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Examining the Role of Glucose Metabolism in the Macrophage Response to Mycobacterial Infection

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ABSTRACT

Background: The metabolic state of macrophages has been implicated in Mtbpathogenesis. Macrophages are the key host phagocytic immune cells that support the growth and proliferation of Mtb early post infection. Two main macrophage types that are involved in early Mtb are the alveolar macrophages (AM) and the interstitial macrophages (IMs). In Mtb infection models, IMs utilise glycolysis by metabolising glucose to become activated whereas Alveolar macrophages (AMs) utilise fatty acid oxidation (FAO) and provide the ideal niche to support the replication of Mtb by providing fatty acids and cholesterol.
Aim: We do not know the availability of glucose within the lung during Mtb infection and we wanted to test the hypothesis that glucose availability impacts the response of macrophages to Mycobacterial infection.
Methods: In order to test our hypothesis, we used bone marrow derived macrophages (BMDM) as model cells and cultured them in various concentrations of glucose, we then exposed them to *Mycobacteria*l infection and compared the response of the macrophages to this exposure.
Results: In different glucose concentrations of 5.5 mM, 11.1 mM and 25mMthe response of activated macrophages was altered. We found that cytokine IL-6 and TNF-αresponse was reduced in 25 mM glucose. Next, we examined macrophage polarization and we found that cells treated with IFN-γ and IL-10 were able to inhibit nitrite and TNF-α production. Our results have shown by altering the availability of glucose we can significantly reduce the pro-inflammatory cytokine TNF-α response of macrophages. We have also implicated FAO as a mediator of macrophage responses when glucose is altered.
Conclusions: Our data support our hypothesis that altering glucose availability impacts the response of macrophages to cytokines and *Mycobacteria*. We also implicated FAO in this response and are investigating the role of these metabolic pathways in bacterial killing.

Keywords: Fatty acid oxidation (FAO), Glycolysis Mycobacterium tuberculosis (Mtb), Glucose Alveolar macrophages (AM), Interstitial macrophages (IM)

Abbreviations: AAM: Alternatively Activated Macrophage, AM: Alveolar Macrophages, ATP: Adenosine Triphosphate, BMDM: Bone Marrow Derived Macrophages, C2: beta carbon, C3: gamma carbon, CAM: Classically Activated Macrophage, CFU: Colony Forming Units, ETC: Electron Transport Chain, FADH2: Flavin adenine dinucleotide (Reduced), FAO: Fatty Acid Oxidation, IFN-γ: Interferon gamma, IL-6: Interleukin-6, IL-1β: Interleukin 1 beta, IL-10: Interleukin-10, IM: Interstitial Macrophage, LPS: Lipopolysaccharide, *Mtb: Mycobacterium Tuberculosis*, MOI: Multiplicity of Infection, NADH: Nicotinamide adenine dinucleotide, NO: Nitric Oxide, OXPHOS: Oxidative Phosphorylation, ROS: Reactive Oxygen Species, TB: Tuberculosis Bacterium, TCA: Tricarboxylic Pathway Cycle, Th1: T helper 1, TNF-α: Tumour Necrosis Factor- alpha

INTRODUCTION

Tuberculosis (TB) - Understanding TB pathogenesis

Tuberculosis (TB) is a highly infectious disease caused by the deadly bacterium *Mycobacterium tuberculosis* (*Mtb*) [1,2]. It has co-existed with humans for over 50,000 years and is known to be one of the oldest diseases to affect humans [3]. Although the incidence of TB has declined overall in the technologically advanced countries, in the less developed part

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of the world, the number of TB cases is on the rise [4,5]. Currently, it is estimated that 1.7 billion of the world's population are infected by *Mtb* and an estimated 3 million deaths are caused by TB worldwide [6,3]. Therefore, TB is a serious global health problem [5,1].

Multifactorial circumstances have contributed to an increase in the spread of TB worldwide. These range from new cases of HIV and diabetes, which along with the continuous decline in living standards in many parts of the world, have accentuated the seriousness of the global health problem [3,5,7]. New cases of *Mtb* infection are on the rise especially in India, Pakistan, Indonesia, Philippines and China which contribute 56% of new TB cases globally [3, 8].Considering the data on TB, the question that must be asked is - what action is required to curb the rise in TB cases globally? Are current treatment regimens and strategies failing to manage the TB epidemic?

History of Tuberculosis

Establishing the origins and evolution of *Mycobacterium tuberculosis* (*Mtb)*is like entering a minefield of information. It is an area which has caused a fair amount of controversy where researchers have not been able to agree a date for the emergence of *Mtb*. However, the genus *Mycobacterium* is a slow growing organism with a slow rate of mutation [4], making it possible to estimate its origin and evolution. Some studies have proposed the genus existed in the Jurassic period, about 150 million years ago [4,9]. DNA evidence extracted from Egyptian mummies suggest that early Egyptians may have died from TB [4,9]. More recently, evidence uncovered from the DNA of *Mtb* from an infected lesion in the lungs of a 1000-year-old Peruvian mummy provide further evidence that the disease was in existence in pre-Columbian New World [10].

In ancient Classical Greek records, the famous Greek Physician Hippocrates and his student Plato and Aristaeus, referred to pulmonary TB as phthisis [4]. The disease was accurately described along with its symptoms and the characteristic tubercle lesion in the lungs [9]. Evidence of bony TB was uncovered from archaeological sites throughout Europe indicating that TB was widespread in the 8th and 19th century as evidenced from archaeological findings [9,4].

The isolation of the *Mtb* bacillus

The discovery of *Mtb* has been credited to Robert Koch in his seminal work where he isolated the bacillus using coagulated bovine serum heated to 40˚Cand identified it using the staining method, methylene-blue [9,4,3]. However, there are earlier records dating back to 1626 where Jean-Antoine Villem in, a French military surgeon, was able to prove the infectious nature of *Mtb*. He extracted *Mtb* from the pulmonary cavity of a patient that had died of the disease and infected a rabbit. After 14 days post infection, the rabbit was sacrificed and he observed how quickly the disease had spread [4,9].

Tuberculosis pathology

The lungs are the main targets for *Mtb* [7,11]. The bacterium starts its infectious life cycle trapped in a liquid aerosol droplet. When an infected individual with active TB coughs, they produce an aerosol droplet encapsulating the bacterium [12]. If the droplet is inhaled by the unsuspectinghost, it travels to the lungs where it is ingested by the alveolar macrophage's resident in lung tissue [12,5].

Bacteria are ingested by macrophages via receptor mediated phagocytosis which uses the recognition of pathogen associated molecular patterns (PAMP) on the bacteria by pattern recognition receptors (PRRs) on the macrophage [13]. Once ingested *Mtb* manipulates the host macrophage response. A key process that occurs during *Mtb* infection, is the formation of a phagosome, which encapsulates the microbe, thus enabling the *Mtb* to avoid detection by the host innate and adaptive immune response. *Mtb's* complex lipid cell envelope, composed of a diverse medley of lipids, disrupts phagosome maturation and prevents acidification of the phagosome and fusion with lysosomes [14]. The ability of *Mtb* to disrupt this mechanism establishes a permissive niche for the bacterium to grow and replicate and inhibits bactericidal killing [15,13]. The formation of the phagosome is a key event in the pathogenesis of TB and ensures the survival of the microbe in its new environment [13].

Alveolar macrophages

Alveolar macrophages (AM) are the main phagocytic cells found in lung tissue located on the luminal surface of the alveolar space [16]. AM cells are the first immune cells that detect and phagocytose the invading *Mycobacterium*. However, alveolar macrophages also have a critical role in tissue repair mechanisms and homeostasis [17,18]. The immune cells carry out a number of important functions as part of their defensive immune response to infection with *Mtb*. AM are highly pliable cells that react to their immediate tissue environment and can adapt their function depending on the health and disease status in the host [16,19,20]. Alveolar macrophages are thought to be alternatively activated macrophages (AAM) also known as M2 macrophages. These phagocytic cells are part of the early innate immune response to *Mtb* invasion. AM provide a permissive environment for *Mtb* bacterium to replicate without activating the innate and adaptive immune system [13]. Studies suggest that *Mtb* "joyrides" in the AM using these cells to travel into the lung interstitium, this process requires an intact ESX-1 secretion system in the bacteria and leads to the establishment of a niche in lung tissue that is conducive to mycobacterial growth and proliferation [21,18].

Macrophages – A niche for *Mtb* replication

Macrophages have a diversified set of functions in the host immune response such as tissue homeostasis and repair, host defence and in pathology [22,23,19]. As macrophages carry out such diverse and opposing functions, macrophages are

highly plastic in their phenotype and can change and adapt their function depending on their microenvironment [24,2,17]. Studies exploring the role of macrophages have suggested two types, M1 and M2 macrophages. M1 macrophages are also known as the classically activated macrophage (CAM), which produce pro-inflammatory cytokines. The alternatively activated (AAM), also known as M2 macrophages, upon activation trigger the production of anti-inflammatory cytokines [19,25]. Macrophages that assume an M1 phenotype are stimulated either by interferon gamma (IFN-γ), tumour necrosis factor (TNF) or lip polysaccharides (LPS) or other pathogen associated molecular patterns which bind to pattern recognition receptors on macrophages [23,26]. These macrophages have bactericidal properties and secrete nitric oxide (NO) to eliminate the pathogen from the host. Conversely the M2 macrophages are activated by cytokines IL-4, IL-10 or IL-13 produced by T-helper cells 2 (Th2) [27,28]. These cytokines drive the M2 phenotype and cause up regulation of specific markers such as dectin-1, mannose receptor or IL-10 found on the surface of M2 macrophages [26,19]. As part of their function in tissue repair and remodelling, M2 macrophages generate ornithine and polyamines via the arginase-ornithine decarboxylase pathway [19] **(Figure 1)**.

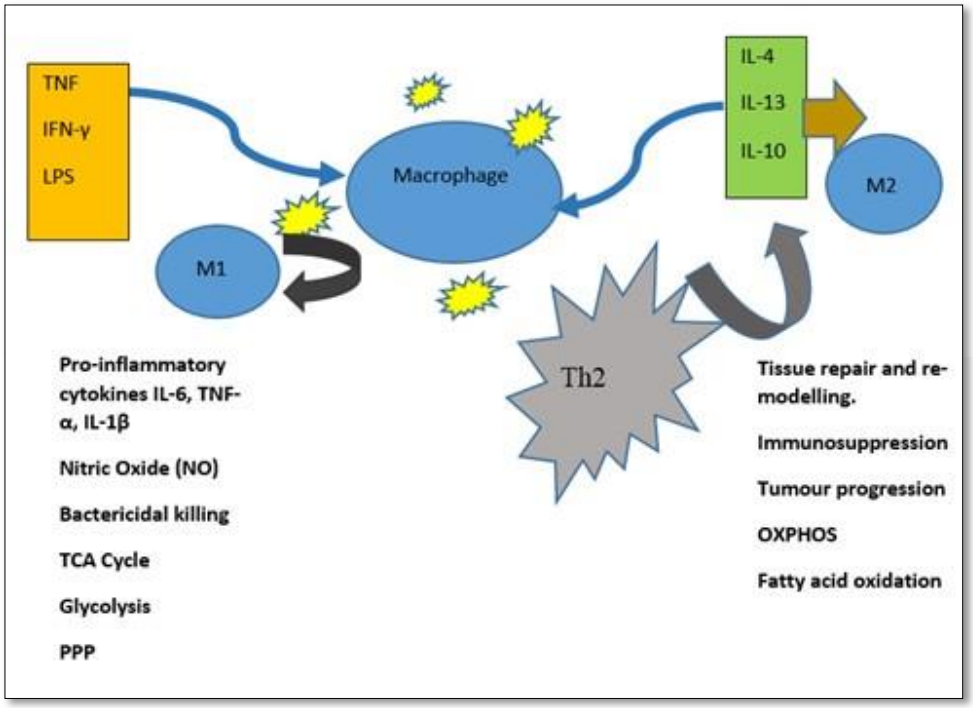

Figure 1. Activation of macrophages. Dependent on the stimulus, macrophages will either polarize towards M2 state and will be involved in tissue repair mechanism or towards the M1 phenotype and will take on bactericidal properties such as releasing ROS intermediates e.g. Nitric oxide (NO) into the environment to kill Mtb.

Glycolysis in M1 macrophage and role of glucose

Glucose is an essential energy source for immune cells and a critical player in the activation of the pro-inflammatory response of macrophages [29]. Without glucose the immune system cannot function correctly. Glucose is a key step in cellular metabolism and activation of signaling pathways which activate the immune response [30,31].

After invasion, glucose is rapidly utilized by *Mtb* raising the requirement for glucose availability. The simple carbon molecule is a key fuel source not just for pathogenic bacteria but also for immune cells [30,31]. M1 macrophages depend on glucose to generate ROS (reactive oxygen species) as well as NO (nitric oxide) in the antimicrobial response [31,25]. Glucose is also required to generate pro-inflammatory cytokines IL-6, TNF-α and IL-1β [32].

Aerobic glycolysis is a key metabolic process occurring in M1 macrophages. It results in an increase in the uptake of glucose via the GLUT1 uniporter to generate enough energy in the form of ATP fueling macrophages to deal with the infectious pathogen [33, 32]. During glycolysis one molecule of ATP is generated via the glycolysis pathway, where a single molecule of glucose is converted to pyruvate [25]. Once generated, pyruvate enters the tricarboxylic pathway (TCA) cycle [31].

The TCA cycle occurs in the mitochondrial matrix and its function is to generate several carbon intermediates, while at the same time generating reducing molecules of NADH and FADH2. Once generated, the reducing agents enter the Electron Transport Chain (ETC) which is found on the inner mitochondrial membrane of Complex I and Complex II. The transfer of electrons is linked to the pumping of protons from

the mitochondrial matrix to the intermembrane space of the mitochondria [31].

The electrochemical gradient that is generated, via the process of transfer of electrons, generates a proton gradient where an enzyme, ATP synthase, produces increasing amounts of ATP. The actual numbers generated are about 36 to 38 ATP molecules per glucose. Eventually the electrons generated are transferred along the ETC and use molecular oxygen to generate water. Therefore, the success of OXPHOS in this pathway crucially depends on the availability of oxygen molecules [31].

When oxygen is limited, pyruvate is metabolized to lactate in the cytoplasm, instead of entering the mitochondria, therefore, the yield of ATP is only two molecules per glucose via the lactate pathway. However, insights into this key metabolic process have been able to identify the aerobic glycolysis pathway, in which pyruvate is metabolized to lactate, despite the availability of oxygen. The process termed "Glycolytic Reprogramming" is a molecular switch occurring in activated immune cells like switches in lipid and amino acid metabolism which regulate immune function [31].

Role of fatty acid oxidation in macrophages

Fatty acid oxidation (FAO) is a key metabolic process in alveolar macrophages (AM), also known as M2 macrophages [34]. These AM cells provide an ideal permissive environment for *Mtb* bacilli to grow and replicate. FAO regulates the metabolic process in response to *Mycobacterial* infection such as the macrophage inflammatory response and macrophage polarization [35]. A number of studies into fatty acid metabolism have shown that fatty acids are a significant player during TB infection. Studies have shown that host lipids are also a source of nutrition for the pathogen [13]. The release of fatty acids (FAs) from host triacylglycerol (TAGs) and sterols are known to act as the only carbon source during an infection with TB [36], therefore, providing a clear rationale for the study of fatty acid oxidation in TB infection. There are a number of processes which are required for fatty acid metabolism. Beta oxidation is a biological process that occurs in cells for the metabolism of fatty acids. The process occurs in the mitochondria and involves the catabolism of fatty acids into acetyl-CoA using redox factors NAD and FAD. Carnitine facilitates the entry of acetyl-CoA into mitochondria where the process of beta oxidation occurs. The result is the removal of two carbon atoms in the form of acetyl-CoA and acyl-CoA at the carboxyl terminal. This metabolic process involves the cleavage of a bond between the second carbon/beta carbon (C2) and the third carbon/gamma carbon (C3), which is why the reaction takes the name of beta oxidation [37].

The reaction results in the removal of acetyl-CoA from acyl-CoA leaving two molecules of acetyl-CoA. When the reaction reaches the end odd-chain fatty acids are cleaved from acetyl-CoA and propionic acid. Acetyl-CoA enters the citric acid cycle and further oxidation occurs which results in the release of one $FADH_2$ molecule and one NADH molecule. The result is the synthesis of four-high-energy phosphate bonds in the electron transport chain (ETC). The net effect of the beta oxidation reaction is the generation of ATP where a single palmitic acid molecule generates approximately 106 molecules of ATP.

TB and diabetes

A factor that increases the risk of TB is diabetes, which was discovered as far back as 1000 A.D. However, the mechanisms mediating increased susceptibility have not been elucidated [38]. Diabetes is a disease that is associated with dysregulated glucose levels. Unregulated levels of glucose resulting from the inability of the body to produce insulin can be detrimental to health and wellbeing. Diabetes has been linked to a higher susceptibility to TB via the main immune cell in TB – macrophages. Studies have shown the macrophages are altered in diabetes and undergo a shift from anti-inflammatory to pro-inflammatory states [38]. The shift in phenotype and metabolic state of macrophages may be why TB patients cannot eliminate the bacterium. Continuous low levels of chronic inflammation develop defective macrophages that are unable to carry out their defensive role [38].

Project aims

This thesis examines the fundamental relationship between metabolism, macrophages and *Mycobacteria*. In particular, the role of glycolysis and fatty acid oxidation (FAO), two key processes occurring in macrophages that are essential for providing the energy to fuel macrophage activation and function [39] will be addressed. As well as glycolysis, FAO is an essential process providing the carbon source for *Mycobacteria* to persist in the human host [40]. Indeed, beta oxidation of fatty acids has been linked with *Mycobacterium* persistence where even numbered carbon atoms are processed into acetyl coenzyme A (CoA), fatty acids and the odd numbered carbons are broken down to acetyl-CoA and propionyl-CoA [40]. The plasticity of macrophages in their immune response to bacterial infection can be observed by changes to its phenotype and metabolic state. Recent advances in immune metabolism link metabolism to the metabolic state and function of immune cells [32].

This study will investigate the role of metabolism in macrophage activation and in *Mycobacterium* infection. It will be achieved by altering glucose availability for activated macrophages infected with a model organism *M. smegmatis* representing *Mtb* and measuring the impact of glucose availability on the activation and cytokine production of macrophages.

Hypothesis 1: Altering glucose concentration and inhibiting FAO will impact the cytokine response of macrophages exposed to *M. smegmatis* Hypothesis 2: Altering glucose

availability can inhibit macrophage polarization into M1 or M2 states via cytokines IFN-γ or IL-10.

MATERIALS AND METHODS

Mice strains

Wild type (WT) C57BL/6 mice were housed in the Preclinical Research Facility (PRF) within the Division of Biomedical Services at Leicester University. Mice were sacrificed according to ethical standards set by the Home Office and femur bones and lungs of euthanized mice were used as part of the experiments as part of a tissue sharing scheme.

Reagents and antibodies

All chemicals were purchased from Thermo Scientific or Sigma Life Sciences. Cell culture media, Dulbecco's modified eagle's medium (DMEM) was purchased from Sigma-Aldrich. ELISA kits were purchased from Bio Legend. *Mycobacterium smegmatis* strain MC^2155 was used to infect bone marrow derived macrophages (BMDM) and was obtained from Prof Barer's Lab.

Mouse bone marrow derived macrophages (BMDM)

Cells were harvested from the femur bones of C57BL/6 mice. The femur bones were cut from sacrificed mice according to approved method. Muscle tissue was removed from bones and the bone marrow was flushed out from each bone using a 5ml syringe attached to a 25-gauge needle that was inserted into the cut end of the bone. Media in the 5ml syringe was used to flush the marrow into a sterile 50ml tube. The needle was moved up and down to scrape the marrow out of the bone until the bone appeared clear, used bone was discarded. The cell suspension in the tube was centrifuged at 1200 rpm, 4˚C for 7 min. Supernatant was discarded and the pellet was re-suspended in 2 ml of lysis buffer to lyse red blood cells and kept at room temperature for 4 min. Extra media was added before centrifuging at the same settings. Supernatant was discarded and 3 ml of fresh media containing 10ng/ml GM-CSF was added. The pellet was re-suspended by vigorously tapping the outside of the tube. A cell count was taken with a haemocytometer (Brightline - cat no 89760) and 10 µl of cell suspension was added to 10 µl (0.04%) Trypan blue (Sigma Life Sciences). Cell density was counted in each of the 1 × 16 squares on the grid and results were recorded. The average cell count was multiplied by 10^4 and then multiplied by the dilution factor. After counting, the cell suspension was plated into a single well of a 6 well plate and kept in an incubator at 37˚C, 5% CO_2, 19% O_2. The next day, non-adherent cells were collected in a 15 ml tube and centrifuged at the same settings. Supernatant was discarded and the pellet suspended in media containing 10 ng/ml GM-CSF. Cells were counted with a haemocytometer and the cell count was recorded. After counting, a cell density of 0.5×10^7/ml of cells were seeded into tissue culture plates and extra GM-CSF containing media was added to each well. The Plate was returned to the incubator and kept at the same settings. On day 3 and 6 of the harvest, cells were washed and media replaced with fresh media containing GM-CSF and returned to the incubator. On day 9 of cell culture, media was replaced with complete DMEM without GM-CSF. Experimental procedures were carried out on day 9 of the harvest. On day 10 the cells were infected with *Mycobacterium smegmatis* from a stock concentration of 2.98×10^9 CFU/mL. In each well 5×10^6/mLof bacteria was added. The plate was kept in an incubator at the same setting for 36 hours. Supernatant was collected after 36 h in labelled vials and stored in a -80 freezer before carrying out ELISA and Greiss assays.

Enzyme-linked immunosorbent assay (ELISA)

Cell culture media stored in vials and kept in a -80 freezer were thawed at room temperature in the culture hood. Capture antibody specific for each cytokinewas diluted in coating buffer (1:200) and added to plates and placed on a shaker (500 rpm with 0.3 cm circular orbit) for 1 h and washed 4 x with wash buffer. Supernatants (0.2 ml) was added to capture antibody coated wells and placed on a shaker for 2 h, the plates were washed with wash buffer (PBS containing 0.02% tween 20). Detection antibody was titrated in assay diluent (1:1000) and added to the wells,placed on a shaker for one hour and washed 4X with wash buffer. Avidin-HRP was titrated in assay diluent (1:200) and incubated in the wells at room temperature with shaking for 30 min. After washing, the plate 5×, TMB (3'3'5'5' Tetramethylbenzidine) substrate (100 µl) was added to wellsand theplate was incubated in the dark for 30 min or until a blue colour developed. A volume of 100 µl ofStop solution (0.16. M sulphuric acid) was added to each well. A blue colour was formed from the reaction of TMB and peroxide in the presence of horseradish peroxidase (HRP) enzyme conjugate. After adding sulphuric acid (stop solution) the absorbance was read at 450nm with a Varioskan microplate reader. ELISA assay was performed to detect antibodies IL-6, IL-1β and TNF-α according to manufacturer's instructions. Concentration of antibody was determined using a standard curve.

Greiss assay

A Greiss assay was performed on supernatants to measure levels of nitrite produced in each of the experiments that were tested and the same method was employed for each experiment. Greiss reagent was prepared with 1% sulphanilamide, 0.1% napthylethylene diamine dihydrochloride and 2.5% phosphoric acid at a concentration of 85%. A 100 ul of supernatant was added to eachwell of a 96 well plate. After adding Greiss reagent (100 µl) to each well containing supernatant the plate was incubated for 30 min at RT or when a colour change was observed. Absorbance readings were measured at 540 nm with a Varioskan LUX Multimode Microplate reader. Standards were prepared with sodium nitrite according to manufacturer's instruction and levels of nitrite concentration produced in each well was determined by a standard curve. Fresh complete DMEM

media was used as a blank and the value was subtracted from each of the absorbance readings to obtain the corrected absorbance values for each well. Supernatants were arranged in triplicates on the ELISA plate for each experiment.

Staining of BMDM with Kwik-Diff staining

BMDM macrophages were harvested (see protocol on BMDM harvest) and on day 9 of cell culture, media was replaced with either 5.5 mM, 11.1 mM or 25 mM glucose. On day 9 macrophages were treated with either 1 mM BSA palmitate, or 40 µM Etomoxir or both. On day 10 cells were infected with *Mycobacterium smegmatis*at a bacterial concentration of 5 X 10^6, after infecting the cellsthe plate was returned to the incubator for 36 h at a temperature of 36˚C. Post 36 h, supernatants from each well was discarded and 100 µl of Kwik-Diff fixative was added to wells using a, Multi-channel pipette (Gilson). Stain solution was immersed briefly before discardingwhich was followed by Kwik-Diff eosin (100 µl) and Kwik-Diff methylene blue (100 µl), the plate was air dried at (RT). Cell morphology of stained cells was observed with a CXK41 phase contrast microscope at ×40 magnification.

Bacterial strains and media

Mycobacterium smegmatis strain MC^2155 was used in this study. It was grown in Middle brooks 7H9 medium (Difco) with 0.2% of glycerol and 10% (vol/vol) OADC (M0678) growth supplement (containing oleic acid, bovine albumin, sodium chloride, dextrose, and catalase). Agar media was sterilized via autoclaving at a pressure (121˚C) for 15 min and then cooled to 50˚C before plating 5 ml of agar on to culture dishes. Plates were stored in a -20 fridge until ready.

Cell viability estimation

Briefly, supernatant was discarded from tissue plate and 0.6 ml of lysis buffer was added to wells and incubated for 10 min at RT to lyse the cells. Lysis buffer (0.5 ml) was added to tube 1 containing 0.5 ml of buffered saline. From Tube 1 (10^0) 5 ml of diluted bacterial suspension was transferred to tube 2 (10^1) and a 100 µl aliquot from tube 2 was plated on to petri dishes containing semisolid agar. Plates were briefly rotated to swirl suspension and then incubated at 37˚C for 48 h. After 48 h colonies were counted and recorded. In a table, Negative control for *Mycobacterium smegmatis*, saline buffered solution and lysis buffer were carried out to detect for contaminants.

Total viable count (TVC)

Mycobacterium smegmatis colonies were counted using a Reichert Quebec Darkfield Colony Counter. Counts were recorded in a table and graphed using Graphpad Prism **(Table 1)**.

Statistical analysis

The data was presented as mean ± SEM (standard error of the mean). A one-way analysis of variance (ANOVA) followed by a Dunnet's multiple comparison test was performed on the data as well as Student's t- test to analyse the results. A P value of <0.05 was statistically significant.

MATERIALS

Preparation for complete DMEM (cDMEM)

cDMEM was prepared with Dulbecco's Modified Eagle Medium (without L-glutamine) – High glucose by Sigma Life Sciences (Lot no #RNBG7040) and supplemented with 20% Fetal Bovine Serum (Gibco cat no FC438), 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 5% HEPES solution, 10% MEM amino acids (50×) and 5% sodium pyruvate. Media was filter sterilised using a 0.22 µm filter system (CORNING) .and kept in a -20 fridge until use.

Preparation for Stop solution

1 Molar (2N) stop solution was prepared by adding 5.5 mL concentrated H_2SO_4 to 100 mL of distilled water.

Preparation cell lysis buffer

Cell lysis buffer was prepared with Tween 20 (0.05%), Sigma Aldrich, (Lot no# mkbz7746V) and diluted into 100 mL distilled water and placed on a magnetic stirrer at 900 rpm for 30 min.

RESULTS

Determining the role of glucose and fatty acid metabolism in the macrophage response to *Mycobacteria*

Hypothesis 1. Altering the availability of glucose in cell culture will alter the activation of bone marrow derived macrophages stimulated with *Mycobacterium smegmatis*.

To test our hypothesis, bone marrow derived macrophages (BMDM) were harvested and grown in complete DMEM (cDMEM) media containing 10 ng/mL of granulocyte stimulating colony stimulating factor (GM-CSF). On day 9 post-harvest, media was replaced with fresh media containing different glucose concentrations.

We knew that in a healthy individual the normal fasting blood glucose levels are between 4.0 – 5.4 mM/L but below 5.5 mM/L. Patients, who are pre-disposed to type 2 diabetes, have a higher fasting blood glucose level ranging from > 7.8 to > 11.1 mM/L.

Based on these previous studies [41] we decided to incubate murine BMDM in 5.5 mM (normal fasting glucose levels) and 11.1 mM (doubling the fasting blood glucose concentrations of pre-diabetic patients) and 25 mM (doubling the fasting blood glucose concentrations of diabetic patients).

Table 1. Number of colonies counted after plating bacteria.

Name of reagent	(Manufacturer)	Lot no#	Cat no
Naphthylethylene diamine dihydrochloride	Fisher Scientific	1870048	M0428
Sulphanilamide	Sigma Aldrich	SLBX8231	S9251
Phosphoric acid (85%)	Merck	181215683	1812156813
DMEM (Dulbecco's modified Eagles Media– High glucose	Fisher Scientific	RNBH3153	D5671
HEPES	Sigma	RNBH4191	H0887
Sodium Pyruvate	Sigma	RNBH0196	S863
MEM Amino acid (50x)	Sigma	RNBG4068	
BSA Palmitate/BSA control	Seahorse Bioscience	20161219	102720-100
Etomoxir Salt Hydrate	Sigma Aldrich	116M4742V	E1905
Middlebrook 7H11		Bcbq1773V	M4028
Bovine Serum Albumin (BSA)		170419-0461	

By having a range of glucose concentrations, we could investigate the impact of glucose levels on the activation state of macrophages that were stimulated with *Mycobacterium smegmatis*. BMDM were infected with *Mycobacterium smegmatis* at a concentration of $5x10^6$colony forming units (CFU) per mL. We measured the levels of pro-inflammatory cytokines IL-6, IL-1β and TNF-α produced by activated macrophages 48 h post infection.

The literature shows that the cytokines TNF-α, IL-6 and IL-1β are major inflammatory cytokines involved in the pro-inflammatory response to infection with *Mycobacterium tuberculosis* [28,29]. Furthermore, TNF-α has a pivotal role in the immune response to *Mycobacterial* infection by acting as a key regulator of the immune response to *Mycobacterium tuberculosis* [29,28].

We also wanted to test whether altering the glucose concentration by either increasing or decreasing glucose availability in macrophages would alter the anti-microbial activity of macrophages. To do this, we measured the ability of macrophages to produce nitric oxide (NO) in response to infection with *Mycobacterium smegm*atis. To measure levels of nitric oxide produced by activated macrophages a Greiss assay was carried out, a technique that measures levels of nitrite, a stable metabolite produced by activated macrophages, which represents the end state of an earlier nitric oxide (NO) reaction.

We also wanted to test the role of fatty acid metabolism using Etomoxir, an inhibitor of fatty acid oxidation on the activation of BMDM. We measured the response of activated macrophages in terms of cytokines IL-6, TNF-α and nitric oxide (NO) production in the presence and absence of excess fatty acid (palmitic acid) and etomoxir **(Figure 2)**.

We found that the production of IL-6 by *M. smegmatis*-infected macrophages was reduced when macrophages were cultured in 25mM glucose and the result was significant. We further observed that in 11.1 mM IL-6 was increased compared to the other two glucose concentrations (**Figure 2**, top left). When Etomoxir was added to inhibit FAO, IL-6 was marginally reduced in the 25mM compared to 5.5mM glucose. We observed that in 11.1mM IL-6 increased compared to other glucose concentrations (**Figure 2**, top right). When palmitate was added the amount of IL-6 was reduced in all conditions but significantly more in 25 mM than 5.5 mM (**Figure 2,** bottom left). Finally, when both palmitate and etomoxir were added the level of IL-6 was very low but variable so that although a trend toward reduced IL-6 in 25 mM was observed it did not reach significance (**Figure2**, bottom right).

Next, we examined the effect of glucose on TNF-α, which is a key cytokine activated in the early immune response to *Mtb* infection **(Figure 4)**. We found that in 11.1 mM glucose, the TNF-α response was significantly increased relative to the 5.5mM (**Figure 4** top left) but the addition of etomoxir (**Figure 4** bottom right) or palmitate (**Figure 4** top right) or both (**Figure 4** bottom left) removed this effect. TNF-α was marginally reduced when both palmitate and etomoxir were added compared to 5.5 mM glucose (**Figure 4** bottom left).

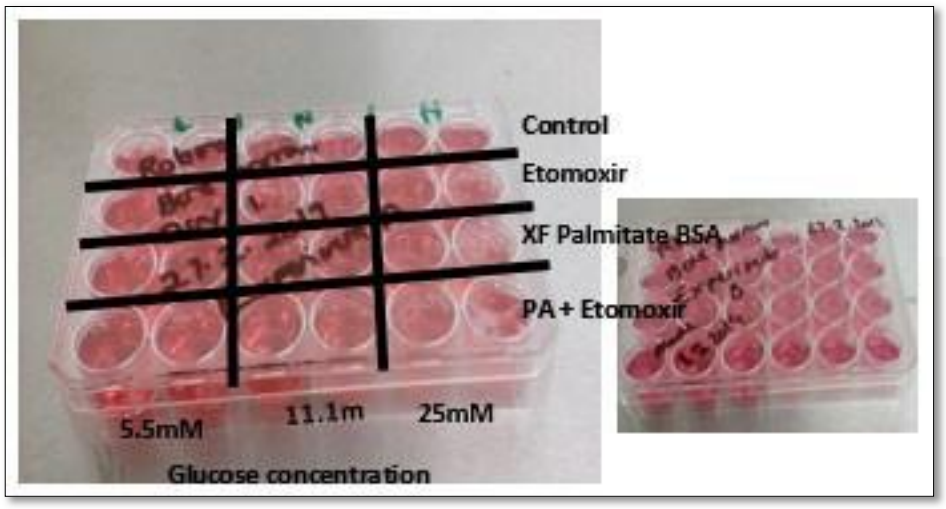

Figure 2. Harvest of BMDM in different glucose.

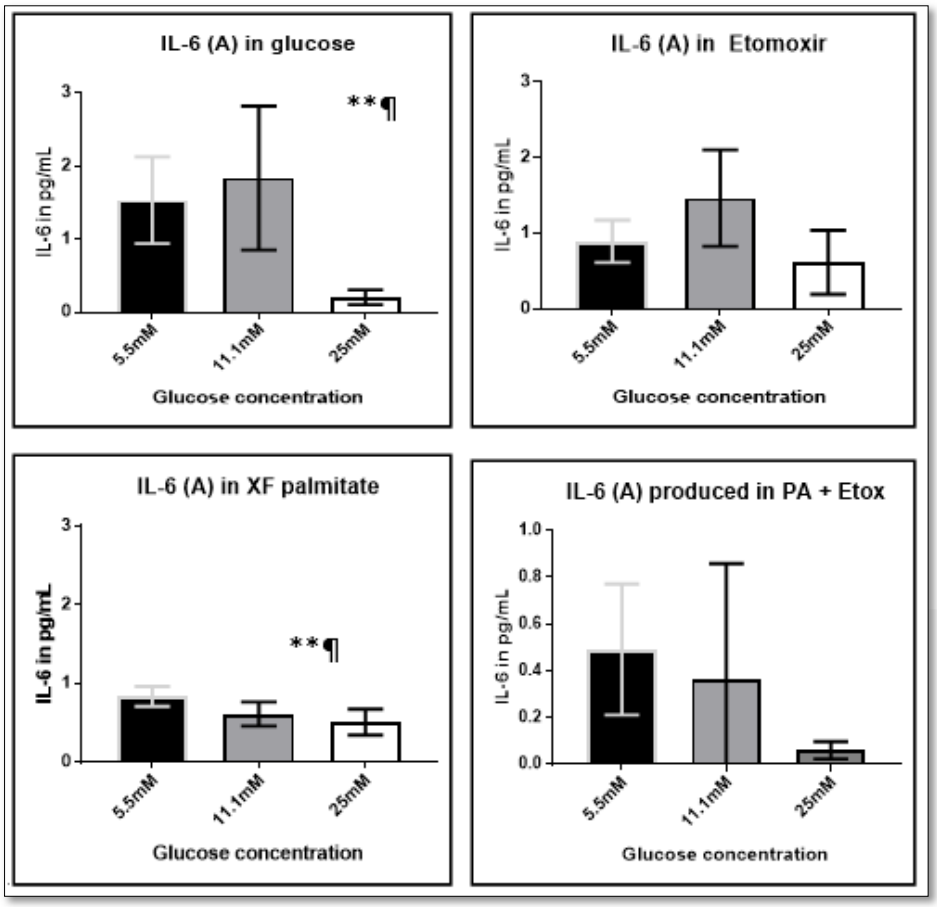

Figure 3. Glucose concentration of 25 mM caused a decrease in IL-6 production in response to *Mycobacterium smegmatis* compared to 5.5 mM. Addition of Etoximir or Palmitate reduced IL-6 in 5.5 mM glucose and the impact of 25 mM glucose was maintained when palmitate was added. Though not significant, a trend to lower IL-6 in 25 mM glucose was also seen in the presence of etomoxir and etomoxir plus palmitate. A one-way ANOVA test used to compare variances between the three groups and if found to be significant was followed by a Dunnet's multiple comparison. One-way ANOVA followed by Dunnet's *<0.05, **<0.01, Graph pad Prism.

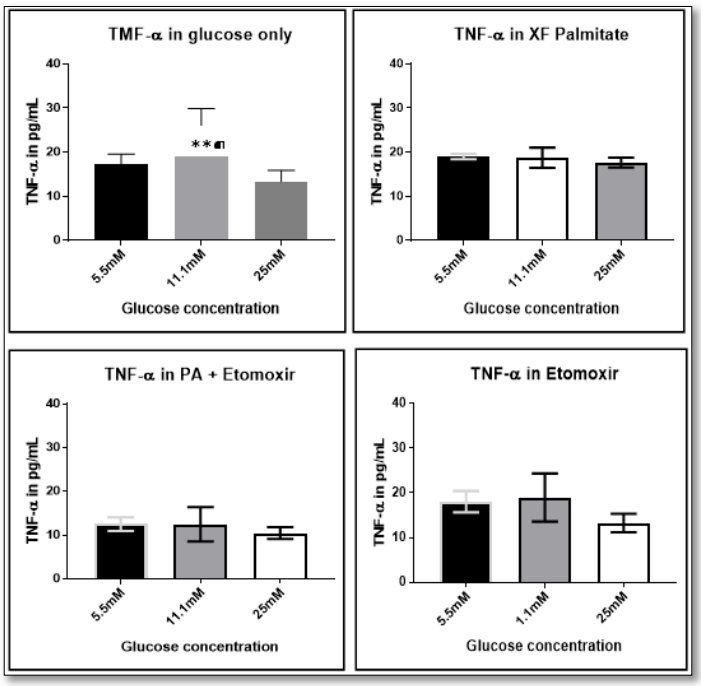

Figure 4. Glucose impacts TNFα production by *M. smegmatis* exposed macrophages to a modest degree. BMDM were cultured in 3 different concentrations of glucose and the amount of TNF produced determined by ELISA. Each condition was tested in two independent wells and the supernatants were analysed in 3 technical replicate wells on the ELISA. Values for each set of conditions were compared using ANOVA followed by Dunnet's multiple comparison test and a result <0.05 was statistically significant. *<0.05, **<0.01, Graph pad Prism. A one-way ANOVA was performed (glucose only) p value *** <0.005 and (etomoxir) only p value * <0.05.

Cytokine response of BMDM treated with Etomoxir, Palmitate or both and in different glucose concentrations

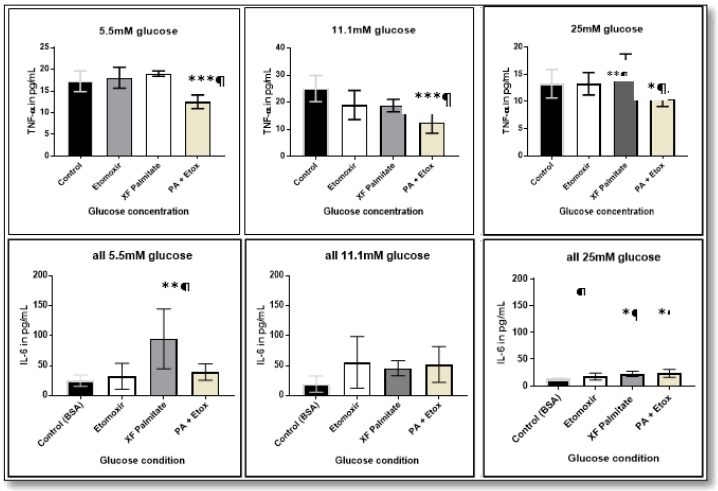

Figure 5. TNF-α response by activated macrophages is inhibited by adding palmitate and etomoxir. The effect of palmitate and etomoxir inhibits TNF-α activation reaching levels of significance in the different concentrations of glucose. BMDM were incubated in different glucose concentrations (5.5 mM, 11.1 mM and 25 mM) cells were further treated with 1mM palmitate or 40 µM of etomoxir or both. An ELISA assay was performed on culture supernatants and absorbance values were read at 450 nm with a microplate reader from triplicate wells on a 96 well plate. A blank was used to subtract the value from the final absorbance readings. A one-way ANOVA followed by a Dunnet's multiple comparison test was carried out and a p value of <0.05 was statistically significant. One-way ANOVA *<0.05, **<0.01, ***<0.001, Graph pad Prism.

We found that the response of TNF-α in macrophages stimulated with *Mycobacterium smegmatis* was reduced when both palmitate and etomoxir were added in 11.1mm glucose (**Figure 5** top middle). This result was similar to the effect we had observed in **Figure 4** (top right and bottom left). We found that in 25 mM glucose the effect of palmitate was mitigated resulting in an increase in TNF-α. We observed in 25 mM glucose the effect of etomoxir was moderate in reducing TNF-α response in activated macrophages compared to 5.5 mM.We found that in all the glucose concentrations the combined effect of palmitate and etomoxir reduced the TNF-α response in activated macrophages reaching significance in different glucose concentrations especially in 5.5 mM and 11.1 mm glucose (top left, middle and right). We had observed the same combined effect of palmitate and etomoxir on TNF-α response in (**Figure 4** bottomright).

When we examined the response of IL-6 by macrophages we found that in 5.5mM glucose,when palmitate was added, the IL-6 response was increased reaching levels ofsignificance compared to control (**Figure 5** bottom left). We observed that in 11.1mM the effect of etomoxir and palmitate reduced TNF-α response by activated macrophages (bottom middle) which was similar to (**Figure 3** top right and bottom left). In 25mM glucose the inhibitory effect of etomoxir and palmitate was maintained reaching significance. These results support the data in **Figure 3**. The effect of palmitate and etomoxir combined had a moderate impact on reducing IL-6 response in 5.5 mM and 11.1 mM glucose (**Figure 5**, left and middle) however, in 25 mM glucose the impact had reached significance. Our data agreed with the results from **Figure 3** (bottom right).

Next, we wanted to examine the effect of different glucose concentrations on nitrite production by macrophages infected with *Mycobacterium smegmatis.* To measure levels of nitric oxide produced we used a Greiss assay that measured levels of nitrite. We knew that activated macrophages secrete reactive oxygen species (ROS) in the form of intermediates nitrite (NO_2) and nitrate (NO_3) in response to bacterial infection. Research in to Tuberculosis had identified that diabetes patients were three times more likely to be infected with tuberculosis (TB). Some studies have suggested that a high glucose environment can increase the virulence factor in pathogens [42].

Nitriteresponse of macrophages in different glucose

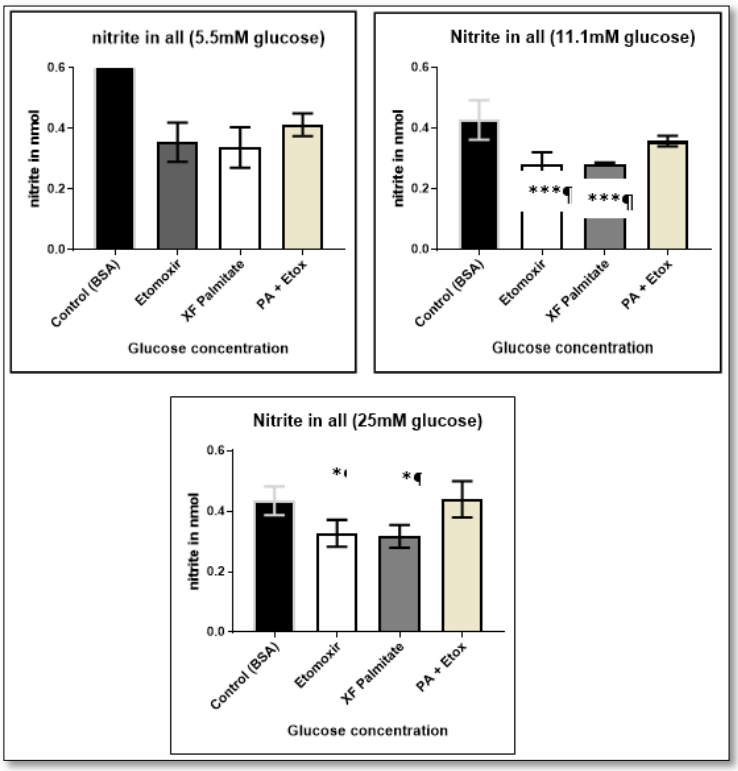

Figure 6. Macrophages treated with palmitate or etoximir or both decrease nitrite response in activated macrophages compared to control. In 11.1 mM glucose, nitrite levels were reduced in the etoximir and palmitate treated cells with results reaching levels of significance. In 25 mM glucose, similar reduced levels of nitrite were observed however, the results were moderately significant compared to 11.1 mM glucose concentrations. A one-way ANOVA test was performed followed by a Dunnet's multiple comparison and a value of <0.05 was statistically significant. One-way ANOVA *<0.05, **<0.01, ***<0.001, Graph pad Prism.

We found that etomoxir and palmitate impacted macrophage activation and reduced nitrite response in all the different glucose conditions (**Figure 6**, left, right and bottom). Reduced levels of nitrite in 11.1 mM glucose were significant (**Figure 6** top left) however, in 25 mM glucose nitrite response was moderately reduced but reached significance (**Figure 6** bottom). We also observed that in 5.5 mM glucose when etomoxir and palmitate were added it reduced nitrite levels but results were not significant (**Figure 6** top). The combined effect of palmitate and etomoxir had a moderate impact on nitrite produced by macrophages but did not reach significance. Our data suggest that palmitate and etoximir impact nitrite response in activated macrophages stimulated with *Mycobacterium smegmatis* with the strongest effect in 11.1 mM glucose concentrations (double the fasting blood glucose concentrations of pre-diabetic patients).

Our results suggest that the pro-inflammatory cytokine response is altered in high (25 mM) and moderate (11.1 mM) glucose. Moreover, our data support the hypothesis that the effect of inhibiting FAO oxidation with etomoxir and palmitate, would appear to impact the macrophage response to *Mycobacterial* infection.

HIGH GLUCOSE (25 MM) ALTERS MACROPHAGE MORPHOLOGY IDENTIFIED BY CELL STAINING

We performed a staining technique on harvested bone marrow macrophages incubated in different glucose concentrations (5.5 mM, 11.1 mM and 25 mM) and treated cells with palmitate and inhibitor of beta oxidation, etomoxir to observe changes in the morphology of macrophages. Our studies had shown that the morphology of macrophages was altered by increasing glucose concentrations [43] **(Figures 7-11)**.

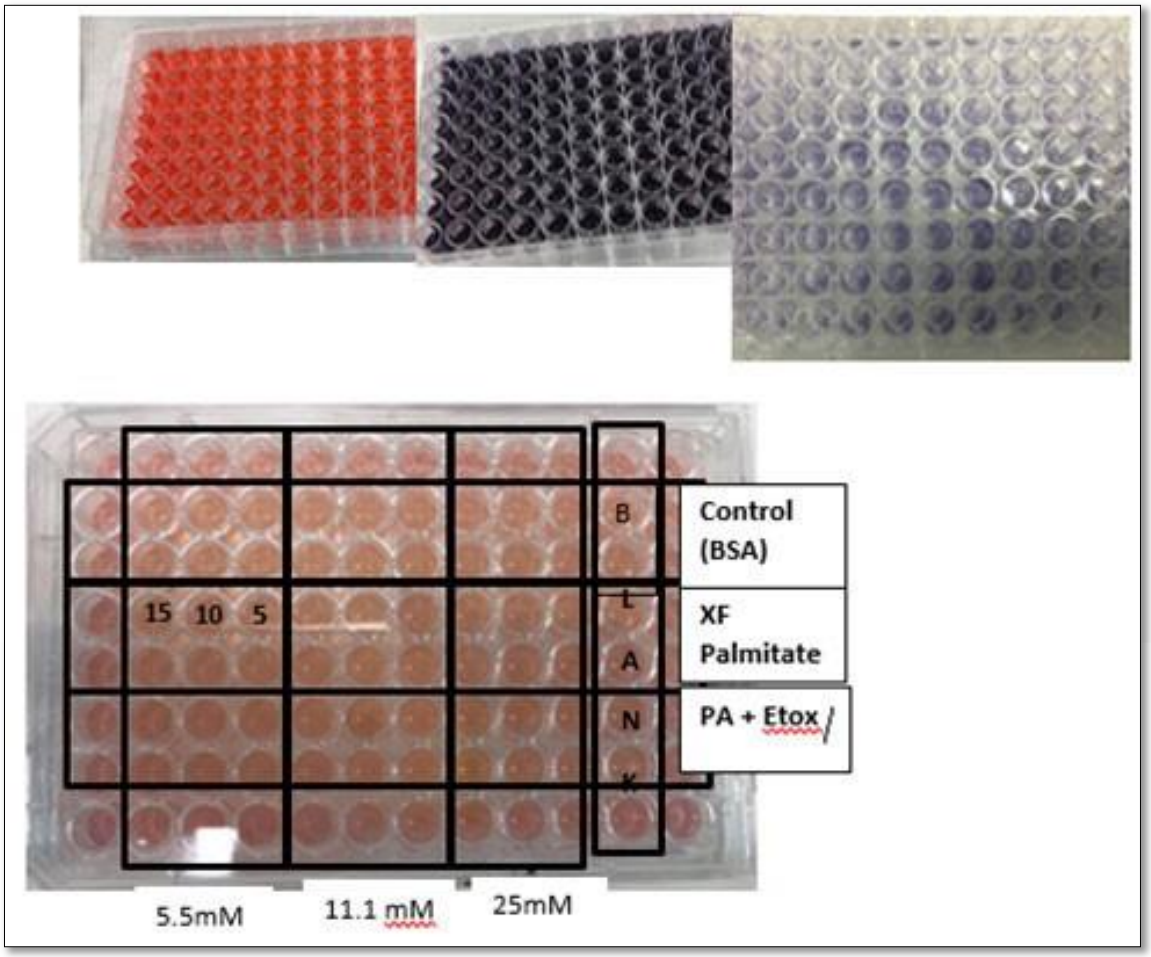

Figure 7. Day 3 adherent BMDM were grown in 5.5 mM, 11.1 mM and 25 mM of glucose. Cells were treated with either 15, 10, 5 µl/ml of etomoxir or XF palmitate or both in each glucose condition. Blank lane cells were untreated and were used as controls. Images of macrophages in each condition were taken with a CKX41 phase contrast microscope at x40 magnification. Top. Picture of 96 well plates treated with Kwik diff staining methylene blue, eosin and fixative.

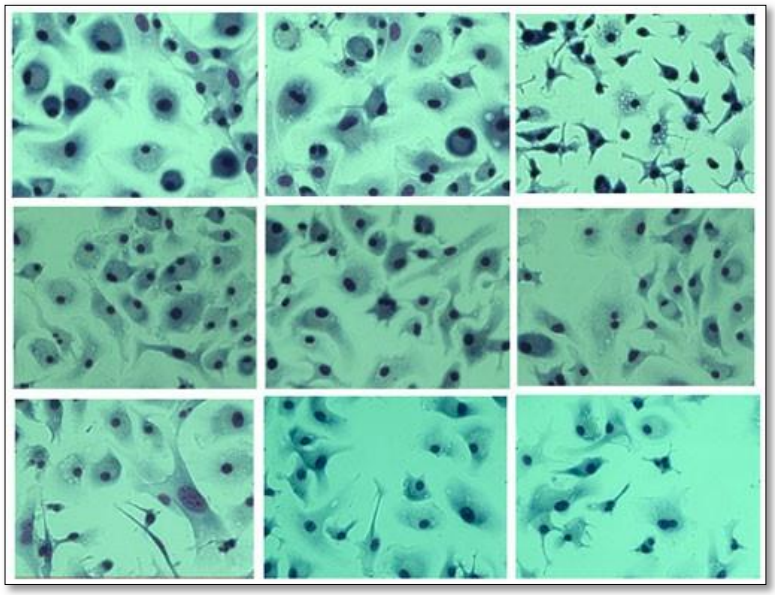

Figure 8. Top panel images of macrophages in 5.5 mM glucose, middle panel-11.1 mM glucose and bottom panel 25 mM glucose. Images were taken in x40 magnification. Cells in 11.1 mM glucose (middle panel) have a higher cell density. In 25 mM glucose (bottom panel) macrophages cells are sparse and assume an irregular shape. In 5.5 mM glucose (top panel) monocytes appear smaller in size and have a round shape. Image show differences in morphology in the different glucose concentrations.

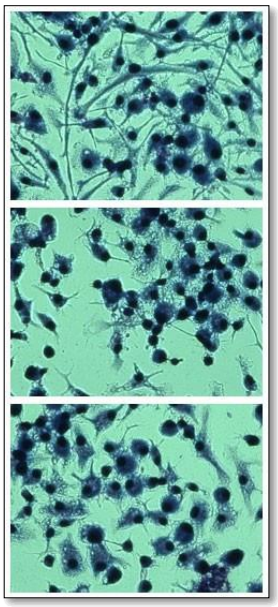

Figure 9. BMDM incubated in 5.5mM glucose. Top Control, middle PA + Etox (10 µl), bottom Palmitate (10 µl). Images were taken with a CXK41 microscope at x40 magnification. Cells appear to be smaller in size with a rounded appearance, (middle). Some of the cells have an irregular shape (bottom panel). Some cells appear to show an elongated spindle morphology (top panel).

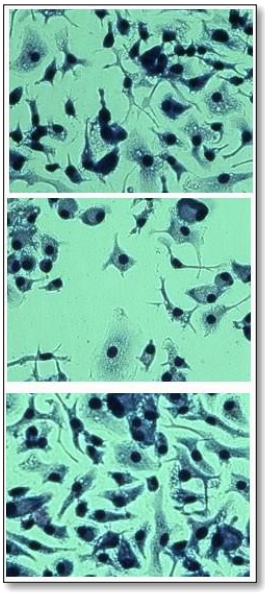

Figure 10. BMDM in 11.1 mM glucose. Top (control), middle (palmitate) 15 µl and bottom (PA + Etox) 15µl. Cells appeared to show an elongated appearance (bottom). When cells were treated with palmitate (middle) the morphology of the cells was altered. The shape of the cells, compared to control (top) are irregular, small and cell numbers sparse as evidenced by large gaps between cells. The increase in spreading (bottom) show cells with an elongated appearance. Cell morphology appears to have altered in higher glucose concentrations.

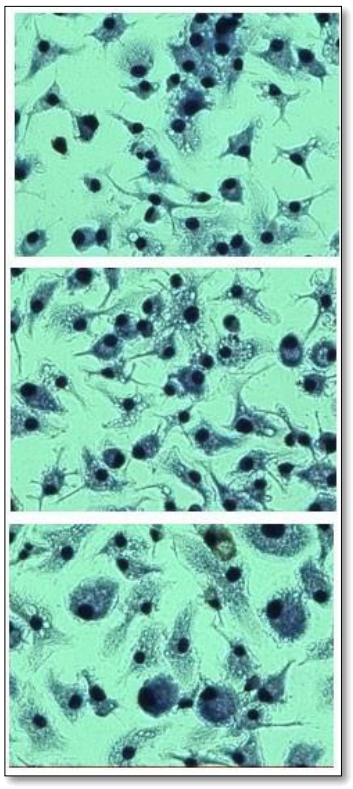

Figure 11. BMDM in 25mM glucose. Top (control), middle (palmitate) 5 µl, bottom (PA + Etox) 5 µl. In high glucose BMDM have an altered morphology the cells look deformed, elongated and do not have a round shape, also appear to show macrophage spreading.

Macrophages upregulate pro-inflammatory cytokine response in normal (11.1 mM) glucose

We wanted to isolate the variable that was impacting macrophage activation. We also wanted to measure the effect that fatty acid and etomoxir had on macrophage activation without altering the glucose concentration. We therefore kept glucose constant using the concentration normally used for cell culture (11.1 mM) and added the palmitate and etomoxir which would influence FAO in the macrophages exposed to *M smegmatis* **(Figure 12).**

We found that when glucose was kept constant (11.1 mM) there was an up regulated response in pro-inflammatory cytokines by activated macrophages (**Figure 12**). We observed that by adding palmitate and etomoxir caused an increase in the response of IL-6 reaching significant levels (**Figure 12** top left). We observed a similar effect on TNF-α (**Figure 12** top right) although the increase was moderate and did not reach significance. We found that IL-1β response was also increased when palmitate and etomoxir were added however, only reaching levels of significance when palmitate was added (**Figure 12** bottom left). We observed a mild increase in nitrite response but the results were not significant (**Figure 12** bottom right). We observed that when palmitate and etomoxir were added together it reduced the TNF-α and nitrite response (**Figure 12** top and bottom left) this effect was not observed for cytokine IL-6 and IL-1β. TNF-α + nitrite response was reduced but not to significance.

NO by activated macrophages in real time with DAF-FM

While the Greiss assay provided an indication of the amount of nitric oxide produced we wanted to see whether nitric oxide was directly impacted. In order to observe nitric oxide being produced by activated macrophages stimulated with *Mycobacterium smegmatis* in real time we stained BMDM with DAF-FM Diacetate (4-Amino-5-Methylamino-2', 7'-Difluoro fluorescein Diacetate) in 5.5mM, 11.1mM and 25mM glucose concentrations. We wanted to compare levels of nitric oxide in macrophages stimulated with + and – *Mycobacterium smegmatis* to see if there were differences in florescence. When DAF-FM, a non-fluorescent reagent reacts with NO, it forms a fluorescent benzotriazole which can be used to measure and detect the presence of low concentrations of nitric oxide (NO) **(Figure 13)**.

Our results had shown that in high glucose (25 mM) the NO response by activated macrophage was the highest in 25 mM glucose compared to the other glucose concentrations (**Figure 14** bottom). A higher level of florescence was observed in 25 mM glucose (**Figure 14** bottom image). This level of intensity in florescence was not observed in 5.5 mM and 11.1 mM glucose **(Figures 14-16)** however, there were mild levels of florescence observed.

Impact of polarising cytokine IFN-γ and IL-10 on pro-inflammatory macrophage activation

Our studies thus far were performed on BMDM without the influence of polarizing cytokines. We wanted to determine the effect of IFN-γ (M1 polarizer) and IL-10 (M2 polarizer) would have on the activation of macrophages and the pro-inflammatory response. We wanted to find out whether the impact of IFN-γ and IL-10 would activate or inhibit the pro-inflammatory response of activated macrophages. Our experimental design was exploratory as we did not know the type of effect.

Our results had shown that in high glucose (25mM) the NO response by activated macrophage was the highest in 25 mM glucose compared to the other glucose concentrations (**Figure 14** bottom). A higher level of florescence was observed in 25 mM glucose (**Figure 14** bottom image). This level of intensity in florescence was not observed in 5.5mM and 11.1mM glucose (**Figures 14-16**) however, there was mild levels of florescence observed.

We observed that IFN-γ and IL-10 decreased IL-6 response in activated macrophages with results reaching significance in all the conditions tested. We observed that IL-10 (M2 polarizer) had the strongest effect on IL-6 response. IL-10 reduced TNF-α response and the results were significant in high (25 mM) and moderate (11.1 mM) glucose. We observed

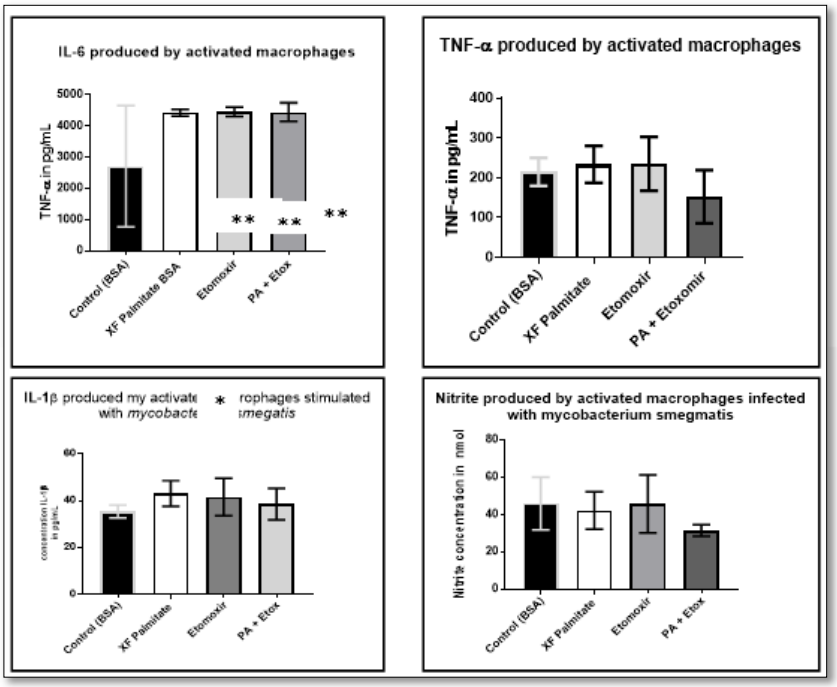

Figure 12. BMDM cultured in cDMEM containing (4500mg/L glucose) increase macrophage pro-inflammatory response. Activated macrophages stimulated with *Mycobacterium smegmatis* caused an increase in the production of pro-inflammatory cytokines (IL-1β, TNF-α, and IL-6 and nitrite). Macrophages that had palmitate and etomoxir added were able to inhibit TNF-α + Nitrite response without reaching significance. A one-way ANOVA test was performed and t test. A p value of < 0.05 was statistically significant. This was followed with a Dunnet's multiple comparison test * <0.05 ** < 0.01 (p values). TNF-α (* p value <0.01), IL-1β (ns), nitrite (ns), IL-6 (** p value <0.01). One-way ANOVA *<0.05, **<0.01, Graph pad Prism.

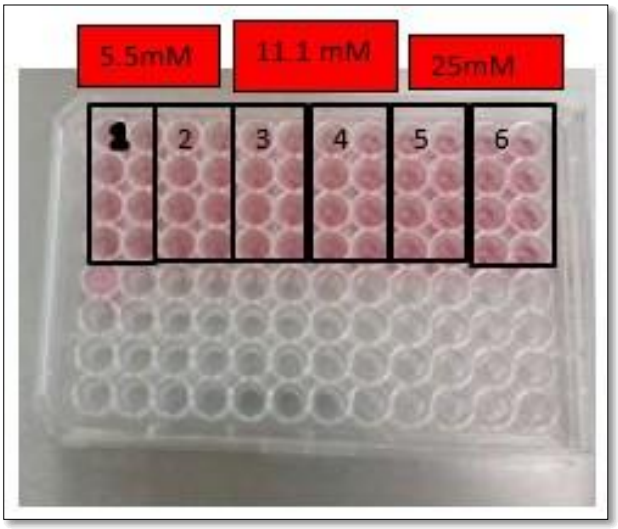

Figure 13. DAF-FM on BMDM. Lane 1 + 3 + 5 normal media minus smeg (control). Lane 2 + 4 + 6 normal media plus *M smegmatis* (treated). Macrophages were treated with IFN-γ at a concentration of 1 µg/mL. On day 9 of cell culture cDMEM media was replaced with either 5.5 mM, 11.1 mM or 25 mM glucose. Day 10 *Mycobacterium smegmatis* was added to each well at a MOI of 5 x 10^6.

a moderate decrease in 5.5mM glucose but it did not reach significance **(Figure 17)**. We observed that IFN-γ reduced IL-6 cytokine and the strongest effect was induced by IL-10. We observed that the polarizing cytokines did not impact IL-1β response as we could only see a moderate increase in the response. We found that IL-10 moderately decreased IL-1β response.

Glucose and cytokines influence bactericidal killing by activated macrophages

We wanted to measure the effect of altering glucose concentrations and treating BMDM with palmitate, etomoxir and cytokines IFN-γ and IL-10 would have on the activity of bactericidal killing by activated macrophages. To measure the effect on bactericidal killing we harvested BMDM and treated the cells with palmitate or etomoxir. Glucose was altered to 5.5 mM, 11.1 mM and 25 mM. In each glucose group a control group was set up which had no intervention **(Figures 18, 9, 10 and 11)**. Colonies that formed on bacterial plates were counted and the results were recorded **(Table 1)** and graphed using graphapd prism **(Figure 20).**

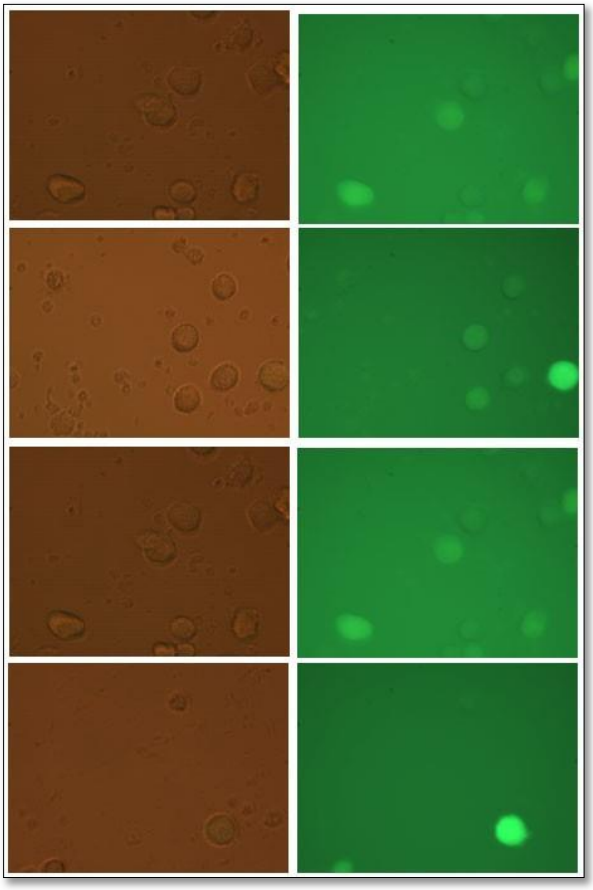

Figure 14. BMDM in 25 mM glucose. Top panel left light, right fluorescence (-smeg) Bottom panel left light, right fluorescence (+smeg) middle panel left light, right fluorescence (-smeg) x40 magnification with CXK41 phase contrast microscope.

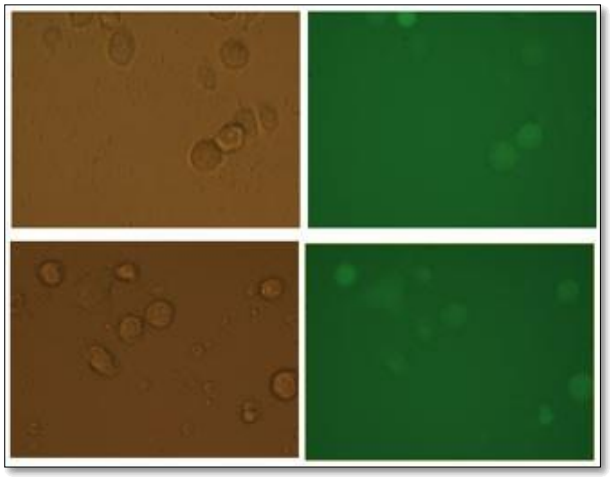

Figure 15. BMDM in 11.1 mM glucose. Top panel right light, left fluorescence (+ smeg). Bottom panel right light, left fluorescence (- smeg) x 40 magnification with CXK41 phase contrast microscope.

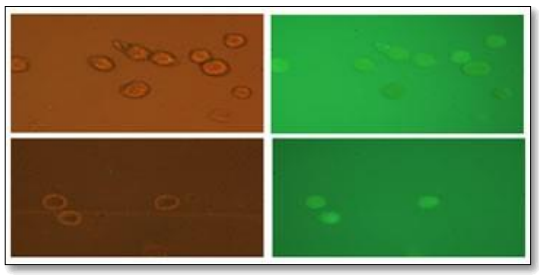

Figure 16. BMDM in 5.5 mM glucose. Top panel left light, right fluorescence (-smeg). Bottom panel left light, right fluorescence (+smeg). Images at x40 magnification with an Olympus CKX41 inverted phase contrast microscope.

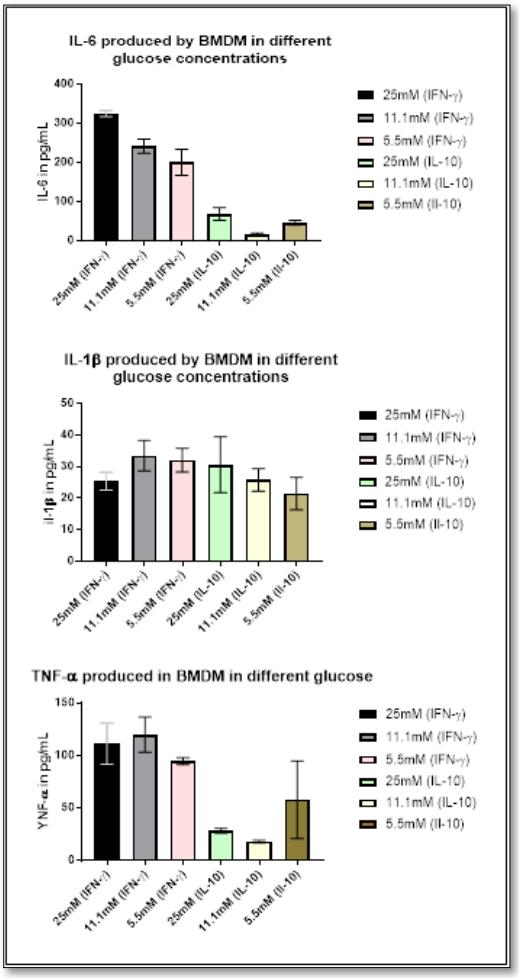

Figure 17. BMDM – impact of IFN-γ and IL-10 macrophages were harvested (see methods) and on day 9 of culture the cells were treated with polarizing IFN-γ (1mg/mL) and IL-10 (3 µg/mL). On day 10 cells were infected with 5 x 10^6 /mL of bacterial stock concentration of *M smegmatis*. Cells were incubated for 36 h and supernatants collected in vials and stored in -80 freezer until ready. ELISA and Greiss assays were performed on triplicate wells on a 96 well plate and cytokine IL-6, Il-1β and TNF-α responses were determined.

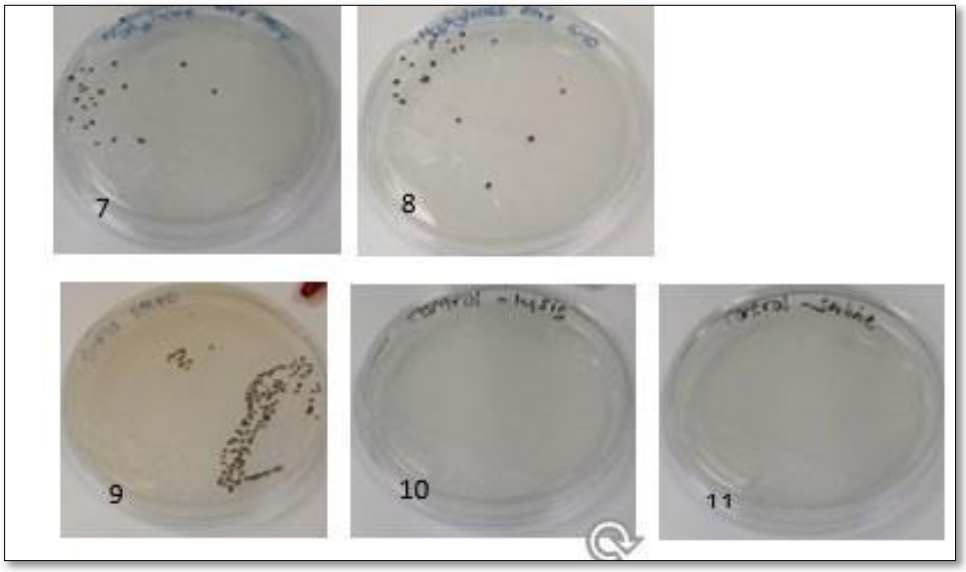

Figure 18. Bacterial plating. Supernatant from each well were discarded and replaced with 0.5 ml lysis buffer to lyse the cells. After 10 min incubation a 1 in 2 serial dilution (1:1000) was carried out in tubes containing 0.5 ml saline solution. A volume of 500 µl from tube 1 was transferred to tube 2 in the dilution and 100 µl from tube 2 was plated onto agar plates. After plating, bacterial plates were kept in the incubator at 37˚C for 36 h before counting the colonies. Controls for *M smegmatis*, lysis buffer and saline solution were carried out to check for contamination.

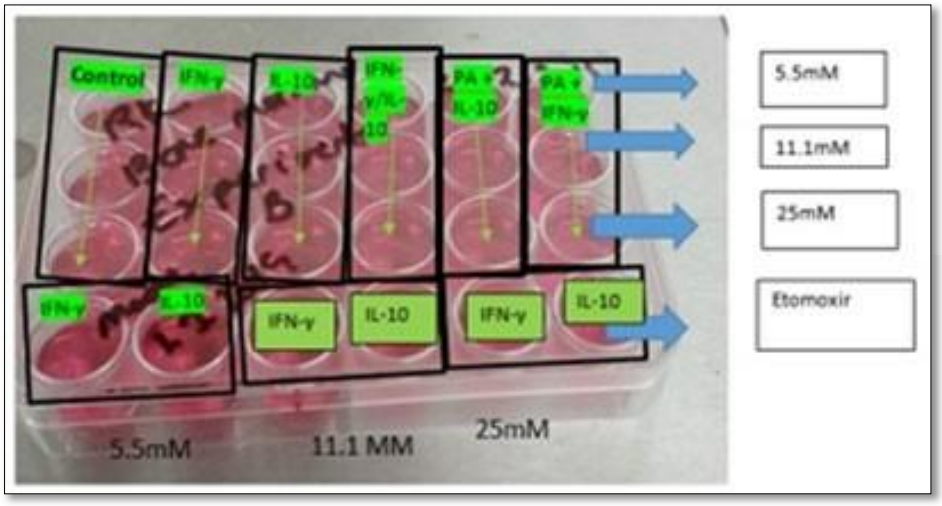

Figure 19. BMDM experimental design Plates prepared for bacterial plating. In lane 1 5.5 mM glucose (across), lane 2 glucose at 11.1 mM and lane 3 25 mM (across). The bottom 6 wells were treated with 40 µM etomoxir and glucose was altered to 5.5 mM (2 wells), 11.1 mM (2 wells) and 25 mM (2 wells). Macrophages were stimulated with IFN-γ (1 mg/mL) and IL-10 (3 µg/mL). 6 wells were treated with palmitate (1 mM). We wanted to measure the effect of different variables on bactericidal killing. All the wells were infected with *M smegmatis* at a concentration of 5 x 10^6 /mL and the plate was returned to the incubator for 36 h at a temperature of 37˚C prior to plating.

Our results had shown that IL-10 had the biggest effect on bactericidal killing on *Mtb* compared to the other treatments tested (**Figure 18** top, middle and bottom graph). We observed that cells that were treated with palmitate and/or IFN-γ and IL-10 increased bacterial colony numbers in 25 mM and 5.5 mM glucose. Interestingly, in 11.1 mM glucose bacterial numbers were reduced **(Table 1)**.

We wanted to observe the macrophages in the different treatment conditions, in particular we wanted to observe the morphology. We knew from studies that high glucose stimulated the production of pro-inflammatory cytokines and altered the phenotype and function of macrophages. Images were taken with an Olympus phase contrast microscope. We observed that in 25mM glucose macrophages appeared to

show signs of damage and an altered shape (**Figure 23**, E+F and **Figure 22** (F+G)).

Table 1. Number of colonies counted after plating bacteria.

	Control	IFN-γ	IL-10	IFN-γ	PA IL-10	PA IFN-γ
5.5mM	32	29	9	3	45	80
11.1Mm	9	11	2	4	8	2
25Mm	1	11	6	29	31	56
	5.5mM		11.1mM		25mM	
	IFN-γ	IL-10	IFN-γ	IL-10	IFN-γ	IL-10
Etomoxir	76	51	23	30	23	29

Lung macrophages treated with IFN-γ inhibit production of TNF-α and IL-1β by activated macrophages stimulated with *Mycobacterium smegmatis*

TB is a disease infecting the lungs. We knew that there were two types of macrophages found in the lungs. Alveolar macrophages (AM) and interstitial macrophages (IM). Our experiments stressed bone marrow derived macrophages however, we wanted to find out if we stressed the lung macrophages would it have the same impact on pro-inflammatory cytokine response from activated macrophages in the lungs.

We found that IFN-γ inhibited IL-1β response in activated lung macrophages compared to controls. We saw a similar effect on TNF-α response. The effect on nitrite was variable however there was a trend towards a reduced nitrite response. (**Figure 23** top, middle and bottom).

High glucose (25mM) and IFN-γ reduces IL-6 and TNF-α in lung macrophages

We wanted to stress lung macrophages further. Therefore, we wanted to examine the effect of glucose at (25 mM) on lung macrophages and whether high glucose alone could inhibit pro-inflammatory cytokine response. We found that in high (25 mM) glucose reduced TNF-α response and IL-6 cytokine was observed. We observed no effect on Il-1β on lung macrophages (**Figure 25** top, middle and bottom).

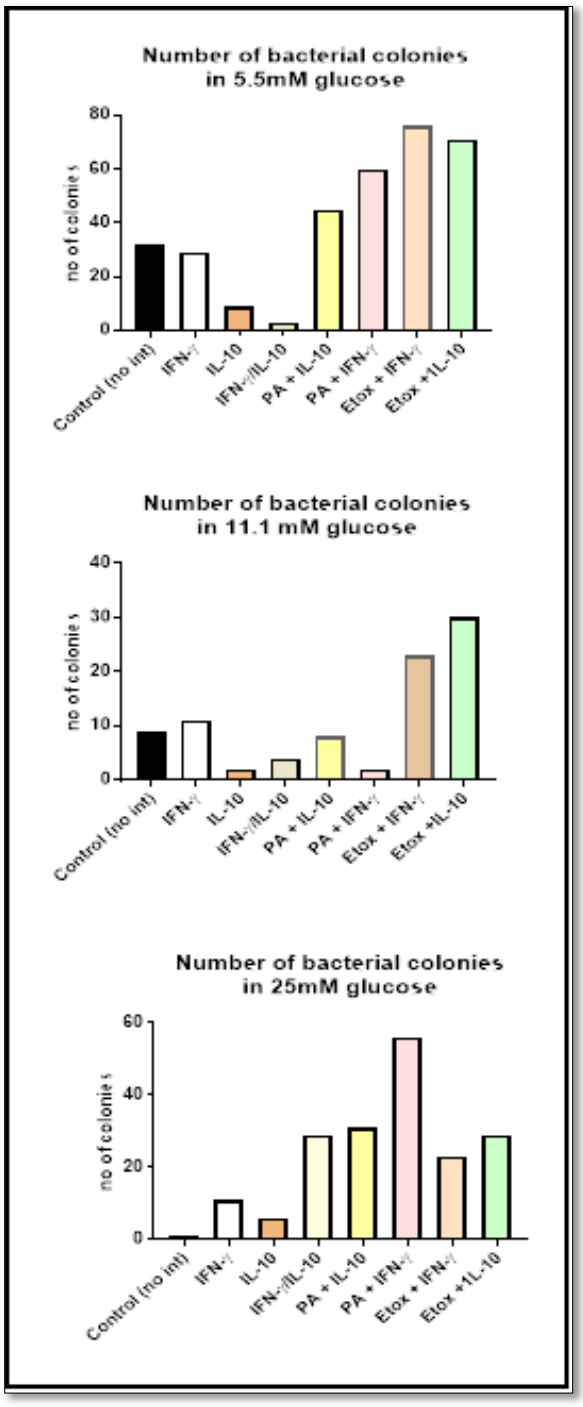

Figure 20. IFN-γ and IL-10 have the strongest impact on bactericidal killing in macrophages in all glucose conditions (5.5 mM, 11.1 mM and 25 mM). We observed that in cells with added palmitate had an increase in bacterial colonies compared to control. A similar response was observed in BMDM treated with etomoxir.

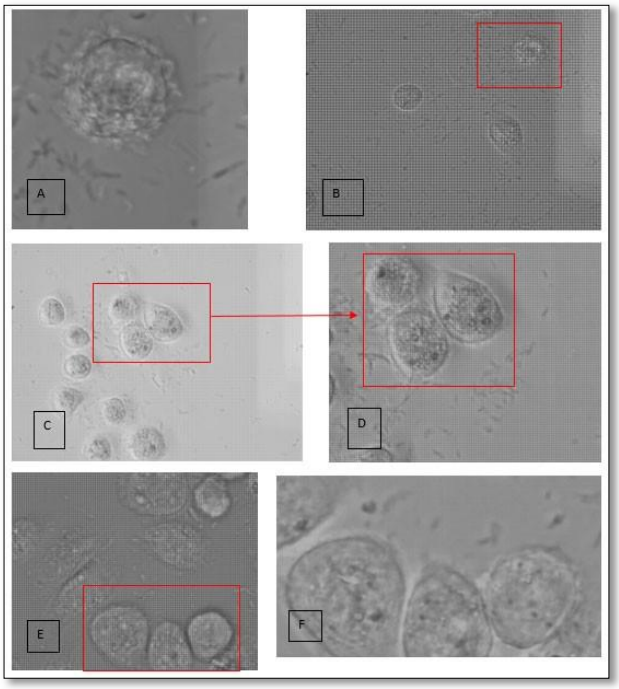

Figure 21. In 5.5 mM glucose. IL-10, IFN-γ and IFN-γ/IL-10 treated cells had lowest bacterial colony count. Macrophages appear rounded with no signs of distortion to shape. A+B (IFN-γ treated), C+D (IL-10 treated) and E+F (IFN-γ/IL-10).

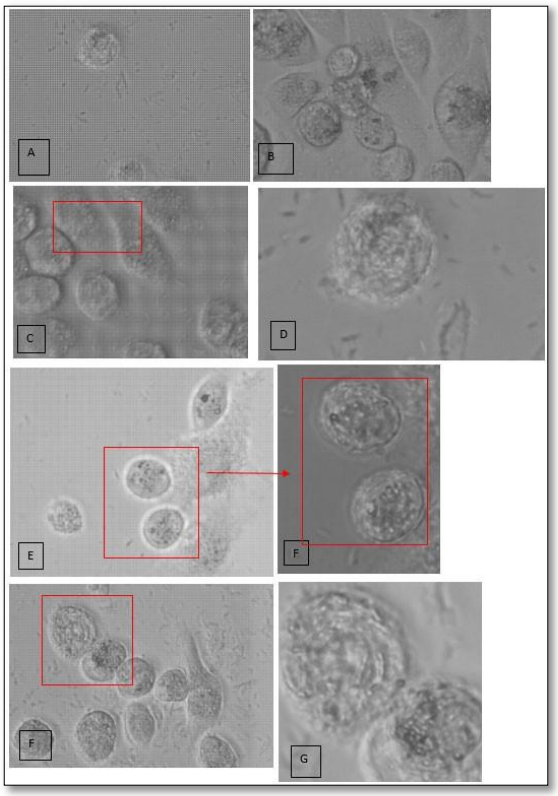

Figure 22. BMDM in 11.1 glucose. Images taken with CXK41 inverted microscope at x20 and x40 magnification. A (control), B (IFN-γ) C + D (IL-10), E + F (PA + IL-10), F + G (PA + IFN-γ). Our results had shown that IL-10 treated macrophages inhibit bacterial growth.

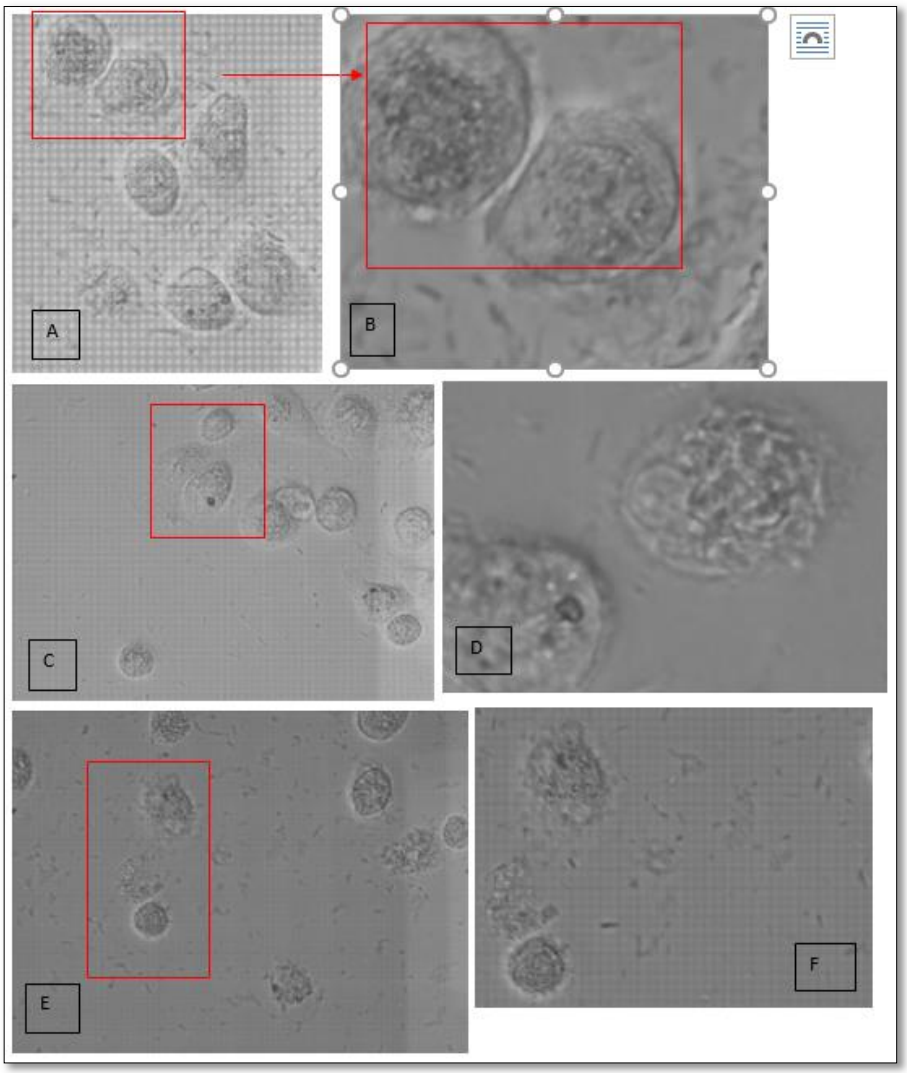

Figure 23. BMDM in 25 mM glucose. BMDM were treated in different conditions see **Figure 17**. A + B (control), C + D (IFN-γ), E + F (IL-10). Treating BMDM with IL-10 and IFN-γ/IL-10 killed bacteria and lower numbers of bacterial colonies were counted on plates. Images suggest damage to macrophages with a distorted morphology in 25 mM glucose.

BMDM treated with inhibitor Etomoxir and Palmitate will reduce TNF-α, IL-6 cytokine and nitric oxide

We wanted to re-examine the effect of Palmitate (fatty acid) and Etomoxir (inhibitor of β oxidation) on BMDM infected with *Mycobacterium smegmatis*. Secondly, we had seen that IL-10 was effective at inhibiting bacterial growth (**Table 1 and Figure 20**) similarly IFN-γ also had an inhibitory effect on bacterial growth. We decided to re-examine the effect of these variables to confirm our previous findings.

Etomoxir and palmitate induce IFN-γ and IL-10 activation of macrophages and trigger NO response

We observed that nitrite response of macrophages was increased in higher glucose (11.1 mM and 25 mM) compared to 5.5 mM glucose. By adding IFN-γ nitrite response was increased compared to no intervention and palmitate only. There was little difference in nitrite response between IFN-γ control and IFNγ with palmitate. When IL-10 was added it had minimal effect on nitrite response however, when INF-γ was added it relieved the inhibitory effect of IL-10 cytokine. We found similar results for 25 mM glucose however in 5.5 mM glucose INF-γ did not increase nitrite response.

By adding etomoxir its increased nitrite response in macrophages. We found that the results were similar to palmitate. Etomoxir and IL-10 had little effect on nitrite response, after adding IFN-γ the inhibitory effect was removed. The impact of etomoxir on macrophages was the same for all glucose (5.5 mM, 11.1 mM and 25 mM). Results for both plates were very significant **(Figure 27).**

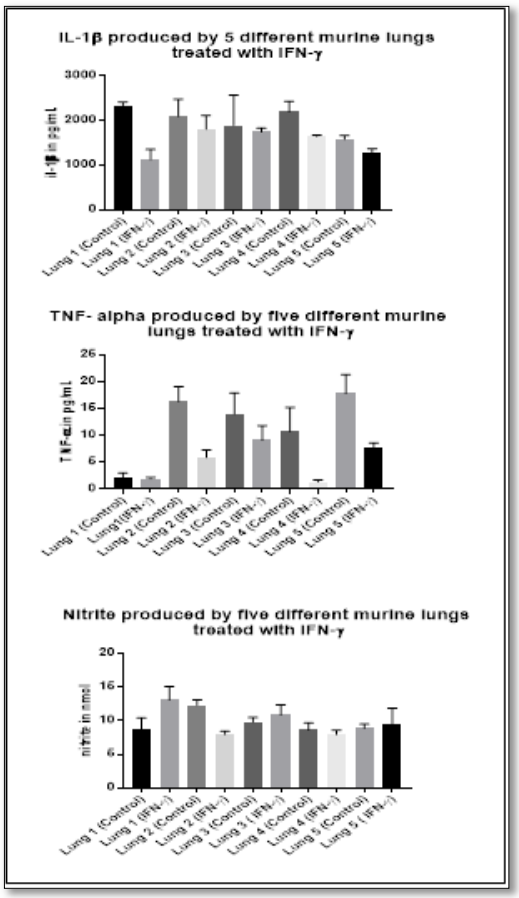

Figure 24. Five murine lungs were homogenised and grown in complete DMEM without GM-CSF. On day 3 of harvest cells were treated with IFN-γ and infected with *Mycobacterium smegmatis*. After 24 h ELISA assays were performed to measure cytokine response IL-1β, TNF-α and nitrite. IFN-γ was able to inhibit TNF-α and IL-1β in activated macrophages. ELISA was carried out on triplicate wells in a 96 well plate and absorbance was read at 450 nm with plate reader.

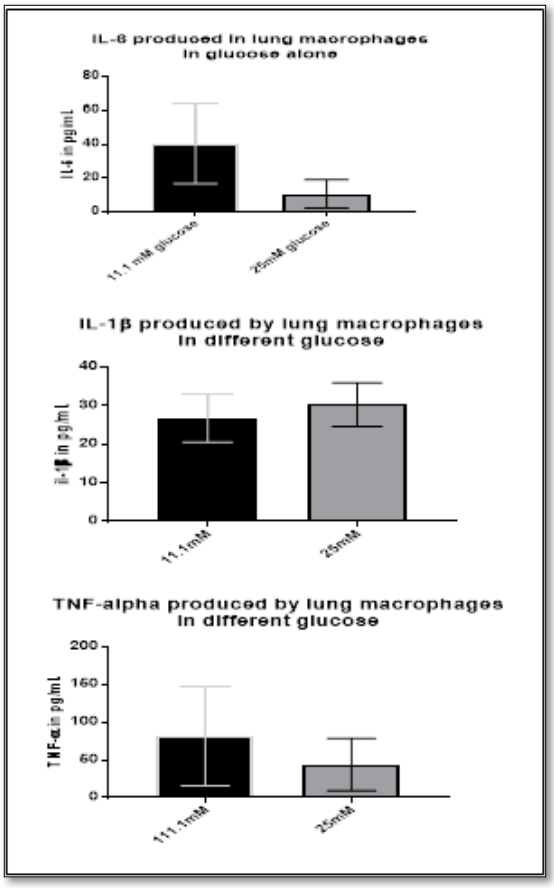

Figure 25. Impact of 25mM glucose on cytokines. Lung macrophages were cultured (see methods) and on day 3 of harvest media was replaced with either 11.1 mM glucose or 25 mM glucose and infected with Mycobacterium smegmatis at a concentration of $1X10^6$. After 24 h post infection with *M smegmatis* ELISA assays were performed and absorbance values were taken from triplicate wells in the 96 well plate. In 25 mM glucose TNF-α and IL-6 cytokine production were reduced.

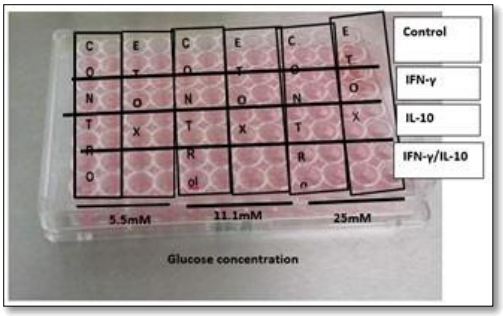

Figure 26. BMDM were harvested and seeded into two times 96 well plates. One plate was treated with Etomoxir and a second plate was treated with Palmitate. To compare the effect of both on macrophage activation and the production of key inflammatory cytokines (IL-6, TNF-α) and nitric oxide. The lay out was the same but instead of etomoxir in each lane palmitate was added at a concentration of 1 mM, Etomoxir (1 mM), IL-10 (3µg/ml), IFN-γ (1 mg/mL). Cells were treated on day 9 of the harvest and infected with *Mycobacterium smegmatis* on day 10. Post infection plates were kept in an incubator at settings temp 37°C, CO_2 5.0%, O_2 18.3% for 36 h. Supernatants were collected in labelled vials and stored in a -80 freezer until required for use.

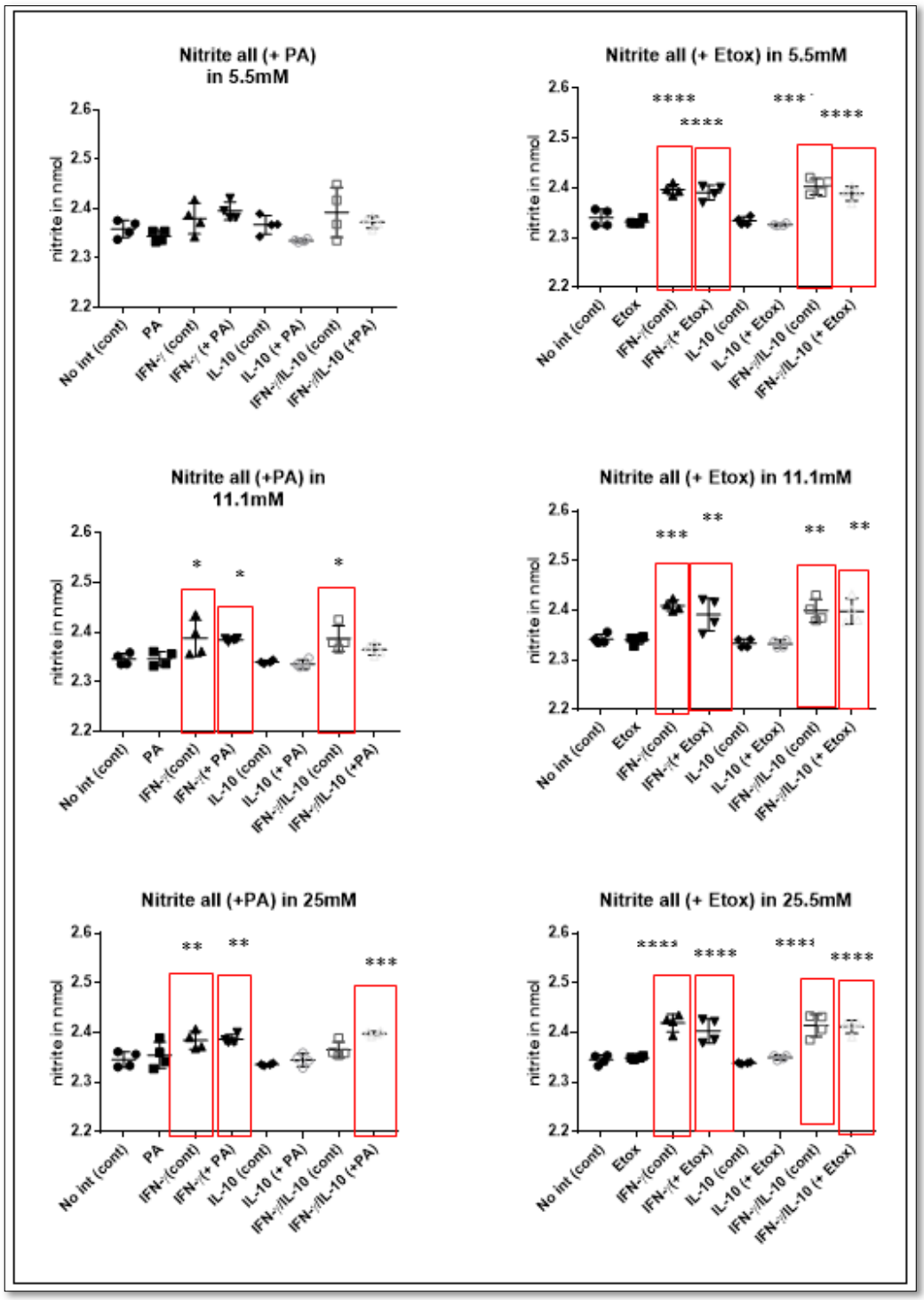

Figure 27. Nitrite produced by activated macrophages in different glucose concentration with either IFN-γ or Etomoxir. In high glucose 25 mM and 11.1 mM there was a trend towards increase in nitrite when palmitate was added with results reaching statistical significance. The nitrite levels increased significantly when etomoxir was added in all glucose conditions. A one-way ANOVA followed by Dunnet's comparison test. A p value of <0.05 was significant.
**p value 0.05, **p value <0.01, ***p value <0.001 ****p value < 0.0001*

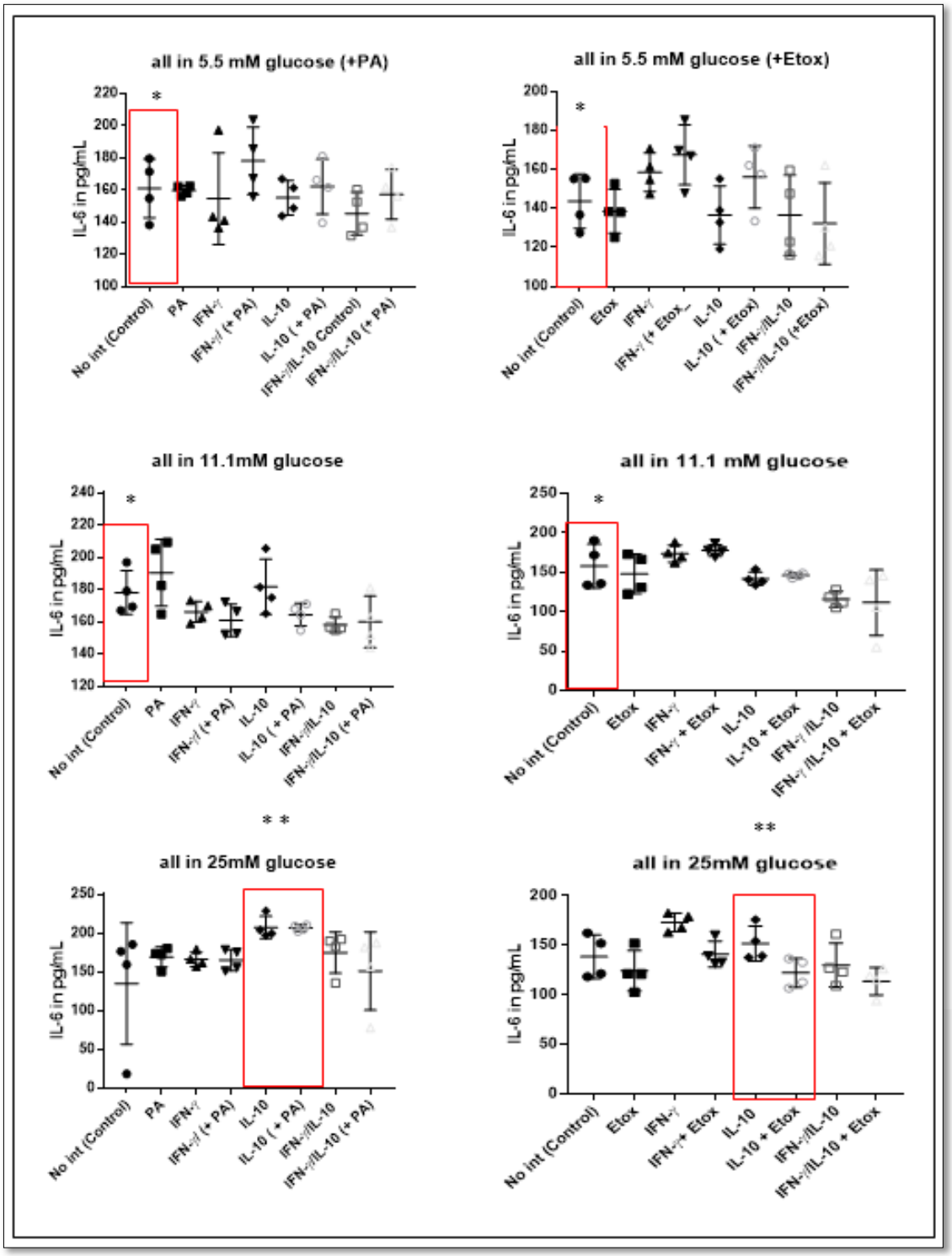

Figure 28. IL-6 response. BMDM cultured in different glucose (5.5 mM, 11.1 mM and 25 mM) were treated with IFN-γ or IL-10 or both. To test polarization of M1/M2 states. We observed a trend towards high IL-6 in high glucose when IL-10 was added. This was true in the + PA and + Etox experimental plates. A one-way ANOVA was performed and a p value of <0.05 was significant. *p value 0.05, **p value <0.01. Supernatants were performed in triplicates on a 96 well plate and absorbance was read at 450 nm.

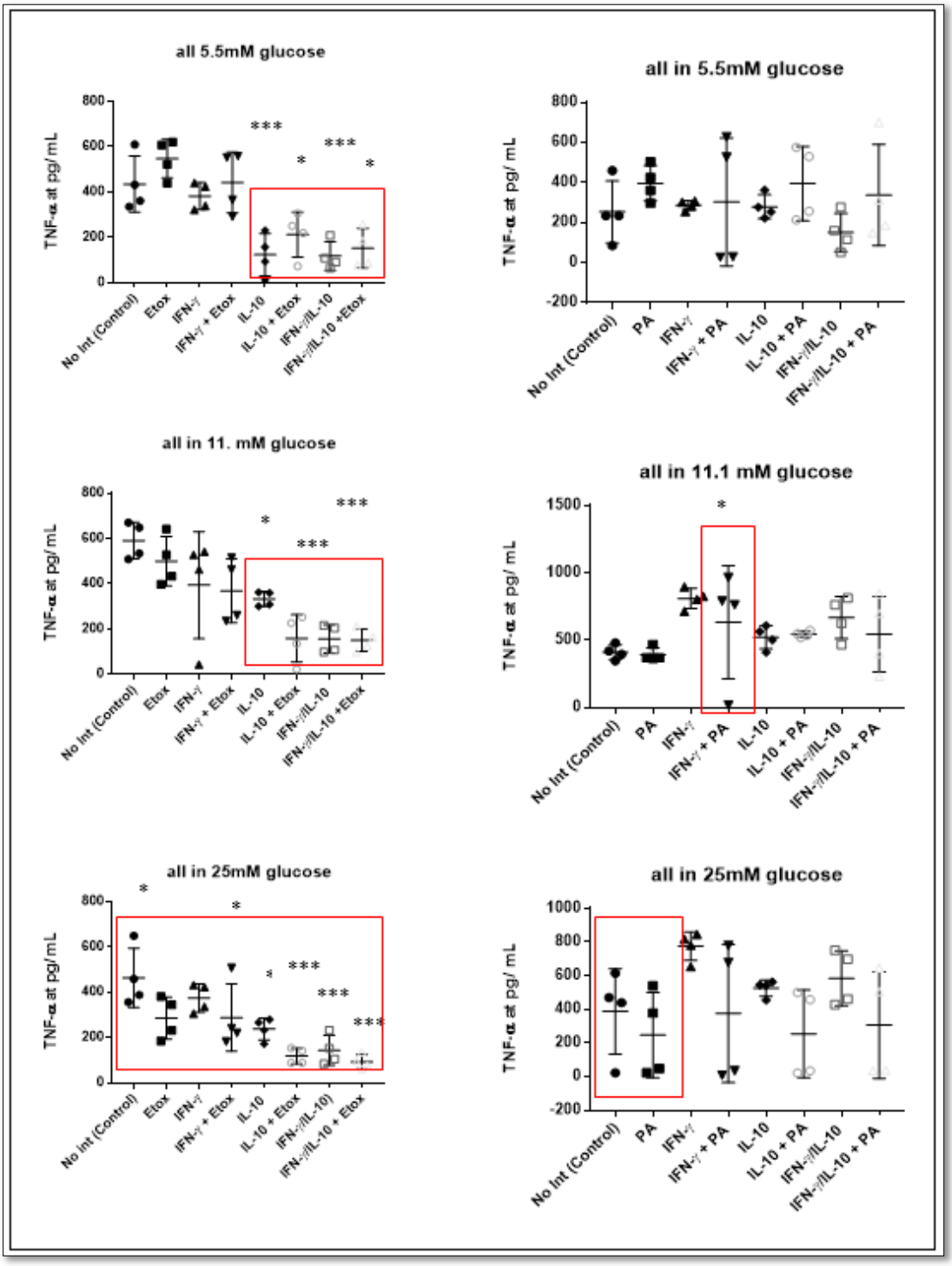

Figure 29. TNF-α response BMDM cultured as described in methods. A higher TNF-α was observed in 25mM, 11.1mM and 5.5mM glucose with results reaching statistical significance when etomoxir was added. Interestingly, when IL-10 was added it reduced the TNF-α response significantly in the etomoxir plate. We observed that in the +PA plate the results were variable. We saw a trend towards higher TNF-α response in all glucose groups. A one-way ANOVA was performed and a p value of <0.05 was significant. * P value 0.05, ** p value <0.01, *** p value <0.001 **** p value < 0.0001.

IL-6 response – The effect on IL-6 was variable in the control, after adding palmitate IL-6 was reduced. It was observed that IL-6 response was increased when palmitate and IFN-γ were added however, the effect was removed by adding IL-10. We found that in higher glucose concentrations (11.1 mM) appeared to reduce IL-6 response even when palmitate was added it had little effect on IL-6 response. Interestingly, in 25mM glucose IL-10 increased IL-6 response, this effect was not observed in other glucose concentrations. By adding etomoxir a trend was observed where its increased IL-6 response in the presence of IFN-γ and after adding IL-10 was moderately reduced **(Figure 28**).

TNF-α response – In 5.5mM glucose etomoxir and IFN-γ increased TNF-α response. The effect was observed in 5.5 mM and 11.1 mM glucose. After adding IL-10 the response was reduced in all glucose concentrations reaching significant levels. IL-10 (control) reduced TNF-α and the effect was pronounced, after adding etomoxir this inhibition was not relived by adding IFN-γ. Etomoxir enhanced TNF-α inhibition and reduced the response **(Figure 29)**.

DISCUSSION

Our study examined the role of macrophages in TB pathogenesis. We knew that one of the key immune cells that play a fundamental role in TB and other diseases such as Atherosclerosis, diabetes and pathogenic infections are macrophages [28,44]. Therefore, a central aspect of our study was the role of macrophages in TB pathogenesis. A number of studies have pointed to the dual role of macrophages in TB. Not only do macrophages provide a niche for *Mtb* bacilli to grow and proliferate in a safe and protective vacuole, but macrophages can assume a pro-inflammatory state, an M1 phenotype, which drives the production of antimicrobial agents such as nitric oxide (NO) and pro-inflammatory cytokines such as TNF-α to eliminate the pathogen from the host [28,34].

Our results support most of the available data on the dual role played by macrophages, namely M1 and M2 types. A key part of our study was the role played by glucose in the activation of macrophages and in the production of pro-inflammatory cytokines infected with *Mycobacterium smegmatis*. Another equally important element was the metabolic processes of glycolysis and fatty acid oxidation (FAO) in macrophages that were essential in regulating polarization of macrophages towards an M1 or M2 state.

We knew that glucose concentrations exceeding physiological levels in the blood (between 4.0-5.4 mmol/L) are known to alter macrophage phenotype and function in diabetes [44,41]. Therefore, our aim was to examine the relationship of glucose on macrophage activation and its ability to regulate *Mtb* bacilli.

A fundamental point is the determination of M1 and M2 states depended on the environment and the signals received by the macrophages. Such that if the environment required a pro-inflammatory response, triggered by the invasion of a pathogenic bacterium such as *Mtb,* macrophages would assume an M1 phenotype and secrete pro-inflammatory cytokines such as TNF-α, IL-6, IL-1β into the milieu [41,38,45]. Once the threat was averted macrophages would be induced by signals from the environment polarizing M1 macrophages to adopt an M2 anti-inflammatory state. M2 macrophages function in tissue repair and healing following a powerful earlier inflammatory response [41]. Therefore, it was the tissue environment that determined the metabolic and polarization state of macrophages as well as their function in response to *Mycobacterium smegmatis* stimulation.

Our results support our hypothesis that by altering glucose availability in the environment impacted the macrophage response and influence either an M1 or M2 metabolic state.

25mM (High) glucose impactsIL-6 and TNF-α response to infection with *M smegmatis*

Our results showed that in 25 mM (high glucose) (double fasting glucose- diabetic) altered the pro-inflammatory response of activated macrophages. It resulted in decreased levels of cytokine IL-6 and TNF-α compared to glucose concentrations of 5.5 mM (normal fasting glucose). Interestingly, we observed that the cytokine response of IL-6 and TNF-αin 11.1 mM (double fasting glucose - pre-diabetic) increased pro-inflammatory cytokines IL-6 and TNF-α reaching levels of significance. We also measured nitrite in glucose alone (data not shown) and found a trend where the levels of nitrite decreased in higher glucose. When we measured nitrite in 11.1 mM and 25 mM glucose, we found that by adding etomoxir and palmitate or both reduced nitrite response reaching significant levels. A similar trend was observed in 5.5mM glucose but results did not reach significance.

Our results support studies examining the priming effect on macrophages in patients with diabetes. In diabetes macrophages assume a pro-inflammatory phenotype and secrete pro-inflammatory cytokines resulting in multiple complications associated with diabetes [44]. Furthermore, diabetes decreases the bactericidal function of macrophages which leads to defective macrophages that are unable to function in tissue repair and wound healing [44]. Moreover, macrophages in high levels of glucose (25mM) have an altered metabolic function and their inflammatory potential is also compromised. These defective macrophages fail to function adequately in diabetes patients and the cells are unable to attach and internalise the bacilli and therefore fail to control the*Mtb* bacterium in the host. This is especially noticeable in more virulent *Mtb* strains [38]. Furthermore, a study by [44] observed that in significantly high glucose the effect of exposure to long term glucose impedes the phagocytic and bactericidal function of macrophages [38]. This would suggest why diabetic patients, who become infected with *Mtb* are unable to control the bacilli and often with fatal consequences [38].

We observed the cell morphology in different glucose (5.5 mM, 11.1 mM and 25 mM) using a phase contrast microscope and found that the morphology of macrophages was altered in 25mM glucose **(Figures 9, 10 and 11)**. In 25mM and 11.1mM macrophages appear to have a deformed morphology compared to macrophages in 5.5 mM. The cells appeared smaller in size, had a less rounded appearance and were sparse in numbers. Cells appeared elongated showing evidence of spreading. This may be a reason why NO produced at 25 mM was reduced. Our findings agree with studies that suggest higher glucose levels alter macrophage function and induce polarization toward an M1 phenotype. Further, studies suggested that in higher glucose the expression of TNF-α was upregulated at a transcriptional and translational level [16]. We found TNF-α response was upregulated at 11.1 mM glucose.

Nitrite by macrophages is modestly reduced in high glucose

A study by [44] found that in high glucose levels of NO were significantly reduced in long term exposure to high glucose. This concurs with our results however; our results were not statistically significant in relation to production of NO by activated macrophages infected with *Mycobacterium smegmatis*. That long term exposure to high glucose can impair macrophage function and alter the macrophage response. Another study [44] found high glucose stimulated low levels of NO but increased levels of TNF-α and IL-1β production. This may explain why exposure of macrophages to a long-term high glucose environment impacts the glycolysis pathway in macrophages, leading to an impaired ability to generate ROS and a reduced bactericidal capacity [38].

Treating BMDM with Palmitate or Etomoxir impacts pro-inflammatory response

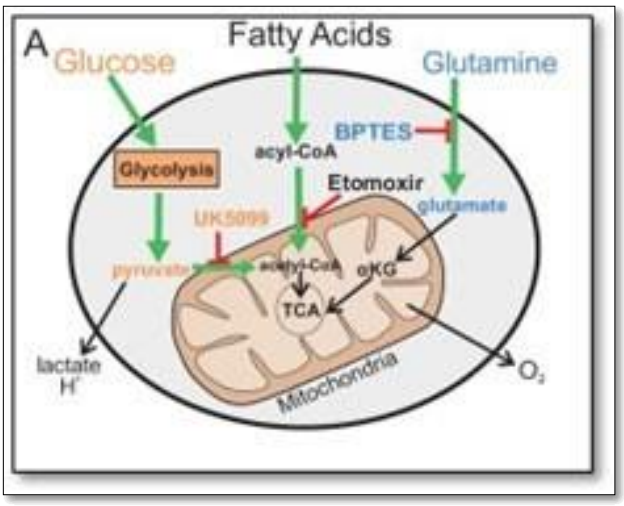

Figure 30. Schematic representation of FAO in M2 macrophages. Cummings et al. [46].

Studies have suggested *Mtb* persistence in the host depends on the availability of host lipids in the environment as well as the ability of *Mtb* to metabolise host lipids [46]. Immune cells have a requirement to generate ATP in order to carry out their function. ATP is generated in the mitochondria. Three substrates are utilized by the mitochondria for OXPHOS (oxidative phosphorylation) to generate ATP which is the fuel source for cells of the immune system. These three substrates are: glucose, fatty acid and glutamine [46]. However, by blocking fatty acid with etomoxir, an inhibitor of carnitine palmitoyl-transferase 1A, which transports fatty acid from the cytosol to the mitochondria. Mitochondria can utilise glucose or glutamine as an alternative for OXPHOS to generate ATP. Mitochondria are flexible in utilising different substrates for ATP [46].

Our results showed that BMDM treated with palmitate reduced levels of IL-6 and TNF-α cytokine compared to BMDM that were exposed to just glucose only. This effect was noticeable in 11.1 mM glucose (**Figure** 4 top left). We found that the cells that were treated with etomoxir decreased IL-6 and TNF-α marginally. When cells were treated with both palmitate and etomoxir levels of IL-6 were reduced but not to significance (**Figure** 3 bottom right). We observed similar results for TNF-α in 11.1 mM glucose and found it was higher compared to other glucose groups. We observed that palmitate removed the impact of glucose (**Figure 4** top left). When palmitate and etomoxir was added it had an effect on TNF-α by reducing the response in all the different glucose conditions (**Figure 4** bottom left). Our results support the idea suggested [46] that mitochondria are flexible in utilising substrates required for OXPHOS. By inhibiting fatty acid metabolism with etomoxir, mitochondria were able to utilise glucose instead to generate ATP to drive macrophage activation **(Figure 27)**. TNF-α, is a key cytokine which plays a role in the early inflammatory immune response as well as in the long-term control of TB [47]. Our results are interesting for diabetes as well as TB. Studies have shown that high glucose induces a pro-inflammatory phenotype in macrophages [44,41]. These macrophages secrete pro-inflammatory cytokines IL-6, TNF-α and IL-1β into the tissue environment. However, the issue that arises is that M1 inflammatory macrophages, in high glucose, remain as M1 macrophages secreting pro-inflammatory cytokines into the environment. The inability of macrophages to receive the signals that would trigger polarization towards an M2 state impairs wound healing in diabetes patients which can be fatal [47]. It leads to a status quo in cell biology of a low grade chronic inflammatory state causing tissue damage, activation of M1 macrophages and a vicious spiral which can ultimately be fatal [41]. In view of the tissue damage caused by chronic pro-inflammatory macrophages, these cells are a key therapeutic target for diabetes patients in the management and cure of the disease [41].

Similar to IFN-γ TNF-α is required for macrophage activation as well as recruitment of immune cells to the site of infection.

Studies have demonstrated its important role in triggering apoptosis in infected cells [47]. Moreover, KO mice deficient in TNF-α, are unable to regulate *Mtb* infection [47]. Therefore, TNF-α plays a critical role in controlling *Mtb* infection. Another cytokine is IL-6 which is secreted early in the infection process. Studies in to the role of IL-6 have shown that the cytokine, together with TNF-α and Il-1β are responders of the early immune response to *Mtb* invasion. Secondly, IL-6 has a role in activating B and T cells as part of the immune response to infection with *Mtb*. Furthermore, IL-6 has shown to induce a priming effect on TB subunit vaccine. In addition, IL-6 KO mice has shown an increased susceptibility to infection with *Mtb* and are eventually overwhelmed by the bacilli. More importantly, IL-6 is known to activate IFN-γ induced protection against TB.

These results have important implications for diabetes and for TB. One of the problems of macrophages in high glucose is that the cells are unable to function due to high glucose in the environment. The macrophages assume a chronic pro-inflammatory state which is damaging to the host. Therefore, if macrophages could be manipulated through inhibiting pro-inflammatory cytokines, either through glucose availability or via inhibiting fatty acid oxidation, could it offer relief to the chronic inflammatory state?

Cytokine response is enhanced in BMDM grown in 4500 mg/mL of DMEM

Our results have shown that activated macrophages grown in constant glucose of 11.1 mM, normal glucose range for cell culture, produced higher levels of cytokine IL-6 compared to control when palmitate or etomoxir were also added. The results were significant (**Figure 12** top left). We observed that BMDM increased pro-inflammatory response after adding etomoxir and palmitate and produced higher levels of TNF-α, IL-1β and nitrite (**Figure 12** top left, bottom right and left). We also observed that macrophages that were treated with palmitate and etomoxir combined, inhibited cytokine response (nitrite, TNF-α + IL-1β), however, we did not observe similar effects for IL-6.

The role of fatty acid in M1/M2 polarization states

Studies had shown that IFN-γ was an important cytokine in activating macrophages to secrete TNF-α, an important cytokine with a pivotal role in the defence against *Mycobacterium* invasion. In addition to activating TNF-α, IFN-γ was also important for the maturation of immune cells [16]. IFN-γ was secreted by IL-6 for activation of macrophages. Cytokine IL-10 was shown to be an important player in suppressing the function of macrophages in *Mtb* infection and functioned in regulating and initiating the immune response to *Mycobacterium* infection [16].

Our results have shown that when cells were treated with IL-10 the production of IL-6, TNF-α and IL-1β were reduced. The results were especially noticeable for IL-6 and that the (M2 polarizer), IL-10 was able to inhibit M1 polarization which resulted in reduced levels of a key pro-inflammatory cytokine.

When BMDM were treated with etomoxir or palmitate and were also stimulated with cytokine IL-10 or IFN-γ, we found that TNF-α was reduced in cells that were treated with IL-10 in the etomoxir plate. However, in the palmitate plate the TNF-α response was reduced when IL-10 was added but the results were not statistically significant.

For IL-6 we found that in high glucose (25mM) when IL-10 was added it increased IL-6 response in both the etomoxir and palmitate treated plates. The results were statistically significant. In 5.5 mM and 11.1 mM we found that when IL-10 was added the cells reduced the response of IL-6 compared to 25 mM glucose. This would explain why in high glucose, as in diabetes, the macrophages are in a constant M1 state [38] and cause a chronic low grade pro-inflammatory state in the environment which is damaging to the host.

High glucose (25mM) impacts pro-inflammatory response of lung macrophages

We observed that in high glucose (25 mM) in lung macrophages TNF-α and IL-6 cytokines produced by activated macrophages were reduced but we did not observe an impact on IL-1β response. The results suggest that in 25 mM glucose impacts the pro-inflammatory response. This may explain why diabetes patients are highly susceptible to *Mtb* infection and once infected, are unable to regulate and regulate *Mtb* progression with fatal consequences.

IL-10 HAS STRONGEST EFFECT ON BACTERICIDAL KILLING IN MACROPHAGES

We found in glucose alone the number of colonies formed was higher in 5.5 mM glucose compared to other glucose groups. In 11.1 mM glucose we found that bacterial colonies dropped by 55% compared to 5.5 mM. When IL-10 was added the lowest number of bacterial colonies was observed compared to all other groups tested. We observed low colony counts for BMDM that were treated with IL-10/IFN-γ combined. In high glucose (25 mM) the highest colony numbers were observed in cells that were treated with palmitate. However, we found the highest colony count in 5.5 mM glucose. This would suggest that palmitate was driving an increase in bacterial numbers via the FAO pathway providing nutrition supporting bacterial growth.

Our study poses interesting results however, a key limitation was the issue of osmosis whether the results obtained in higher glucose were caused by osmotic effects. As we did not use mannitol as a control, we were not able to eliminate osmotic effects. We would need to address osmotic effects in a future study. Another limitation was that as macrophages are heterogeneous the results were specific to the macrophages in the different experimental conditions and any

variations between groups was due to the heterogeneity of macrophages.

CONCLUSION

In conclusion, the study of macrophages from a therapeutic potential holds significant promise in the quest to find a cure for TB. Our study was able to show that at least in an in-vitro system, it was possible to manipulate the immune system by artificially altering glucose and FAO. By determining the impact on activated macrophages and by measuring the levels of pro-inflammatory cytokines, we have shown that high glucose can impact the activation and pro-inflammatory response of macrophages. We also found that IL-10 and IFN-γ were able to inhibit TNF-α and nitrite. The results of the study have implications in the management of TB and diabetes.

Future directions would need to address the impact of glucose on infected lung tissue in mouse models. Our data support the hypothesis that macrophage activation can be altered in-vitro. Further experiments would need to address glucose inin-vivo mouse models.

It is important that the global international science community do not take their eye off this disease. Tuberculosis is a challenge in our century and requires a re-evaluation of current treatment and approaches in the management of the disease. A world free of TB would be a major undertaking and its accomplishment would be a pivotal milestone for the human race. Tuberculosis requires a united and global exchange of ideas from world leading researchers on the optimum pathway to tackle and eliminate this insidious bacterium.

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