our findings suggest that EtG may carry out its inhibitory effects mainly on oxidative phosphorylation resulting to a reduction in anti-inflammatory response.

In conclusion, our study demonstrate that EtG (alcohol derived metabolite) inhibits an anti-inflammatory cytokine IL-10 production, enhances a pro-inflammatory chemokine MIP-1 β production and may ownregulate oxidative phosphorylation of γ -*Mtb* stimulated mice lung macrophages. Our findings suggest that EtG may promote lung injury by decreasing IL-10 production, enhancing MIP-1 β production and downregulation of oxidative phosphorylation in macrophages of alcoholic mice infected with *Mtb*. Further studies are needed to be performed to determine *in vivo* relevance to our current findings to develop better methods to treat alcoholic individuals infected with *Mtb*.

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