

**INFLAMMATORY DENDRITIC CELLS IN THE
HOST IMMUNE RESPONSE TO
MYCOBACTERIA: THE ROLE OF CELLULAR
METABOLISM**

A Thesis Submitted to the University of Dublin, Trinity
College, for the Degree of Doctor of Philosophy in the
Faculty of Medicine

By

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DECLARATION OF AUTHORSHIP

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Summary

Mycobacterium tuberculosis (Mtb) causes an estimated 1.5 million deaths annually and BCG, the only available vaccine, is ineffective at preventing adult pulmonary TB. Inflammatory dendritic cells (DCs) play an essential role in both innate and adaptive immunity and are potential vaccine targets. A metabolic switch from oxidative phosphorylation to glycolysis occurs in murine inflammatory DCs stimulated with TLR agonists and is required for optimal activation.

Glycolysis is the process of degradation of glucose which occurs in both anaerobic cellular respiration and aerobic respiration. It breaks glucose into pyruvate and energy is also released in the form of NADH and ATP. In the presence of oxygen, pyruvate can enter the TCA cycle to generate macromolecules and energy via mitochondrial respiration or, under anaerobic conditions, can be fermented to lactate and secreted by the cell. Activated immune cells often undergo aerobic glycolysis, where pyruvate is converted to lactate in the presence of oxygen, similar to the Warburg effect in cancer cells. This thesis explores the role of metabolism in the maturation and survival of human monocyte-derived dendritic cells (moDCs) infected by BCG.

First, the role of glycolysis in the maturation and viability of BCG-infected moDCs was investigated. The DCs were infected with different amounts of GFP-tagged BCG or treated with the TLR4 agonist LPS in the presence of the glycolysis inhibitor 2-deoxy glucose (2DG). The impact of glycolysis inhibition on the upregulation and secretion of inflammatory cytokines, expression of cell surface maturation markers and viability was assessed. Phenotyping of mo-DCs as well as cell death assessment via Annexin/7AAD assays was carried out by flow cytometry. In cell-free supernatants, cytokine secretion was evaluated with the help of multiplex ELISA and expression of cytokine genes in cell lysates was determined by quantitative RT-PCR. Glycolysis was necessary for optimal CD86 cell surface expression, IL1 β and IL-10 mRNA expression and IL-13 secretion by BCG-infected moDCs. Furthermore, glycolysis had a role in promoting BCG-induced necrosis, suggesting that it is not the main route of energy generation in this setting. Other parameters also related to maturation, including CCR7 and HLA-DR cell surface expression, were unaffected by 2DG.

Second, metabolic flux of moDCs at rest and following infection with BCG at different multiplicities of infection and various time points after infection was measured via the Seahorse Metabolic Flux Analyser. Infection of moDCs with BCG resulted in increased flux through glycolysis under stressed conditions but minimal changes in basal rates of

glycolysis. In addition, the effects of BCG on moDC metabolic flux were compared to those of the virulent H37Rv strain of Mtb. Infection with Mtb shows reduced mitochondrial respiration levels, which may reflect mitochondrial damage during Mtb infection. The glycolytic rate of infected moDCs could be increased by treatment with IFN γ and the anti-diabetes drug metformin, raising the possibility that metabolism could be manipulated to alter the immunogenicity of the BCG vaccine.

Third, the Nanostring nCounter platform was utilized in order to measure the mRNA level of 180 genes involved in cellular metabolism in live and killed BCG-infected moDCs. Nanostring analysis revealed that infection with killed or live BCG stimulates the downregulation and upregulation of various metabolic genes in moDCs. Subsequent metabolic flux analysis utilizing metabolic modulators showed that BCG-infected moDCs increase their dependency on fatty acid oxidation compared to uninfected cells. This is the first report investigating the role of cellular metabolism in human DCs infected with mycobacteria. Overall, the results suggest that glycolysis plays an important though limited role in maturation of human moDCs after BCG infection and that mitochondrial respiration is maintained via fatty acid oxidation. Further investigation will be needed to determine whether manipulation of DC metabolism could enhance the protective effects of the BCG vaccine.

Posters & Presentations

Karima Elayati, D. Triglia, S. O’Leary, J. Keane, M. O’Sullivan. 2016, “The role of glycolysis in the activation of human monocyte-derived dendritic cells following exposure to BCG”. The EMBO Conference on Tuberculosis 2016, Institute Pasteur (September 19th-23th, 2016), Paris, France. Poster presentation.

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GLOSSARY OF ABBREVIATIONS

2-DG	2-deoxyglucose
7-AAD	7-aminoactinomycin D
ACC	Acetyl-CoA carboxylase
ACC1	Acetyl-CoA carboxylase 1
ACC2	Acetyl-CoA carboxylase 2
ACLY	ATP-citrate synthase
ACTB	Actin beta
AFB	Acid fast bacilli
AKT	Serine/threonine kinase 3
ALDOC	aldolase, fructose-bisphosphate C
AM	Alveolar macrophage
ANOVA	Analysis of variance
APC	Antigen presenting cells
ARNT	Aryl Hydrocarbon Receptor Nuclear Translocator
ASC	adaptor protein
ATP	Adenosine triphosphate
BCG	Bacillus Calmette-Guerin
BMDM	Bone marrow-derived macrophage
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation
cDNA	Complementary DNA
CFU	Colony forming units
CIC	Mitochondrial citrate carrier
CO ₂	Carbon dioxide
CPT1	carnitine palmitoyltransferase 1
CPT1A	carnitine palmitoyltransferase 1A
cRPMI	Complete RPMI
Ct	Cycle threshold
CV	Crystal violet
DAMP	Danger-associated molecular pattern
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule -3 – grabbing non integrin (also: CD 209)
DCs	Dendritic cells
DGAT1	Diacylglycerol O-acyltransferase 1
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECAR	Extracellular acidification rate
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay

ENO3	Enolase 3
EPAS1	Endothelial PAS domain protein 1
ETC	Electron transport chain
FAO	Fatty Acid Oxidation
FASN	Fatty Acid Synthase
FBS	Foetal bovine serum
FBS	Foetal bovine serum
FCCP	Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazine
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte -monocyte colony -stimulating factor
GALM	Galactose mutarotase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GLUT1	Glucose transporter1
GP	Glycogen phosphorylase
GR	Glycolytic reserve
GYS	glycogen synthase 1
H3F3A	H3 histone family member 3A
H6PD	hexose-6-phosphate dehydrogenase/glucose 1-dehydrogenase
HIF-1 α	Hypoxia-inducible Factor alpha
HIG2	Hypoxia-inducible protein 2 HIG2
HIV	Human Immunodeficiency Virus
HK1	Hexokinase 1
HKII	Mitochondrial hexokinase II
IBTS	Irish Blood Transfusion Service
IDH	Isocitrate Dehydrogenase
IDH1	isocitrate dehydrogenase (NADP (+)) 1
IFN	Interferon
IFN- γ	Interferon gamma
IL	Interleukin
iNOS	Inducible nitric oxide synthase
iRv	γ -irradiated H37Rv
LAM	Lipoarabinomannan
LDHA	Lactate Dehydrogenase A
LN	Lymph node
LPS	Lipopolysaccharide
LTBI	Latent TB Infection
MDMv	Monocyte-derived macrophage
MDR	Multiple drug resistant
MET	Human MET proto-oncogene
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
MPC -1	Mitochondrial Pyruvate Carrier 1
MPC	Mitochondrial Pyruvate Carrier
mRNA	Messenger RNA
mROS	Mitochondrial ROS

Mtb	<i>Mycobacterium tuberculosis</i>
mTOR	Mechanistic target of rapamycin
mTORC1	mTOR complex 1
NK	Natural killer
NLR	nucleotide-binding oligomerization domain-like receptor, or NOD-like receptor.
NO	Nitric oxide
NOS2	Nitric oxide synthase
OCR	Oxygen consumption rate
OD	Optical density
OMM	Outer membrane mitochondrial
OXPHOS	Oxidative phosphorylation
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PK2	pyruvate dehydrogenase kinase 2
PK3	Pyruvate dehydrogenase kinase 3
PET	Positron emission tomography
PFA	Paraformaldehyde
PFK2	Phosphofructokinase-2
PG	Prostaglandin
PGE ₂	Prostaglandin E ₂
PI	Propidium iodide
PKFM	Phosphofructokinase
PLD ₂	Phospholipase D ₂
PMA	Phorbol myristate acetate
PPAR	Peroxisome proliferator-activated receptor
PPARGC1A	PPARG coactivator 1 alpha
PPIA	peptidylprolyl isomerase A
PPP	Pentose phosphate pathway
PRR	Pattern recognition receptor
PS	Phosphatidylserine
RBSs	Red Blood Cells
RET	Reverse electron transport
RNA	Ribonucleic acid
RNF11	ring finger protein 11
ROS	Reactive oxygen species
RPL19	ribosomal protein L19
RPMI-1640	Roswell Park Memorial Institute-1640 medium
rRNA	Ribosomal RNA
RT-qPCR	Quantitative reverse transcription PCR
SDH	Succinate dehydrogenase
SDHA	succinate dehydrogenase complex flavoprotein subunit A
SHMT1	serine hydroxymethyltransferase 1

SLC2A3	solute carrier family 2 member 3
SLC7A11	Solute carrier family 7 member 11
SRC	Spare respiratory capacity
SUCLG2	Succinate-CoA ligase GDP-forming beta subunit
TB	Tuberculosis
TBHP	Tert-butyl hydrogen peroxide
TCA	Tricarboxylic acid
TLR	Toll-like receptor
TNF α	Tumour necrosis factor- α
WHO	World Health Organisation
YWHAZ	tyrosine3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta

CHAPTER 1:

Introduction and Literature Review

1.1 The Host Response to *Mycobacterium tuberculosis* Infection

1.1.1 Tuberculosis Epidemiology

Tuberculosis (TB) has been plaguing mankind for centuries and is leading cause of death due to an infectious disease. It is caused by *Mycobacterium tuberculosis* (Mtb) which was first isolated on 24th March 1882 by Robert Koch [1]. Approximately one quarter to one third of the world's population is thought to be latently infected with Mtb. Tuberculosis has been given the title of a pandemic because it affects about 8 million people per year irrespective of geographical location or race and ethnicity, with approximately 1.5 million of them actually dying [2], [3]. One-fourth of the population in Europe, comprising mainly of adults, was wiped out in the 19th century by TB, and it is again on the rise due to immunodeficiency (for example, co-infection with HIV) and the development of multi-drug resistant strains of Mtb (MDR-TB) [2]. According to the shared notions of the United Nations Sustainable Development Goals and WHO's End TB strategy the epidemiological burden of TB should to be reduced by 80% and deaths by 90% by 2035 [4]. Nevertheless, in 2018, 10.0 million new cases were reported, out of which 500,000 patients showed resistance towards rifampicin (the most potent first-line drug) [5]. Two thirds of the adult population of eight countries, namely, Nigeria, Pakistan, Indonesia, China, Philippines, Pakistan, South Africa and India were among the ones most affected. Sex ratio of adult male, 57% of all TB cases in 2018. While adult women accounted for 32% and children for 11% and 8.6% of patients were also HIV positive [5]. But positive progress was also seen from 2015 to 2018, the incidence rate fell 15% and the global deaths per year from TB decreased by 24% [5]. The burden of this fatal disease can be decreased only if newer antibiotics and vaccines are developed in line with evolution of new drug resistant bacterial strains.

1.1.2 Bacillus Calmette-Guerin (BCG)

BCG is a live vaccine against tuberculosis which was derived from a strain of *Mycobacterium tuberculosis*. The vaccine was developed over more than 13 years of the period from 1908 to 1921 at the Pasteur Institute by French Bacteriologists Albert Calmette and Camille Guérin who named it Bacillus Calmette Geurin or BCG [6]. The virulence was of *M. bovis* was attenuated by 230 successive passages on a media impregnated with Ox bile [7] and patients were first vaccinated with BCG in 1921 [8]. BCG immunization is carried out in new-born children in countries with a high incidence of TB and protects the babies from TB meningitis.

BCG does not dependably prevent Mtb infection or prevent pulmonary TB disease among adults [9]. Estimates of the efficacy of the BCG vaccine in protecting adults against pulmonary TB have ranged from 80% protection to no benefit. It seems that the protective efficacy of BCG varies depending in which geographical location it is administered, but the reasons for this are not fully understood [10]. The benefits of revaccination later in life among those with negative tuberculin skin tests are yet not proven [11], [12]. Generally, the BCG vaccine is safe but it can cause disseminated mycobacterial disease in infants with defects in the interferon gamma (IFN- γ)/Interleukin-12/23 (IL-12/IL-23) pathway [13]. BCG has other non-specific but beneficial therapeutic effects that are unrelated to the prevention of TB including in autoimmune and allergic disease, cancer and childhood infectious diseases. Recently these therapeutic benefits have been attributed to the induction of trained immunity (i.e. innate immune memory) [14] and appear to be dependent on metabolic reprogramming of innate immune cells. In type 1 diabetes, BCG vaccination rebalances monocyte metabolism from an over reliance on oxidative phosphorylation to glycolysis which reduces the levels of glucose [15]. High doses of BCG are used as immunotherapy for bladder cancer, apparently stimulating an immune response that eliminates the tumour cells [16].

Much work has been done in recent years to develop a safer, more effective TB vaccine, especially one that will be safe for HIV positive neonates [17], [8]. The attenuation of the BCG vaccine is due in large to the deletion of a region of its genome called Region of Difference 1 (RD1) that is present in virulent strains of *M. bovis* and Mtb. This encodes for several immunogenic proteins including ESAT-6 and HspX: two Mtb antigens that are important for activating dendritic cells (DCs). As a result of this deletion BCG has a reduced ability to induce DC maturation, stimulate release of cytokines and CD4⁺ lymphocyte proliferation and to activate NK cells [18]. Several approaches have been taken to develop a new vaccine including the development of more immunogenic live recombinant BCG vaccines [19] or subunit vaccines expressing Mtb antigens missing from BCG designed to boost the immune response to BCG [12]. For example, the M72/AS01E vaccine developed by GlaxoSmithKline contains the M72 recombinant fusion protein derived from two immunogenic *M. tuberculosis* antigens (Mtb32A and Mtb39A), combined with the AS01 adjuvant system is currently being tested in a Phase 2B clinical trial in Kenya, South Africa and Zambia. Results published recently indicate that this vaccine is providing around 54.0% protection against active pulmonary TB disease in latently infected adults, without any

safety concerns [20], [21]. Another subunit vaccine candidate, H4:IC31 - containing mycobacterial antigens Ag85B and TB10.4 and IC31 adjuvant, signalling through toll-like receptor 9 (TLR9) – prevented infection in 30.5% of adolescents who had previously undergone neonatal BCG vaccination. Interestingly, in this clinical trial carried out in South Africa, BCG revaccination had a 45.4% efficacy at preventing infection. It is heartening to know that BCG has some protective efficacies. A better understanding of the interaction of BCG with immune cells may aid in the development of improved vaccines.

1.2 Dendritic cells

Dendritic cells (DCs) connect the innate and adaptive immune system; the main function of DCs is to capture, process and present antigens on cell surface MHC molecules to T cells [22]. The stellar discovery of DCs was made in the late 70s by Steinman and Cohl [23], [24]. It was later proved that several cells in non-lymphoid tissues could also travel via the lymphatics to the lymphoid organs and prime T-cells when exposed to a foreign antigen [25]. In the mid-90s murine lymphoid organ DCs were shown to be divided into 2 main subsets, with and without the presence of CD8 receptors and showing distinct immunogenic functions [26]. But it took another 20 years to recognise non-lymphoid tissue DCs that were characterised by expression of CD103 integrin [27]. Later another subset of plasma like cells were discovered that produced high amounts of IFN- α on applying external stimuli and also differentiated into immunogenic DCs that helped in priming T-cells against viral antigens; these cells were named **plasmacytoid DCs** (pDCs) and the ones discovered by Steinman came to be known as **classical DCs** (cDCs) or myeloid DCs [28].

It is now known that DCs are a category of cells that are involved in presenting antigens and bear the capability of stimulating naïve T cells by means of antigen presentation. They are found in the vascular system and lymph nodes (LN) and in organs, including the lung. In general, they serve as a mediator between the innate and adaptive immune system. In essence, they recognize and capture pathogens or other danger signals such as necrotic cells and react to their signals and in turn command the adaptive immune system to produce factors that will process the pathogens proteins and launch an adaptive immune response against the pathogen.

In response to danger signals, DCs mature and release different combinations of cytokines to induce cytotoxic CD8⁺ T cells and Th1, Th2, Th17 and/or Treg CD4⁺ T cell responses,

depending on the type and strength of the inciting stimulus. In the mouse it has been established that individual DC subsets have different functions and are equipped to respond to different stimuli. DC subsets achieve this by differential expression of pattern recognition receptors such as TLRs, c-type lectins (such as CLEC9a and Dec205), activity of specific transcription factors and chemokine receptors. For example, pDCs, as a consequence of expressing TLR7 and TLR9, recognise nucleic acids and play an important role in maintaining tolerance and in the anti-viral response through their secretion of type 1 interferons [279].

Murine cDCs are divided into three subsets; pDCs and two subsets of classical or myeloid DCs – which are referred to as cDC1 and cDC2. Human pDCs can be distinguished from cDCs by their cell surface expression of CD123 [24]. Myeloid DCs exhibit CD45, MHC-II, and CD11c but show absence of markers unique to T cell, B cell, natural killer cell and other cells of the erythrocyte lineage and distinct Toll-Like receptor and C-type lectin pattern recognition receptor expression [29]. The cDC1 subset express the transcription factors IRF8, Batf3 and Id2 during their development (**Figure 1.1**) [26]. In lymphoid tissue murine cDC1 also express the chemokine receptor XCR1, Clec9a (a receptor for dead cells) and the cell surface marker CD8 α . They can be identified by their expression of CD103 in non-lymphoid tissues such as the lung [279], where they are found mainly in the epithelium and vascular wall [280]. In the mouse this subset of DCs specialise in cross-presentation of antigen to prime CD8 $^{+}$ cytotoxic T cells in an MHC class I-dependent manner and tend to induce a more tolerogenic CD4 $^{+}$ T cell response. The mouse cDC1 are also specialised in cross-presentation of antigens from apoptotic cells which they traffic to the LN to cross-tolerise CD8 $^{+}$ T cells in the steady state or, in the presence of PAMPs, cross-present and stimulate an immunogenic T cell response to activate CD8 $^{+}$ T cells [281]. In humans, cDC1 cells do not express CD8 α but are characterised by expression of CD141 (BDCA3) and XCR1 [27].

The murine cDC2 subset express IRF4, and the cell surface marker CD11b in both lymphoid and non-lymphoid tissues. Murine cDC2 express CD11b, TLR2 and can release inflammatory mediators such as TNF α and nitric oxide and specialise in the stimulation of CD4 $^{+}$ T cells in an MHC1I-dependent manner. Human cDC2 are present at ten times greater numbers than the cDC1 subset in the blood and can be identified by cell surface expression of CD1c (BDCA1) as well as the myeloid antigens CD11b, CD11c, CD13 and CD33 and

they require IRF4 for their development [279]. The DC2 subset is also found in the lymph nodes and tissues, including the lung, where they reside mainly in the lamina propria [280]. Evidence so far suggests that the response of human cDC subsets is not as restricted as that of their murine counterparts. For example, unlike murine cDC2, the human cells secrete a range of proinflammatory cytokines including high levels of IL-12 after TLR stimulation and can activate Th1, Th2, Th17 and CD8⁺ T cells. Therefore, human cDC responses may vary depending on the type and strength of the stimulus rather than on DC ontogeny [279].

Epidermal Langerhans cells are present in the epidermal layer of the skin, similar to macrophages but after migration to lymphoid organs can display properties unique to cDCs [1]. Langerhans cells were thought to be DCs but are now thought to be tissue resident macrophages although they have many of the characteristics of DCs, including migration to the lymph nodes [279].

1.2.1 Monocyte-derived Dendritic Cell Ontogeny

The most common model used to study DC-pathogen interactions consists of monocyte-derived DCs (moDCs) that have been differentiated in the presence of GM-CSF and IL-4. Human blood monocytes are categorized into three subsets; CD14⁺ CD16⁻, CD14^{lo} CD16⁺ and an intermediate form, CD14⁺ CD16⁺. The most common monocyte subset is CD14⁺ CD16⁻, known as the Classical monocytes, that differentiates into macrophages or DC-like cells at the time of inflammation [31], [32]. An equivalent subset to the human classical monocytes in mice is the LyC6^{hi} monocytes, called inflammatory monocytes previously. The LyC6^{lo} monocytes are the equivalent of non-classical monocytes. They are formed in the bone-marrow and are recruited to sites of inflammation. Classical monocytes, which are usually recruited first, can differentiate into pro-inflammatory **monocyte-derived macrophages or moDCs** [33].

In the mouse, **moDCs** in vivo are mostly detected at the time of inflammation when monocytes start differentiating into TNF α and iNOS producing DCs (TIP DCs). But there always has been some debate regarding the extent to which these cells embody the characteristics of dendritic cells. Murine in vitro derived CD11c⁺ DCs with high expression of MHC II molecules from bone-marrow, cultured in GM-CSF are somewhat similar to in vivo monocyte derived DCs (moDCs) [30], [36 – 41].

Thus, at present there are three broad categories of DC subsets, namely, classical DCs (cDCs), plasmacytoid DCs (pDCs) and **moDCs** and they have a common point of origin, i.e. the Common Myeloid progenitor cell (CMPs) (**Figure 1.1**).

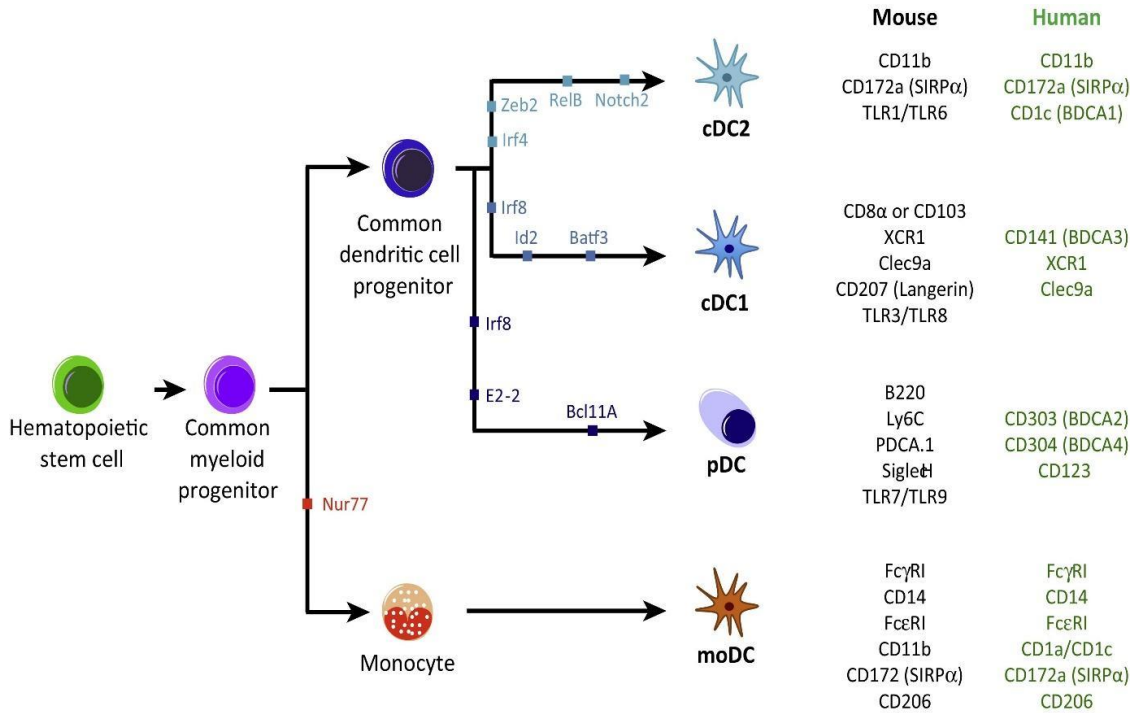


Figure 1.1: Differentiation of DC. DCs are derived from hematopoietic stem cells, and then further differentiate into common myeloid progenitors (CMPs). CMPs differentiation into monocytes is regulated by the transcription factor Nur77, which differentiate into monocyte DCs (moDCs) under inflammation [42].

1.2.2 Maturation of dendritic cells and activation of T cells

DC progenitors arise in bone marrow, enter the blood and finally establish themselves in non-lymphoid organs like the gut, skin epidermis, lung parenchyma, etc. wherein, they give rise to immature DCs that are capable of antigen uptake and processing but are not capable of priming T cells [43]. Depending on their expression of PRRs, different PAMPs can elicit different DC responses (immunogenic or tolerogenic) and consequently, different T cell responses. After the uptake of antigens in presence of PAMPs, DCs travel through blood to LNs or the spleen. They undergo a maturation process during this migration and when they arrive in these secondary lymphoid organs, their antigen uptake and processing capacity reduces, but they present the antigens effectively to CD4⁺ and CD8⁺ T cells (**Figure 1.2**). This is attributed to the upregulation of co-stimulatory cell surface markers like CD80, CD86 and CD40 along with CD54 and CD83 and Class I and II MHC molecules and a high rate of secretion of cytokines like IL-12 [44]. They present antigens within class I and II MHC molecule clefts (Signal 1) and co-express co-stimulatory molecules (Signal 2) and secrete

cytokines and chemokines (Signal 3) (**Figure 1.3**) [47]. Co-stimulatory molecules cooperate with MHC molecules in antigen presentation to naïve T-cells and memory cells that reside in lymph nodes (**Figures 1.2 & 1.3**) [48].

Once activated, cytotoxic CD8⁺ T cells can directly eliminate infected cells by lysis. Antigenic determinants influence the proliferation of CD4⁺ T cells into different effector subsets; Th1 cells are stimulated by viruses and intracellular bacteria and produce IFN- γ to activate macrophages to an M1 phenotype, while Th2 cells secrete IL-4 and IL-13 in response to helminth infection [282]. Infections against fungi and extracellular bacteria stimulate a Th17 response, secreting IL-17, IL-21, and IL-22 to stimulate innate immune cells to secrete IL-8 and attract neutrophils. T follicular helper cells (Thf) initiate the humoral response providing help to B cells during germinal centre reaction, and IL-10 and TGF β produce a regulatory T cell (Treg) population that suppresses effector T cell function [283]. Using cytokines, the Th cells also regulate the production of antibody via the maturation of B cell into effector B cell and plasma B cells. Antibodies function as opsonins that stimulate clearance of infected cells from the system. When an infection has been cleared, the populations of effector cells contract and only long-living memory T cells survive. If the appropriate antigen is encountered at a later date these memory cells can rapidly expand once more to quickly eliminate the infection [47].

Immature monocyte-derived DCs can be generated *in vitro* from human peripheral monocytes using granulocyte/macrophage colony stimulating factor (GM-CSF) and interleukin 4 (IL-4) [1] and induced to undergo maturation by treatment with stimuli such as LPS, TNF α , INF- γ , CD40L or exposure to pathogens such as bacteria and viruses [2]. This process is associated with a dramatic change in morphology and function (**Figure 1.2**).

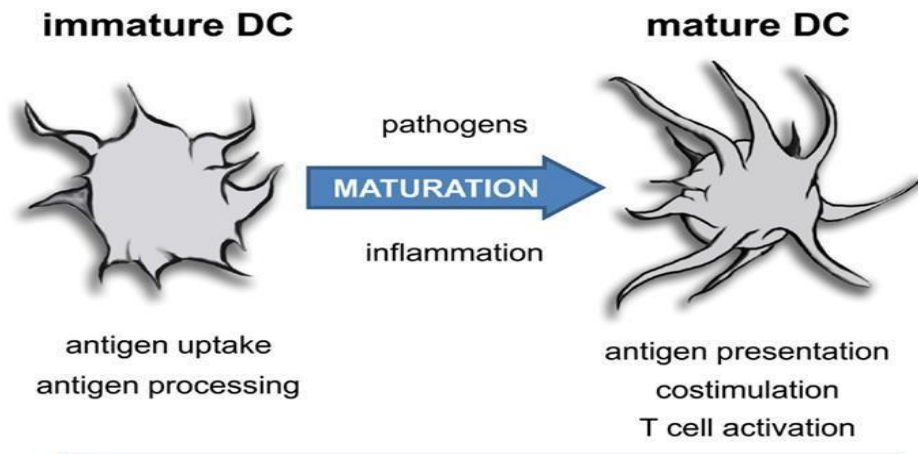


Figure 1.2: Maturation of DCs. Immature DC (CD80 low, CD86 low and CD83 low) stimulation by PAMPs such as LPS leads to fully matured DC (CD80 high, CD86 high and CD83 high) with dramatic changes in morphology and function. Figure from Hubo [22].

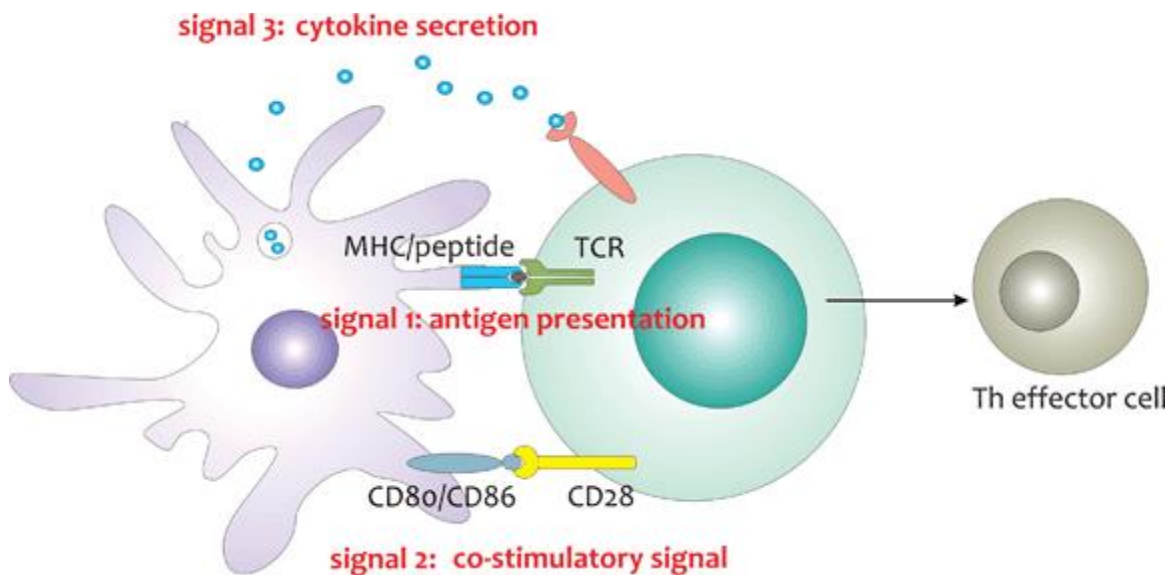


Figure 1.3: CD4+ T cells are activated by antigen presented by antigen-presenting cells (APC) such as dendritic cells. The “signal 1” for T cell activation, occurs following the recognition of MHC-peptide complex on an antigen-presenting cell (APC) by the T cell receptor (TCR) on a T cell. The “signal 2” for T cell activation is provided by binding of B7 molecules (CD80/ 86) on the APC to CD28 on the T cells. Following this interaction, T cells are activated and perform various effector functions. Figure from De Koker et al [71].

Another classification defines DCs as “immunogenic” and “tolerogenic”. Immunogenic DCs mature, release pro-inflammatory cytokines and stimulate effector T cell responses. Tolerogenic cells are immunosuppressive cells that are able to tolerate the pathogens they encounter and do not launch immune responses. In the steady state DCs promote peripheral tolerance and maintain immune homeostasis. In response to tolerogenic signals, like the immunosuppressive cytokines IL-10 and TGF β , immature DCs are matured toward an

alternate phenotype, expressing lower levels of costimulatory and MHC-II molecules than immunogenic DCs. Tolerogenic DCs act on the adaptive immune response by stimulating the differentiation of Tregs, by inducing T cell anergy, or by causing T cell apoptosis (important in the induction of T cell clonal deletion) [284]. In addition, they can generate an anti-inflammatory milieu in their immediate micro-environment which can inhibit pro-inflammatory innate immune cells such as neutrophils, macrophages and innate T cells. To maintain tolerance these DCs release immunosuppressive cytokines, express inhibitory surface molecules such as programmed cell death ligand (PD-L1/2), express the enzyme indoleamine 2,3-dioxygenase (IDO) which catabolizes the amino acid tryptophan and also produce retinoic acid (RA), which induces differentiation of Treg. Tolerogenic DCs can be generated *in vitro* by treatment of immature moDCs with the pharmacological agents rapamycin, dexamethasone and/or vitamin D3. The immunosuppressive cytokines IL-10 and TGF β can also drive the development tolerogenic DCs [285].

1.3 The immune response to TB

Individuals generally become infected with virulent mycobacteria by inhaling droplet nuclei of the tubercle bacilli - measuring 1 to 2 microns - that reach the lower respiratory tract near the lungs, overcoming barriers like nasopharynx or the upper respiratory tract. The first immune cells that come in contact with such bacilli are the macrophages in the alveoli that take them up via phagocytosis. Once engulfed bacteria can be killed by different microbicidal processes like, phagolysosome fusion wherein, the organisms get killed by intra-lysosomal acidic hydrolases via generation of Reactive Oxygen and Nitrogen Species (ROS and RNS) [45]. Macrophages can also process and present antigen to antigen-specific effector T cells to stimulate adaptive immunity but Mtb can inhibit many of the anti-microbial mechanisms of macrophages such as phagolysosome fusion and antigen presentation to persist in macrophages. Infected macrophages release cytokines and chemokines to attract other immune cells including neutrophils, DCs and lymphocytes, eventually resulting in the formation of a granuloma, which contains the infection. Although neutrophils are the most commonly infected phagocyte found in sputum and BAL from patients with active TB, their role in TB pathogenesis is not well understood; they may be protective in the early stages of infection but in chronic TB infected neutrophils appear to promote a type I interferon response that correlates with disease severity [286]. Mycobacteria can survive for years or even decades in the granuloma in a latent state without

causing disease but can reactivate to cause TB if the individual becomes immunosuppressed [46].

1.3.1 The role of dendritic cells in the immune response to *M. tuberculosis*

Dendritic cells in their resting state express germ-line encoded pattern recognition receptors (PRRs) like toll-like receptors (TLRs) which are quick to respond to inflammatory stimuli as well as pathogenic products [47]. Immature early dendritic cells encounter bacteria and are equipped well enough to engulf them, whereas the late DCs (after maturation in the LNs) are suitable for priming and presenting microbial peptides to T-cells and imparting protective immunity [48]. The major T cell subsets required for an effective immune response to mycobacteria is the Th1 cells and CD8⁺ cytotoxic T cells or CTLs that kill the infected cells. In the case of TB, B cells and antibodies do not seem to play an important role in combating the infection [48]. When allogeneic T-cells were cultured together with BCG treated DCs they showed high expression of CD25, CD54 and CD71 on both CD4⁺ and CD8⁺ T cell surface along with increased secretion of IL-2, IL-10 and IFN- γ by both helper and cytotoxic T-cells. CD71 is used by the body to help transport iron from transferring to the cells. This also acts an immunosuppressive molecule and deters the immune cells from getting recruited to the infection site. CD54 is also known as a cell adhesion molecule as it helps in the formation of immune synapses and has also been shown to possess immunosuppressive actions of macrophages. Similar to the above two, CD25 is a marker expressed on the regulatory T cells hence it is also involved in immunosuppression. This showed that DC infected with mycobacteria could stimulate potent T cell responses [50]. A study was also carried out to further implicate the significance of dendritic cells in mice infected with TB. Herein, DCs were depleted using diphtheria toxin/GFP transgenic mice that showed significant lowering of T-cell response and increased load of bacteria in the lungs showing that these cells are essential in controlling Mtb infection in vivo [51]. In addition to their role in initiating adaptive immunity DCs can also directly inhibit the intracellular growth of mycobacteria after stimulation by IFN- γ in vitro [52].

The DCs receptors implicated in capturing mycobacteria include the mannose receptors (MRs), CD11b and CD11c. But the most crucial lectin receptor that is required to engulf the bacteria is the DC-specific ICAM-3 Grabbing Non-Integrin (DC-SIGN) which is a Pattern

Recognition Receptor (PRR) interacting with bacteria like *Mycobacteria* and *Salmonella* spp [49]. The residues recognized by DC-SIGN are mannose residues of lipoarabinomannan and other lipomannans that mycobacteria generally express on their surface, also stimulating the production of IL-10. As mentioned in Section 1.2.2 above, after maturation the phenotype and functionality of DCs alter and other receptors like CD80, CD86, CD40, CD54 and CD83 along with Class I and II MHC molecules are upregulated.

DCs present the mycobacterial antigens loaded on Class I MHC molecules to naïve CD4⁺ T-cells to stimulate a Th1 response. These cells then get activated and secrete IFN- γ , IL-2 and TNF α . TNF α continues controlling the bacterial population by activation of macrophages and initiating granuloma formation [55]. Individuals with rare mutations in the IFN- γ –IL-12/23 signalling pathway are extremely susceptible to mycobacterial infections indicating that a Th1 response is essential in anti-TB host immunity [13]. Induction of Th17 cells, which secrete IL-17 and TNF α are thought to be necessary for protection in the early stages of mycobacterial infection, although this T cell subset can contribute to immunopathology in the later stages of infection [161].

CD8⁺ T-cell activation during tuberculosis is more complicated owing to the fact that these cells get activated only when triggered by antigenic peptides loaded onto Class I MHC molecules. But this event occurs in the cytosol and mycobacteria usually reside in the phagosome. It is thought that virulent *Mtb* strains can escape from the phagosome (this is dependent on the ESX1 secretion system, which is not present in BCG due to a large deletion of RD1 of the original *M. bovis* genome). Therefore, DCs come into play via two pathways for CD8⁺ T-cell activation. Firstly, when the bacteria directly enter into the cytosol of infected DCs and antigens are presented by Class I MHC molecule and secondly, when the infected macrophages undergo apoptosis that results in uptake of vesicles carrying antigenic peptides by the DCs and cross presentation of the mycobacterial antigens to CD8⁺ T-cells [56–59].

Adaptive immunity to *M. tuberculosis* residing intracellularly within macrophages is a complicated process as it requires the aid of multiple T-cell subsets in addition to CD4⁺ and CD8⁺ $\alpha\beta$ T-cells, including $\gamma\delta$ T-cells, CD8⁺ mucosal-associated invariant T (MAIT) cells and CD1 restricted T-cells [54]. Therefore, as well as exhibiting foreign peptides on their surface via both Class I and II MHC molecules, DCs also present non-peptide antigens to

innate T-cells circulating in the LN vicinity [53]. CD1 restricted T cells bear characteristics of both Natural Killer cells and the T cells and therefore share commonalities with both adaptive and innate immune cells. They are polyclonal, unlike CD4⁺ and CD8⁺ $\alpha\beta$ T-cells and proliferate after they encounter mycobacterial lipid antigens (which are abundant in the mycobacterial cell wall). They are also sometimes capable of conventional means of innate stimulation, for example, with the aid of cytokines. Innate T cells can respond to mycobacteria in the early stages of infection, before conventional T cells become activated [287].

In mouse models of TB, inflammatory moDCs derived from infiltrating classical LyC6^{hi} monocytes play an important role in host defence [61]. During Mtb infection, IL-1 β plays an essential role in host resistance and does not require TLR signalling or caspase-1 activity. IL-1 β signalling in macrophages *in vitro* normally requires the induction of IL-1 β expression by TLRs in concert with the triggering of the inflammasome signalling with caspase 1 activation. However, Mayer-Barber et al. showed in 2012 that this combined activity is not required *in vivo* in mice infected with TB even though they require IL-1 β to control Mtb infection. It was suggested that this is a major means of acquiring host resistance since the IL-1 β can now be invoked with the help of some other mechanism that does not require these two pathways to act. This IL-1 β is secreted primarily by infiltrating monocytes which differentiate into DCs in the mouse lung [60]. Alternative pathways for inflammasome independent IL-1 β biosynthesis have been identified including serine protease cleavage of pro-IL-1 β by neutrophils but the mechanism has not been elucidated in DCs [288]. Inflammatory moDCs also transport live Mtb bacteria from the site of infection to the draining LN. Subsequently the DCs transfer invading antigens to classical dendritic cells to initiate the proliferation and polarization of naive CD4⁺ T cells to Th1 cells [61- 63].

Stimuli like IFN- γ or Toll-Like Receptor (TLR) ligands play an important role in activating such monocyte-derived macrophages (MDMs) and DCs [34]. In one study, when human monocytes were activated with TLR ligands like the 19kDa lipopeptide (TLR 2/1 ligand) from Mtb, two cell subsets were formed, a macrophage-like one expressing DC-SIGN and the other consisting of more DC-like cells expressing CD1b⁺, which were dependent on IL-15 and GM-CSF respectively. A subset of monocytes stimulated with a combination of IL-15 and GM-CSF were positive for both CD1b and DC-SIGN, which is similar to the phenotype of mo-DCs generated *in vitro* by IL-4 and GM-CSF [35].

TB is characterised by a delay in activation of adaptive immunity which takes around 2-3 weeks in mice (**Figure 1.4**). In humans, it takes 6 weeks before antigen-specific T cells can be detected. Consequently, Mtb grows logarithmically until the appearance of T cells in the lung inhibits its growth. BCG vaccination has no influence on the early logarithmic growth of Mtb in mice up to around day 12 although the bacterial load stabilises at a lower level than in unvaccinated mice once adaptive immunity comes into effect [64], [65]. Impaired DC function has been implicated in the delay in the development of the T cell response. MoDCs undergo a necrotic form of cell death when infected *in vitro* [66] which may reduce the numbers of infected DCs available to migrate to the draining LN. In addition, in Mtb infection moDC migration is impaired, their ability to migrate depends on the virulence and strain of Mtb [67]. This impaired migration contributes to the delayed activation of T-cells [68] and may be compounded by a delay in the arrival of CD11b⁺ and CD103⁺ cDCs subsets at the mediastinal LN [69].

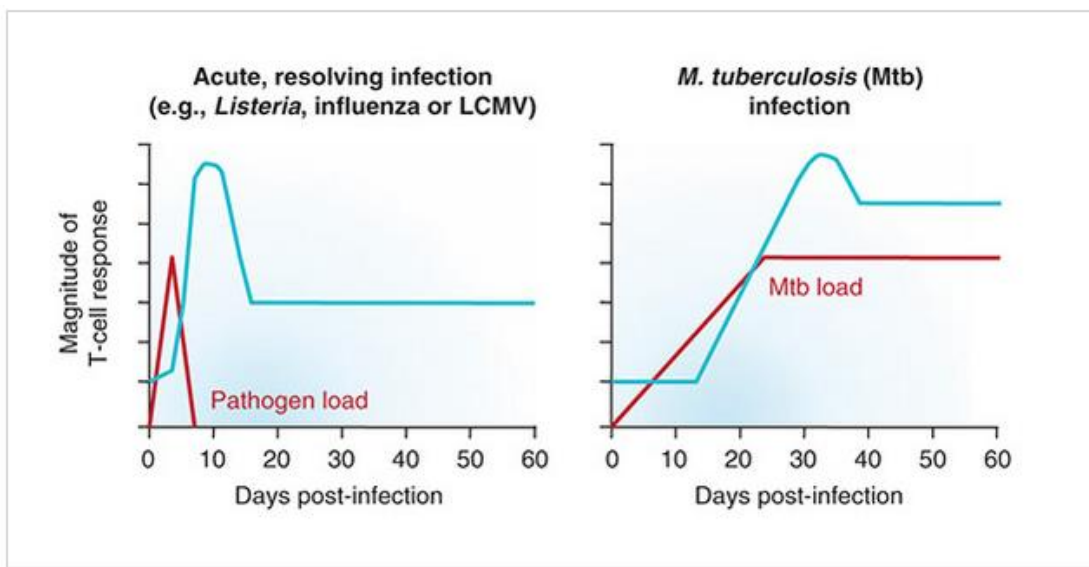


Figure 1.4: Delayed T- Cell Response in TB. In the response to acute resolving infections, on the left, such as Listeria, where T-cell responses (blue) start three days after the initial infection and peak at seven to eight days. The pathogen load (red) rapidly peaks on the third day and then the bacteria are eliminated. On the right, in Mtb infection, T-cell responses (blue) do not start until eleven to fourteen days and Mtb load (red) plateaus but is not eliminated. Figure from Kevin Urdahl [70].

Though studies have proved that infection with mycobacteria activates DCs and establishes an immune response [72–75], there is evidence that states the opposite as well [49]. Normally, bacteria infect DCs, activating the nuclear factor kappa-B family of transcriptional activators followed by secretion of cytokines and chemokines and production of Reactive Oxygen and nitrogen species [76]. The cytokines that show anti-mycobacterial

immunity are IL-12, IL-6, TNF α and IL-1 β . But interaction between DCs and mycobacteria can also lead to the production of IL-10 that downregulates IL-12 production inhibiting cell-mediated responses against the bacteria [77].

Mycobacteria successfully bypass the host immune response by impairing maturation of dendritic cells. This can occur when DC-SIGN receptor on immature DCs interacts with the mannose caps of the Lipo-arabino-mannose (LAM) residues on surface of slow growing virulent strains like *M. tuberculosis* and *M. avium* [78]. Due to these interactions DC maturation is impaired along with induction of IL-10 that inefficiently stimulate T-cells and creates an antigen specific tolerance cascade [78], [79]. Another recent study showed how a chaperone like protein (GroEL2) in Mtb, when cleaved, can modulate the pro-inflammatory response, hampering DC maturation and antigen presentation [80]. Another study suggested that inflammatory DCs, because of their impaired ability to migrate when infected, become trapped in the lung and are responsible for spreading Mtb to uninfected areas of the lung [81]. It has also been shown that the virulent H37Rv strain of Mtb does not allow human DCs to fully differentiate from human monocytes in presence of IL-4 and GM-CSF. These DCs did not have detectable expression of the family of CD1 molecules, and weakly expressed the co-stimulatory molecule CD80, along with low Class I and II MHC molecules and were unable to stimulate the expansion of IFN- γ -secreting CD4⁺ T-cells [82]. Interestingly, high level of Tregs are found in the blood and urine of patients who were given the BCG vaccine to treat bladder cancer. Treg levels in the urine correlated with recurrence of the cancer [289], suggesting that the efficacy of BCG as an anti-TB vaccine may also be compromised by its induction of immune tolerance.

1.4 Metabolic reprogramming in dendritic cells

Immunometabolism is a newer area of extensive research that concentrates on the metabolic changes in immune cells of the host during diseases like bacterial infection or cancer. The metabolic state of immune cells changes with respect to normal cells at the time when the body is compromised. There are variable metabolic changes that help initiate effector function in these cells [84].

1.4.1 Cellular metabolism

Oxidative phosphorylation (OXPHOS): The process providing ATP (Adenosine triphosphate) that is used by higher animals and plants for survival and growth and to

maintain the overall metabolic homeostasis in our body is called oxidative phosphorylation, an important part of the entire cellular respiration. It takes place in mitochondria, which have evolved due to an endosymbiotic event in which a free-living organism capable of OXPHOS was engulfed by a higher cell [85]. After the TCA (Tricarboxylic acid) cycle in which acetyl-coA gets oxidized to produce NADH and FADH₂, these reducing equivalent are used up during oxidative phosphorylation to reduce oxygen to water via a number of electron transfer reactions occurring within a complex set of membrane proteins called the Electron Transport Chain (ETC). The main function of OXPHOS is to convert the electron transferring potential of FADH₂ and NADH into the phosphoryl transfer potential of ATP [86]. The OXPHOS machinery is located in the inner-mitochondrial membrane with 13 essential proteins encoded by the mitochondrial genome; and acts as a unit that transfers electron via a number of macromolecular complexes leading to cells respiring and synthesising ATP. It also produces a vast number of radicals and is involved in apoptosis [86 – 88] (**Figure 1.5 and Figure 1.6**).

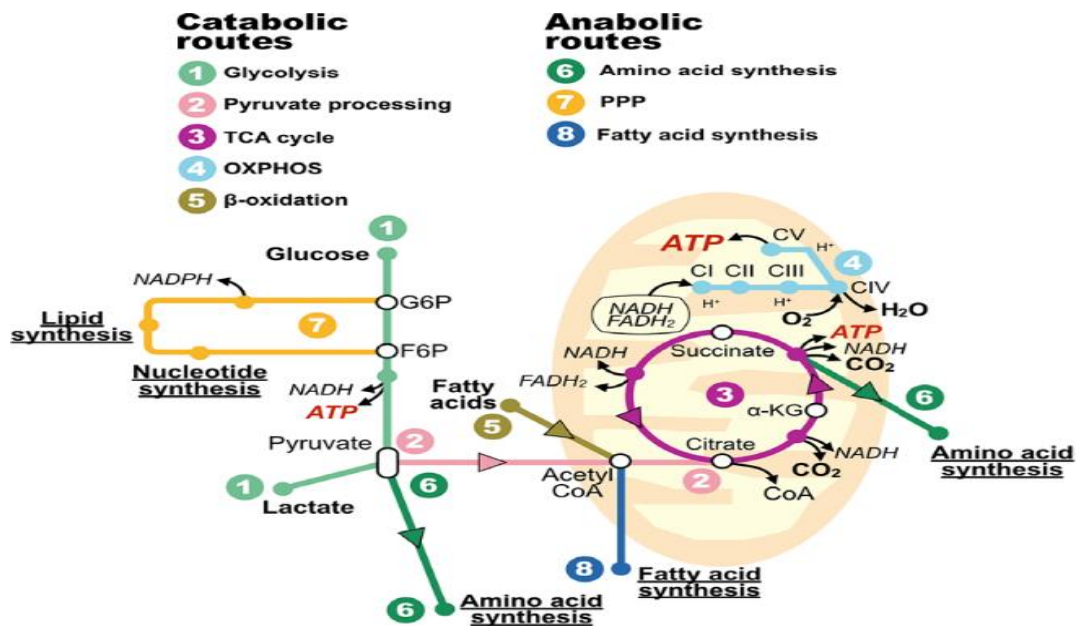


Figure 1.5: Overview of metabolic pathways. The major catabolic pathway used by cells to break down molecules to generate ATP is OXPHOS (4). Anabolic pathways lead to the biosynthesis of biomolecules (6, 7, 8). Catabolic and anabolic ways collaborate to provide the metabolic requirements for cellular functions. Figure from Pedro Escoll [89].

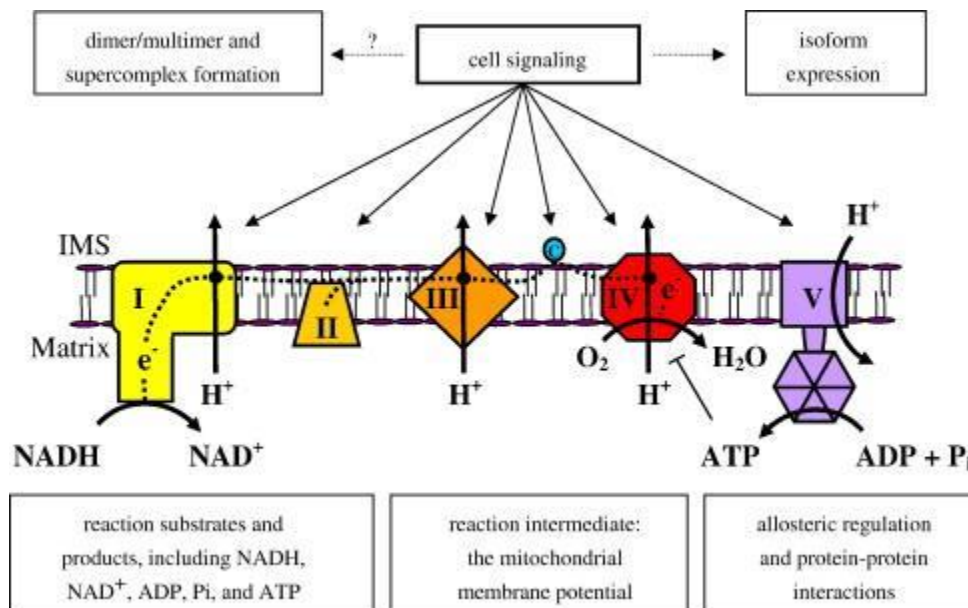


Figure 1.6: The mechanism via which ATP is produced by OXPHOS. Firstly, the OXPHOS unit consists of the electron transport chain (ETC) consisting of NADH dehydrogenase (**complex I**), succinate dehydrogenase (**complex II**), ubiquinone, bc1 complex (**complex III**) and cytochrome c (cyt c) along with cytochrome c oxidase (ccO; **complex IV**). Electrons from NADH enter the ETC via complex I, whereas complex II feeds electrons into the ubiquinone/ubiquinol pool. Other sources of electron input are the electron transferring flavoprotein (ETF), ubiquinone oxidoreductase and glycerol-3-phosphate dehydrogenase. The electrons get transferred to oxygen with simultaneous transfer of protons across the inner mitochondrial membrane (Δp) that helps create a gradient that is further utilized by **complex V** or ATP synthase to provide the ultimate energy currency of the cell. Figure from Hüttemann [88].

Glycolysis: Although glycolysis is a major energy production pathway in prokaryotes, in eukaryotes it is only utilized during adverse conditions or to provide energy in specific organs like the brain, liver and muscles [90]. OXPHOS and glycolysis are tightly regulated with the former being the major ATP producer in humans and the latter producing 2 molecules of ATP from a molecule of glucose compared to 36 molecules produced by OXPHOS. The end product of glycolysis - pyruvate - enters mitochondria to initiate the tricarboxylic acid cycle as well as oxidative phosphorylation [91].

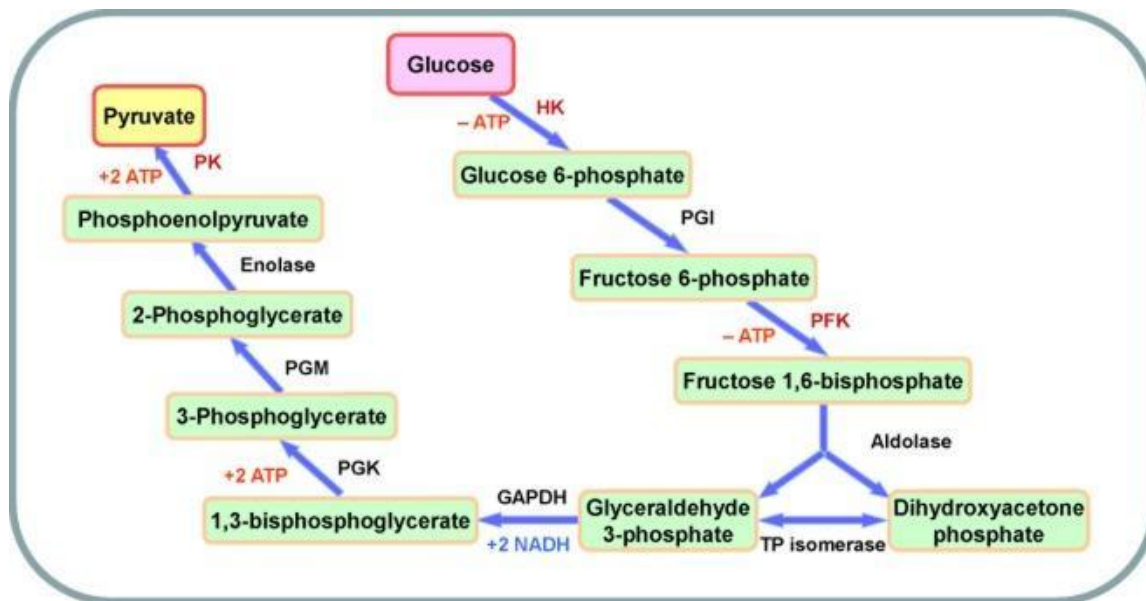


Figure 1.7: The glycolytic pathway showing key enzymes involved and the ATP producing steps. The entire process is mediated by 10 enzyme catalysed reactions in the cytoplasm namely, hexokinase, phosphoglucose isomerase, phosphofructokinase, aldolase, triosephosphate isomerase, glyceraldehyde-3 phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, enolase and pyruvate kinase. Under low oxygenic conditions both normal as well as diseased or tumour cells preferably reduce pyruvate to lactate but tumour cells, even in presence of oxygen, preferentially convert pyruvate to lactate rather than it entering the TCA cycle. Figure from Li X [92].

Warburg effect: The Warburg effect was discovered in the 1920s by a German scientist named Otto Heinrich Warburg who observed that tumour cells require more energy for biosynthetic processes than normal cells. In tumour cells aerobic glycolysis takes place at a high rate followed by lactic acid fermentation as opposed to the low rate of glycolysis followed by oxidation of pyruvate that occurs in normal cells [93]. The number of pyruvate molecules generated by glycolysis remains the same, but the only difference under low oxygen concentration is seen when pyruvate does not enter mitochondria and gets converted to lactate in the cytosol itself, giving 2 molecules of ATP and generating a pool of NAD^+ , that can be used by cells to perform glycolysis at a high rate. This concomitant increase in lactic acid production via glycolysis and the lowering of OXPHOS was found to occur in

malignant tumours even under normal oxygenic conditions [94]. The main aim of proliferating cancer cells is to increase biomass production, for which amino acids, nucleotides and fatty acids have to be synthesised at a high rate as they are the building blocks of cellular macromolecules. Intermediates of aerobic glycolysis are used in the pentose phosphate pathway that provides amino acids, nucleotides and NADPH. Moreover, fatty acids required for cellular membrane lipid synthesis are generated from citrate in the cytosol via ATP citrate lyase which produces acetyl-coA. These fatty acids then combine with glycerol that is in turn produced from a glycolytic intermediate, dihydroxyacetone phosphate (DHAP) [94]. On activation by pathogenic ligands immune cells such as macrophages and DCs undergo metabolic changes similar to tumour cells in order to produce pro-inflammatory cytokines [95], [96].

1.4.2 Metabolic regulators in the differentiation of DCs from monocytes

Receptor signalling pathways that activate DCs regulate gene expression that alter DC function and also change the metabolism of the cells so that cellular processes are modulated during activation [30]. During differentiation from monocytes, DCs require mitochondrial biogenesis and fatty acid synthesis, evident from the upregulation of two transcriptional activators, namely peroxisome-proliferator activated receptor- γ (**PPAR γ**) along with the PPAR γ co-activator 1 α (**PGC1 α**) [97], [98]. It was observed that mitochondrial citrate was exported to the cytosol, wherein it underwent conversion to acetyl-coA, a crucial intermediate in fatty acid synthesis that fuelled both *in vitro* as well as *in vivo* DC differentiation in lymphoid organs as well as peripheral tissues [99]. In contrast, during activation by TLR agonists, murine DCs were shown to consume more glucose to perform glycolysis and produce lactate [98].

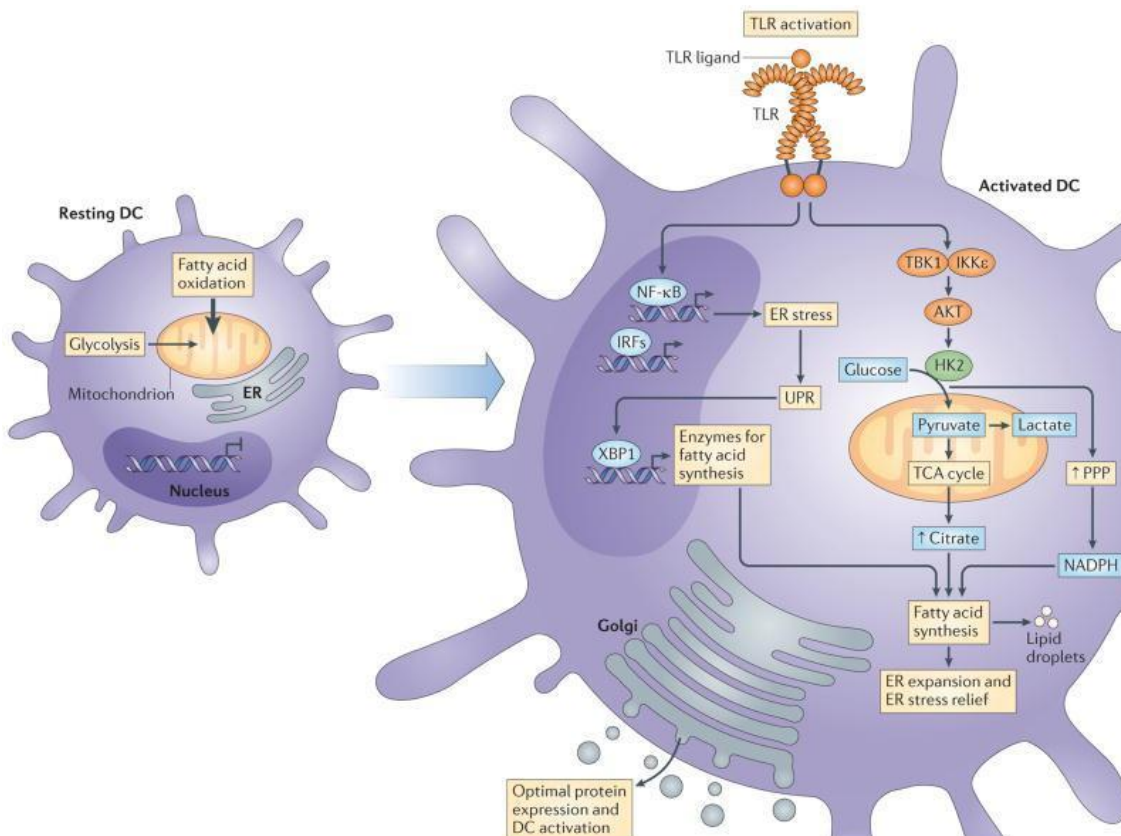


Figure 1.8: The shift in metabolism from resting to activated murine bone marrow-derived DCs. On the left, i.e. in the resting cell, normal glycolytic and oxidative pathways are underway with fatty acid oxidation. Whereas on the right, once the DC is exposed to the TLR ligand downstream signalling pathways are switched on that induce expression of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) and interferon regulatory factor genes. As the cells prepare to secrete cytokines they increase protein translation that puts a stress on the ER. This switches on unfolded protein response or UPR pathway that activates genes for fatty acid synthesis. When other TLR agonists come into play cellular kinases like AKT and hexokinase II get activated that results in increased flux through glycolysis and thereby increasing citrate export to carry on fatty acid synthesis. This is called a Warburg like phenotype and is a very important switch from OXPHOS for proper activation of the DCs. Figure from Pearce E [30].

TLR-induced glycolytic reprogramming: When TLR agonists like LPS or pathogens like Mtb activate immature murine bone marrow-derived DCs (BMDCs) generated in presence of GM-CSF there is a rapid (within minutes) increase in glycolysis that is mediated by a TBK1/IKK/Akt pathway. This is followed later by an almost complete switch in metabolism from OXPHOS to robust glycolysis which is regulated by a mTOR/HIF1 α /iNOS-driven signalling pathway [100], [101].

Early events in glycolytic reprogramming: Previously Akt was thought to promote translocation of glucose transporters to the cell membranes that would increase glycolytic flux. It also promotes activity of a crucial glycolytic enzyme, PFK2 by stimulating activity

of PFK1, its allosteric enhancer. But work by Everts et. al, [100] has proved that neither increased surface expression of GLUT-1 nor knocking down PFK2 had any effect on DC activation after LPS stimulation. When HK-II was competitively inhibited from binding to the outer membrane mitochondrial (OMM), the LPS-induced burst in glycolysis was absent. All these observations suggest that TBK1 and IKK ϵ affected Akt and increased glycolytic flux after TLR ligand stimulation that helped in binding HK-II with the mitochondrial membrane.

The pyruvate generated by glycolysis in the cytosol enters the mitochondria with help of the mitochondrial pyruvate carrier-1 (MPC-1), getting converted to acetyl-coA which can then enter the Krebs's cycle (TCA cycle) [30]. Mitochondrial stability was seen to be maintained due to increased membrane potential and spare respiratory activity after stimulation with LPS. This indicated that the influx of glycolytic carbon in the form of pyruvate through MPC-1 was important in maintaining cellular integrity. The activity of MPC-1 was downregulated using short hair pin RNA specific for *Mpc-1* gene and then the activity was seen to be decreased after 6 hours of stimulation as compared to the control [100]. Therefore, from these experiments it was clear that pyruvate was important for GM-DCs activation and MPC-1 was a major player that aided in the entire process.

Akt kinase has also been shown to commit activated human moDC towards increased glycolytic programming. After stimulation with TLR ligands Akt was phosphorylated at both Thr308 and Ser473 residues necessary for its activation. After this its direct downstream targets were also phosphorylated and it was also shown that when inhibitors of P13K and mTOR (normal activators of Akt), though phosphorylation of Akt was impaired but there was no decrease in the overall rate of glycolysis. On the other hand, inhibition of both TBK1 and IKK ϵ demonstrated a more substantial effect on the glycolytic response after LPS stimulation [100], [102]. Activated Akt was shown to increase glycolytic flux by aiding in associating Hexokinase-II (HK-II) with the outer mitochondrial membrane, so that it can use the mitochondrial ATP [103].

Later events in glycolytic reprogramming: After 24 hours of activation with TLR ligands endogenous NO redirects the cell almost entirely towards glycolytic metabolism [47]. In both murine macrophages and bone marrow-derived DCs, stimulation by TLR agonists shows a complete change of metabolism with the aerobic glycolysis rate increasing and

OXPHOS and fatty acid oxidation (FAO) decreasing [104], [105]. TLR stimulation switches on expression of iNOS gene that codes for the nitric oxide synthase enzyme. The toxic nitric oxide (NO) produced then inhibits mitochondrial ETC and thus OXPHOS and ATP production (discussed later). It was also shown that only those murine DC subsets that express the iNOS gene are able to undergo the Warburg effect [100]. Early glycolytic reprogramming of DCs is independent of NO right after stimulation and, as outlined above, is dependent on downstream pathways set in motion by activation of kinases like Akt/phosphatidylinositide-3 kinase, or the TBK1-IKK ϵ with the latter controlling Akt-driven reprogramming towards glycolysis [101].

Nitric oxide produced by the NO synthase enzyme (iNOS), has important roles in host defense against different of pathogens including Mtb [106], [107]. After 12 hours of stimulating DCs with TLR agonists it was seen that mitochondrial function was repressed due to initiation of NO function [30]. So it is now well established that most inflammatory monocyte derived DCs can generate ATP through glycolysis in response to impaired OXPHOS, but this is not the case for DC subsets that do not express the iNOS gene. Inflammatory splenic moDCs display an iNOS-dependent by LPS stimulation ex vivo while cDCs isolated from the spleens of mice did not express iNOS after activation by LPS [101].

mTOR is a protein kinase phosphorylating serine or threonine residues and belongs to the family of P13K related kinase family [30]. It is a target protein for Rapamycin, a macrolide produced from *Streptomyces spp.* [108]. mTOR generally forms 2 complexes, mTORC1 and mTORC2, and depending on nutrient signalling, growth factors and other stimuli, they regulate anabolic metabolism. mTOR also induced MYC protein expression that upregulates glycolysis related genes [109]. Although, during development of DCs, MYC expression is downregulated because of one of its paralogues (MYCL) being expressed in cDC progenitors [109]. mTOR is activated in macrophages by infection with mycobacteria [110].

Another important regulator of DC activation in the later stages of metabolic reprogramming is the **Hypoxia Inducible Factor-1 α (HIF-1 α)**, a transcription factor that regulates cell functions under low oxygen concentration (hypoxia) and also promotes the bactericidal properties of phagocytes [111]. But HIF-1 α has also been shown to get upregulated even under normoxic conditions and plays a role in DC maturation and cytokine production. It is

a constitutively expressed gene that under normal oxygen concentration is degraded by post-translational modifications. In inflammation myeloid cells like macrophages and DCs depend on it to promote bactericidal responses after migrating to the required location. This dependency has been attributed to the characteristic change in metabolism to aerobic glycolysis in activated cells [112].

LPS activates HIF-1 α through the mammalian target of rapamycin (mTOR) pathway. After TLR stimulation, this Ser/Thr kinase enzyme helps cells meet the demand of being metabolically active. This increases the HIF-1 α expression that in-turn upregulates glycolytic function in macrophage as well as inflammatory genes [112], [113]. HIF-1 α gets regulated both at the transcriptional and post translational level. mTOR promotes the unique 5'-terminal oligopyrimidine signals (5'-TOP) that are present on mRNAs of the hypoxia inducible factor. Also there is another motif within the HIF-1 α mRNA that on interaction with the raptor component in mTOR upregulates transcription of this factor [114]. In DCs too, LPS stimulation induces greater expression of HIF-1 α protein as well as mRNA. HIF-1 α then targets the promoters of genes involved in glucose transport and glycolysis and increasing their expression. The HIF-1 α protein under normal conditions rapidly undergoes degradation by hydroxylation of its proline residues, using oxygen as a substrate, targeting it for ubiquitin mediated proteasomal degradation [115]. There is a conserved HIF1 α binding site in the *Il1B* gene promoter sequences of both humans and mice. Under hypoxia LPS-induced IL-1 β production increased by many fold thereby relating HIF-1 α to the production of this important cytokine. Succinate impairs the activity of the hydroxylase enzyme that in turn stabilises HIF-1 α . The prolyl hydroxylase inhibitor stabilised HIF-1 α protein and induced higher level of expression of IL-1 β mRNA [116]. Therefore, it influences murine and human dendritic cell activation in response to LPS and under hypoxic conditions [115].

Among the positive regulators of HIF-1 α , succinate is one that stabilises it post-translationally by inhibiting prolyl hydroxylase domain containing enzymes that in presence of oxygen catalyse hydroxylation of the factor followed by its proteasomal degradation. But it has been shown that 2-deoxyglucose inhibits accumulation of HIF-1 α , and this can be an important target in developing therapeutics that would actually lead to reduced survival of cancer cells [117]. In case of dendritic cells, hypoxic conditions actually promote inflammatory activities, helping them migrate to the secondary lymphoid organs. But LPS stimulated DCs under hypoxic conditions showed downregulation of CD40, CD80, CD86,

CD83 that reduced their T cell priming activity [118]. In contrast high amounts of pro-inflammatory cytokines were formed, i.e. TNF α and IL-1 β which were characteristic of DCs in response to early infections when a strong inflammatory environment was crucial to prevent any more progression of the disease [118].

Increased glucose uptake and glycolytic metabolism is also essential for CCR7 oligomerization and dendritic cell migration [119]. However; cell intrinsic glycogen metabolism also supports the glycolytic reprogramming needed for DCs immune responses [120]. As well as increased glucose uptake, glutamine uptake is also increased during glycolysis and is used for the synthesis of nucleotides and non-essential amino acids [89]. Glutamine is converted into glutamate, aspartate, and alanine by undergoing partial oxidation to CO₂, in a process called glutaminolysis.

1.4.4 TCA cycle intermediates as signalling molecules

TCA cycle intermediates are important sources for generating signalling molecules that affect immune-inflammatory pathways. Several breakpoints can occur in the TCA cycle in LPS-stimulated monocytes or macrophages leading to accumulation of metabolic intermediates which can be diverted to other pathways. For example, oxidation of **succinate** by succinate dehydrogenase activates macrophages and reverses the flow of electrons to Complex-I of the ETC, producing ROS, that in turn leads to activation of HIF-1 α [121]. Succinate accumulation in the cytosol inhibits prolyl hydroxylase enzymes that are dedicated in maintaining homeostasis of HIF-1 α by degrading it. This allows HIF-1 α to translocate to the nucleus, wherein, it binds to hypoxia response elements in the promoter regions of its target genes. These include genes coding for enzymes involved in suppressing mitochondrial function. Pyruvate dehydrogenase kinase is one such gene that gets upregulated and inactivates PDH which prevents pyruvate being converted into acetyl-coA and entering the Krebs cycle [116], [122].

Itaconate is an anti-inflammatory modulator that accumulates in LPS activated moDCs and that is produced due to the accumulation of citrate. Itaconate generally inhibits isocitrate lyase enzymes of bacteria (including Mtb) and kills them. Its role has been elucidated well in macrophages and was seen to be under regulation of *Irg1* gene. Another important point is that itaconate inhibits succinate dehydrogenase and is anti-inflammatory, causing decreased production of NO, IL-1 β , IL-18 and HIF-1 α [123], [124].

Another breakpoint in the TCA cycle during DC activation is during the isocitrate dehydrogenase (IDH) reaction, to withdraw **citrate** from the cycle. The citrate is used in lipid biosynthesis for expansion of both ER and Golgi so that the cells can compensate for increased protein demand during activation [125]. It has pro inflammatory influences in immune system and it is able to increase the capability of macrophages and the endothelial cells to present antigens. Citrate also stimulates production of inflammatory mediators for example, (TNF α) and IL-1 β [125]. Mitochondrial citrate carrier (CIC) exports the citrate from TCA cycle in exchange for malate. Citrate in cytosol is broken down further into acetyl-coA and oxaloacetate by ATP citrate lyase (ACLY). The oxaloacetate gets converted to malate by MDH and re-enters the mitochondria for the above process to take place again. Alternatively, in the cytosol the acetyl-coA gets converted to malonyl co-A by acetyl-coA carboxylase (ACC) and gets incorporated into cholesterol and fatty acids with the latter getting incorporated into phospholipids to form membranes or as triacylglycerides which accumulate in lipid bodies [126]. It has now been proved that in both LPS stimulated macrophages and DCs increased level of citrate leads to production of pro-inflammatory mediators like NO, ROS and prostaglandin E₂ (PGE₂). When fatty acid synthase was inhibited LPS induced PGE₂ production was hampered along with pro-IL-1 β , thereby implying the critical role for citrate in IL-1 β production [124], [127].

Proteins can be functionally modulated by post-translational changes like acetylation, glycosylation and methylation. All of these processes have been shown to be very important in dendritic cell biology as well. Citrate entry into the cytosol from mitochondria was converted to acetyl coA that acts a major donor for acetyl groups that can help in histone acetylation leading to epigenetic modifications [124], [127]. Some key participants of the glycolytic and fatty acid metabolic pathways can also get acetylated, such as Pyruvate kinase M2 [128]. In BMDCs action of deacetylases like sirtuins; SIRT1 can deacetylate interferon (IFN) regulatory factor-1 that inhibits production of IL-27 and IFN- β [129] and controls autoimmune inflammation. There also has been evidence of methylation of histones H3 and H4 that could promote or inhibit genes involved in the inflammatory response of DCs [130]. Therefore, these are some areas which still need a lot of investigation if they are to be targeted for the development of new therapeutics.

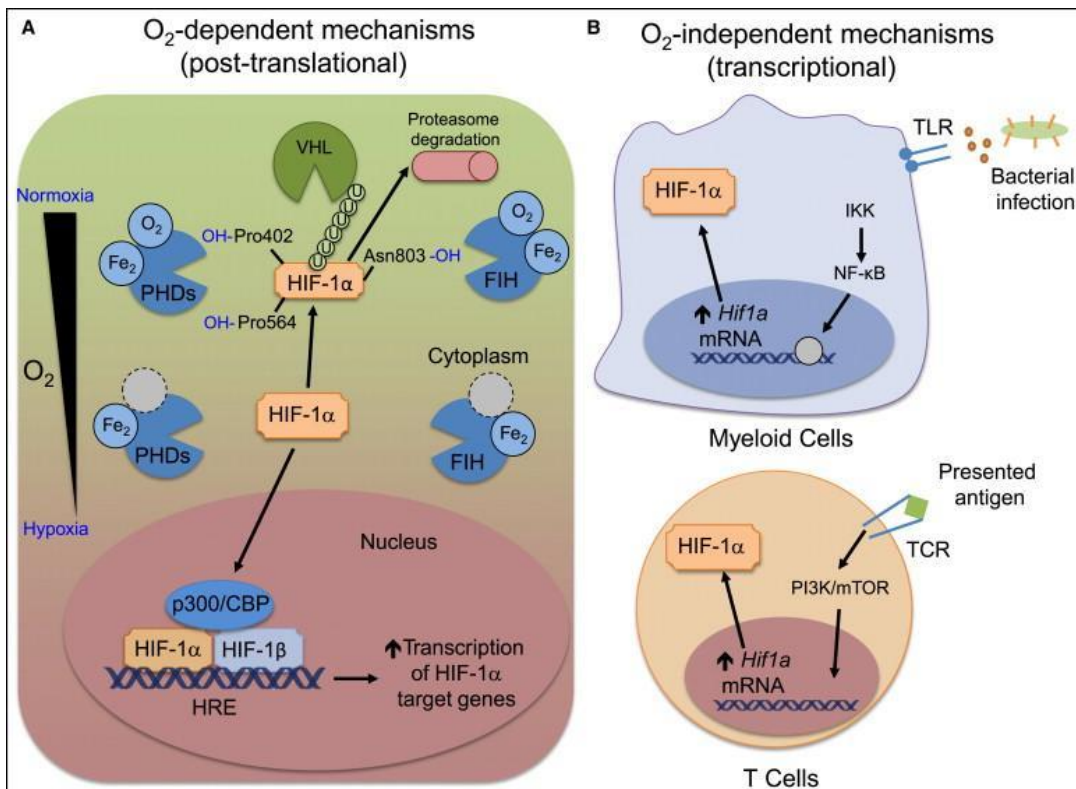


Figure 1.9: The oxygen independent transcriptional and oxygen dependent post-translational regulation of HIF-1 α subunits. The molecular mechanism of HIF-1 α regulation is depicted in myeloid cells that shift to perform aerobic glycolysis as a result of inflammatory stimuli. Figure from Asis [131] HIFs -Hypoxia-inducible factors, HREs - hypoxia response consensus sequences, TCR - T cell receptor, PHDs - prolylhydroxylases, VHL - von Hippel-Lindau tumor suppressor protein, FIH - Factor inhibiting HIF, CBP - CREB binding protein.

1.4.5 Exogenous metabolites as modulators of DC function

From every detail regarding immunometabolism in activated moDCs that has been discussed so far it is fairly clear that glycolytic reprogramming is not only important in anabolism, supporting moDC differentiation and function, but various cellular metabolites whether exogenous or endogenous are crucial in altering cellular responses. Exogenous metabolites can also be sensed by DCs during their activation and consequent functions. Intracellular succinate has already been shown as a crucial player in maintaining the Warburg effect in DCs, but recently evidence regarding extracellular succinate that binds to a G-protein coupled receptor, namely succinate receptor-1 has also been provided [132].

Butyrate - a short chain fatty acid that is generally synthesised by commensal bacteria in the gut by fermenting dietary fibres - can bind to the GPCR GPR109 on the DC surface and promote expression of IL-10 along with aldehyde dehydrogenase that converts retinol to retinoic acid [133]. IL-10 and retinoic acid are known to induce tolerogenic and anti-inflammatory effects as a part of the normal interaction between inherent immune cells and

gut microbiota [133]. Another pathway through which butyrate can enter DCs is by the sodium coupled monocarboxylate transporter SLC5A8. Butyrate then inhibits histone deacetylase (HDAC) activity and modulate expression of genes that are responsible for the GM-CSF induced development of DCs *in vitro* [134]. Butyrate also influence the immune response to Mtb [135].

There are a number of extracellular ligands that bind by different receptors in DCs and accentuate anti-inflammatory actions by not responding to TLR agonists [136]. Some of these ligands include poly-sterols, retinoic acid and oxidised and polyunsaturated fatty acids. The receptors that get activated by such ligands are retinoic acid receptors (RARs), liver X receptors (LXRs) and vitamin D receptors. Dexamethasone and vitamin D3–treated human mo-DCs (tolerogenic moDCs) have a greater glycolytic capacity and reserve than mature moDCs, despite mature moDCs shifting to glycolysis. Glycolytic rates were similar in mature DCs and tolerogenic [137]. Sometimes during activation DCs can themselves make ligands for these receptors to drive T cell responses [136].

ATP and adenosine can be sensed by DCs through A2A GPCRs on its surface that limits its activation and mounts an anti-inflammatory response [138]. Lactate can also activate macrophages and this is exhibited by genes that stimulate IL-4 production, like *Arg1* coding for arginase *Mg11* encoding macrophage galactose type lectin, etc [138]. Moreover, the role for HIF-1 α was also proved for the above phenomenon, and we know that HIF is stabilised by lactate under normoxic conditions. High lactate concentrations favour the production of tolerogenic DCs that produce high levels of IL-10 with no production of pro-inflammatory cytokines on TLR stimulation [139].

Role of cellular metabolism in other immune cells

Macrophages are plastic cells that can adopt a pro-inflammatory (M1) or anti-inflammatory (M2) phenotype in response to cues from their immediate environment. Polarizing cytokines give rise to two distinct phenotypes called the M1 and M2. M1 macrophages are classically activated by IFN- γ from Th1 cells, while alternatively activated M2 macrophages are induced by Th2 cytokines [290]. Combination of IFN- γ and LPS polarize macrophages from resting (M0) to classically activated M1 cells and IL-4 polarize M0 to M2 macrophages. M1 macrophages are pro-inflammatory and microbicidal while M2 macrophages promote wound healing and tissue homeostasis [291]. These phenotypic differences are regulated by

changes in their metabolism. M1 macrophages show increased glycolytic flux with reduced OXPHOS compared to M0 cells and a broken TCA cycle, with accumulation of citrate and succinate [292]. M2 macrophages show higher OXPHOS with intact TCA cycle and increased fatty acid oxidation (FAO) in the mitochondria, production of glutamine dependent α -KG along with uridine diphosphate N-acetylglucosamine is also elevated [105].

Non proliferating and differentiated mammalian cells are metabolically quiescent and metabolize glucose mainly using the TCA cycle coupled with the electron transport system to maximize ATP synthesis. Naïve T cells follow this pattern but, once activated to undergo rapid expansion, antigen-activated T lymphocytes undergo metabolic reprogramming and mainly depend on aerobic glycolysis as do NK cells activated by IFN- γ [294]. However, mitochondrial biogenesis, which is dependent on MTORC1, is also important to initiate T cell activation. Mitochondria localise to the immune synapse which the T cell interacts with the APC. These mitochondria maintain a punctate morphology which is characteristic of cells with a high rate of glycolysis. Tregs on the other hand upregulate expression of enzymes involved in OXPHOS and rely on fatty acid oxidation for energy [293]. When effector T cells contract, the surviving memory cells undergo a reverse metabolic shift and return to OXPHOS (fuelled by fatty acid oxidation) as their main energy [294]. This is accompanied by a change in mitochondrial morphology from punctate to elongated. As a result, memory lymphocytes increase their spare respiratory capacity and have the ability to respond rapidly to restimulation with rapid proliferation and cytokine production [293]. The metabolic requirements of $\gamma\delta$ T cells are less well understood. However, a recent report showed that IFN- γ production by $\gamma\delta$ T cells is dependent on glycolysis and regulated by mTOR [295].

1.4.6 Metabolic reprogramming in the host response to Mtb infection

Macrophages and not DCs have been the main focus of study on host cell metabolism in Mtb infection. Alterations in macrophage lipid metabolism in the TB granuloma have been recognised for many years [149]. However, more recently, in a mouse model of TB, lipid laden dendritic cells were also identified in the lung [150]. Genes involved in synthesis and catabolism of lipids were among the granuloma-associated transcripts that were significantly increased in expression in a study that used laser assisted microdissection and genome wide microarray analysis of TB in human lung tissue [151]. Foamy macrophages, due to the influence of mycolic acids from the mycobacterial cell wall, were originally thought to

promote survival of the bacteria providing nutrients for intracellular Mtb [152]. Knight and colleagues found that host lipids are not required for the growth of mycobacteria in an *in vitro* murine model using an inhibitor of lipid droplet (LD) formation. Instead LD formation was driven by IFN- γ signalling and required HIF-1 α and its target gene Hypoxia inducible protein 2 (Hig2) [153]. De novo fatty acid synthesis (FAS) in macrophages and classical DCs (cDC1) are elevated after infection with Mtb and BCG in macrophages and DCs *in vitro*. However, deletion of Acetyl-CoA carboxylase 1 (ACC1, the enzyme controlling the rate-limiting step in fatty acid synthesis) in either DCs or macrophages had no effect on cytokine production or bacterial burden indicating that FAS is not required to control mycobacteria infection in these cells [154].

In addition to alterations in lipid metabolism there is also evidence for a Warburg-like metabolic shift in Mtb infection *in vivo*. Indeed, ^{18}F -Deoxyglucose Positron Emission Tomography (FDG-PET), an imaging technique that uses the glucose analogue FDG to detect increased glucose uptake, has been used for *in vivo* imaging of the lungs of TB patients. This technique can be used to determine the severity of TB disease, although the cells responsible for the high glucose uptake cannot be identified using this method [155]. A metabolomics study of tissue from granulomas of guinea pigs infected with the H37Rv strain of Mtb indicated there was increased flux of metabolism through the glycolytic, pentose phosphate, and TCA pathways compared to normal lung tissue with the virulent H37Rv strain of Mtb [156]. In a gene expression study, Shi and colleagues found increases in expression of transcripts of the glucose transporters including Glut1 and Glut3 by two fold at day 30 (when growth of bacteria had ceased) compared to day 12 (during the acute phase of growth) and powerful induction (around seven fold) was detected for a Glut6 splice variant in Mtb infected mice. This coincided with enhancement of the expression of glycolytic enzymes and enzymes in the pentose phosphate pathway and down regulation of OXPHOS and TCA-related genes, along with increased expression of HIF-1 α the granulomas [157]. In several other studies, mice lacking HIF-1 α in their myeloid cells were shown to be very susceptible to infection with Mtb and produced lower levels of inflammatory cytokines and nitric oxide in response to IFN- γ , demonstrating the importance of HIF-1 α in regulating Warburg shift in the immune response to Mtb [158-160].

Our group showed that there is a shift towards glycolytic metabolism in macrophages infected with the irradiated virulent H37Rv strain of Mtb (iH37Rv) *in vitro*. Increased

glycolysis was further confirmed by using live H37Rv and H73Ra (an avirulent strain) in human MDM by measuring lactate secretion. Glycolysis inhibition with 2 deoxy glucose or substitution of glucose with galactose in the cell culture medium reduced the secretion of IL-1 β and increased the formation of IL-10 leading to increased replication of intracellular mycobacteria [158]. A more recent report from Cummings and colleagues found that live H37Rv reduced mitochondrial function and glycolytic flux in human MDM cultured *in vitro*. Infection with BCG on the other hand increased the rate of glycolysis of these cells and led to the accumulation of the TCA intermediate citrate intracellularly, suggesting that a break occurs in the TCA cycle. In addition, they found that BCG infection increased the dependency of MDM on fatty acid oxidation [159].

As mentioned in **Section 1.2**, BCG vaccination protects against autoimmunity and unrelated infectious diseases and this has been attributed to trained immunity. Trained immunity refers to increased responsiveness of previously infected monocytes/macrophages to a secondary infection or inflammatory stimulus (e.g. LPS). *In vitro* there are increases in glycolysis, OXPHOS and flux through the pentose phosphate pathway when monocytes are infected with BCG and LPS. There was also evidence of increased expression of glycolysis genes in peripheral blood monocytes of BCG-vaccinated individuals stimulated *ex vivo* several months after vaccination [160].

Glutamine metabolism has also been implicated in the macrophage response to Mtb infection [161] and trained immunity [160]. Human peripheral blood mononuclear cells infected with Mtb displayed a decreased secretion of cytokines (IFN- γ , IL-1 β and IL-17), when the cell culture medium lacked glutamine. Inhibition of the glutamine pathway enzymes also caused diminished cytokine responses and genetic polymorphisms of genes encoding for proteins involved in glutamine metabolism including SLC1A5, GLS2 and SLC7A5 also affected these cytokine responses [161].

1.5 Dendritic cell apoptosis and the immune response

All physiological processes in our body are controlled by a proper balance between cell division and cell death. The mechanism of cell death - apoptosis or necrosis - can have profound effects on an organism, both locally and systemically. Apoptosis is induced either through intrinsic triggers (signals from inside the cells that permeabilize mitochondrial outer membranes and activate caspases) or extrinsic signals (arising due to cytokine signalling,

infectious organisms or cancer, sepsis, UV irradiation, etc.) which also result in caspase activation, allowing for an orderly dismantlement of the cell without releasing its intracellular contents [181], [182]. In the steady state DC apoptosis is important in maintaining tolerance, and any defects in it can cause autoimmune diseases [181]. Tolerance is characterised by an increase in T-regulatory cells (Treg) and an overall immunosuppressive environment [183].

Importantly, DC apoptosis can also result in tolerance even during an inflammatory response [184] indicating that apoptosis of activated DCs helps regulate the balance between tolerance and immunity [179], [182], [184], [185]. DCs may also undergo apoptosis to control spread of intracellular pathogens as seen in case of *Legionella pneumophila*. This bacterium evades destruction in host phagosomes by forming a vacuole and replicating within it. But host DCs undergo an early apoptotic pathway via caspase 3 activation, thereby not allowing further replication [186]. Uninfected bystander DCs also play a very important role in cross-presenting mycobacterial peptides transferred to them after APCs like macrophages and other DCs undergo apoptosis. The antigens are presented on Class I MHC to cytotoxic CD8+ T cells which secrete IFN- γ , which, in turn, checks bacterial growth by stimulating other uninfected bystander macrophages to release antimycobacterial agents [187], [188]. Recently, Kumar et al showed that BCG (at low multiplicity of infection) inhibits spontaneous apoptosis of mouse BMDCs by activating NF κ B, which increases the expression of the anti-apoptotic proteins Bcl-2 and Bcl-xL. The authors hypothesise that extending the lifespan of DCs in this way could contribute to the efficacy of the BCG vaccine [189] by increasing the time that they interact with T cells.

Necrotic cell death

Necrosis was initially thought to be an accidental, unregulated form of cell death but is now known to occur through several highly regulated pathways. It generally is characterised by loss of integrity of the cell membrane that eventually leads to expulsion of cytoplasmic and nuclear contents into the extracellular space. *In vitro* infection of monocyte derived macrophages with the attenuated H37Ra strain of Mtb results in some of the cells dying by necrosis [190]. Virulent strains of Mtb also inhibit apoptosis and induce caspase-independent form of macrophage cell death with features of programmed necrosis [191]. Necrosis is not desirable for the host because it releases viable mycobacteria for transmission

that will lead to re-infection. Necrosis is also deemed as an important factor in granuloma formation and inflammatory tissue damage [185].

Necroptosis and pyroptosis are the most studied forms of programmed necrosis. Necroptosis occurs when signalling through receptors of the TNF α superfamily and their ligands occurs in cells where caspases are inhibited. Macrophages infected with Mtb are primed to undergo necroptosis but mycobacteria appear to subvert this cell death process [304]. Pyroptosis depends on activation of caspase-1 and caspase-11 by the inflammasome [192]. The inflammasome complex consists of a Nod like receptor (NLR), an adaptor protein (ASC) and inactive pro-caspase 1 protein [193]. When the NLRs such as NLRP3 sense PAMPs, the inflammasome complex is assembled to activate caspase-1 and gasdermin D. Caspase-1 activation results in IL-1 β being cleaved to its mature form. Cleaved gasdermin D forms pores in the cell membrane which allows secretion of IL-1 β and causes pyroptotic cell death [193].

Alternative pathways of cell demise in activated DCs have not been researched to the same extent as in macrophages. Our group has shown that human mo-DCs undergo caspase-independent cell death when infected with Mtb, similar to macrophages, ruling out the apoptosis and pyroptosis pathways in this process through the use of pharmacological inhibitors [66]. In 2012, using murine BMDM deficient in inflammasome components, Abdalla et al showed that the NLRP3 inflammasome is responsible for IL-1 β secretion by H37Rv-infected murine BMDCs but confirmed that DC death did not occur via pyroptosis. They attribute Mtb-induced DC death to apoptosis and showed that it was dependent on the ESX-1 secretion system of Mtb [194] which is absent in BCG [195].

Cell death and metabolism: While discussing the change in metabolic profile of DCs and macrophages we should consider the effects of these changes on the eventual cell death pathways that occurs as a result of bacterial infection [140]. Mitochondrial dependent apoptotic pathways are triggered in response to cellular stress, such as induction of DNA damage, production of ROS etc. Under these circumstances, in DCs mitochondrial release of cytochrome c and proteins of the Bcl-2 family can lead to apoptosis [141]. Cytokines play a major role in maintaining a homeostatic balance of DC populations. When murine BMDC differentiation is stimulated by GM-CSF, and then the latter is removed there is an upregulation in the pro-apoptotic Bim protein that accelerates DC death [142]. IL-10 on the

other hand shows the opposite effect and leads to cell death by inhibiting anti-apoptotic molecules Bcl-2 and Bcl-xL [143]. Another anti-inflammatory cytokine, TGF- β 1, also mediates apoptosis in monocyte derived DCs [144].

Mycobacterial infection of macrophages and DCs can eventually lead to cell death. When the mycobacterial load increases to approximately 20 bacilli inside macrophages they undergo cell death independent of caspase activity [66], [145]. Our group has shown that human moDCs also undergo caspase-independent cell death following treated with the pan-caspase inhibitor Q-VD-OPh and infected with live, but not killed, Mtb [66]. However, it was observed that BCG stimulation enhanced DC survival and prolonged DC lifespan in a dose-dependent manner. Live BCG led to a better DC survival compared with heat-killed BCG. FITC-Annexin V staining showed that BCG promoted DC survival by inhibiting apoptosis [146].

It has been shown that T-cells can also play a role in regulating DC cell death. After the DCs have primed and activated T-cells they disappear from the LNs which may suggest that they have migrated out or they are killed due to T-DC interaction, the latter being shown *in vitro* on interaction with CD4⁺ T cells [147]. Killing of antigen presenting DCs by activated effector T cells may sometimes be beneficial as feedback regulation in antigen specific immune responses so that hyperactivity of T-cells does not occur [147]. One way by which DC based vaccines might be improved would be to prolong the immune response by inhibiting DC apoptosis.

The loss of energy production in tumour cells while they are undergoing the Warburg metabolism is related to a caspase dependent decrease in ATP [148]. Caspases had a specific inhibitory effect on glycolytic rate limiting enzymes, phosphofructokinase and pyruvate kinase along with other metabolites [148]. It is not known whether this is the case in activated DCs. Therefore, all these interdependent metabolic and cell death pathways need to be investigated to further assess the role of metabolites in activation and functioning of immune cells like DCs *in vitro* and *in vivo*. This could open avenues for developing better vaccines and new drug targets that may enhance glycolysis in these cells and augment their survival allowing them to mount a better response against pathogens like Mtb.

1.6 The Knowledge Gap: Reasons for investigating the role of metabolic programming in DCs

The BCG vaccine is ineffective at preventing pulmonary TB in adolescents and adults, so there is clearly a need to either design new vaccines or improve BCG in some way. Both of these approaches are being investigated but, even if new vaccines are approved, it seems likely that they will be used as booster vaccines for BCG [12], [19], [20], [21]. DCs play a pivotal role in initiating the immune response to BCG vaccination or *Mtb* infection. However, the outcome of BCG vaccination is lacking in terms of protection against subsequent infection. As discussed in this **Introduction**, DC function in response to BCG challenge appears to be less than optimal in terms of the kinetics and magnitude of DC maturation, migration and in the stimulation of effector T cells [49], [77], [81], [82]. Therefore, it is important to obtain a better understanding of how these cells respond to infection and cellular metabolism is likely to be an important aspect of this response. While many studies have been carried out about the role of immunometabolism and *M. tuberculosis* infection in macrophages, nothing is published so far regarding the effects of mycobacteria on DC metabolism. Understanding the role of cellular metabolism in DC activation could reveal new targets to improve vaccine efficacy.

The overall hypothesis of this work at the outset was that an increase in glycolytic flux is required for optimal DC maturation and survival after infection with BCG. Consequently, the overall aim of the current work is to determine whether metabolic reprogramming is required for the maturation of human moDCs in response to BCG infection. Accordingly, this work was carried out with the following specific aims:

Aim 1 (Chapter 3): Define the role of glycolysis in the maturation of BCG-infected human moDC. Specifically, to

1.1. determine whether inhibition of glycolysis with 2-DG in moDCs infected with BCG influences their capacity to: i) upregulate cell surface expression of co-receptors such as CD86 and CD40 and the antigen presentation molecule MHC class II, ii) upregulate and secrete inflammatory cytokines, iii) maintain viability.

1.2. Investigate whether glycogen metabolism plays a role in DC maturation and viability.

Aim 2 (Chapter 4): Measure changes in metabolic flux in moDCs infected with BCG and *Mtb* using the Seahorse Analyser to

- 2.1 Assess OXPHOS and glycolytic rates of moDCs infected with killed BCG under basal and stressed conditions.
- 2.2. Compare the effects of BCG and Mtb-H37Rv on moDC metabolic flux under basal and stressed conditions.
- 2.3 Evaluate the effect of potential modulators of glycolytic reprogramming on the metabolism of BCG and Mtb-infected moDCs.

Aim 3 (Chapter 5): Identify unique metabolic gene profiles that are associated with BCG infection in human moDCs

- 3.1 Investigate differential expression of metabolic genes in moDCs infected with live and killed BCG compared to uninfected cells using the Nanostring nCounter platform.
- 3.2 Investigate the dependence of immature and mature moDC for ATP production on selected metabolic pathways identified as being differentially expressed in BCG-infected versus uninfected cells.

Chapter 2: Materials and Methods

2. Materials and Methods

This chapter provides a comprehensive list of the materials and methods used throughout the present work. A number of methods also reference the particular chapters in which they are discussed.

Safety and containment measures employed to culture mycobacteria and human peripheral blood-derived cells

Monocyte-derived DCs were generated from monocytes isolated from buffy coats supplied by the Irish Blood Transfusion Service. Universal precautions were observed during isolation of monocytes and subsequent culturing of these cells. Cell isolation procedures and cell culture were carried out in Class IIA microbiological safety cabinets (MSC) which were serviced and validated every six months. All operations with open containers containing live cells were performed within the MSC.

BCG, which is classified as a Hazard Group II pathogen, must be used at Containment Level II. BCG was subcultured and used to infect DCs in a designated Class IIA MSC within the TTMI Containment Level II laboratory. Centrifuge buckets were sealed with lids before centrifugation of live BCG. Before analysis by flow cytometry or fluorescence microscopy the cells were fixed with 2% paraformaldehyde to inactivate BCG. The Seahorse Analyzer measures metabolic flux in live cells (so the DCs could not be fixed with paraformaldehyde for these experiments) and the lid of the plate is removed before placing it in the instrument. The use of antibiotic-killed BCG was necessary to avoid exposure of personnel to BCG during these experiments because the Seahorse Analyser is not in an MSC.

All waste (including discarded DCs and mycobacteria, used cell culture medium, used pipette tips and containers) was decontaminated by immersion overnight in a sodium dichloroisocyanurate solution to sterilise it. Liquid waste was then disposed of down the sink. Solid waste was placed in biohazard bags and sent for incineration.

The use of sharps was avoided where possible. However, if used, sharps were disposed of in a Sharps container which was subsequently incinerated.

Specific cell culture protocols used in the current work are described in the following sections.

2.1 Media for mycobacteria

BCG-GFP was propagated in a liquid culture of Middlebrook 7H9 broth prior to infection of DCs.

Materials:

- Middlebrook 7H9 broth (Difco)
- Middlebrook ADC enrichment (albumin, dextrose, catalase; Difco)
- Tween 80 (Difco)
- Glycerol (Difco)

7H9 broth:

7H9 powder (0.940g), Tween 80 (100µl) and Glycerol (1ml) were added to 180 ml ultra-pure water. The solution was autoclaved for 10 minutes at 120°C and 1 bar pressure, allowed to cool to 45°C, and a 20 ml ADC enrichment was added aseptically. The solution was mixed, filtered (0.2 µm), allowed to cool, and stored at 4°C for a maximum of 8 weeks.

2.2 Mycobacteria

2.2.1 *Mycobacterium tuberculosis* Bacillus Calmette-Guerin (BCG)

BCG expressing green fluorescent protein (GFP) was obtained from V. Deretic (University of New Mexico, Albuquerque, NM) and a frozen stock was prepared in 1 ml aliquots by Dr. M. O'Sullivan and stored at -80°C. An aliquot was thawed and grown by inoculating 1 ml of BCG-GFP in 9 ml Middlebrook 7H9 broth (Difco / Becton Dickinson, Sparks, MD) and supplemented with an albumin-dextrose-catalase supplement (Becton Dickinson), 0.05% Tween 80 (Difco), and 10µg/ml of kanamycin from Sigma Aldrich and then incubated under 5% CO₂ at 37°C. The medium was replaced every week by adding 9 ml of fresh medium to 1 ml of BCG and mycobacteria were used to infect cells in mid-log of the growth phase. The bacteria were discarded after a period of 2 months. **All waste which had been in contact with Mtb (including discarded Mtb suspensions) was inactivated overnight in a sodium dichloroisocyanurate solution (NaDCC; 5 g/L; Johnson & Johnson, New Brunswick, NJ).**

Preparation of GFP-BCG for infection of Dendritic Cells

On the day of infection, the mycobacteria were suspended in Middlebrook broth, and the BCG-GFP was passed through a 25 and 27 G gauge needle a minimum of 8 times each. This suspension was centrifuged at 800 rpm (72 rcf (relative centrifugal force or x g)) for 3 minutes in order to pellet any remaining clumps. The supernatant was then placed in a sterile 5 ml glass nephelometer tube.

Before infection an estimate of the number of bacilli per ml of medium was required to ensure that moDCs were challenged with a known multiplicity of infection in each experiment. In this thesis multiplicity of infection (MOI) is defined as the ratio of number of bacilli versus the number of moDCs in each well of a cell culture plate. A BD CrystalSpec nephelometer (BD Diagnostic Systems, Sparks, MD) was used to estimate the BCG-GFP bacterial concentration in bacilli/ml of suspension (McFarland, 1907). The BD CrystalSpec nephelometer used was a small portable device designed to estimate the relative turbidity of bacterial suspension in a clear medium, such as Middlebrook 7H9 broth, by passing a beam of light through the sample. The light reflected from the bacteria in suspension was then read by a detector. The greater the concentration of bacteria in the sample, the higher the turbidity of the suspension. The relative turbidity of the sample compared to a clear blank liquid standard was assigned a McFarland value, which correlated with a known

concentration of bacteria per milliliter of liquid suspension (CFU/ml) (McFarland, 1907). The McFarland reading of the BCG suspension was recorded in the nephelometer. This was repeated three times and the average was used for calculation.

A table of concentration ranges was supplied as part of the CrystalSpec nephelometer manual which enabled the number of bacilli/ml to be determined from the sample. The concentration ratio was then calculated as bacteria per cell to determine the amount of BCG-GFP required for each BCG ratio (50,100 and 250 bacteria per DC). In order to calculate the precise volume of bacterial suspension to apply to cells, the following steps were used:

For example, if MOI 100 was needed and the number of DCs in culture per millilitre of medium was 1×10^6 cells, that equated to mean 100×10^6 , and counting the resultant colony forming units (CFUs). The numbers of CFUs were comparable to those predicted by the nephelometer readings, 6×10^8 CFU/ml. Therefore, to calculate the volume of bacterial suspension to apply to cells in this instance, the following formula was used:

$$\frac{\text{Cell concentration (cells/ ml)}}{\text{Bacterial concentration (CFU/ml)}}$$

$$\frac{100 \times 10^6 \times 10^3}{6 \times 10^8}$$

In short, the sum of 166.666 µl would be added to 1×10^6 cells.

The BCG-GFP were then centrifuged at 3,800 rpm (2890 rcf) for 10 minutes and the supernatant discarded. The supernatant was replaced with an equal volume of RPMI 1640 supplemented with 10% defined FBS to re-suspend the bacterial pellet, and the bacteria mixed well, before adding them to each well of DCs at the required multiplicity of infection (MOI).

2.2.2 Killed BCG

When using the Seahorse Analyzer, it was necessary to use killed BCG to avoid any risk of infection. To test whether dead BCG can induce DCs maturation, the BCG-GFP was killed by treating a mid-log phase culture with Rifampacin (100ug/ml) for two days. The BD CrystalSpec nephelometer (BD Diagnostic Systems, Sparks, MD) was also used to estimate the killed BCG bacterial concentration and calculate the bacilli/ml of suspension.

The killed BCG was syringed a minimum of 8 times each through a 25G and 27G needle. This was then centrifuged at 800 rpm (72 rcf) for 3 minutes in order to minimize clumping and estimate the BCG-GFP bacterial concentration. In order to calculate the bacilli/ml of suspension by BD CrystalSpec nephelometer as outlined above, the BCG-GFP were then centrifuged at 3,800 rpm (2890 rcf) for 10 minutes and the supernatant discarded. The supernatant was replaced with an equal volume of RPMI 1640 supplemented with 10% defined FBS to re-suspend the bacterial pellet, and the bacteria mixed well before adding the calculated volume of killed BCG to each well of DCs to obtain the required MOI.

2.2.3 Gamma-irradiated *Mycobacterium tuberculosis* iH37Rv

Gamma-irradiated H37Rv (iH37Rv) was obtained through the NIH Biodefense and Emerging Infections Research Resources Repository under the license, NIAID, NIH: *Mycobacterium tuberculosis*, Strain H37Rv, Gamma-Irradiated Whole Cells, NR-14819. On receipt, the iH37Rv was prepared in 1 ml aliquots in sterile PBS by Dr. M. O'Sullivan and stored at -80°C for future use.

The re-suspension of the iH37Rv was conducted as follows:

1 ml aliquot of iH37Rv was thawed and transferred to a sterile 50 ml skirted conical tube. 5 ml of sterile Middlebrook 7H9 medium were added and the solution then sonicated in a sonicating water bath for 15 minutes to disperse clumps. Bacteria were syringed at least 8 times through a 25G needle to break up clumps. 2 ml volumes of the bacterial suspension were transferred into 2 ml centrifuge tubes (well mixed before each aliquot) and centrifuged for 3 min at 800 rpm (72 rcf) on the microcentrifuge to remove remaining clumps. The bacterial suspension from each of the tubes was carefully transferred to a new tube. The BD CrystalSpec nephelometer (BD Diagnostic Systems, Sparks, MD) was then used to estimate the iH37Rv bacterial concentration and calculate the bacilli/ml of suspension McFarland value. The iH37Rv was centrifuged at 3,800 rpm (2890 rcf) for 10 minutes and the supernatant discarded. The supernatant was replaced with an equal volume of complete RPMI in order to re-suspend the bacterial pellet, and the bacteria mixed well before adding the calculated volume of iH37Rv to the DCs.

2.3 Cell Culture

Human monocyte cells monocyte-derived dendritic cells (mo-DCs)

Donors

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of anonymous healthy donors. The buffy coats were obtained with permission from the Irish Blood Transfusion Service (IBTS) and used to generate monocyte-derived dendritic cells (moDCs).

Recommended isolation buffer

Phosphate-buffered saline (PBS) 500 ml sterile (PBS ($\text{Ca}^{2+}/\text{Mg}^{2+}$ -free, endotoxin-free), 0.29g EDTA and 2.5g Bovine serum albumin (BSA) were supplied from Sigma, left until dissolved, and then mixed slowly and filtered by vacuum pump through a sterile disposable 500 ml filter unit with PES Membrane, pore size 0.2 μm .

2.3.1 Isolation of PBMCs from Buffy Coats

The buffy coat was mixed 1:1 with isolation buffer. Polypropylene tubes (50 ml, Bio-One, Australia) were then filled with 15 ml Lymph prep (STEMCELL Technologies). The diluted blood was carefully poured into the 50ml tubes (25 ml blood per tube) and centrifuged at 1500 rpm (400 rcf) for 20 min at 18°C with the brake off. The mononuclear cell fraction (lymphocytes and monocytes) accumulated as a white band at the interface between the plasma and the Lymphoprep layer and was aspirated using a plastic Pasteur pipette. The mononuclear cells were collected in the tube up to 25 ml and diluted with 25 ml of isolation buffer and centrifuged at 300 x g for 7 minutes. The supernatant was carefully discarded and the pellet was re-suspended in 30 ml of isolation buffer which was centrifuged at 200 x g for 7 minutes. This wash was repeated twice. To lyse any remaining red blood cells Ammonium Chloride Solution (STEMCELL Technologies) was added to the cells at a volume ratio of 9:1 for lysis of red blood cells (RBCs) and the cell suspension was thoroughly mixed by inverting the tube and then placed on ice for 10 minutes.

The cells were washed twice with the isolation buffer and centrifuged at 200 x g for 7 minutes. They were then re-suspended in 20 ml of isolation buffer and counted using a glass haemocytometer. Monocytes were isolated from the mononuclear cells using STEMCELL Technologies EasySep® Human Monocyte Enrichment Kit.

2.3.2 Isolation of human monocytes from PBMC using immunomagnetic beads (STEMCELL Technologies)

EasySep® Human Monocyte Enrichment Kit Catalog #19059

The EasySep™ Human Monocyte Enrichment Kit (STEMCELL Technologies) was used to isolate classical CD14⁺/CD16⁻ monocytes from peripheral blood mononuclear cells by positive selection. This kit targets non-monocytes (CD14⁺) for removal using antibodies which recognize specific cell surface markers, including CD16. Unwanted cells (including CD16⁺ monocytes) are labelled with antibodies and magnetic particles, and separated without columns using an EasySep™ magnet, while the CD14⁺ cells are poured off into a new tube. The resulting cells consist of highly purified (CD14⁺CD16⁻) monocytes, representing classical monocytes which can later be differentiated into inflammatory DCs. The recommended protocol was followed as outlined below:

The PBMCs was prepared at the recommended concentration of 5×10^7 cells/ml in the recommended buffer in a 5 ml sterile polystyrene tube (BD Biosciences, Franklin Lakes, NJ).

The EasySep Human Monocyte Enrichment Cocktail was added at 50 µl/ml cells, mixed well, and the cells incubated at 4°C for 10 minutes. The EasySep Magnetic Particles for Monocytes were vortexed before use and added to the cells at 50 µl/ml cells, mixed well, and incubated at 4°C for 5 minutes. The cell suspension was brought to a total volume of 2.5 ml by adding the recommended buffer, mixed well, and placed in an EasySep magnet (STEMCELL Technologies). The tube was left in the magnet for 2.5 minutes at room temperature (15-25°C), and one continuous motion used to invert the magnet and tube and pour the enriched cell suspension into a new tube. The monocytes were then counted and plated at a concentration appropriate to the parameters of the experiment (500,000 to 1×10^6 cells/ml).

2.3.3 Human monocyte-derived dendritic cells

GM-CSF/IL-4 supplementation of the cell culture medium was maintained to generate monocyte-derived DCs, and to preserve the DC phenotype in culture thereafter and to prevent spontaneous cell death [162].

Recombinant Human Granulocyte Macrophage Colony Stimulating Factor (rh GM-CSF)

GM-CSF stimulates the growth and differentiation of hematopoietic precursor cells from various lineages, including granulocytes and macrophages. A GM-CSF stock was prepared by reconstituting the lyophilized human recombinant (rh) GM-CSF (50µg; Cat. No. 11343125 ImmunoTools, Friesoythe, Germany) in sterile H₂O. This was stored in aliquots at -30°C. Working concentrations of 50 ng/ml GM-CSF were prepared as needed by diluting the stock in complete RPMI medium.

Recombinant Human Interleukin-4 (rh IL-4)

IL-4 suppresses the generation of macrophages in GM-CSF-supplemented cultures. The IL-4 stock was prepared by reconstituting the lyophilized human rh-IL-4 (50µg; Cat. No. 11340045; ImmunoTools, Friesoythe, Germany) in sterile H₂O. This was stored in aliquots at -30°C. Working concentrations of 50 ng/ml IL-4 were prepared as needed by diluting the stock in complete RPMI medium.

To generate human monocyte-derived dendritic cells (DCs), the PBMCs were isolated from buffy coats as outlined above in **section 2.3.1**. Monocytes were purified from PBMC by immunomagnetic separation using the STEM CELL Technologies' EasySep® Human Monocyte Enrichment Kit. Cells were then incubated in DC medium: RPMI 1640 (provided by Gibco) supplemented with 10% defined FBS (containing the cytokine concentration 40 ng/ml recombinant human IL-4 and 50 ng/ml recombinant human GM-CSF (both from ImmunoTools, Friesoythe, Germany)). The monocytes were seeded on 6-24- or 48 well culture dishes at a density of $0.5 - 1 \times 10^6$ cells/ml and cultured for 6 days under 5% CO₂ at 37°C. Medium containing fresh cytokines was added every 2 to 3 days. To do so, half the medium was carefully removed from the surface of the liquid to avoid disturbing the cells. The medium was then gently replaced with an equal volume of fresh medium containing double the cytokine concentration (80 ng /ml IL- 4 and 100 ng /ml GM-CSF) to allow them to differentiate to immature DCs. The DCs were used for experiments on Day 6. At this stage the DCs were CD14⁺ and DC-SIGN⁺ (constituting approximately 90% of gated cells), as determined by flow cytometry (see **section 2.5** below). The cells were initially gated based on their size and granularity, measured by forward scatter and side scatter. Unstained cells were used to set the negative portion.

For extracellular flux analyses, the human moDCs were similarly isolated and plated on 6-well plates. On the day of infection, the DCs were centrifuged at 1200 rpm (289 rcf) for 5 minutes. They were then re-suspended in medium RPMI 1640 supplemented with 10% defined FBS containing the cytokine concentration 40 ng/ml IL-4 and 50 ng/ml GM-CSF, and a cell count performed using a haemocytometer. The cells were then plated onto Seahorse Biosciences 24-well plates that had been coated with Cell-Tak (see below) at 250,000-300,000 cells/well, and deemed ready for infection.

Corning® Cell-Tak™ Cell and Tissue Adhesive 1mg; USA

To attach DC cells to the 24 Seahorse plate, 17 µl of Cell-Tak was added to 1.250 µl of sodium bicarbonate (NaBic) for a final coating concentration of Cell-Tak of 3.5 µg/cm², and 50 µl were added to each well and incubated at room temperature for 1 hour. The wells were then washed twice with 250 µl of distilled water, all liquid removed from wells, left to dry, covered with parafilm, and stored at 4°C to use next day.

2.4 Cell treatments

2.4.1 Infection of dendritic cells with mycobacteria

After 6 days in culture DCs were infected with BCG-GFP, killed BCG or iH37Rv as appropriate to the experiment, and incubated at 5% CO₂ at 37°C for 24 or 48 hours depending on the parameters of the test.

2.4.2 Cell stimulation/inhibitors

Recombinant human IFN-γ, R&D Systems, Catalogue number: 285-IF-100

The IFN-γ was reconstituted in Sterile PBS (containing 1mg/ml BSA) at a concentration of 1mg/ml (2 x 10⁷ International units (IU)/ml) and stored at 4°C. Before use, an intermediate stock was made up by diluting 1/1000 in complete medium. Then 10 µl of intermediate stock was added per ml of RPMI 1640 medium to give a final concentration of 10 ng/ml (or 200 IU/ml).

Metformin (Merck Chemicals, Catalogue number: 317240)

Metformin was dissolved in DMSO to a concentration of 200mM stock and stored in aliquots at -30°C. Diluted to 5mM in RPMI 1640 complete medium.

Lipopolysaccharide (LPS)

LPS produced by *E. coli* bacteria from Alexis Biochemicals (1mg/ml) was used as a positive control for maturation and metabolic profile. As such, cells were isolated, cultured and prepared as outlined in **Section 2.2.1**, and on the day of experimentation, a working concentration of the LPS was made up in a standard culture media and added to the DCs to a final concentration of 1µg/ml.

Glycolytic inhibition using 2-deoxyglucose (2DG)

The compound 2-deoxyglucose (2-DG) is a competitive inhibitor of phosphoglucose isomerase (PGI), the enzyme which catalyzes the second step of the glycolytic pathway. The 2-DG is converted to 2-deoxyglucose-phosphate (2-DG-P) by hexokinase and the 2-DG-P then inhibits PGI. The 2-DG powder (Sigma-Aldrich) was reconstituted in PBS to make a stock solution of 1M, which was then filter-sterilized and stored at -30°C. The stock solution was diluted in a culture medium on the day of experimentation in order to achieve a working concentration.

The cells were isolated and cultured as outlined in **Section 2.3** and 30 minutes prior to infection with BCG-GFP, the cells were treated with culture medium supplemented with 2-DG, and cells incubated at 37°C in 5% CO₂ for the duration of experiment. Optimization experiments for different concentrations of 2-DG (1mM, 5mM, 10mM) were tested for their effect on the maturation and viability of DCs infected with BCG-GFP at a ratio of 250 mycobacteria per DC.

CP-91149

CP-91149 is a selective glycogen phosphorylase (GP) inhibitor (Santa Cruz Biotechnology). The tube of powder was centrifuged for a few seconds to bring it to the bottom and 500.2 µl of DMSO added to 10 mg of CP-91149 to produce a 50 mM stock solution. This was left to stand for 10 minutes, mixed well, small amounts were aliquoted into sterile tubes, and stored at -30°C. To dilute the inhibitor to a 100 µM solution, 2 µl of inhibitor was added to 1 ml of warm RPMI medium.

Rifampicin

Rifampicin (Melford Laboratories Ltd, UK, Cat. no.: R64000)

Rifampacin stock was made up in sterile water to a concentration of 50mg/ml, filtered through a 0.2um filter and stored in aliquots at -30°C. This stock solution was diluted in Middlebrook broth at a final concentration of 100µg/ml to generate killed BCG for selected experiments.

2.5 Flow Cytometry

2.5.1 Antibodies and Zombie Red™ Fixable Viability kit

The following antibodies and viability stains were used:

FITC anti-human CD14 (Clone HCD14), ImmunoTools and Biolegend), PE anti-human CD209 (DC-SIGN, Clone DCN48), PE anti-human CD86 (Clone 2331 (FUN-1) and APC-HLA DR (Clone G46-6) (all BD Pharmingen), Zombie Red™ Fixable Viability kit (Biolegend), Annexin V-PE Apoptosis Detection Kit (ImmunoTools), and 7-AAD (Sigma).

The cells were isolated, cultured, prepared and infected as outlined in **Sections 2.3 and Section 2.2** Where indicated, supernatants were removed following centrifugation of the cells* (i.e. 24 hours post-treatment / infection), and retained and stored at -80°C for subsequent measurement of lactate, glucose, or cytokines.

In order to detect cell surface expression of proteins the cells were harvested, washed with Phosphate-buffered saline (PBS), centrifuged at 2000 rpm (800 rcf) for 4 minutes to remove extracellular bacteria, and re-suspended in diluted antibodies 1:20. They were then incubated in the dark at 4°C for 30 min, re-washed at 2000 rpm (800 rcf) for 4 minutes, and re-suspended in 2% paraformaldehyde (PFA) to fix and analyzed by flow cytometry on a CyAn ADP from Beckman Coulter (with 3 lasers: Blue, Red, Violet, and 9 fluorescent detectors). The cells were gated on both forward and side scatter to exclude clumps and debris. Doublets were excluded from the analysis by gating on singlets on a FSC-Area against FSC-Height plot. Dead cells were excluded from the analysis based on Zombie red staining. Unstained cells were used to set the negative portion (which corresponded also with negative controls). Also, the relevant isotype controls were used for each antibody, One Comp (Thermofisher) compensation beads were used to set the compensation. Subsequent results were analyzed using Summit software version 4.3.

2.5.2 Staining cells with Annexin V-PE and 7-Amino-Actinomycin (7-AAD) to detect apoptosis

Cells were isolated, cultured and infected as outlined in **Section 2.3.** and **Section 2.2** Where indicated, supernatants were removed at this point* (i.e. 24 hours post-treatment / infection) and retained and stored at -80°C for subsequent measurement of lactate, glucose, or cytokines. The cells were harvested by centrifugation, washed in binding buffer (0.008 mol of Ca²⁺/L PBS), centrifuged at 250 rcf for 5 minutes, the supernatant discarded to remove extracellular bacteria, and the cell pellet re-suspended in 100 µl binding buffer. Annexin V PE - and 7-AAD were then added, and the cells were gently vortexed and incubated for 15 min at room temperature in the dark. Thereafter 400 µl of Binding Buffer was added and the cells analyzed by flow cytometry within 1 hr on a CyAn ADP flow cytometer from (Beckman Coulter). The cells were gated on both forward and side scatter to exclude clumps and debris, and subsequent results analyzed using Summit software version 4.3.

In live cells, phosphatidylserine (PS) is located on the cytoplasmic side of the cell membrane. During apoptosis, PS moves to the outer leaflet of the cell membrane where it can be detected using a fluorescent annexin V conjugate. Necrotic cells can also be stained by annexin V entering through pores in their cell membrane and binding to intracellular PS. Therefore, it is necessary to use a dye like 7-AAD, which is excluded from live and apoptotic cells but, similarly to PI, can enter necrotic cells through their compromised cell membrane, to discriminate between apoptotic and necrotic cells. Cells which are positive for Annexin V but exclude 7-AAD are considered to be apoptotic. Cells positive for Annexin V and 7-AAD may be in late apoptosis or necrotic and cells which are negative for 7-AAD and negative for Annexin V are considered to be viable [163].

2.6 Fluorescent microscopy

2.6.1 BCG-GFP quantification using Phase-contrast Inverted Fluorescent Microscopy

The cells were isolated, cultured, and prepared as outlined in **Section 2.3**, and infected as outlined in **Section 2.2**. Following infection, the DCs were transferred to microcentrifuge tubes, centrifuged and resuspended in 500 µl of PBS. The DCs were fixed by the addition of 500 µl 4% paraformaldehyde for 10 min, centrifuged for 5 minutes at 2000 rpm (800 rcf), the supernatant discarded, re-suspension of the cells in 50 µl of PBS, applied to a poly-lysine coated glass slides, and left to air-dry overnight. The cells were then stained with modified auramine O stain (Scientific Device Laboratory) for acid-fast bacteria and the DC nuclei

counterstained with 10 µg/ml of Hoechst 33342. The slides were analyzed using a 100X lens on an Olympus IX51 Fluorescent Microscope.

2.6.2 Viability staining using Propidium Iodide exclusion assay

In addition to the flow cytometry, the propidium iodide (PI) exclusion assay was used to assess cell viability. PI stain penetrates the leaky cell membrane of the dead cells and is excluded from the viable and healthy cells. This dye used to evaluate cell viability and usually combined with other dyes to distinguish between dead and live cells in a population of cells used with other dyes that stain live cells or cells that are early stage of apoptosis. In our assay, the nuclei of viable cells were counterstained with two Hoechst dyes. In unfixed cell cultures, Hoechst 33342, a blue fluorescent dye, stains all healthy nuclei while Hoechst 33258 preferentially stains the nuclei of dead cells blue. Between them they stain all of the cell nuclei blue.

Stock solutions

Stock solutions included a propidium iodide 1mg/ml solution in water (Sigma P4864). In addition, Hoechst 33258 (Sigma B1155), powder was dissolved in ultrapure water at a concentration of 25 mg/ml and stored at 4°C (or at -20°C for long-term storage). Hoechst 33342, (Sigma B2261) powder was dissolved in ultrapure water at a concentration of 10 mg/ml and stored at 4°C (or at -20°C for long-term storage).

Working solution

Just before staining the cells, the stock solutions were mixed together in complete RPMI at the following dilutions: PI 1/20 (50µl PI per ml of RPMI), Hoechst 33258 1/50 (20µl per ml of RPMI) and Hoechst 33342 1/50 (20µl per ml of RPMI). The cells were stained by adding the diluted dye (100µl/ml) directly into the medium in the tissue culture plate wells. The plate was protected from light and kept at room temperature before and during analysis.

The nuclei of live cells stain blue with Hoechst, while dead cell nuclei stain red (PI) and blue. The cells were stained just before imaging on a Cytell Cell Imaging System (GE Healthcare) and the percentage of PI positive cells was determined using the Cell Viability Bioapp (GE Healthcare).

2.7 Gene expression analysis

2.7.1 Isolation of RNA

Human monocyte-derived dendritic cells were cultured and plated at 0.5×10^6 /mL on 24 well plates as outlined in **Section 2.3** and infected with or without 5mM 2-DG, as outlined in **Section 2.2**. The cells were centrifuged at 2000 rpm (800 rcf) for 5 minutes, and supernatants removed. Cells were then lysed and homogenized by adding 350 μ L denaturing Buffer RLT (QIAGEN) to each well to ensure immediate inactivation of RNases while permitting isolation of intact RNA. Lysates were stored at -80°C.

RNA isolation was performed using the RNeasy Mini Kit (QIAGEN) according to manufacturer instructions. On the day of RNA isolation, the lysates were quickly thawed by placing the tubes in a beaker of water and lysates passed through a 20-gauge needle attached to a sterile RNase-free plastic syringe at least 5-10 times or until a homogeneous lysate was achieved. The lysates were then diluted by adding 350 μ L of 70% ethanol before being passed through the RNeasy Spin Column by centrifugation at 8000 x g for 15 seconds. RNA binds to the membrane of the spin column. Contaminants were then washed away by adding 700 μ L Buffer RW1, followed by centrifugation at 8000 x g for 15 seconds (to remove biomolecules such as carbohydrates, proteins, fatty acids that are non-specifically bound to the silica membrane while RNA molecules larger than 200 bases remain bound). After the further addition of 500 μ L Buffer RPE, the column was centrifuged at 8000 x g for 2 minutes, placed in new 2 ml tube, and centrifuged at full speed for one minute (to remove salts and traces of other buffers). Then the column was placed in a new 1.5 ml tube to elute RNA by adding 30 μ L of RNase-free water and centrifuging at 8000 x g for one minute, stored at -80°C. In some experiments repeated elution with the RNA eluate was carried out. Purified RNA was stored at -80°C.

2.7.2 Reverse transcription reaction

The Thermo Scientific RevertAid RT Kit (Thermo Scientific) was used to affect synthesis of the first strand of cDNA from the total RNA templates mix. The components of the kit were briefly centrifuged, and stored on ice. Firstly, the RNA concentration in each sample was measured using the NanoDrop TM ND-8000n Spectrophotometer with ND-8000 Software Version 1.0 (Thermo Fisher Scientific). The 260nm/280nm absorbance ratio was also noted to assess purity of RNA. For all experiments, a 260/280 ratio of ≥ 2 was

considered acceptable. After checking the purity of the RNA, it was diluted by RNAase-free water according to each sample calculation.

According to the protocol, 1µg of RNA was added to each cDNA reaction microtube and 1 µL Random Hexamer primer, in addition used water control for the reverse transcription reaction. A total of 12 µL were mixed gently, centrifuged briefly and incubated at 65 °C for 5 min. Thereafter they were chilled on ice, spun down, and the vial placed back on ice with the addition of the following components: 5X Reaction Buffer 4 µL, RiboLock RNase Inhibitor (20 U/µL) 1 µL, 10 mM dNTP Mix 2 µL and RevertAid RT (200 U/µL) 1 µL with a total reaction volume of 20µL per sample. These were mixed gently and centrifuged briefly, incubated for 5 min at 25 °C followed by 60 min at 42 °C. The reaction was terminated by heating at 70 °C for 5 min.

The reverse transcription reaction was carried out on a Techne TC-3000 Thermal Cycler (Bibby Scientific Ltd, UK) and the prepared cDNA was stored at -80°.

2.7.3 Reverse transcription reaction for RT-PCR using SYBR Green detection (carried out in UCD)

High quality RNA was purified using standard protocols and quantified using the NanoDrop. In the samples for nanostring used (Qubit™ RNA HS Assay Kit, Invitrogen) was a kind gift from Dr John Brown.

Samples were kept on ice while the assay was being set up and all reagents and all plastic ware was RNase/DNase free. The amount of RNA to be converted to cDNA was calculated (100-500 ng), the RNA was diluted to a volume of 10.0 µL in a labelled clean RNase free LoBind tube using water, a representative number of duplicates for performing minus RT controls were also prepared at least 1 per treatment group.

An RT master mix was prepared using the Life Technologies cDNA conversion kit (4368814). According to the protocol, 2 µl Random primers, 2 µL RT Buffer, 0.8 µL dNTPs, 1 µL Enzyme, 4.2 µL Water were combined. A total of 10 µL were mixed gently, added to each of the RNAs samples, vortex and spun, incubated at 37 °C for 120 min. The minus RT control are prepared in the same manner except the reverse transcription is omitted from the master mix and replaced with water. Following the incubation step, cDNA pool was created

by pooling an aliquot from each of the samples (2.5-5 μ l), this cDNA pool can be used to prepare standard curves, positive controls and interpolate calibrators as needed. The remaining cDNA was diluted to the appropriate concentration using water and store at -20 °C, (concentration of 1 ng/ μ l of RNA equivalents), the cDNA diluted a minimum of 1:10

2.7.4 Quantitative PCR

The relative gene expression was then examined by cDNA amplification using quantitative real-time polymerase chain reaction (Q-RT-PCR) with Taqman® probes, gene expression Taqman primer-probe sets (Thermofisher) were used IL-1 β ; Hs01555410_m1, TNF α ; Hs00174128_m1, IL-10; Hs00961622, which anneal to cDNA of specific genes of interest and are incorporated into new DNA strands (Applied Biosystems). Diluted cDNA was used at a ratio of 1:10 with nuclease-free water. Each RT-PCR sample was performed in triplicate.

Q-RT-PCR experiments used a master mix consisting of 5 μ L Taqman reaction mix and 0.5 μ L primer mix (used GAPDH, IL-1 β , TNF α , IL-10, ACT-B), 5.5 μ L of the master mix was applied to each appropriate well on 384 well plates. The well plate was gently tapped on the bench to ensure mix was at base of well and 4.5 μ L cDNA added. Once again, each well plate was gently tapped on bench, an optical cover applied to plate, and run on QuantiStudio PCR machine, in addition used water and minus reverse transcriptase(-RT) as control.

The ACT-B and GAPDH was measured in triplicate for each sample, and this was used to normalize variations in cDNA concentrations between samples by calculating the Δ Ct (i.e. Ct value for target gene mRNA for one sample - Ct value for 18S rRNA for corresponding sample). For each gene, the infection / treatment-induced expression were then compared to expression in uninfected (control) samples, generating the $\Delta\Delta$ Ct (i.e. Δ Ct value for infected sample - Δ Ct value for control sample). Finally, the fold-change in mRNA expression relative to the control sample was calculated (i.e. $2^{-\Delta\Delta CT}$).

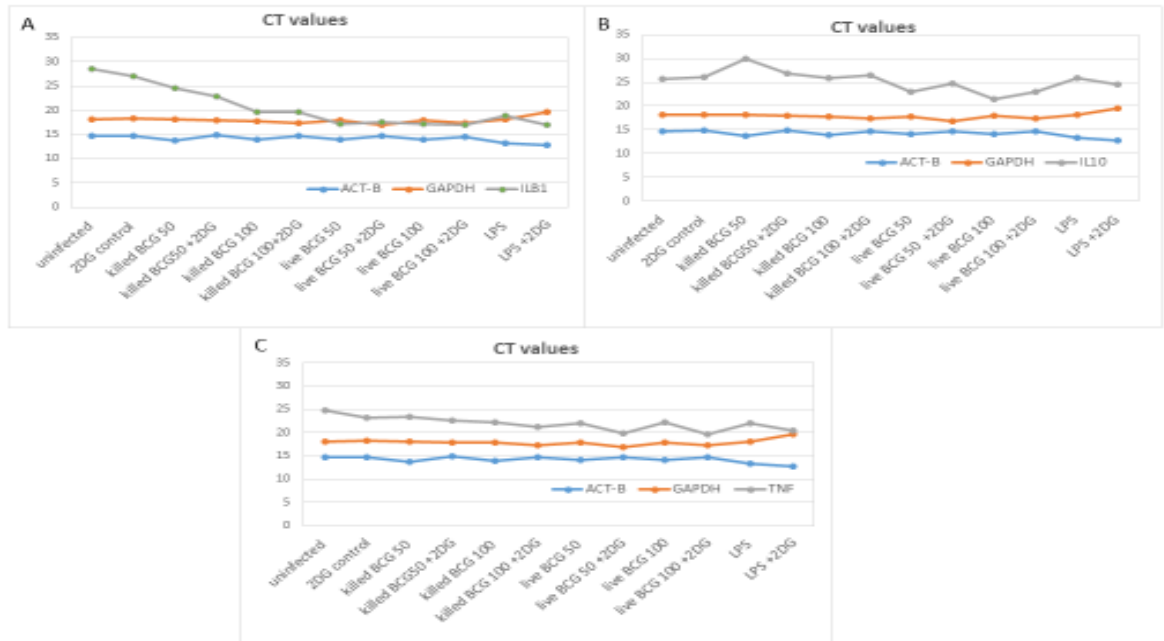


FIGURE 2.1: Comparison between GAPDH and ACT-B as reference genes. Human monocytes were separated from buffy coats using negative selection. On day 6 infected moDCs were untreated or treated with 5mM 2DG30 minutes before infection with live and killed BCG at different MOI for 24 hours. Uninfected moDCs were treated with lipopolysaccharide (LPS) (1µg/ml) as a positive control or left uninfected (control). At 24 hours post-infection, supernatants were removed. Cells were lysed in RLT buffer, RNA extracted and cDNA generated, and qPCR was performed. mRNA expression fold-change relative to uninfected calculated using the $2^{-\Delta\Delta C_t}$ method used GAPDH and ACTB (β -actin) as reference gene. Panels A -C CT value of IL-1 β (A), (B) IL-10 and TNF α (C) for two individual donors.

2.7.5 Quantitative PCR SYBR Green detection (carried out in UCD)

Preparation of qRT- PCR assay. calculated the total number of samples to be assayed, including the appropriate controls, all samples were analysed in duplicate. Sufficient master mix for all samples plus 10% excess was prepared. According to the protocol, 10 µL SyBR Green (4385617) (Thermo Fisher), 1.2 µL Forward Primer (5uM), 1.2 µL Reverse Primer (5uM), all primers supplied from Sigma- Aldrich, Germany, 2.6 µL Water were combined, vortexed and spun. 15µL of master was distributed in to each well as per the plate design, added 5 µL of the diluted cDNA template to each well as per the plate design, applied an adhesive seal to the plate. The plate was spun for 1 minute at 1000 rpm (177 rcf) in centrifuge, and then loaded into the instrument (7500 Fast Real- Time PCR). Primers from Sigma-Aldrich (table 1) were designed and diluted 1:20.

Table 2.1: Primer information of selected genes

Gene	Accession number	Primer Sequence (5' – 3')
Solute carrier family 7 Member 11 (SLC7A11)	BC012087	Forward GAGATTCAAGTATTACGCGGTTG Reverse TGACAATGGCCATGGATATACA
Serine/threonine kinase 3 AKT3	AF135794	Forward TGGTTCGAGAGAAGGCAAGTG Reverse GCTTTCAGTTAGAGTGTGTGCC
Galactose mutarotase (GALM)	BC019263	Forward GTCAACCTGACCAACCATTCT Reverse: GGTTTCATCCACAGGCAAATAAG
Fatty Acid Synthase (FASN)	BC007909	Forward CAATACAGATGGCTTCAAGG Reverse GATGTATTCAAATGACTCAGGG
Phospholipase D2 (PLD2)	AF033850	Forward ATCTATGAGCAGATCTTCCG Reverse AAGACTCATCCTCTAGGAAC
Human MET proto-oncogene	J02958	Forward ATGAGAGCTGCACCTTGACT Reverse GTCCCGTGGCCATTTGAAAT

Optimal reference target selection

Optimal reference target selection used 8 potential reference genes including ACTB, GAPDH, H3F3A, PPIA, RPL19, YWHAZ, RNF11 and SDHA (**Figure 2.2**)

Data analysed used qbase+ software by Biogazelle. A significant difference in gene expression was analyzed by one-way ANOVA ($p < 0.05$).

Reference target stability

Very high reference target stability (average geNorm $M \leq 0.2$). This is typically seen when evaluating reference targets using genomic DNA as input, or when reference targets are very stably expressed.

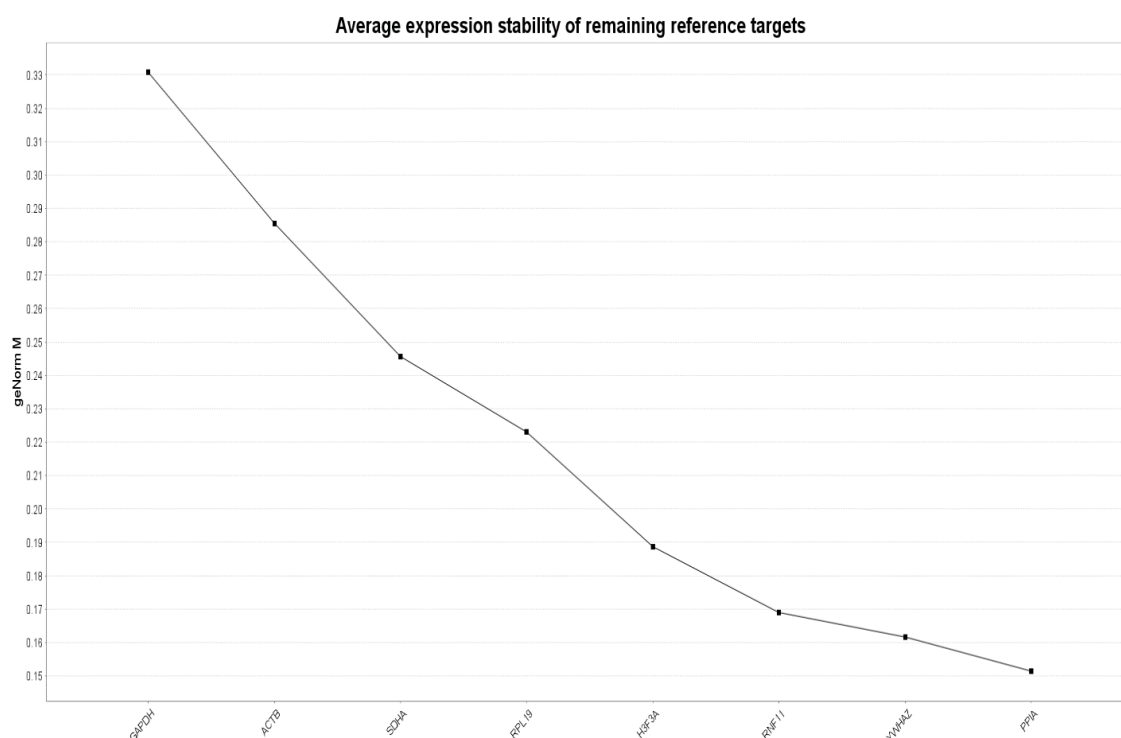


FIGURE 2.2: Optimal reference target selection.

The normalization of relative quantities with reference genes depend on the hypothesis that the reference genes are stably expressed to all tested samples. When using only one reference gene, its stability can not be evaluated. while use of multiple reference genes does not only produce more reliable data, but also permits an evaluation of the stability of these genes as well. The optimal number of reference targets in this experimental situation is 2 (geNorm $V < 0.15$ when comparing a normalization factor based on the 2 or 3 most stable targets). As such, the optimal normalization factor can be calculated as the geometric mean of reference targets YWHAZ and PPIA.

2.8 Agilent RNA 6000 Nano Assay Protocol for gel electrophoresis

Ribonuclease (RNase) is an important enzyme that helps in the breakdown of Ribonucleic Acid (RNA) in oligonucleotides. Therefore, the procedure of Ribonuclease decontamination was performed to prevent degradation of sample RNA during electrophoresis. Each well of an electrode cleaner was filled with 350 µl Ribonuclease ZAP (RNaseZAP) and placed in the Agilent 2100 bioanalyzer. With the lid closed for one minute. Then 350 µl RNase- free water was added to one well of another electrode cleaner and left for 10 seconds in the Agilent 2100 bio analyzer. Water that accumulated on electrodes was allowed to evaporate for about 10 seconds before closing the lid.

2.8.1 Gel Preparation

The reagents were allowed to equilibrate to room temperature for about 30 minutes. Then 550 µl of Agilent RNA 6000 Nano gel matrix was placed in the top vessel of a spin filter. The spin filter was placed in a micro centrifuge and centrifuged at 1500 g $\pm$ 20 % for 10 minutes (for the Eppendorf micro centrifuge, this corresponds to 4000 revolution per minute). Filtered gel (65 µl) was aliquoted into 0.5 ml Ribonuclease- free microfuge tubes that are incorporated in the kit. Then the gel was stored in aliquots (at 4 °C) for further use within a month after preparation.

2.8.2 Gel-Dye Mix Preparation

All the reagents were equilibrated for about 30 minutes prior to use, the dye was protected from light at room temperature, The RNA 6000 Nano dye concentrate was vortexed for 10 seconds and spun down. Then 1 µl of RNA 6000 Nano dye concentrate was added to a 65 µl aliquot of filtered gel. The tube was closed, rigorously vortexed, then visually inspected to confirm the mixture of dye and gel. Then the dye concentrate was stored in the dark room at 4°C. Before use the mixture was incubated for about ten minutes at room temperature and centrifuged at 13000 g (Eppendorf micro centrifuge, this corresponds to 14000 revolution per minutes). The prepared gel dye mix was used within one day.

2.8.3 Loading the Gel-Dye Mix

The gel-dye mixture was equilibrated for 30 minutes at room temperature prior to its use protected from light. A Nano chip was placed on the chip priming station. 9.0 µl of the gel dye was pipetted at the base of the well and loaded according to the manufacturers instructions. Samples were prepared by Karima Elayati and the Quantitative PCR and gel electrophoresis was performed by Karima Elayati and Dr. John Browne.

2.8.4 Loading the RNA 6000 Nano Marker

5 µl of Ribonuclease 6000 Nano marker was pipetted into each well of the Nano chip.

2.8.5 Loading the RNA Ladder and Samples

An aliquot of the RNA ladder was defrosted prior to its use and heated (70 °C, 2 minutes) to denature and decrease secondary structure. Then 1 µl of Ribonucleic Acid ladder or sample was pipetted in to each well, the chip was placed in the IKA adapter of the vortex mixer and vortexed for about 60 seconds at 2400 revolution per minute (rpm).

Inserting a Chip in the Agilent 2100 Bioanalyzer

The chip was inserted into the electrode cartridge of the Agilent 2100 bioanalyzer and the RNA is analysed by gel electrophoresis. After the run the Nano chip was removed and disposed of according to laboratory practices.

2.8.6 Agilent RNA 6000 Nano Assay Results

The main features of an effective total RNA run are:

- One marker peak
- Two ribosomal peaks.

The concentration of RNA, the ratio of ribosomes and the integrity of Ribonucleic Acid. (RIN), were calculated by the software (2100 expert software version B.02.02). Ribonucleic Acid integrity values are between 1-10, 10 is considered excellent, >7.5 is very good.

2.9 nCounter Assay

The Nanostring platform (NanoString Technologies, Seattle, Washington USA) is more sensitive than microarrays and is a new method for gene expression measurement. The

nCounter Assay is based on original digital molecular barcode technology which can count all results. NanoString's nCounter TagSet chemistry consists of target-specific oligonucleotide probe pairs, fluorescently-labeled specific Reporter Tags, and a biotinylated universal Capture Tag, collectively called a TagSet. Each TagSet Reporter Tag has a unique pattern of six spots of colour, generating fluorescent barcodes that can be counted during data collection. For each target gene the Nanostring instrument produces fluorescent counts and tabulates them.

Total RNA produced from moDCs was used for the nCounter assay according to the manufacturers protocol. Samples were prepared by Karima Elayati and the nCounter Assay was performed by Karima Elayati and Dr. John Browne.

2.9.1 nCounter Vantage RNA Assays

The nCounter® Vantage 3D™ Cancer Metabolism Panel (Human) VRXC-CM1-12, was used to measure the expression of 180 human metabolism-related genes. The first step is a hybridization process in which the Tag sets and samples are incubated together overnight to allow the Tags to bind to the mRNAs of interest. The following day the samples and cartridge are placed on the Nanostring workstation where post-hybridization sample processing, data capture and data analysis take place.

Hybridisation reaction

The nCounter XT TagSet aliquot was removed from the freezer and thawed on ice. After mixing by inversion several times the reagent was centrifuged briefly for several seconds at less than 1000rcf. A 30X Probe A Pool working dilution was created by adding of 22 µl of TE buffer to 3 µl Probe A stock aliquot. A 30 X Probe B Pool working dilution was created by adding 3 µl Probe B Pool stock aliquot.

A master mix was created by adding the reagents directly into the TagSet tube; 70 µL of hybridization buffer, along with 7 µL of 30X Working Probe A Pool were added. This mixture was mixed well by flicking the tube, and centrifuged for several seconds at 1,000 rcf, prior to the addition of 7 µL of 30X Working Probe B Pool. This was mixed was spun again. Then 8µl of master mix was aliquoted into each well of a 12-tube strip followed by 7 µl of sample (100 ng total RNA). The samples were hybridised in a pre-heated 65°C thermal cycler for at least 16 hours at 67°C. The lid of thermal cycler was set at the temperature of

5°C above temperature of block. Hybridization time was 16 hours after which the reaction ramped down to 4°C.

The following day the hybridised samples and the cartridge were placed on the Nanostring work station where the hybridization mixture was allowed to bind to magnetic beads complementary to the Capture probes. The beads were washed to remove excess probes and non-target cellular transcripts. The target-probe complexes were then eluted off the beads and allowed to bind to a second set of magnetic beads complementary to the Reporter probes. Wash steps were performed to remove excess Reporter probes. Finally, the purified target-probe complexes were eluted off the beads, immobilized on the cartridge and aligned for data collection.

2.9.2 Data Production

Data collection was carried out automatically on the Nanostring instrument using epifluorescent microscopy and a CCD camera. Automatic digital quantification was performed for each target molecule and count outputs generated for each probe. After sample imaging, data was imported into the nSolver Analysis Software and the Advanced analysis 2.0 plugin for downstream analysis.

2.10 Measurement of secreted cytokine profiles by multiplex immunoassay

Cytokine secretion was measured in cell-free supernatants by ELISA using the V-PLEX Proinflammatory Panel 1 (human) multiplex 96 well plate assay Kit (Meso Scale Discovery (MSD), LLC. U.S). This was capable of detecting IFN- γ -, IL-1 β , IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13 and TNF α in the same well. The MSD instrument measures analytes by electrochemiluminescence detection and readings are linear across a wide dynamic range (3-4+ logs). The IL-4 measurements obtained in the assay were disregarded since the DCs were maintained in a culture with the IL-4 cytokine, rendering it impossible to distinguish levels secreted by the cells themselves.

Sample and reagent preparation

All reagents were used at room temperature. In order to prepare calibration solutions diluent 2 was added to reconstitute the lyophilized calibrator blend. The tube was inverted 3 times, equilibrated for 15-30 minutes at room temperature, vortexed and diluted in a series of 4-fold dilution steps to prepare a calibration curve ranging from 1500 to 0.1 pg/ml. Diluent 2 alone was used as a zero calibrator. Before applying to the 96 well plate, samples were

diluted 2-fold in Diluent 2. The plate was placed on a shaker for 1 hour at room temperature and then incubated overnight without shaking at 4°C. The following morning each 50X detection antibody was diluted 50 fold in Diluent 3 to prepare a combined detection antibody solution. Then 25µl of detection antibody mix was added to each well and the plate was incubated for 2 hours at room temperature with shaking. A solution of 2X Reader Buffer T was prepared by diluting 4X Reader Buffer T by 2-fold with Ultra-pure water. The plate was washed 3 times with 150 µl /well of wash buffer (PBS 0.05% Tween 20). After the third wash, all of the buffer was removed by repeatedly tapping the inverted plate on paper towels. Following the addition of 150 µl/well of 2X Reader Buffer T, the plate was analyzed on a SECTOR Imager 2400 to detect the chemiluminescent signals and the data was analysed using Discovery Workbench 4.0.12 software (MSD).

2.11 Metabolic Analyses

Extracellular Flux Analyses

Extra cellular flux analysis was performed using a Seahorse XFe24 Analyzer (Agilent Technologies), an instrument capable of measuring the oxygen concentration and pH in live cell cultures. This instrument permits the live quantification of OCR (Oxygen consumption rate) and ECAR (Extracellular acidification rate) in a multi-well plate equivalent to the rate of mitochondrial respiration and glycolysis respectively. Adherent cells form a single layer inside the well. Sensor probes, responsible for measuring the oxygen concentration and free protons, are lowered towards the cells to form a transient micro chamber for about two to five minutes.

2.11.1 The Seahorse XF Cell Mito Stress Test Kit

The Agilent Seahorse XF Cell Mito Stress Test (Seahorse Biosciences, Agilent Technologies UK Ltd.) gauges the key parameters of mitochondrial function by directly measuring the oxygen consumption rate (OCR) of cells. Sequential compound injections measure basal respiration, ATP production, proton leak, maximal respiration, spare respiratory capacity, and non-mitochondrial respiration. The Seahorse XF Cell Mito Stress Test Kit use modulators of respiration which target components of the electron transport chain (ETC) in the mitochondria to reveal key parameters of metabolic function.

Compounds included in this kit are oligomycin, Carbonyl cyanide-4 (trifluoromethoxy), phenylhydrazon (FCCP), and rotenone combined with antimycin A. Oligomycin is the first

injection which inhibits the ATP synthase (complex V) leading to a decrease in OCR following injection of oligomycin associates to the mitochondrial respiration and with cellular ATP production. FCCP, the second injection, is an uncoupler of oxidative phosphorylation in mitochondria. Therefore, it disrupts ATP synthesis by transporting protons across cell membranes. As a result, the electron flow through the ETC is uninhibited, and oxygen is maximally consumed by complex IV leading to increases in OCR and used to calculate the Spare Respiratory Capacity (SRC). This is defined as the difference between maximal respiration post-FCCP OCR and basal respiration. Spare respiratory capacity (SRC) is a measure of the ability of the cell to increase energy demand under stresses. The third injection is a mix of rotenone, a complex I inhibitor, and antimycin A, a complex III inhibitor. Both inhibitors act to shut down all ETC activity and all mitochondrial oxygen consumption, and therefore reflect the Non-Mitochondrial Oxygen Consumption Rate of the cells. (**Figure 2.3**)

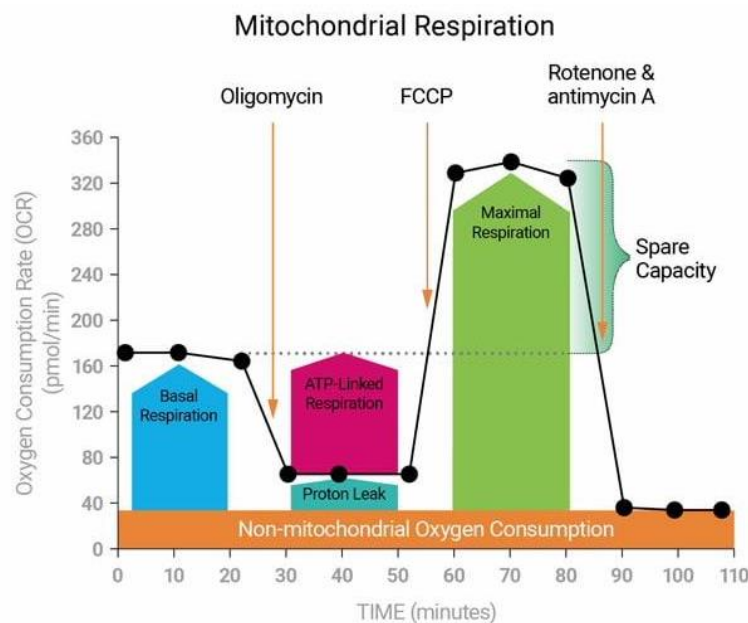


Figure 2.3: Metabolic parameters derived from a XF Extracellular flux assay. OCR measurements (in pmol/min) at baseline and following sequential addition of mitochondrial inhibitors oligomycin; FCCP = Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazine; Rotenone& antimycin A. Illustrating how different oxygen consumption parameters are calculated adapted from Agil. Seahorse XF Cell Mito Stress Test [164].

Extracellular Flux Analysis protocols:

Hydrating the Seahorse XFe24 sensor cartridge

Add 1ml of XF Calibrant solution (Seahorse Biosciences, Aligent Technologies UK Ltd.) into each well of a 24-well utility plate and place the hydro-booster (pink spacer) onto the utility plate, followed by the sensor cartridge, and finally the lid. Place the sensor cartridge, incubated in a 37°C CO₂-free incubator overnight. A non-CO₂ incubator was used to prevent CO₂ contributing to the acidification of media during analysis.

For all extracellular flux analyses, cells were prepared as outlined in **Section 2.3** and plated on Seahorse 24-well microplates in a usual culture medium with 4 designated “blank” wells left free, left for at least 2 hours, and then infected as outlined in **Section 2.2**, except in two experiment where DCs were infected on Nunc plates and transferred to the Seahorse plate on the day of the Seahorse assay to detect any different between two methods.

The Seahorse XF Cell Mito Stress Test Kit Protocol

On the day of interrogation, the Seahorse media (bicarbonate-free DMEM) (Seahorse Biosciences, Agilent Technologies UK Ltd.) was supplemented with 2mM glutamine and 10mM glucose, warmed to 37°C, and filtered through 0.2µm sterile syringe filters (Corning, NY). Thereafter, 200 µl media was removed from all wells of the Seahorse microplate, the cells washed twice by the addition of a supplementary 400µl of Seahorse media, and 450µL of fresh Seahorse media applied to each well (including blank wells by added 500µl of fresh Seahorse media). The microplate was then incubated at 37°C in a CO₂-free incubator prior to interrogation using the Seahorse XFe24 Analyzer.

Optimisation of Inhibitor Dose: Dose optimisation was performed for oligomycin, FCCP and Rotenone and antimycin A. In addition, the optimal count for DCs was determined by comparing the effects of the inhibitors on different cell densities of moDCs. Optimised inhibitor concentrations were 2 µM oligomycin, 0.5 µM FCCP, and 1 µM rotenone with antimycin A [137]. See **Chapter 4 Section 4.2.2**

The interrogation protocol was designed using Seahorse XF Wave Desktop Software. The Seahorse Assay Cartridge was inserted into the instrument and calibration of probes performed. The Seahorse microplate was then inserted, an equilibration step performed, and four baseline measurements of OCR and ECAR made. Mitochondrial inhibitors oligomycin

were added to all wells, followed by FCCP, then rotenone/antimycin A, with two measurements of OCR and ECAR taken following the addition of each compound.

The data initially displayed as real-time graphs of OCR and ECAR using Seahorse XF Wave Desktop Software were then converted to Microsoft Excel format for further analysis. Though three measurements were taken following the addition of each mitochondrial inhibitor, only the minimal (oligomycin, rotenone/antimycin A) or maximal (FCCP) OCR measurement, with its corresponding ECAR measurement, was used for analysis.

Following completion of the metabolic flux assay, a crystal violet (CV) assay was performed to determine cell viability in each well. The media was then removed and 50 μ L 1% glutaraldehyde (Sigma-Aldrich) was added to each well for 15 minutes. Thereafter the glutaraldehyde was removed and wells washed twice with 100 μ L PBS and 50 μ L 0.1% CV (Sigma-Aldrich) added to each well for 30 minutes. The CV was then removed and the microplate inverted and left to air dry for several hours. At this stage, 40 μ L 1% Triton X solution was added to each well and the plate placed on a shaker for 15 minutes to allow full elution of the crystal violet to occur. The contents of each well were then transferred to a 96-well plate and absorbance was read at OD595nm using a Biotek plate reader with Gen 5 Data Analysis software (BioTek Instruments, VT). The results were then used to normalize both the ECAR and the OCR measurements. The normalization for cell viability reduced inter-donor variability and facilitated the pooling of data. The CV assay also facilitated interpretation of data by investigating variation in cell viability between treatment conditions which could impact observed results.

2.11.2 The Seahorse XF Glycolysis Stress Test Kit

The Agilent Seahorse XF Glycolysis Stress Test gauges glycolytic function in the cells by directly measuring the extracellular acidification rate (ECAR) of cells. Sequential compound injections measure the following parameters of glycolytic flux: Glycolysis, Glycolytic Capacity, Glycolytic Reserve, and non-glycolytic acidification.

When required, the cells possess the ability to switch between Glycolysis and oxidative phosphorylation pathways, which are considered the two major energy-producing pathways in the cell. Glucose in the cell is converted to pyruvate (referred to as glycolysis), and then converted to lactate in the cytoplasm, or CO₂ and water in the mitochondria.

First, cells are incubated in the glycolysis stress test medium without glucose or pyruvate, and the ECAR measured by using Seahorse XF Glycolysis Stress Test Kit (Seahorse Biosciences, Agilent Technologies UK Ltd.).

The first injection is a saturated concentration of glucose. The cells utilize the glucose injection and catabolize it through the glycolytic pathway to pyruvate, producing ATP, NADH, water, and protons. This results in a rapid increase in ECAR; the rate of glycolysis reported under basal conditions after glucose-induced response. Prior to the glucose injection, the ECAR is a measure of non-glycolytic acidification.

The second injection is oligomycin. This inhibits ATP synthase (complex V), preventing ATP synthesis by oxidative phosphorylation. Consequently, it shifts the energy production to glycolysis and increases the ECAR revealing the cellular maximum glycolytic capacity.

The final injection is 2-deoxy-glucose (2-DG). This is a competitive inhibitor of Hexokinase, the enzyme which catalyzes the first step of the glycolytic pathway. The resulting decrease in ECAR confirms that the ECAR produced in the experiment is due to glycolysis, which is then used to calculate Glycolytic Reserve (GR) via the difference between glycolytic capacity and glycolysis. Glycolytic Reserve (GR) is a measure of the ability of the cell to increase glycolytic rate under stresses.

The Seahorse XF Glycolysis Stress Test Kit protocol

On the day of interrogation, Seahorse media (bicarbonate-free DMEM) (Seahorse Biosciences, Agilent Technologies UK Ltd.) was warmed to 37°C. Thereafter 200 µl media was removed from all wells of the seahorse microplate, the cells washed twice by the addition of 400µl of Seahorse media, and then 450µL of fresh Seahorse media was applied to each well (including blank wells by added 500µl of fresh Seahorse media). Then the cells were incubated in the glycolysis stress medium without glucose or pyruvate, and the microplate was incubated at 37°C and a CO₂-free incubator prior to interrogation using the Seahorse XFe24 Analyzer.

Compounds used were 10 µM glucose, 1.0 µM oligomycin, and 50 µM 2DG, applied in sequence by injection through the Seahorse ports according to the manual. The interrogation protocol was designed using Seahorse XF Wave Desktop Software. The Seahorse Assay Cartridge was inserted into the instrument and calibration of probes performed. The Seahorse microplate was then inserted, an equilibration step performed, and four baseline

measurements of the ECAR and the OCR made. Glucose was added to all wells, followed by oligomycin, then 2DG, with two measurements of the ECAR and the OCR taken following the addition of each compound.

The data initially displayed as real-time graphs of the ECAR and the OCR using Seahorse XF Wave Desktop Software was then converted to Microsoft Excel format for further analysis.

Following completion of the interrogation, a crystal violet (CV) assay was performed as before.

2.11.3 The Seahorse XF Mito Fuel Flex Test Kit

The Agilent Seahorse XF Mito Fuel Flex Test Kit (Seahorse Biosciences, Aligent Technologies UK Ltd.), measures the dependency, capacity, and flexibility of cells to oxidize three mitochondrial fuels: Glucose (pyruvate) Glutamine (glutamate) and Long-chain fatty acids in real time.

The Seahorse XF Mito Fuel Flex Test determines the rate of oxidation of each fuel by measuring mitochondrial respiration [the oxygen consumption rate, (OCR)] of cells in the presence or absence of fuel pathway inhibitors. Dependency indicates that the cells mitochondria are unable to compensate for the blocked pathway by oxidizing other fuels. Inhibiting the two alternative pathways followed by the pathway of interest enables the calculation of cells mitochondrial capacity to meet energy demand. Flexibility indicates that the cells' mitochondria have the ability to compensate for the inhibited pathway by using other pathways to fuel mitochondrial respiration. The presence of dependency and absence of flexibility demonstrates that the mitochondria require that fuel pathway to maintain basal OCR. Fuel Flexibility is calculated by subtracting the Fuel Dependency from the Fuel Capacity for the pathway of interest.

The compounds included in this kit are UK5099, BPTES and Etomoxir. UK5099, an inhibitor of the glucose oxidation pathway which blocks the mitochondrial pyruvate carrier (MPC). Cells convert glucose to pyruvate through glycolysis. Pyruvate can be transported into the mitochondria and oxidized by the TCA cycle. As an inhibitor of the glutamine oxidation pathway, BPTES is an allosteric inhibitor of glutamines (GLS1). Glutaminase converts glutamine to glutamate, which in turn, is converted to alpha-ketoglutarate, and

oxidized by the TCA cycle. Etomoxir is an inhibitor of long chain fatty acid oxidation. This inhibits carnitine palmitoyl-transferase 1A (CPT1A) which is critical for translocating long chain fatty acids from the cytosol into the mitochondria for beta oxidation.

To perform a Flexibility test, two groups are needed for each fuel included (Glutamine oxidation, Fatty acid oxidation and Glucose oxidation); one Dependency, and one Capacity group. For Glutamine oxidation dependency, the first injection is BPTES and the second injection Etomoxir/UK5099, while conversely, for the Glutamine oxidation capacity, the first injection is Etomoxir/UK5099 and the second injection BPTES. Similarly, for Fatty Acid oxidation dependency, the first injection is Etomoxir and the second injection BPTES/UK5099, while conversely, for Fatty Acid oxidation capacity, the first injection is BPTES/UK5099 and the second injection Etomoxir. Likewise, for Glucose oxidation dependency, the first injection is UK5099 and the second injection BPTES/ Etomoxir, while conversely, for Glucose oxidation capacity, the first injection BPTES/ Etomoxir and the second injection UK5099.

The Seahorse XF Mito Fuel Flex Test Kit protocol

On the day of interrogation, the Seahorse media (bicarbonate-free DMEM) (Seahorse Biosciences, Aligent Technologies UK Ltd.) was supplemented with 1 mM pyruvate, 2 mM glutamine, and 10 mM glucose, three compounds supplied from Sigma, warmed to 37°C and filtered through 0.2µm sterile syringe filters (Corning, NY), Thereafter 200 µl media was removed from all wells of the Seahorse microplate, the cells washed twice by the supplementary addition of 400µl Seahorse media, and 450µL of fresh Seahorse media applied to each well (including blank wells by added 500µl of fresh Seahorse media). The microplate was then incubated at 37°C and 0% CO₂ prior to interrogation using the Seahorse XFe24 Analyzer.

The interrogation protocol was designed using Seahorse XF Wave Desktop Software. The Seahorse Assay Cartridge was inserted into the instrument and calibration of probes performed. The Seahorse microplate was then inserted, an equilibration step performed, and three baseline measurements of OCR made. Inhibitors were added to all wells in order of dependency and capacity comprising first and second injection, and six OCR measurements conducted following the addition of each compound.

The data initially displayed as real-time graphs of the OCR using Seahorse XF Wave Desktop Software was then converted to Microsoft Excel format for further analysis.

Though three measurements were taken following addition of inhibitor, that measurement was used for analysis. Following completion of interrogation, a crystal violet (CV) assay was performed to determine cell viability in each well.

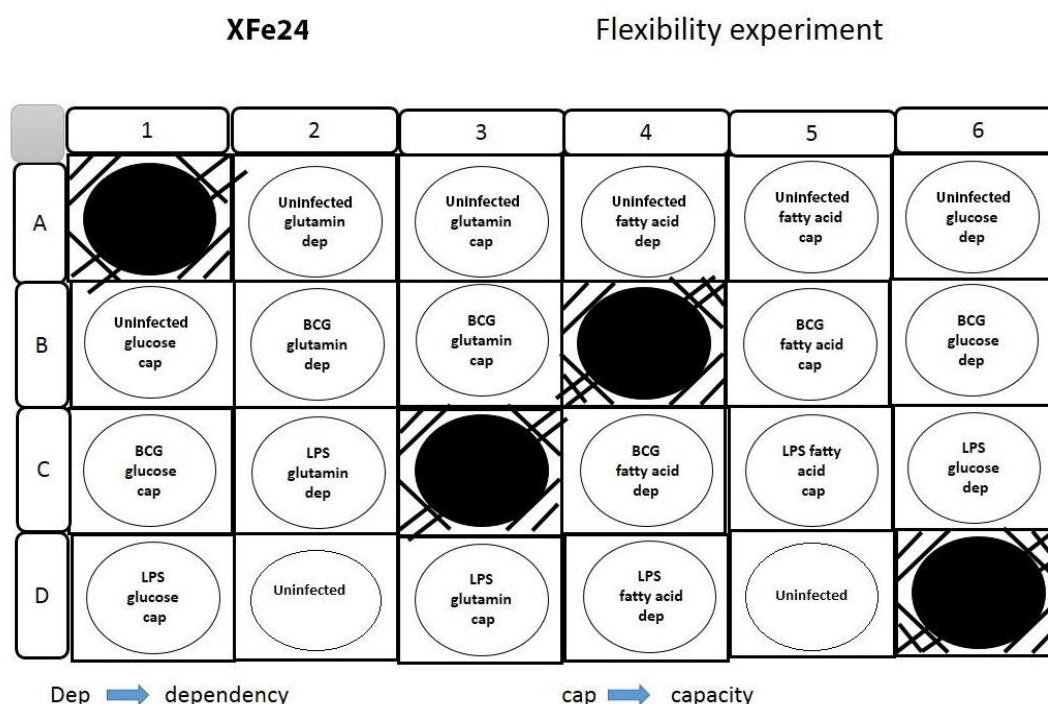


Figure 2.4: Agilent Seahorse XFe24 Flexibility test plate map. moDCs were plated on Seahorse Biosciences 24-well plates at 250,000 cells/well. Cells were infected for 24 hours with 100 MOI of killed BCG. 24 hours after infection inhibitors of the Mito Fuel Flex Test Kit was used. This test involves measuring the rate of oxygen consumption in the presence and absence of inhibitors blocking glucose, glutamine or long chain fatty acid utilization in the organelle. Uninfected moDCs were also treated with 1µg/ml LPS. Extracellular flux analysis was performed and moDC **mitochondrial flexibility in glucose oxidation** was measured using the XFe mitochondrial fuel flexibility test with sequential addition of inhibitors and data normalised using CV assay. Extracellular flux analysis was performed with sequential addition of UK5099 (2 µM), BPTES (3µM) and Etomoxir (4 µM). The oxygen consumption rate (OCR) was measured over time to determine the **dependence of moDCs on glucose oxidation**. The basal OCR was established before the sequential injection of UK5099 and the combination of BPTES and etomoxir. The average OCR was measured over time for moDC capacity to oxidize glucose. The basal OCR was established before the sequential injection of the combination of etomoxir and BPTES followed by UK5099 and then **flexibility** was calculated (the difference between dependency and capacity in uninfected moDCs and BCG infected moDCs). MoDC mitochondrial **flexibility in glutamine oxidation** was measured with sequential addition of BPTES (3µM), Etomoxir (4 µM) and UK5099 (2 µM). The basal OCR was established before the sequential injection of BPTES and the combination of etomoxir and UK5099. The average OCR was measured over time for moDC capacity to oxidize glutamine. The basal OCR was established before the sequential injection of the combination of etomoxir and UK5099 followed by BPTES and then flexibility calculated in uninfected moDCs and BCG infected moDCs. To assess **mitochondrial flexibility of moDC in fatty acid (FA) oxidation**, extracellular flux analysis was performed with sequential addition of Etomoxir (4 µM), BPTES (3µM) and UK5099 (2 µM). The basal OCR was established before the sequential injection of Etomoxir and the combination of BPTES and UK5099. The average OCR was measured over time for moDC **capacity to oxidize glutamine**. The basal OCR was established before the sequential injection of the combination of BPTES and UK5099 followed by Etomoxir and then flexibility was calculated in uninfected moDCs and BCG infected moDCs for three donors.

CHAPTER 3:

The role of glycolysis in the maturation and survival of BCG-GFP infected Human Monocyte-Derived Dendritic Cells

3.1 Introduction

The overall aim of this chapter is to investigate the role of glycolysis in the maturation of BCG-infected monocyte-derived dendritic cells (mo-DCs). The role of metabolism in DC differentiation and activation has been discussed in detail in Chapter 1. To summarise, most of our knowledge in this area comes from studies of LPS-treated murine DCs. In these cells TLR-driven early glycolytic burst is a metabolic sign of activation [165]. When activated by TLR ligands, murine bone-marrow-derived DCs (BMDCs) rapidly increase their consumption of glucose to generate pyruvate; pyruvate enters mitochondria via mitochondrial pyruvate carrier 1 (MPC1) and is converted into acetyl-CoA, which enters the TCA cycle. Alternatively, pyruvate can be converted to lactic acid, which is secreted into the extracellular environment as lactate. This conversion leads to coupled regeneration of NAD⁺ and the production of ATP by the glycolysis pathway [30], [94]. In the early stages of activation, DCs maintain high rates of OXPHOS as well as aerobic glycolysis. In DCs the main function of increased glycolysis in the early stage of maturation appears to be to generate citrate. Citrate is used for the synthesis of fatty acids (required for the expansion of the endoplasmic reticulum and Golgi apparatus associated with maturation events such as cytokine synthesis and secretion) and in the TCA cycle [30] which leads to an increase in spare respiratory capacity (SRC) [166]. SRC is the extra mitochondrial capacity available in a cell to produce energy in response to increased work or stress and is associated with cellular survival and function [167].

At later stages of maturation there is a switch from a combination of glycolysis and OXPHOS to exclusively using glycolysis for ATP production in murine BMDCs stimulated with LPS. This is facilitated by (i) inhibition of the ETC by nitric oxide (NO) [167] which nitrosylates iron-sulfur proteins present in electron transport chain complexes (such as Complex I) [168] cytochrome c oxidase [169] and (ii) upregulation of glycolysis-related genes such as GLUT1 [170] and lactate dehydrogenase (LDH) by the mammalian target of rapamycin (mTOR) [171] and HIF1 α [172].

The most commonly used method to investigate the role of glycolysis in immune cell activation is treatment with the glucose analogue **2-deoxy glucose (2DG)**. 2DG is phosphorylated by the hexokinase II enzyme, which catalyses the first reaction of glycolysis (conversion of glucose to glucose-6 phosphate). The resulting 2DG-6-phosphate cannot be metabolised further by glycolytic enzymes, accumulates in cells and competitively inhibits

glycolysis [173]. This inhibits murine DC activation thus proving that glycolysis is vital for DC metabolism during maturation [30], [100], [104]. Blocking glycolysis with 2DG in LPS-activated murine DCs impairs their ability to produce a range of inflammatory cytokines and stimulate T cells [104], [172], [174]. Treatment with 2DG specifically decreased the production of the inflammatory cytokine IL-1 β in bone marrow-derived macrophages (BMDMs) activated by LPS [116]. In addition, treatment of human and murine macrophages with 2DG reduced the expression and secretion of IL-1 β induced by the H37Ra and H37Rv strains of Mtb [158]. Secretion of IL-1 β by infiltrating monocyte-derived DCs is required for control of Mtb infection in a mouse model of TB [60], [175], however, the role of glycolysis in DC cytokine production after mycobacterial infection is not known.

Mature murine BMDCs depend almost exclusively on glycolysis for ATP production [167]; inhibition of glycolysis by 2-DG completely blocks maturation of these cells and eventually causes cell death [166], [172]. In contrast OXPHOS is maintained in mature human mo-DCs possibly because they do not express *Nos2*, the enzyme responsible for NO production in BMDCs. In fact, Malinarich et. al. recently found that mature moDCs are able to provide 40% of their ATP under glycolysis inhibition. This indicates that ATP production does not depend on solely on glycolysis and that mature human moDCs maintain some level of energetic plasticity for survival [137].

3.1.1 A role for glycogen in the TLR-driven glycolytic burst

Many lymphoid and myeloid cells depend fully on extracellular glucose for glycolysis-dependent effector responses [101], [137], [176]. Glycolytic reprogramming after LPS stimulation to support DC activation occurs via increased expression of glucose transporter, GLUT-1, but this surge in GLUT-1 levels happens after many hours of TLR stimulation [177]. Therefore, there must be some other way by which the cells boost glycolysis, and this was proved to be glycogen reserves [178] that were broken down by the glycogen phosphorylase (PYG) enzyme to produce glucose-1 phosphate, which directly fed the glycolytic cycle [179] (**Figure 3.1**). PYG inhibition also reduced stimulation of CD4⁺ T cells, indicating that glycogenolysis is important for effector immune responses against pathogens [120]. Thwe et al showed that both murine BMDCs and human mo-DCs express the liver isoenzyme of PYG. The PYG inhibitor, CP91149 (CP), inhibited LPS-induced maturation of BMDCs and the accompanying increase in ECAR, as well as TCA citrate

production, indicating that DCs are not completely dependent on extracellular glucose to fuel the TLR-driven glycolytic burst.

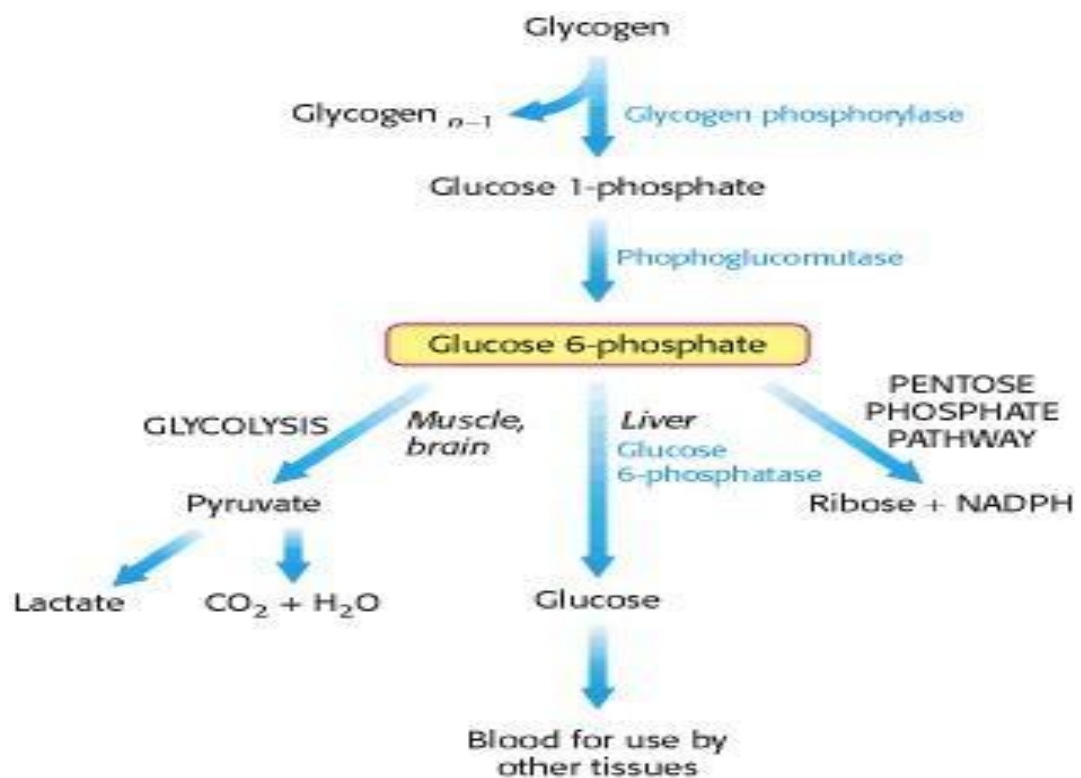


Figure 3.1: Glycogen metabolism. Glycogen synthesis requires an activated form of glucose which can be metabolised to Glucose 6-phosphate by glucose 1-phosphate and used in three ways; as a fuel for anaerobic or aerobic metabolism; processed by the pentose phosphate pathway to generate NADPH or ribose; or converted into free glucose in the liver and then released into the blood. Figure from Berg JM, Tymoczko JL [180].

Recently, several studies have shown that DC apoptosis is closely linked to cellular metabolism [101], [120], [137], [176]. Engaging in increased glycolytic metabolism may act as a protective mechanism to maintain cellular ATP levels and protect DC viability during an immune response [104]. While ATP generated by glycolysis prevents apoptotic cell death [196], blocking of glycolysis may lead to increased apoptosis.

3.1.2 Hypothesis

An increase in glycolytic flux is required for optimal DC maturation and survival after infection with BCG.

Aims of this chapter

The overall aim of this chapter is to define the role of glycolysis in the maturation of BCG-infected monocyte-derived dendritic cells (mo-DCs). Specifically, to

1. determine whether inhibition of glycolysis in DCs infected with BCG influences their capacity to:
 - i) upregulate cell surface expression of co-receptors such as CD86 and CD40
 - ii) present antigen
 - iii) upregulate and secrete inflammatory cytokines
 - iv) maintain viability
2. Investigate whether glycogen metabolism plays a role in DC maturation and viability.

3.2 Methods

While most of the materials and methods used are fully described in **Chapter 2**, the basis for the choice of methods used is briefly outlined in this section.

3.2.1 Cell culture

The current work studied the effect of the glycolysis inhibitor 2-deoxyglucose (2DG) and glycogen phosphorylase (GP) inhibitor on human monocyte-derived DCs that were infected with different MOI of BCG-GFP over a 24-hour period. Changes in the DC expression of maturation markers, chemokine receptors and cell viability were assessed by flow cytometry.

Human monocyte-derived DCs (mo-DC) were generated from CD14⁺ monocytes isolated from IBTS buffy coats and prepared for experimentation as delineated in **Chapter 2, Section 2.3**. Monocytes were seeded on 24-well culture dishes at a density of 0.5 - 1 × 10⁶ cells/ml, and incubated in DC medium. The RPMI 1640 was supplemented with 10% defined FBS, 50 ng/ml recombinant human IL-4 and 50 ng/ml recombinant human GM-CSF for 6 days prior to infection with BCG-GFP under 5% CO₂ at 37°C, and the medium containing fresh cytokines was replaced every 2 to 3 days to allow them to differentiate to immature DCs. Uninfected DCs were treated with lipopolysaccharide (LPS) (1 µg/ml) as a positive control for maturation and staurosporine (20 µg/ml) as a positive control for cell death.

Phenotyping of monocytes and mo-DCs by flow cytometry

In each experiment, the purity of monocyte-derived dendritic cells after 6 days in culture with GM-CSF and IL-4 was checked by flow cytometry of cells stained with antibodies to CD14 and DC-SIGN. Unstained cells were used to set the negative portion (which corresponded also with isotype controls). The DC-SIGN expression was detected using a PE-conjugated antibody (PE-conjugated anti-human CD209 (DC-SIGN) from BD Pharmingen), while the CD14 antibody was FITC-conjugated (FITC-conjugated anti-human CD14 from ImmunoTools and Biolegend). Single-color PE and FITC control samples were used to detect and designate positive staining on the flow cytometer and calculate the compensation. DCs produced in this way expressed DC-SIGN and lost expression of CD14 from their surface after 6 days of differentiation. DC-SIGN⁺, CD14⁻ cells were considered to be DCs and had the characteristic DC appearance on microscopy.

3.2.2 Infection with BCG-GFP and treatment with inhibitors

On the day of infection DCs were co-cultured with different MOI of GFP-BCG for 24 hr or 48 hr for some experiments. The number of mycobacteria per DC (multiplicity of infection; MOI) was calculated using a nephelometer as described in **Chapter 2 Section 2.2**. To inhibit glycolysis DCs were treated with 5mM 2-Deoxy-D-glucose (2DG) 30 minutes before infection. Uninfected DCs were treated with lipopolysaccharide (LPS) produced by *E.coli* bacteria (1 µg/ml) as a positive control for maturation and staurosporine (20 µg/ml) was applied for 24 h as a positive control for cell death.

To inhibit glycogen phosphorylase (GYP) DCs were treated with 100 µM CP-91149 5 minutes before infection. The DCs were also treated with DMSO (2 µl/ml) as the vehicle control.

3.2.3 Flow cytometry to assess DC activation and viability

The following antibodies and viability stains were used: (PE anti-human CD86 and APC-HLA, (all BD Pharmingen) to detect maturation of DCs. Zombie Red™ Fixable Viability kit from (Biolegend) was used for cell death in combination with the antibodies used above and to exclude dead cells from the analysis. Annexin V-PE from (ImmunoTools) and 7-AAD Apoptosis Detection Kit from (Sigma) were used in additional experiments to detect apoptosis (see below). To detect the cell surface expression of proteins cells were harvested 24 or 48 hours post-infection, washed with calcium/magnesium-free phosphate-buffered saline (PBS), centrifuged at 2000 rpm (800 rcf) for 4 minutes to pellet the DCs and remove extracellular bacteria and resuspended in diluted antibodies 1:20. They were incubated in the dark at 4°C for 30 min, washed at 2000 rpm (800 rcf) for 4 minutes, resuspended in 2% paraformaldehyde (PFA) to fix, and analyzed by flow cytometry on a CyAn ADP from Beckman Coulter 3 laser, Blue, Red, Violet and 9 fluorescent detectors.

3.2.4 Fluorescent microscopy to detect phagocytosis of BCG

In addition to using flow cytometry to assess the rate of infection, in some experiments the number of mycobacteria phagocytosed per cell was determined by fluorescent microscopy following the procedure below:

- Cells were removed from the well by pipetting gently up and down to dislodge them from the plate, then transferred to a micro centrifuge tube
- Cells were centrifuged for 5 minutes at 2000 rpm (300 rcf) on an Eppendorf Centrifuge
- Cells were resuspended cells in 500 µl of PBS
- 500 µl of 4% PFA was added, mixed gently and the cells were fixed for 10 minutes
- The cells were centrifuged for 5 minutes at 2000 rpm (300 rcf), the supernatant discarded, resuspended in 50 µl of PBS and 5 µl, 10 µl and 30 µl were pipetted onto separate areas of a poly-lysine coated glass slide (VWR, Randnor, PA)
- Slides were left to air dry in the dark overnight
- Cells were permeabilised in 100% methanol at -30°C for 5 minutes, the slides were air-dried and then stained for acid fast bacteria (AFB).

Acid fast staining procedure

- Slides were washed in tap water, Modified Auramin O stain was applied for 1 minute and slides were washed well with tap water.

- Modified Auramin O Stain Quencher-Decolouriser was applied for 1 minute, and the slide washed with tap water.
- Hoescht 33342 (10µg/ml) was applied for 6 minutes to stain the DC nuclei, then 1 drop of Anti-fade medium (Dako) was added, a coverslip was placed on the slides, and the cells were examined using a 100X oil immersion objective on the fluorescent microscope using the FITC (for AFB) and DAPI filters (for Hoescht 33342). The slides were analyzed using a 100X lens on an Olympus IX51 Fluorescent Microscope. (Olympus Europa GmbH, Germany). For each sample, for 50 DCs were counted and the number of bacteria per cell and the percentage of infected cells were calculated.

3.2.5 Cell death assessment using the PI exclusion assay and Annexin/7-AAD assays

Cell death plays key roles in a variety of biological processes and cells die by different mechanisms, each with its own phenotype. For this reason, different cell death assays were used to identify particular aspects of DC death following BCG-GFP infection.

A) Propidium iodide (PI)

The propidium iodide (PI) exclusion method (quantified by automated fluorescence microscopy) was chosen as an alternative method to flow cytometry to assess viability of BCG- infected mo-DCs. DC were infected and treated with 5mM 2DG as described in **Chapter 2** and staining mo-DCs with propidium iodide/Hoechst to determine viability, and the previously described protocol employed in **Chapter 2 Section 2.6.2**. The nuclei of live cells stain blue with Hoechst, while dead cell nuclei stain red (PI) and blue. PI penetrates the leaky cell membrane of the dead cells and is excluded from the viable and healthy cells. The cells were analyzed and photographed on the Cytell Cell Imaging System (GE) within one hour of staining. The data was analysed using the Cell Death Bioapp (GE).

B) Assessment of DC apoptosis

The PI exclusion assay is a quick cell death screening method but does not allow recognition of the mechanism of cell death as well as Annexin V-PE/7-AAD staining which also detects apoptosis, late apoptosis and non-apoptotic cell death. Therefore, flow cytometry was used to investigate apoptosis wherein DC were infected and treated with 5mM 2DG as described in **Chapter 2** and staining by Annexin V-PE apoptosis / 7-AAD as described in **Chapter 2 Section 2.5.2**.

3.2.6 Cytokine detection

Dendritic cells were infected with BCG-GFP for 24 hours or left uninfected as a negative control. LPS was applied for 24 hours (1 µg/ml) as a positive control for DC maturation and cytokine secretion. Cytokine secretion was measured in cell-free supernatants by ELISA using the MSD Proinflammatory Panel 1 (human) which was capable of detecting IFN- γ , IL-1 β , IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13 and TNF α . IL-4 measurements were disregarded since DCs were maintained in culture with IL-4, rendering it impossible to distinguish levels secreted by the cells themselves.

3.2.7 Statistical analysis

Results are expressed as means $\pm$ the standard errors of the mean (SEM). The data were analyzed with Graph Pad Prism 5 software statistical software using two-way Repeated measures ANOVA with Bonferroni post-tests comparing each condition to the uninfected control. Negative (uninfected) and positive controls (LPS) with and without 2-DG were analysed using Students T-test (two-tailed) for paired data. A p value of < 0.05 considered statistically significant.

3.3 Results

3.3.1 Assessment of the purity of monocyte-derived dendritic cells

Human monocyte derived DCs were prepared from buffy coats using immunomagnetic beads as described in Methods (**Chapter 2**). In all experiments on the day of isolation a population of DC-SIGN⁻ CD14⁺ cells were purified (ranging from 83-95%, mean 90.1%, SD 3.7%, of gated cells) (**Figure 3.2 A**). Following 6 days of culture in the presence of IL-4 and GM-CSF, a population of DC-SIGN⁺ CD14⁻ cells was reliably generated (typically ranging from 80-90% of gated cells) (**Figure 3.2 B**). When observed by phase contrast microscopy, DCs were found to have more dendrites when infected with BCG.

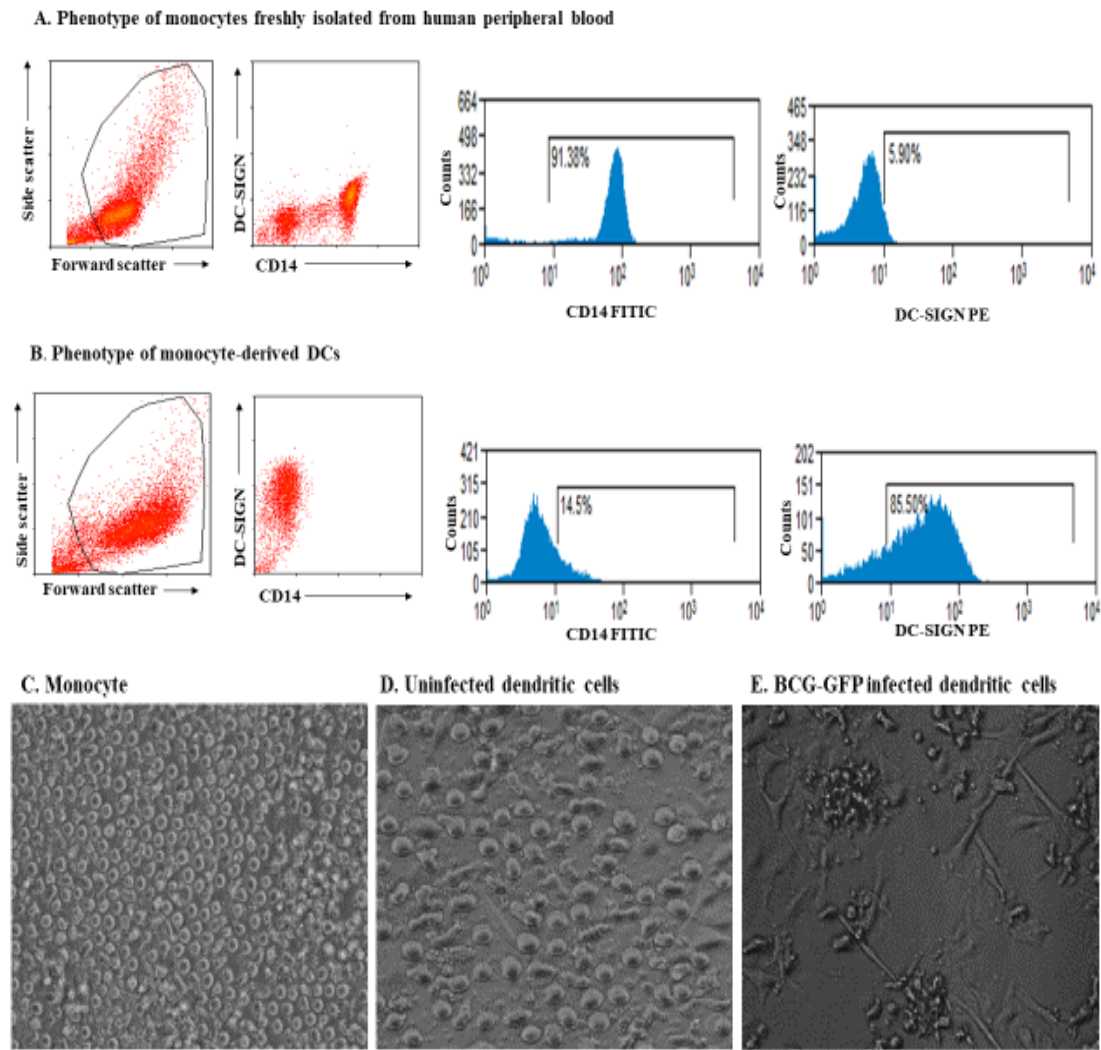


Figure 3.2: Identification of monocytes and dendritic cells before and after incubation with IL-4+GM-CSF for 6 days. Human monocytes were separated from buffy coats using negative selection and analysed for CD14 and DC-SIGN expression by flow cytometry, **A.)** before, and **B.)** after being cultured for 6 days in the presence of recombinant human IL-4 (40 ng/ml) and GM-CSF (50 ng/ml) to allow differentiation to DCs. Scatter plots and histograms are from one donor and are representative of 13 experiment. FS, forward scatter; SS, side scatter. **C.)** Phase contrast micrographs showing on the left monocytes on the day of isolation, **D.)** Uninfected DCs cultured in GM-CSF and IL-4 for 6 days and **E.)** BCG-GFP infected DCs 24 hours after infection.

3.3.2 Dose- and time-dependent responses of monocyte-derived DC to BCG

In a small pilot study to determine the effect of dose and time of infection on DC function, monocytes were obtained from two donors. This was carried out to determine the best MOI, time point and concentration of 2DG to use in subsequent experiments. DCs were generated as described in **Chapter 2 Section 2.3** and were infected with GFP-BCG at different MOI of for 24 and 48 hours. Parallel samples from each donor were analysed for the percentage of cells infected, expression of the maturation marker CD86 and cell death.

Dose response and kinetics of BCG infection

In order to assess the effect of different multiplicities of infection (MOI) of BCG on DC function and the level of infection, ratios of BCG-GFP to DCs of 50, 100 and 250 were used to infect DCs for 24 hours and 48 hours after which the cells were harvested, washed, fixed and the percent of BCG-GFP infection was assessed by flow cytometry. Zombie Red dye was used to exclude dead cells from the analysis. There was a dose-dependent increase in the percentage of BCG infected cells at 24 and 48 hours after initial infection. The level of infection was slightly higher at 48 hours compared to that at 24 hours, increased in BCG-GFP MOI 100 from 27.32% to 36.92% and in BCG-GFP MOI 250 from 52.25% to 67.08%.

Figure 3.3

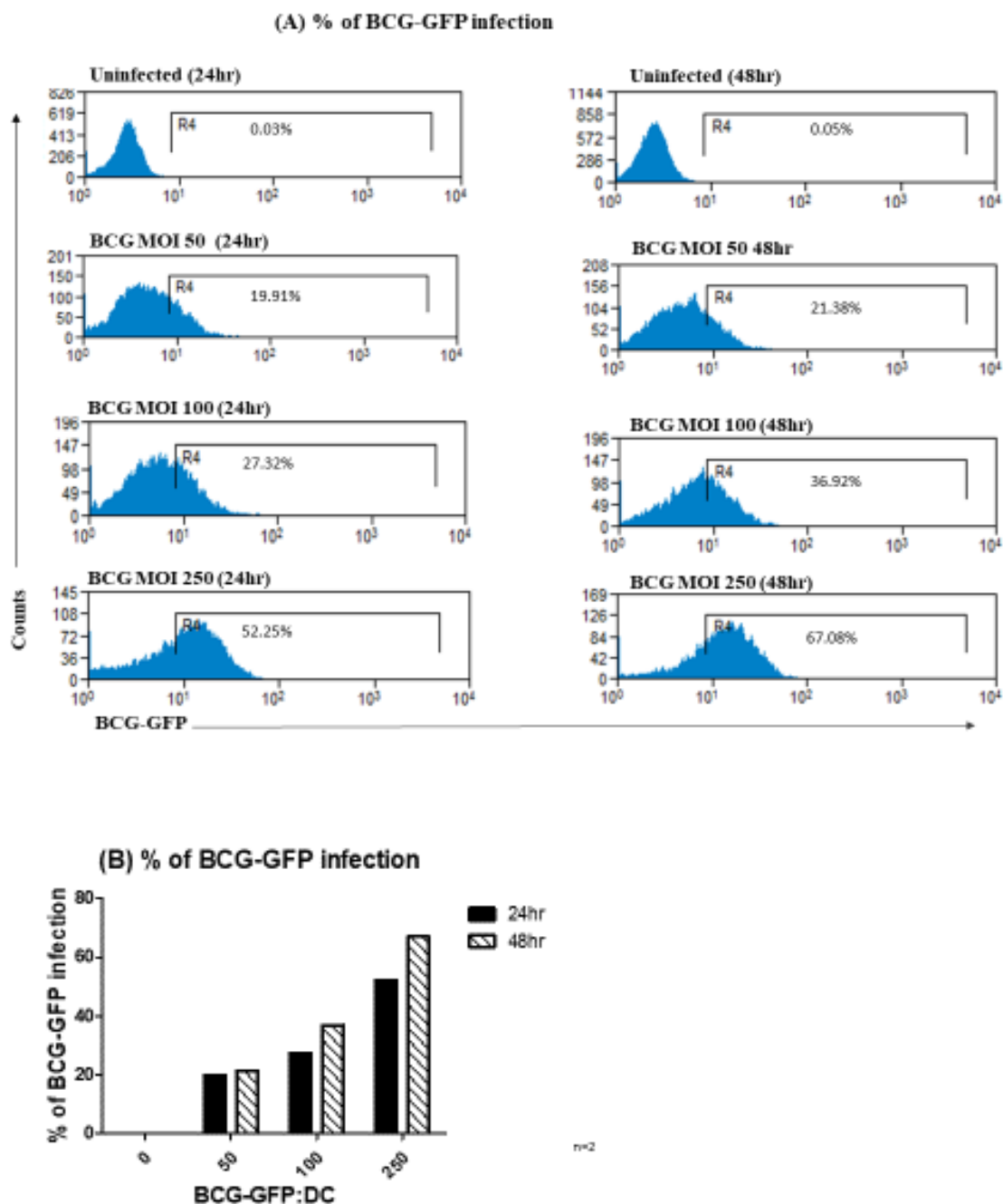


Figure 3.3: Dose response and kinetics of BCG infection. DCs were infected with different MOI of BCG-GFP (MOI of BCG-GFP: DCs was 50,100 and 250). The level of infection was assessed at 24hours and 48 hours by flow cytometry. Zombie Red dye was used to exclude dead cells from the analysis. A.) Histograms showing the percentage of GFP-positive cells from one donor and are representative of two experiments. B.) The percentage of GFP-positive cells was plotted showing the combined results for the 2 donors.

In addition to using flow cytometry to assess the rate of infection the number of mycobacteria phagocytosed per cell was determined by fluorescent microscopy (**Figure 3.4** and **Table 3.1**). DCs were infected with different MOI of GFP-BCG for 24 hours. The number of mycobacteria per DC was calculated using a nephelometer as described in **Chapter 2 Section 2.2** and the cells were incubated with BCG-GFP for 24 hours. The DCs were then washed, fixed with paraformaldehyde and processed for fluorescent microscopy. The level of infection ranged from 82% for DCs infected at MOI 50 to 100% at MOI 250. The average number of bacteria per cell were 2.88, 3.30 and 5.34 at MOI 50, 100 and 250 respectively (**Table 3.1**).

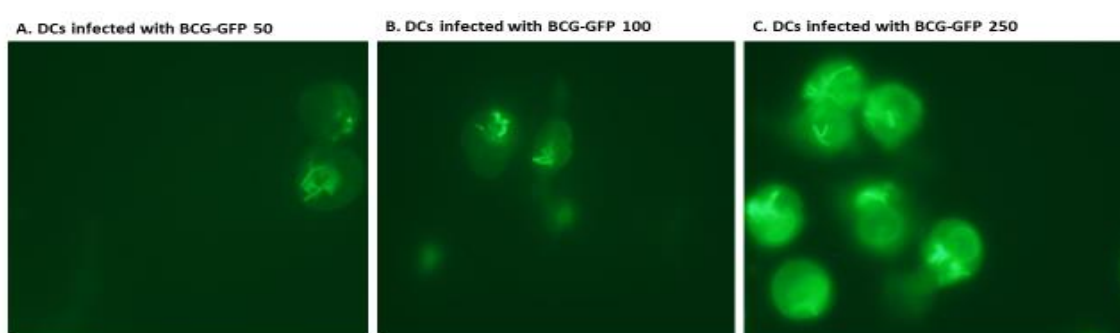


Figure 3.4: DCs were infected with different ratios BCG-GFP. Bacteria were stained with auramine, nuclei with Hoechst, and were visualised by fluorescent microscopy as follows: A.) DCs infected 50 MOI BCG-GFP; B.) DCs infected 100 MOI BCG-GFP; and C.) DCs infected 250 MOI BCG-GFP

Table 3.1: The average number of bacteria per DC. DCs were infected with different MOI of BCG-GFP. The percentage of infected cells and average number of bacteria per cell was calculated by counting the number of bacteria per cell in 50 cells per condition. Data represents means ($\pm$ SEM) of 3 different donors.

Ratio of BCG: DC*	Means	SD	Average number of bacteria/ DC
50	82.2	0.1	2.88 (1 to +10)
100	85.8	0.2	3.30 (1 to +10)
250	100	0	5.34 (2 to +10)

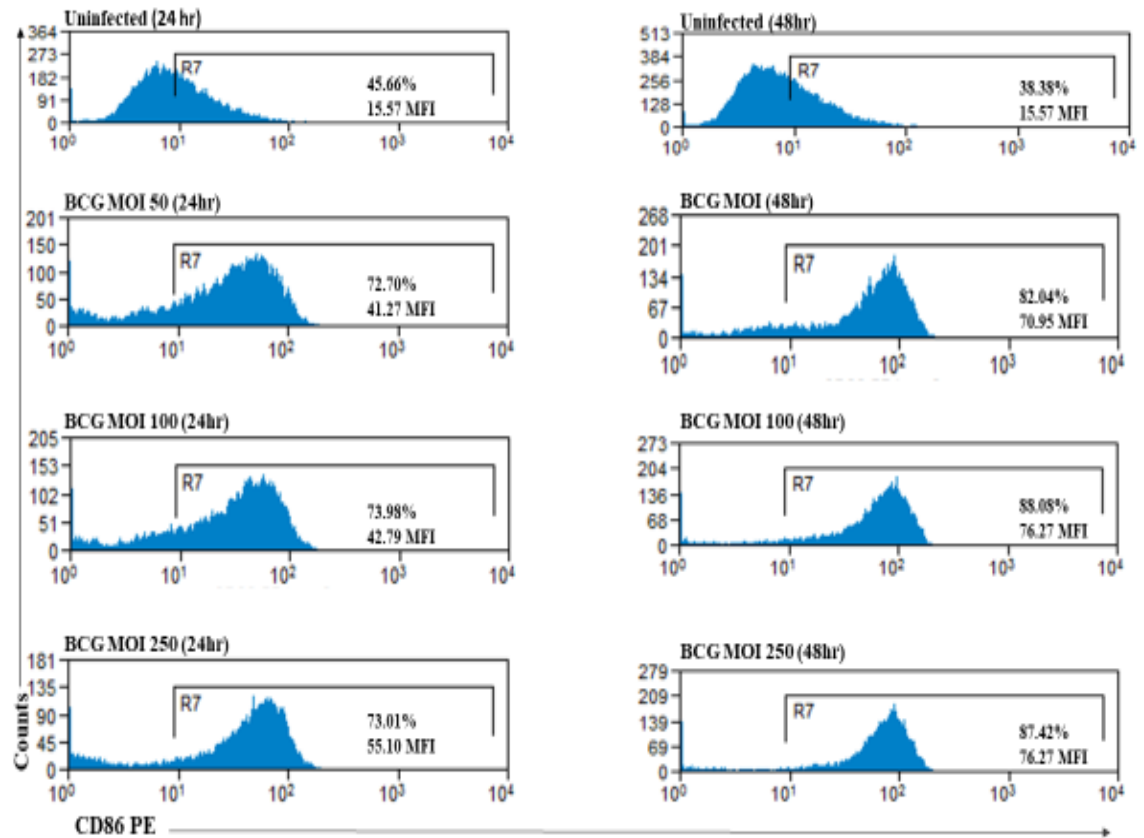
* (based on Nephelometer measurements)

Dose- and time-dependent increase in the expression of CD86 following BCG infection

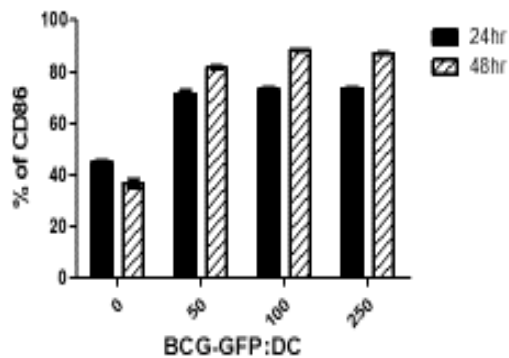
Immature DCs challenged with TLR agonists like LPS or bacteria undergo maturation and display increased migration, which is characterised by down-regulation of the capacity to capture antigen (endocytosis and phagocytosis), up-regulation of antigen processing and presentation, increased expression of costimulatory molecules and increased migratory capacity [120], [176]. In order to investigate the impact of BCG-GFP on human monocyte derived DCs, mo-DC were isolated and prepared as outlined in **Chapter 2, Section 2.3.1 & 2.3.2**. DCs were infected as delineated in **Chapter 2, Section 2.2.1** and maturation was assessed by determining the expression of the co-receptor CD86 (which had previously been shown to be the most robust indicator of maturation in response to mycobacterial infection [66]). Following staining with PE-conjugated anti- human CD86 antibody, the surface expression of CD86 (as indicated by the percentage of cells expressing the marker and the mean fluorescent intensity (MFI) of the total live cell population) was assessed by flow cytometry. Zombie Red dye was used to exclude dead cells from the analysis.

A total of $36.7\% \pm \text{SEM } 1.5\%$ of uninfected cells expressed CD86 and expression was increased to $87\% \pm \text{SEM } 0.4\%$ at a ratio of 250 after 48 hours. Percent expression of CD86 was high compared to uninfected after 24 hours and 48 hours and the MFI of CD86 was increased after 48 hours compared to MFI of CD86 after 24 hours. A total of $15.7\% \pm \text{SEM } 1.6\%$ of uninfected cells expressed CD86 and expression was increased to $76.6\% \pm \text{SEM } 0.3\%$ at a ratio of 250 after 48 hours. (**Figure 3.5**).

A. Expression of CD86



B. Expression of CD86 (%)



C. Expression of CD86 (MFI)

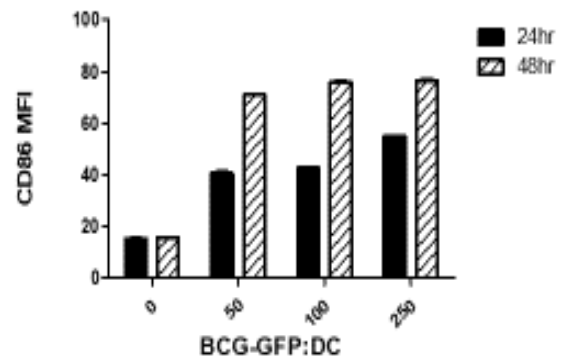


Figure 3.5: Effect of different ratios of BCG on CD86 expression 24h and 48h after infection A. DCs were infected with the indicated ratios of BCG-GFP for 24 hours and 48 hours. The surface expression of CD86 (% of CD86-positive cells and mean fluorescence intensity (MFI)) was assessed by flow cytometry; **B.** Expression of CD86 (% of live cells infected); and **C.** MFI of CD86 gating on live cells.

Dose-dependent increase in cell death following BCG infection

To assess the infected DCs viability, cells were washed to remove extracellular bacteria and stained with Zombie Red™ Fixable dye, and cell death was assessed by flow cytometry. Cell death was induced by BCG infection in a dose-dependent manner; $10.1\% \pm \text{SEM } 1.5\%$ of dead cell death in uninfected cells was increased to $60.4\% \pm \text{SEM } 2.7\%$ following BCG MOI 250 infection for 24 hours. And there was a slight increase in cell death at 48 hr compared to 24 hours (**Figure 3.6**).

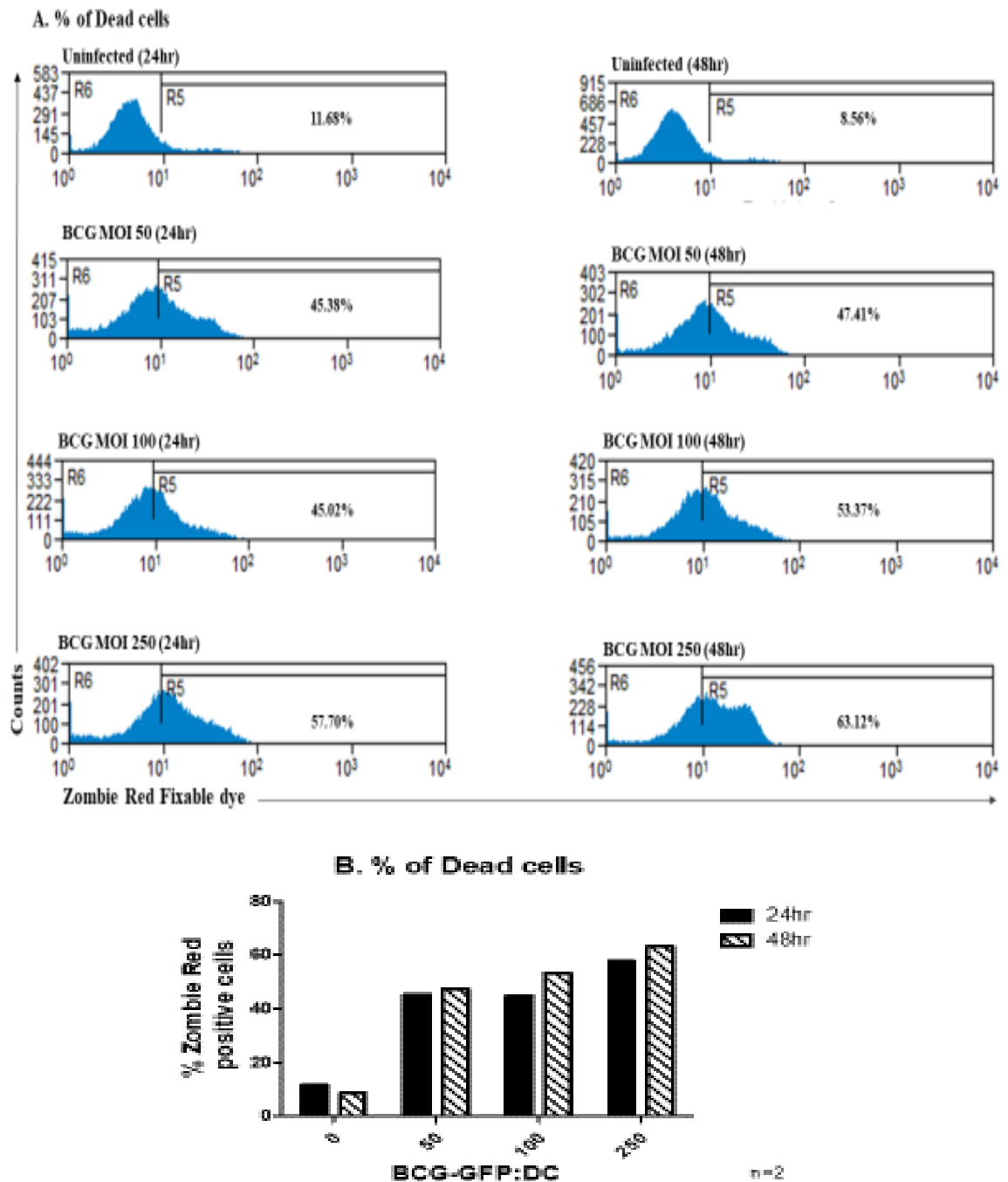


Figure 3.6: Effect of dose and time on DC viability. A.) Human DCs were infected with the indicated MOI of BCG-GFP. Cell death was assessed at 24 hours and 48 hours using Zombie Red™ Fixable Viability dye, and assessed by flow cytometry. **B.)** The graph shows the comparison between the viability of uninfected and infected by BCG-GFP at indicated ratios after 24 hours and 48 hours. The percentage of Zombie Red positive cells showed the combined results for 2 donors.

Taken together these results show that there was a dose-dependent increase in the percentage of BCG-infected mo-DCs at 24h and that the level of infection was not appreciably different at 48h. In addition, maturation of DCs could be detected at both 24h and 48h as measured by CD86 expression. To maximise the number of viable cells for analysis, 24 h post-infection was chosen as the endpoint for subsequent experiments.

3.3.3 Effect of increasing doses of the glycolysis inhibitor 2-DG on monocyte-derived DC responses to BCG infection.

The glycolysis inhibitor 2-DG was used in order to assess the effect of glycolysis on activation of infected DCs. To establish the best concentration of the inhibitor to use going forward, different concentrations of 2-DG (1mM, 5mM,10mM) were tested for their effect on the maturation and viability of DCs infected with BCG-GFP at MOI of 250. In addition, the effects of 2DG on phagocytosis of BCG were assessed.

Effect of 2-DG on the level of infection of DCs with BCG

DCs were infected with BCG-GFP at MOI of 250 bacilli per DC and incubated for 24 hours at 37°C in a CO₂ incubator. DCs were treated with 1mM, 5mM, and 10mM 2DG 30 min. before infection with BCG-GFP. After 24 hours, the cells were harvested, fixed and percent of BCG-GFP infected cells was assessed by flow cytometry. The percentage of infected DCs was not changed by any concentration of 2DG tested (**Figure 3.7**) indicating that pre-treatment of mo-DCs with 2-DG did not inhibit uptake of BCG.

(A) % of BCG-GFP infection

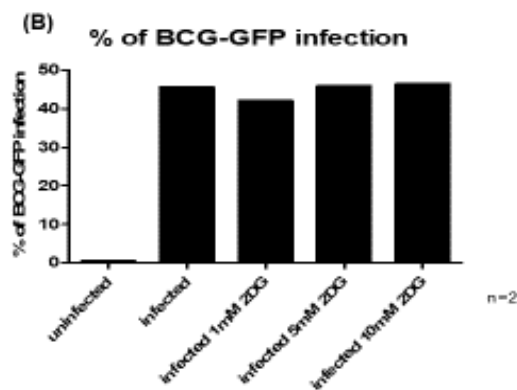
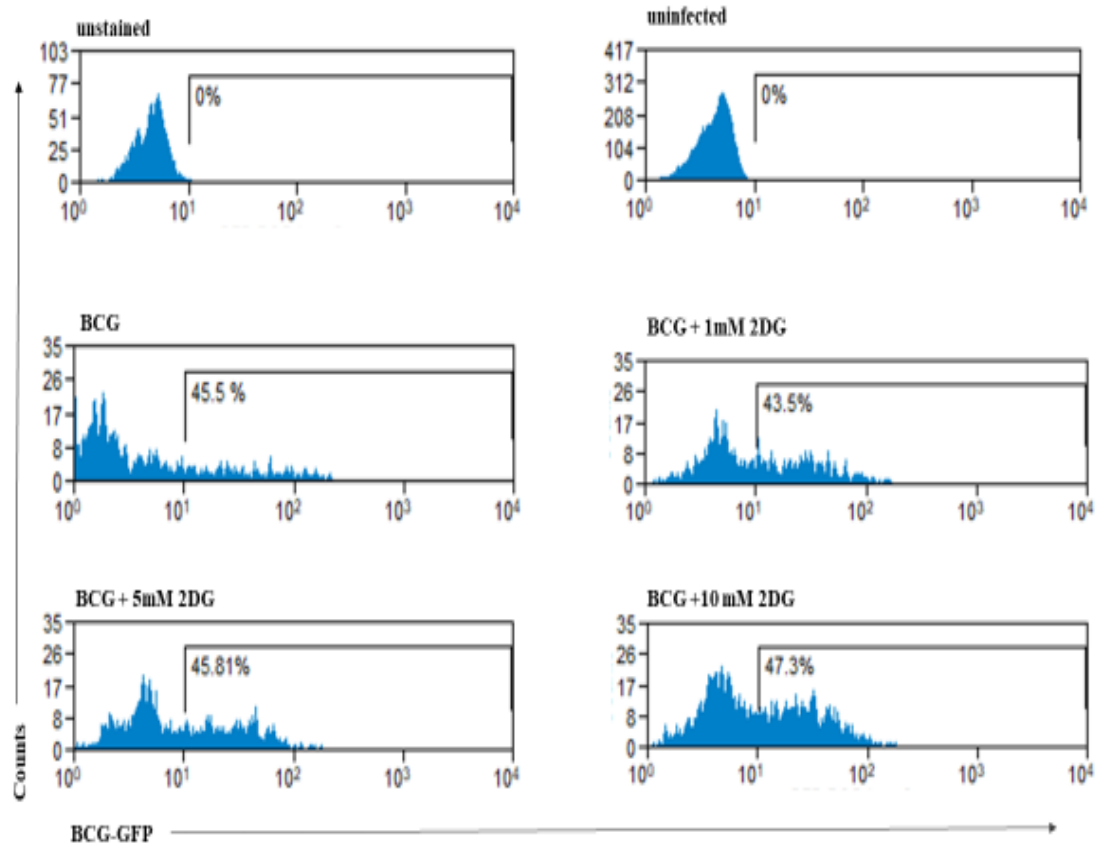


Figure 3.7: Inhibition of glycolysis with 2-DG does not inhibit uptake of BCG-GFP by DCs. DCs were treated with the indicated concentrations of 2DG 30 min before infection with BCG-GFP at MOI 250. The level of infection was assessed at 24 hours by flow cytometry and Zombie Red dye was used to exclude dead cells from the analysis. **A.** Histograms showing the percentage of GFP-positive cells in uninfected, infected treated and untreated with 2DG from one donor and are representative of 2 experiments; and **B.** show the percentage of GFP-positive cells showing the combined results for 2 donors.

Effect of increasing doses of 2-DG on the expression of CD86 following BCG infection

DCs were pre-treated with 2-DG infected with BCG-GFP at MOI 250. Maturation was assessed as before by determining the expression of the co-receptor CD86 in live cells. 16.4% $\pm$ SEM 1.2% of uninfected cells expressed CD86 and expression was increased to 66.5% $\pm$ SEM 1.2% following BCG infection of untreated DCs and to 78.5% $\pm$ SEM 0.5%, 77.9% $\pm$ SEM 0.6%, 67.7% $\pm$ SEM 0.9% in infected DCs treated with 1mM, 5mM and 10mM of 2DG respectively (**Figure 3.8 A and B**). The MFI of CD86 decreased with increasing concentrations of 2-DG (**Figure 3.8 C**).

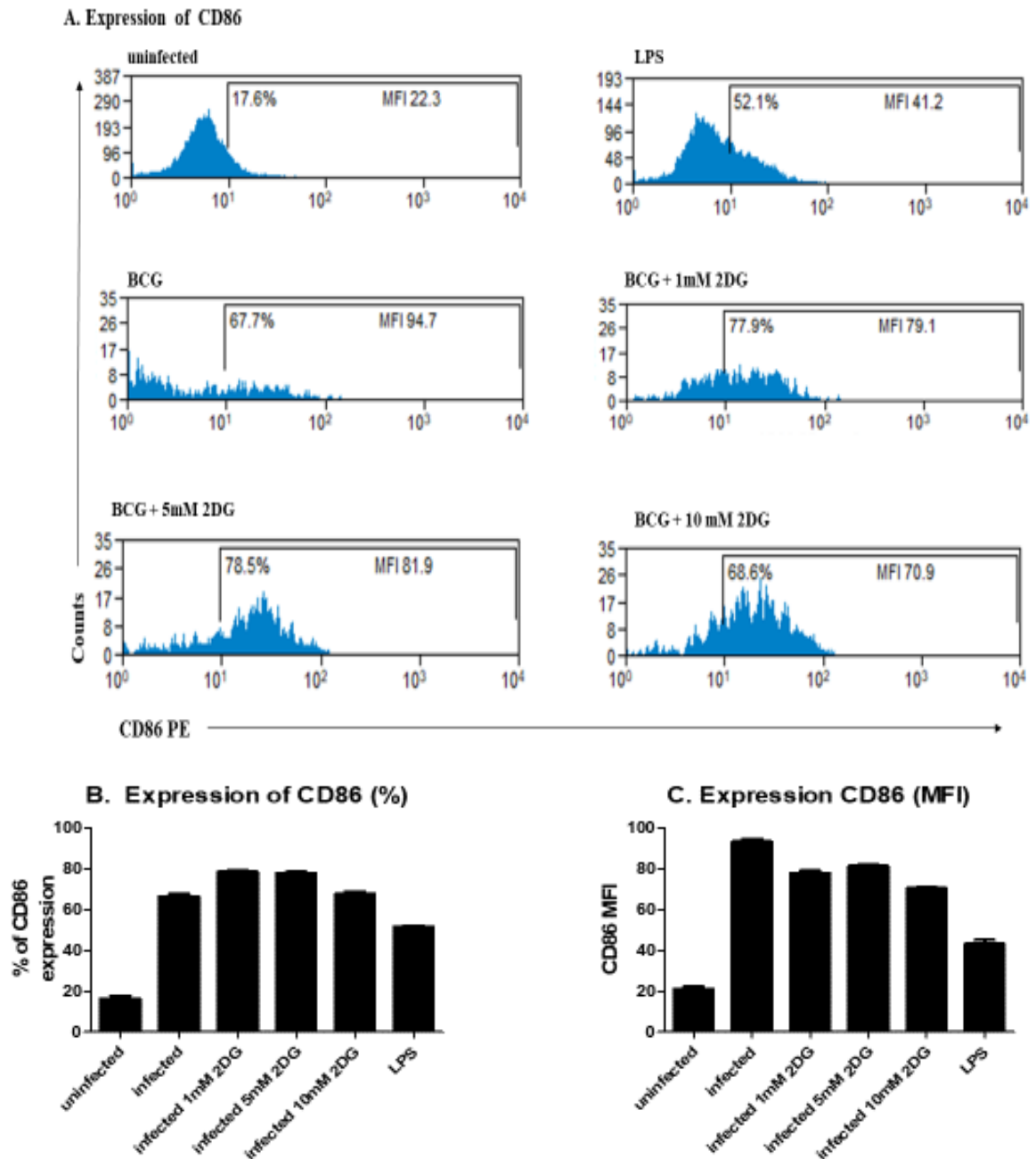


Figure 3.8: Effect of 2-DG on the expression of CD86 following BCG infection. Dendritic cells (DCs) were infected with BCG-GFP at MOI 250. Infected cells were treated with different concentrations of 2DG (1mM, 5mM, 10mM) 30 minutes before infection with BCG-GFP. Uninfected DCs were treated with lipopolysaccharide (LPS) (1µg/ml) as a positive control for maturation. Surface expression of CD86 (% positive cells and MFI) was assessed by flow cytometry and Zombie Red dye was used to exclude dead cells from the analysis **A**. Histograms showing the percentage and MFI of surface expression CD86 in uninfected, treated and untreated with 2DG from one donor and are representative of 2 experiments. **B** and **C**. illustrate the percentage and MFI of surface expression CD86 showing the combined results for 2 donors.

Effect of increasing doses of 2-DG on BCG-induced cell death

To assess DCs viability, cells were washed to remove extracellular bacteria stained with Zombie Red™ Fixable Viability dye, and cell death was assessed by flow cytometry as before. Cell death was increased from 23.02% for uninfected cells to 86.24% following infection with BCG at MOI 250. Cell death was decreased to 72.42%, 63.81%, 62.83% following treatment with 1mM, 5mM and 10mM of 2DG respectively (**Figure 3.9**).

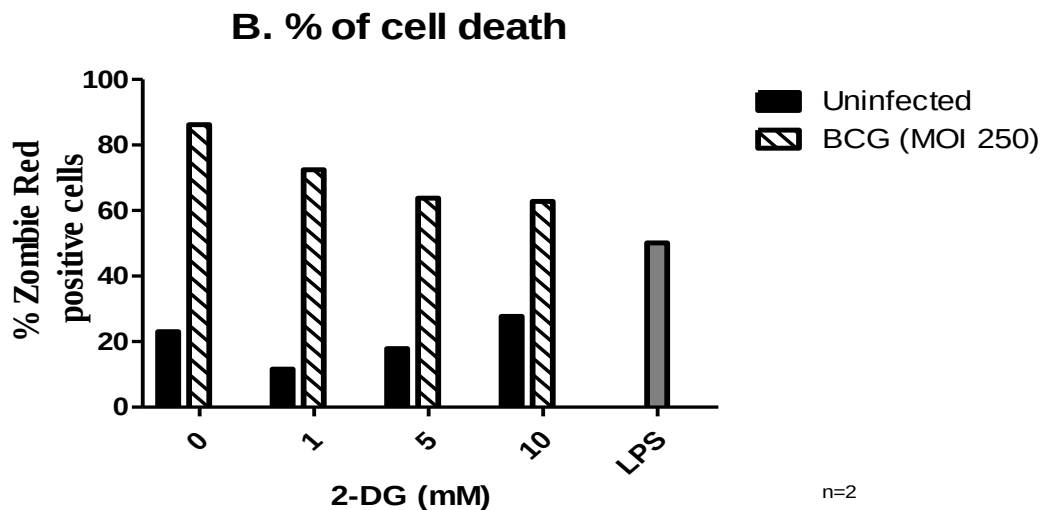
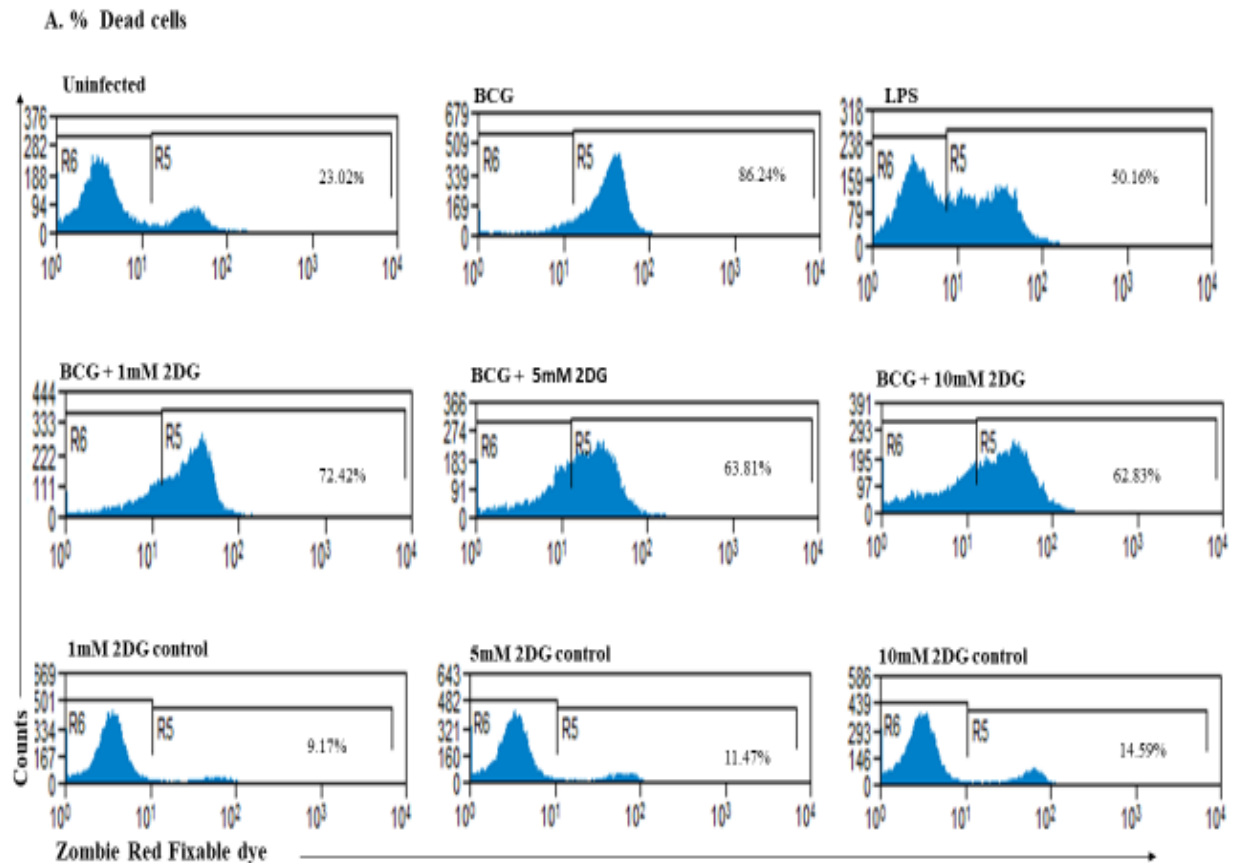


Figure 3.9: Effect of 2-DG on BCG-induced cell death. DCs were infected with MOI 250 of BCG-GFP. DCs were treated with the indicated concentrations of 2-DG 30 minutes before infection with BCG-GFP. Uninfected DCs were treated with lipopolysaccharide (LPS) (1 μ g/ml) as a positive control for maturation. Cell death was assessed at 24h by Zombie Red™ Fixable Viability stain and analysed by flow cytometry. **A.** Histograms showing the percentage of dead cells from one donor and are representative of 2 experiments; and **B.** The percentage of cells death showing the combined results for 2 donors.

3.3.4 Effect of 2-DG on mo-DC viability

To confirm that 5mM 2-DG does not cause cell death a different method was used to assess the viability of Mtb infected mo-DCs. DC were infected and treated with 5mM 2DG as described in Chapter 2. The cells were stained with propidium iodide to detect cells with a compromised cell membrane and with Hoechst to stain the total number of cells. Live and dead cells were then determined by automated fluorescent microscopy using the Cytell Cell Imaging platform as outlined in **Section 2.6.2**. By this method 5mM 2DG had no significant effect on cell viability (**Figure 3.10 A and B**).

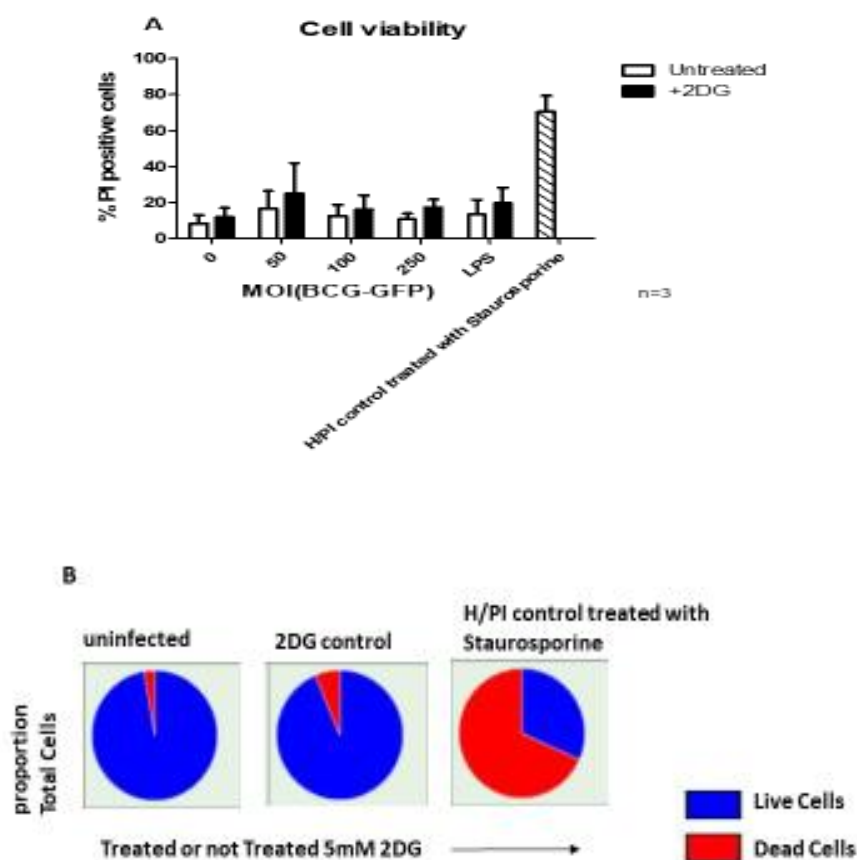


Figure 3.10: 5mM2DG does not reduce mo-DCs viability. DCs were infected with the indicated ratios of BCG-GFP and treated with 5mM 2DG in duplicate wells for each condition. Uninfected DCs were treated with lipopolysaccharide (LPS) (1µg/ml) as a positive control and staurosporine (20 µg/ml) as a positive control for cell death. 24 hours after incubation, cells were stained with PI 50µg/mL, Hoechst 33258 20µg/mL and Hoechst 33342 20µg/mL and analysed using the Cytell Imaging System. **A** depicts cell viability with or without treatment with 5mM 2DG. Data represents means ($\pm$ SEM) of 3 different donors. Panel **B** depicts the proportion of live (blue) and dead (red) cells at 24 hours following treated or not treated with 5mM 2DG. Data equates to one representative donor.

3.3.5 Effect of the glycolysis inhibitor 2-DG (5mM) on monocyte-derived DC responses to different MOI of BCG

Based on the results of the experiments above and previous publications [158] 5mM 2-DG was chosen to inhibit glycolysis in the following experiments as it did not induce cell death above that of untreated cells (**Figure 3.9**). The cells (from 3 donors) were infected with different MOI of BCG-GFP for 24 hours before analysis.

Effect of 2-DG on uptake of different doses of BCG by DCs

The percentage of DCs infected with different MOI of BCG-GFP in the presence or absence of 5mM 2-DG was determined by flow cytometry after 24 hours of infection. There was no significant difference in the percentage of DCs infected with any MOI of BCG-GFP tested, with or without 2-DG treatment (**Figure 3.11 A**).

Effect of 2-DG on the expression of CD86 following BCG infection

DCs were infected with different MOI BCG-GFP as described above. Maturation was assessed in DCs infected with BCG-GFP for 24 hours as before by measuring surface expression of CD86 (% and MFI) by flow cytometry. Zombie Red dye was used to exclude dead cells from the analysis. $36\% \pm \text{SEM } 13.8\%$ of uninfected cells expressed CD86 and expression was increased to $67.080\% \pm \text{SEM } 17.7\%$, $80\% \pm \text{SEM } 13.1\%$, $68.2\% \pm \text{SEM } 8.5\%$ at a ratio of 50, 100 and 250 respectively. There was a slight increase in expression with increasing MOI of BCG-GFP but 2-DG had no effect on the percentage of DCs expressing CD86. In contrast CD86 MFI was decreased by 2-DG but this did not reach statistical significance (**Figure 3.11 C&D**). The donor-to-donor variation in CD86 MFI value was high, therefore the data for untreated and 2DG-treated cells was normalised to the corresponding values for uninfected cells. When the normalised data was analysed by 2-way ANOVA there was a statistically significant inhibition of CD86 expression at the MOI of 250 (**Figure 3.11 E**).

Dose-dependent increase in cell death following BCG infection and treated with 5mM 2DG

DCs viability was determined by staining the cells with Zombie Red™ Fixable Viability dye followed by flow cytometry as before. There was a significant decrease in cell death at MOI of 100, and 250 BCG-GFP with 2DG treatment and 2-DG also reduced cell death induced by LPS (**Figure 3.11 B**).

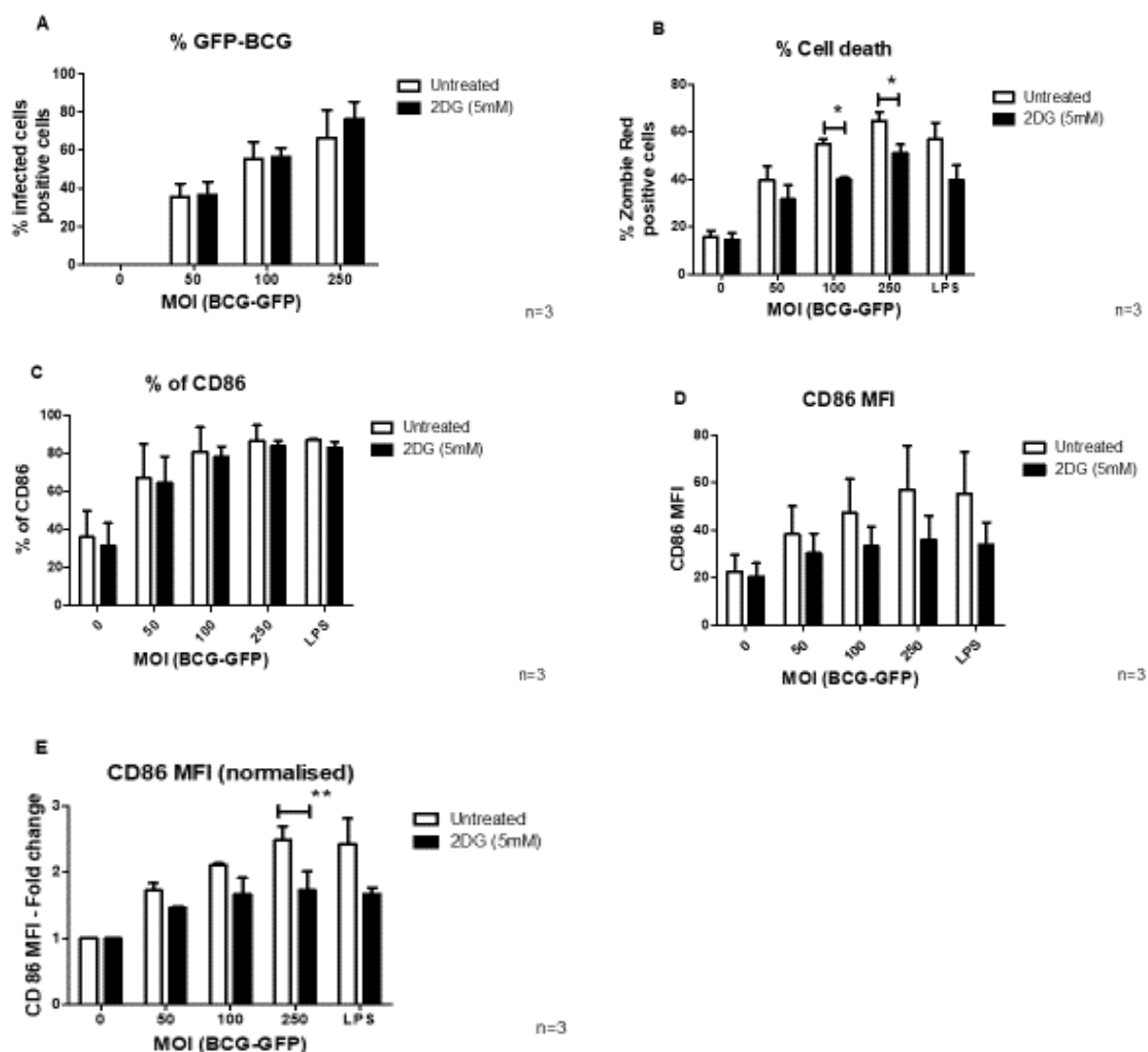


Figure 3.11: Effect of the glycolysis inhibitor 2-DG on monocyte-derived DC responses to BCG infection. moDCs were untreated or treated with 5mM 2DG 30 minutes before infection with varying MOI of BCG-GFP for 24 hours. Uninfected DCs were treated with lipopolysaccharide (LPS) (1 µg/ml) as a positive control for maturation and staurosporine (20 µg/ml) as a positive control for cell death. The level of infection was assessed at 24 hours by flow cytometry. Zombie Red dye was used to exclude dead cells from the analysis. **A.** shows the percentage of BCG-GFP infected DCs; **B.** illustrates the viability of DCs infected with different MOI of BCG-GFP with and without 2-DG treatment. Cell death was assessed at 24h by Zombie Red™ Fixable Viability and analysis by flow cytometry * $p < 0.05$; **C and D.** illustrate surface expression of CD86 (% and MFI); **E.** MFI data normalised to the uninfected control, analysed by 2-way ANOVA showing statistically significant inhibition of CD86 expression at the MOI of 250 $p < 0.01$. Data assessed by flow cytometry. Data represents means ($\pm$ SEM) of 3 different donors.

3.3.6 Effect of 2-DG on the expression of HLA-DR in BCG-infected DCs

DCs were untreated or treated with 5mM 2-DG as before and infected with BCG-GFP at MOI 100 after 24h expression of HLA-DR were assessed by flow cytometry. The percentage of cells infected with BCG and cell viability were assessed in parallel as before. As before, there was no significant difference between the uptake of BCG-GFP in untreated compared to 2-DG-treated cells (**Figure 3.13 C**).

Antigen presentation and migration potential were then assessed by determining the expression of the co-receptor HLA. Following staining with APC-HLA-DR antibody, surface expression of HLA-DR (% and MFI) was assessed by flow cytometry. Zombie Red dye was used to exclude dead cells from the analysis.

The majority of uninfected DCs expressed HLA-DR and the percentage of cells expressing it were not significantly altered by infection with BCG or by 2DG treatment. The fluorescence intensity was increased in BCG-infected DCs compared to uninfected cells indicating that there was increased expression of the protein on the cell membrane of infected cells. Inhibition of glycolysis with 2DG did not significantly influence expression of HLA-DR (**Figure 3.12**).

Effect of 2-DG on cell death

In parallel with the above, cell death was assessed in the same DCs by Zombie Red staining and flow cytometry as before. Cell death was $2.9\% \pm \text{SEM } 0.9\%$ in uninfected DCs, slightly increased in DCs infected at MOI 100 to $5.590\% \pm \text{SEM } 1.5\%$ and treatment with 2DG did not significantly effect on the death of infected cells in this series of experiments (**Figure 3.13 A& B**).

A. Expression of HLA-DR

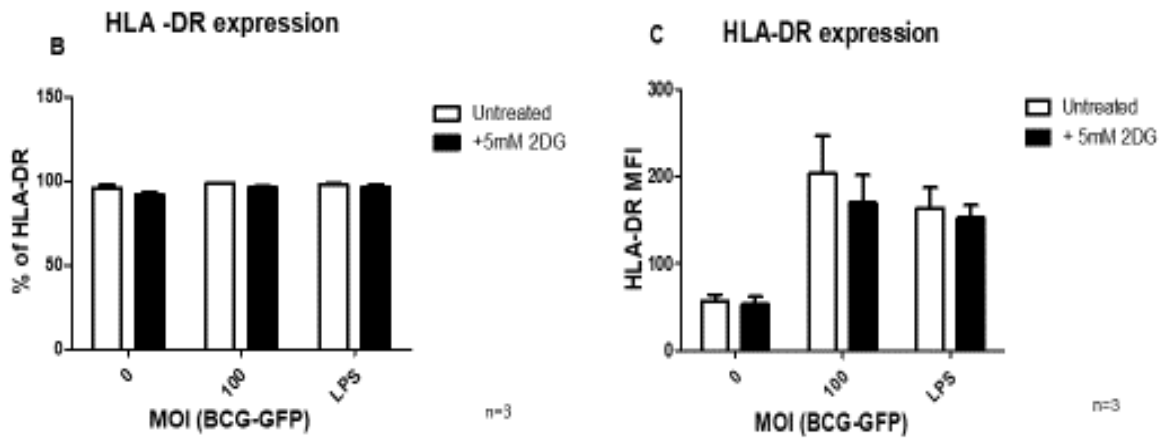
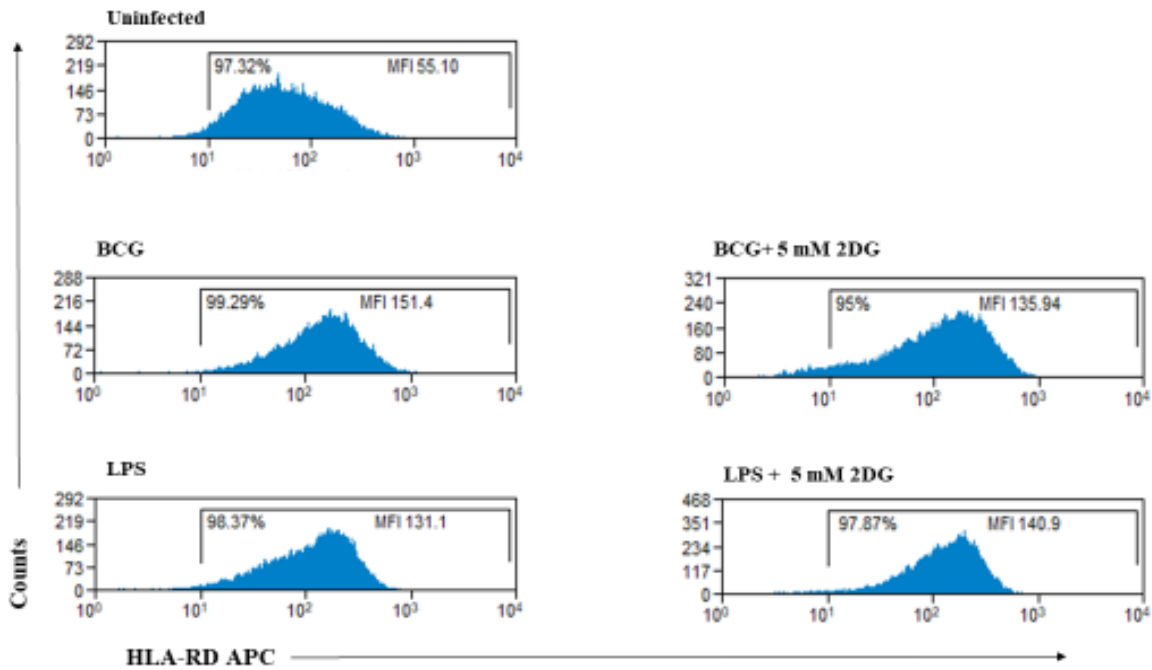


Figure 3.12: Effect of the glycolysis inhibitor 2-DG (5mM) on HLA-DR expression. DCs were treated with 5mM 2DG 30 minutes before and infected with BCG-GFP at MOI 100. Uninfected DCs were treated with lipopolysaccharide (LPS) (1µg/ml) as a positive control for maturation and staurosporine (20 µg/ml) as a positive control for cell death. The level of infection was assessed at 24 hours and analysed by flow cytometry. Zombie Red dye was used to exclude dead cells from the analysis. **A.** Histograms showing the percentage and MFI of surface expression HLA-RD in uninfected, treated and untreated with 2DG from one donor and are representative of 3 experiments.; **B.** and **C.** demonstrate surface expression of HLA-DR (% and MFI) was assessed by flow cytometry. Data represents means (± SEM) of 3 different donors.

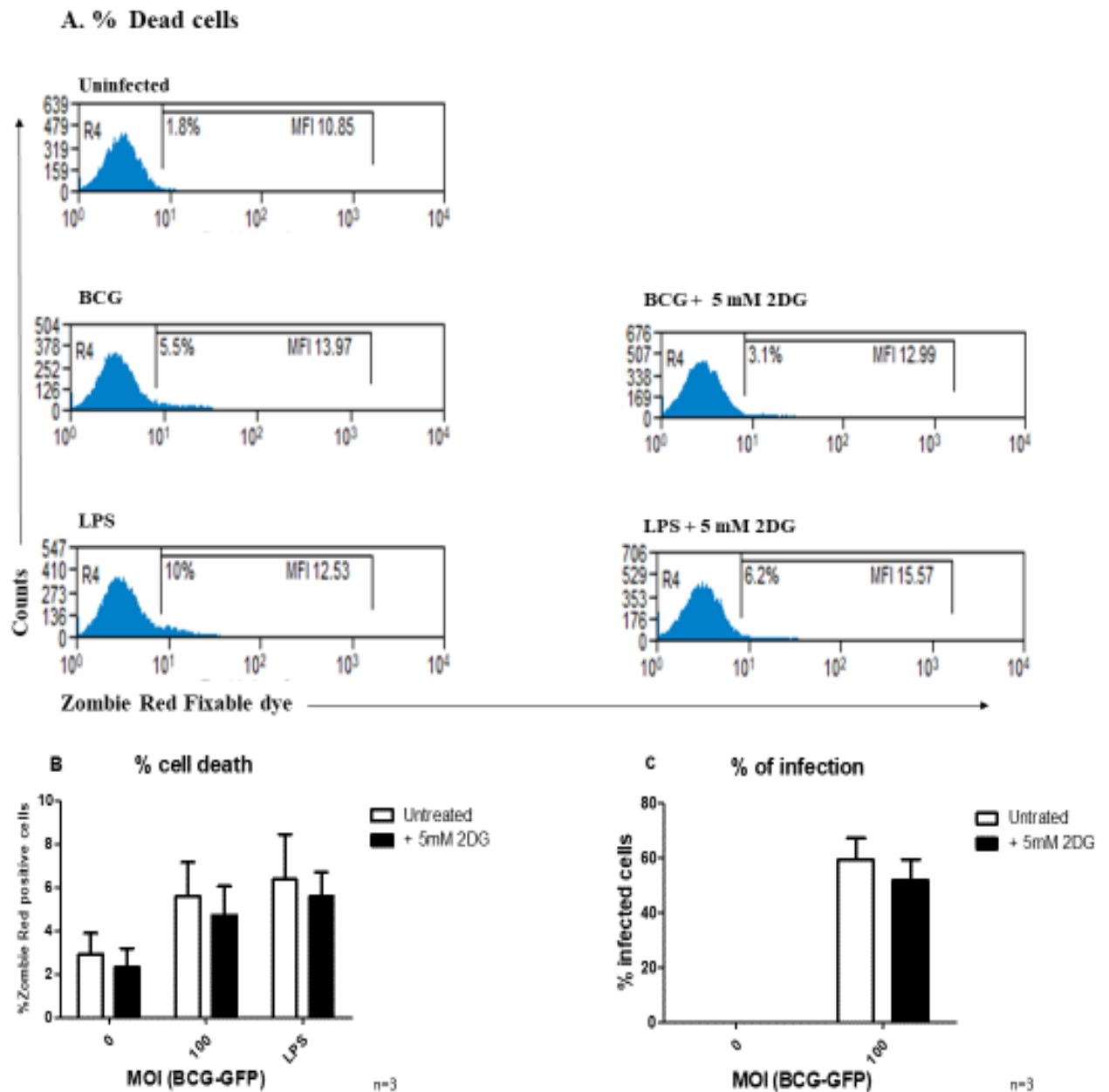


Figure 3.13: Effect of the glycolysis inhibitor 2-DG (5mM) on cell death and uptake of BCG carried out in parallel on those DCs used to assess HLA-DR expression (above). DCs were treated with 5mM 2DG 30 minutes before and infected with BCG-GFP at MOI 100. Uninfected DCs were treated with lipopolysaccharide (LPS) (1 μ g/ml) as a positive control for maturation and staurosporine (20 μ g/ml) as a positive control for cell death. The level of infection was assessed at 24 hours and analysed by flow cytometry. Zombie Red dye was used to exclude dead cells from the analysis. **A.** Histograms showing the percentage of dead cells from one donor and are representative of 3 experiments; **B.** illustrates BCG-induced cell death; and **C.** demonstrates the uptake of BCG-GFP with and without 5mM of 2DG. Data represents means ($\pm$ SEM) of 3 different donors.

3.3.7 Investigation of the mechanism of cell death induced by BCG

The results so far show that BCG induces cell death in DCs 24h post infection but do not provide any information on the mode of cell death that the cells undergo since neither Zombie Red or PI can distinguish between apoptotic and necrotic cells, Apoptotic cells externalize phosphatidylserine which binds fluorescently labelled Annexin V. Necrotic cells can also stain positive for Annexin V as their cell membrane lyses and the Annexin V enters the cell and binds to PS on the inner leaflet of the plasma membrane. The combination of Annexin V with a nuclear dye such as 7-AAD - to stain necrotic cells with a compromised plasma membrane - can be used to identify apoptotic cells which have an intact plasma membrane (7-AAD negative) and are positive for Annexin V. Therefore, to investigate the mechanism of cell death induced by BCG infection and inhibited by 2-DG, monocyte-derived DCs were infected with BCG-GFP at MOI 50, 100 and 250 and untreated or treated with 5mM 2-DG to inhibit glycolysis.

The levels of apoptosis were determined by staining with Annexin V-PE and 7-AAD followed by analysis by flow cytometry. Cells which are positive for Annexin V but exclude 7-AAD are considered to be apoptotic. DC did not undergo apoptosis with or without treatment with 2DG. The percentage of cells undergoing spontaneous apoptosis was decreased by BCG infection compared to uninfected cells although this did not reach statistical significance (**Figure 3.15 A**). Typical apoptosis was only observed in cells treated with the apoptotic stimulus staurosporine. There were dose-dependent increases in late apoptotic/necrotic cells, although this did not reach significance and there was no difference between untreated and 2DG-treated cells (**Figure 3.15 B**).

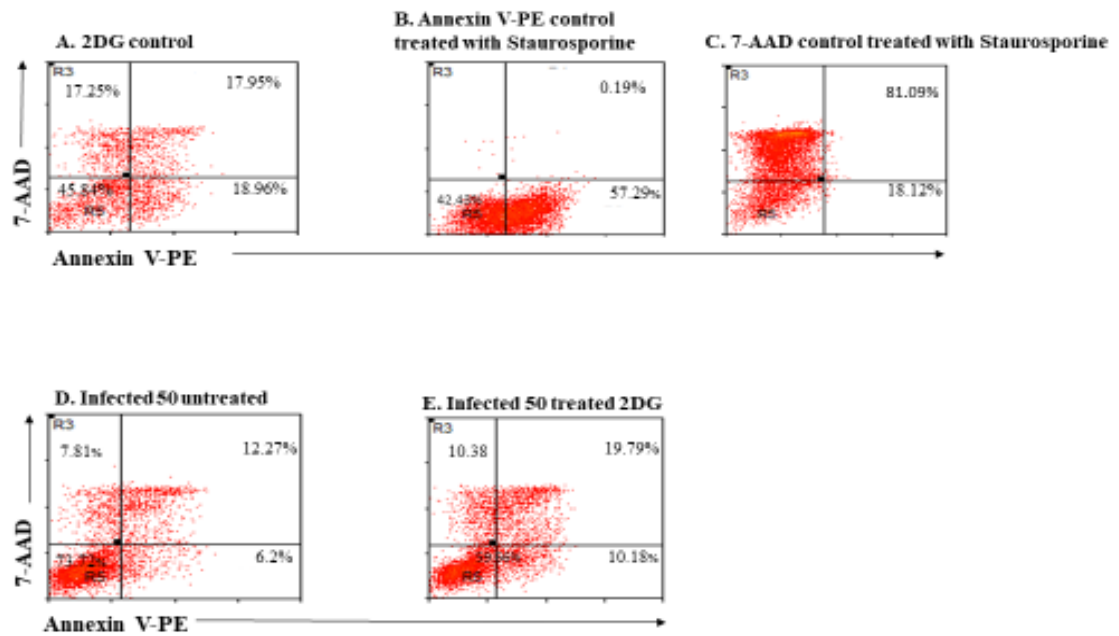


Figure 3.14: Dot plots of mo-DCs stained with Annexin V and 7-AAD in flow cytometry analysis. Annexin V-PE/7-AAD staining of uninfected DCs infected with BCG for **A.** 2DC control, **B.** Annexin V-PE, **C.** 7-AAD control, **D.** infected DCs MOI 50 untreated with 5mM 2DC, **E.** infected DCs MOI 50 treated with 5mM 2DC

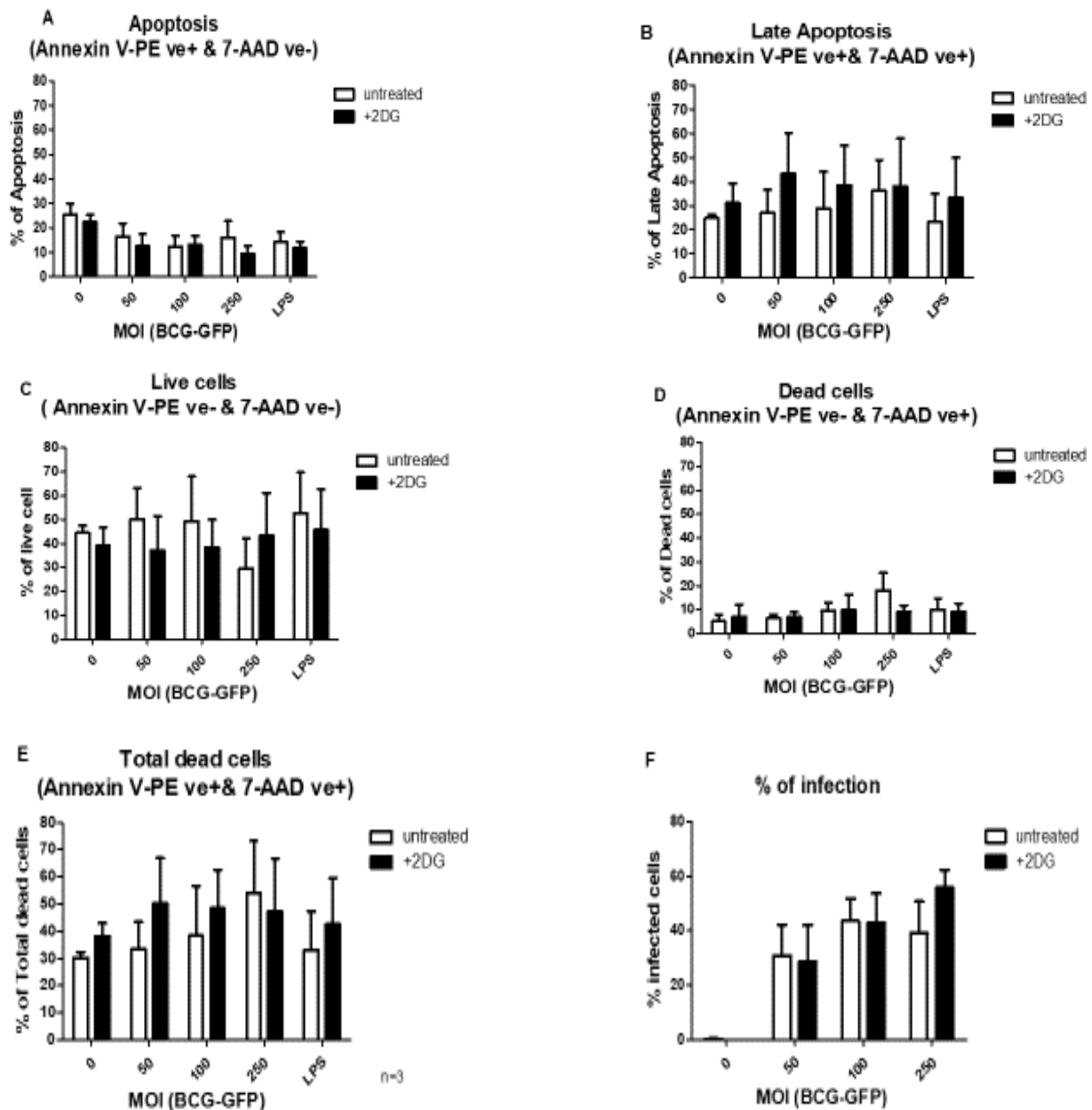


Figure 3.15: Annexin V-PE/7-AAD staining of DCs infected with BCG. DCs (untreated and treated with 5mM 2DG30 minutes before infection) were infected with MOI 50,100 and 250 with BCG-GFP. Uninfected DCs were treated with lipopolysaccharide (LPS) (1µg/ml) as a positive control and staurosporine (20 µg/ml) as a positive control for cell death, and viability of DC was assessed by Annexin V-PE and 7- AAD staining at 24 hours and analysed by flow cytometry. **A.** Apoptotic cells (Annexin V positive, /7-AAD negative); **B.** illustrates late apoptosis (Annexin V+/7-AAD double positive cells); **C.** demonstrates the live cells determined as Annexin V-PE negative and 7-AAD negative; **D.** shows the dead cells determined by percentage of 7-AAD positive /Annexin V negative cells (7-AAD single positive cells); **E.** shows the total dead cells determined by adding the number of late apoptotic cells (Annexin V-PE+ /7-AAD+) to the percentage of 7-AAD single positive cells (B+D); and **F.** shows the level of infection which was assessed at 24 hours and analysed by flow cytometry. Data represents means ($\pm$ SEM) of 3 different donors.

3.3.8 Secretion of cytokines by BCG infected dendritic cells

To determine the cytokine profile of BCG-infected DCs, infected DCs as before at MOI 50, 100, 250 were untreated or pre-treated with 5mM 2DG. Infection of DCs from each donor with BCG-GFP stimulated the release of pro- and anti-inflammatory cytokines including TNF α , IL-6, IL-8, IL-1 β and a modest increase in secretion of IL 2, IFN- γ , IL-10 and IL-12p70. While there was a significant decrease of IL-13 after treatment with 2DG in BCG infected moDCs at MOI 100, 250 and LPS treated moDCs. There was decreased production of IL-1 β , IL-12p70, and IFN- γ when DCs were treated with 2DG, but due to donor variation, these results did not reach statistical significance when the data was pooled (**Figure 3.16**).

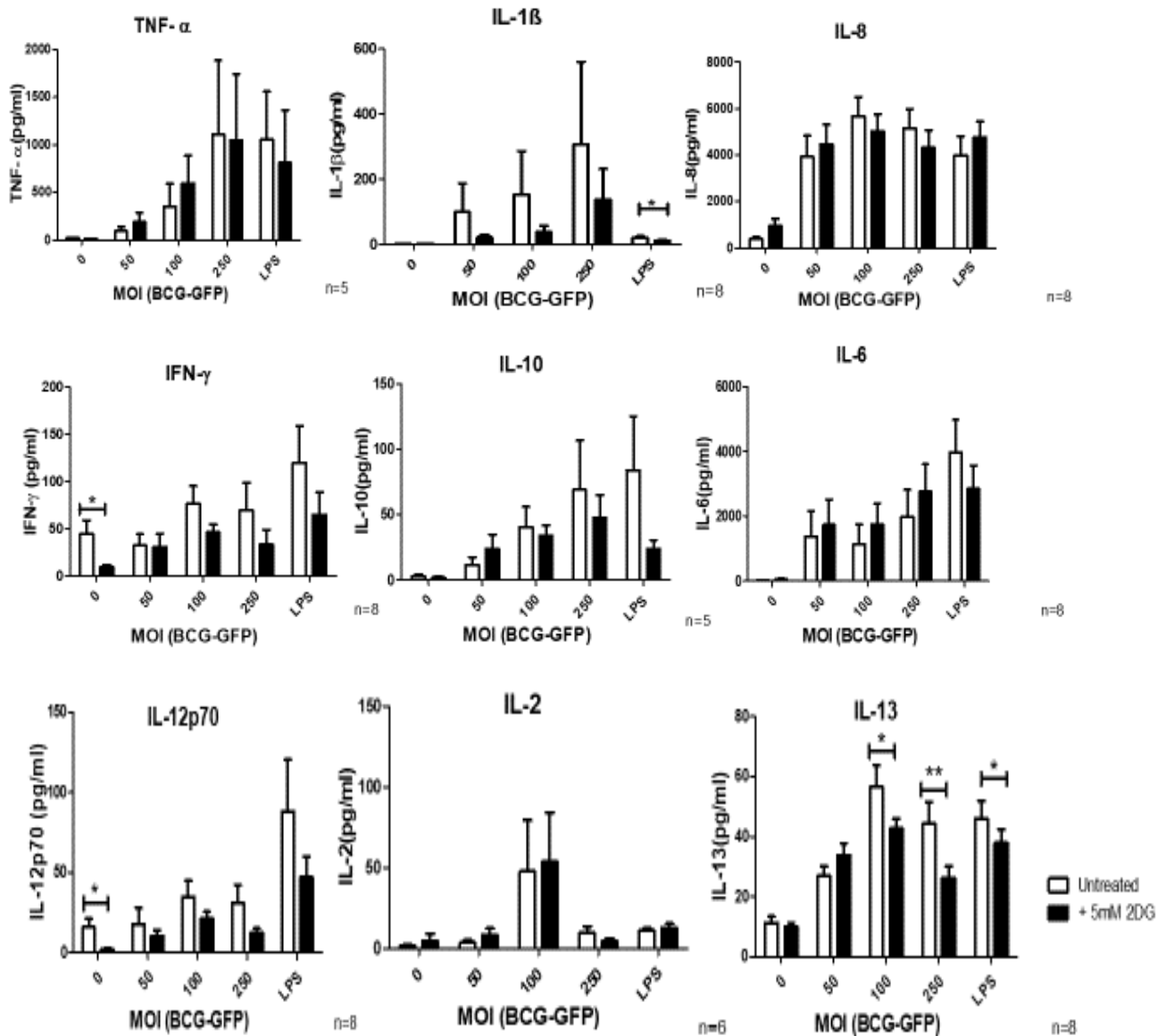


Figure 3.16: BCG-GFP infected DCs secrete cytokines. Human mo-DCs were infected with BCG at different MOI (50,100 and 250) and were untreated or pre-treated with 5mM 2DG for 30 minutes before infection. Uninfected DCs were treated with LPS. Supernatants were harvested after 24 hours and cytokine levels were measured in cell-free supernatants by multiplex ELISA. Data represents means ($\pm$ SEM) of 8 different donors in all cytokines except TNF α (n=5), IL 10 (n=6) and IL 2 (n=6).

3.3.8 BCG-GFP treated or untreated with 5mM2DG induced gene expression patterns in human monocyte-derived DCs

To assess cytokine gene expression, we investigated expression of IL-1 β , TNF α and IL-10 used mRNA levels using GAPDH as the reference gene. Although GAPDH is commonly used as a housekeeping gene its mRNA levels has been reported to change with changes in metabolic flux. Therefore, both GAPDH and B-Actin were initially used in optimisation studies for comparison. However, GAPDH Ct levels did not vary appreciably and similar results were obtained when gene expression was normalised with either housekeeping gene. There was increased IL 1 β , IL-10 and TNF α gene expression in infected cells, indicating that BCG-GFP induces upregulation of these genes in moDCs (**Figure 3.17**).

IL 1 β , IL-10 gene expression decreased with 2DG treatment while TNF α gene expression increased in moDCs infected at MOI 100 and treated with 2DG (**Figure 3.18**) and (**Figure 3.19**)

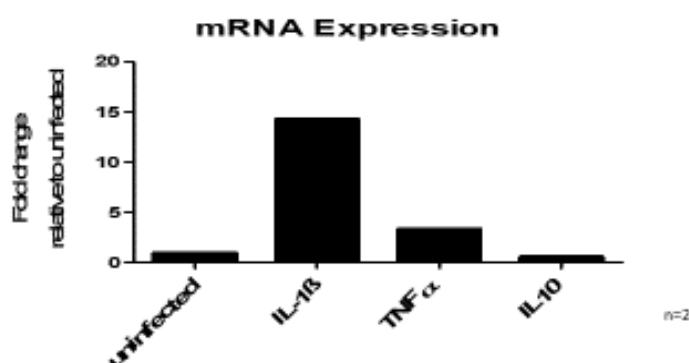


FIGURE 3.17: BCG-GFP infection induces upregulation of cytokine gene expression in moDCs. Human moDCs were infected with BCG-GFP at MOI 50 for 24 hours. Uninfected moDCs were treated with lipopolysaccharide (LPS) (1 μ g/ml) as a positive control or left uninfected (control). At 24 hours post-infection, supernatants were removed. Cells were lysed in RLT buffer, RNA extracted and cDNA generated, and qPCR was performed in triplicate using Taqman primers. mRNA expression fold-change relative to uninfected calculated using the 2- $\Delta\Delta$ Ct method used GAPDH as reference gene for two individual donors

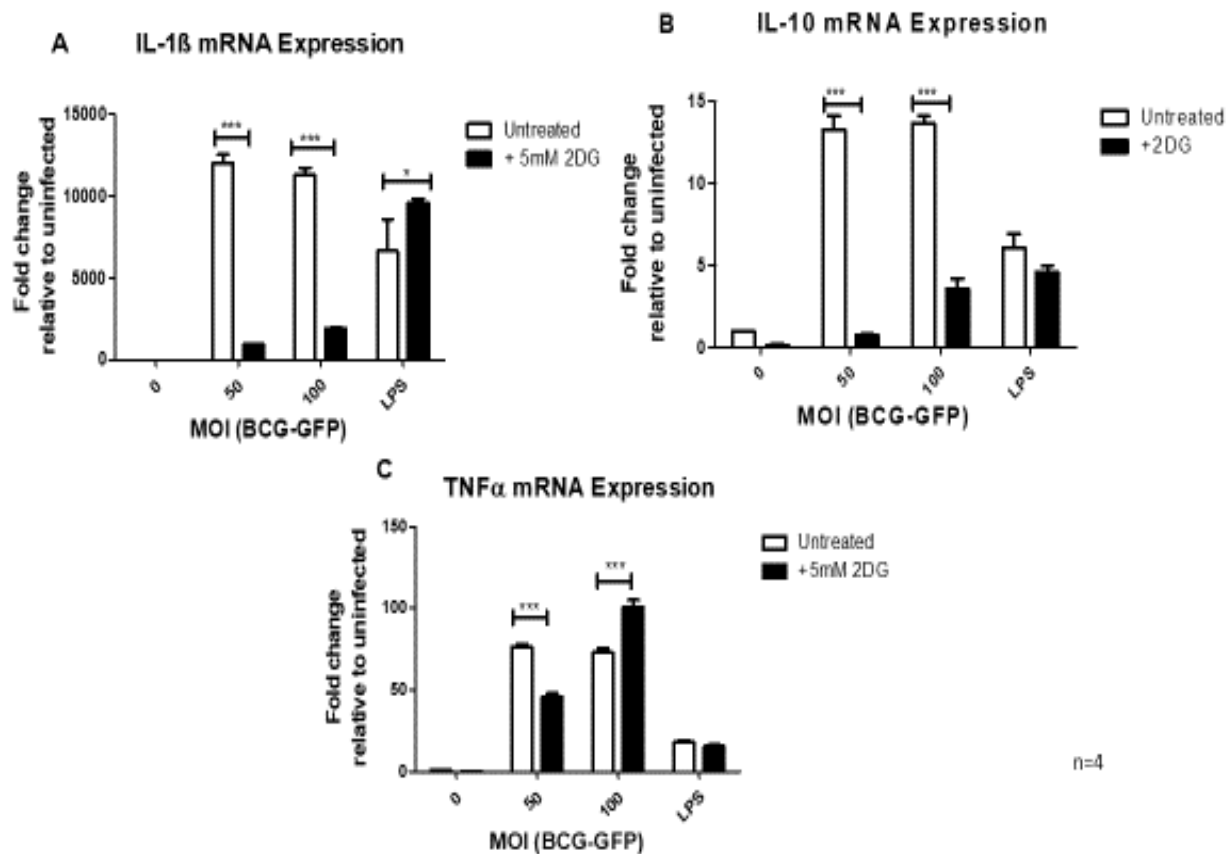


FIGURE 3.18: Using GAPDH as reference gene, 5mM 2DG inhibits upregulation of gene expression in BCG-GFP infected moDCs at different MOI. Human moDCs were untreated or treated with 5mM 2DG 30 minutes before infection with live BCG-GFP at MOI 50, 100 for 24 hours. Uninfected moDCs were treated with lipopolysaccharide (LPS) (1 μ g/ml) as a positive control or left uninfected (control). At 24 hours post-infection, supernatants were removed. Cells were lysed in RLT buffer, RNA extracted and cDNA generated, and qPCR was performed. Data was normalised and mRNA expression fold-change relative to uninfected calculated using the 2- $\Delta\Delta$ Ct method used GAPDH as reference gene. Panels A -C depict relative mRNA expression of IL-1 β (A), (B) IL-10 and TNF α (C). Data represents means ($\pm$ SEM) of 4 different donors, the data were analyzed with Graph Pad Prism 5 software statistical software using two-way Repeated measures ANOVA with Bonferroni post-tests

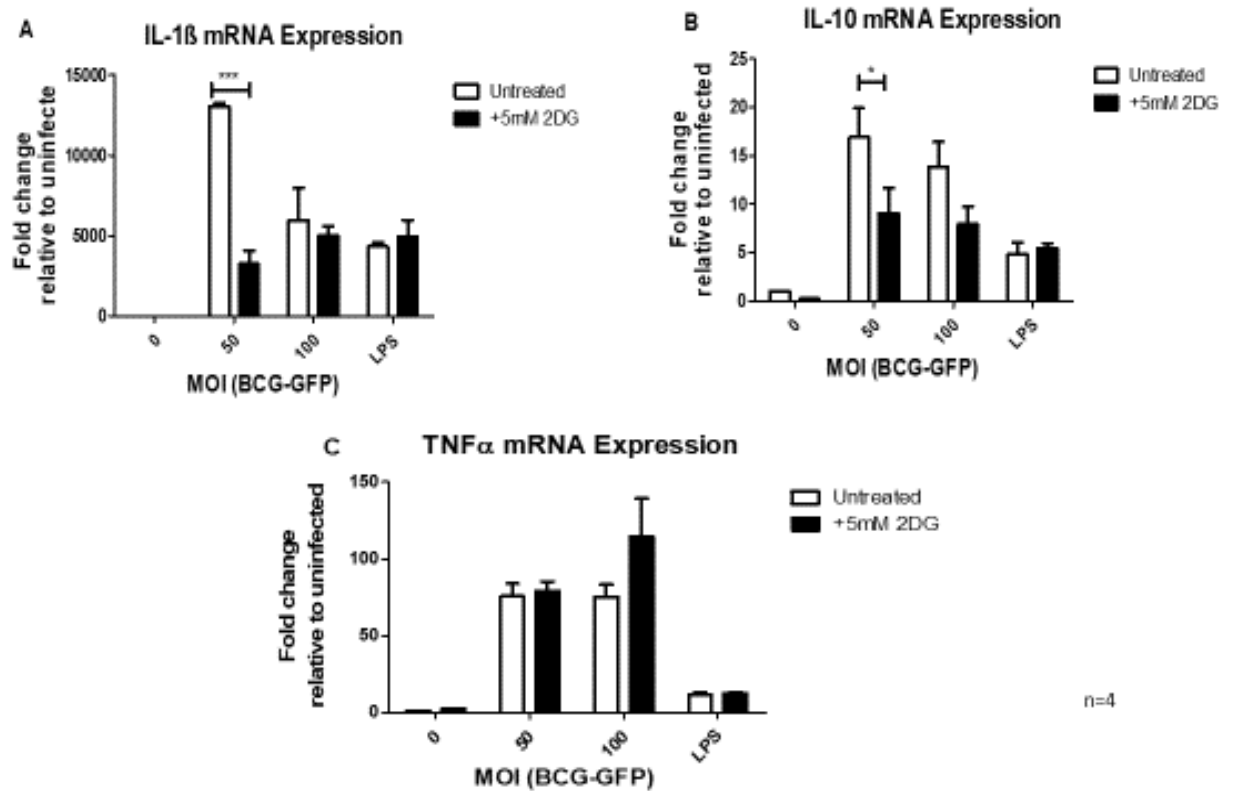


FIGURE 3.19: Using ACTB (β -actin) as reference gene, 5mM 2DG inhibits upregulation of gene expression in BCG-GFP infected moDCs at different MOI. Analysis of the samples shown in Figure 3.18 using a different housekeeping gene. mRNA expression fold-change relative to uninfected calculated using the $2^{-\Delta\Delta C_t}$ method used ACTB (β -actin) as reference gene. Panels A -C depict relative mRNA expression of IL-1 β (A), (B) IL-10 and TNF α (C). Data represent means ($\pm$ SEM) of 4 different donors, the data were analyzed with Graph Pad Prism 5 software statistical software using two-way Repeated measures ANOVA with Bonferroni post-tests

3.3.9 Inhibition of glycogen phosphorylase (GYP) by CP-91149

Many studies have identified an importance of extracellular glucose sourcing to support DC activation, but intracellular glucose stores can also be used in glycolysis. Recently, Thwe *et al*, have demonstrated that a differential utilization of glycogen and glucose metabolism regulates their optimal immune function in DCs [120]. The glycogen phosphorylase inhibitor CP-91149 was therefore used in order to assess whether glycogen degradation contributes to the activation of infected DCs.

Effect of CP-91149 on uptake of different ratios of BCG, maturation and cell viability

Mo-DC were isolated and prepared as described in **Chapter 2, section 2.3**. DCs were then infected with BCG-GFP at MOI 10, 50 and 100 bacilli per DC as outline in **Section 2.2.1**. 5 minutes prior to infection with BCG-GFP, the cells were treated with culture medium supplemented with 100 μ M CP-91149, and cells were infected and incubated at 37°C in 5% CO₂ for 24 hours. Uninfected DCs were treated with lipopolysaccharide (LPS) (1 μ g/ml) as a positive control for maturation and staurosporine (20 μ g/ml) as a positive control for cell death. After 24 hours, the cells were harvested, fixed and the percent of BCG-GFP infected cells was assessed by flow cytometry. Zombie Red dye was used to exclude dead cells from the analysis.

As expected, there was a dose-dependent increase in the percentage of BCG-infected cells. Unexpectedly, CP-91149-treated DCs phagocytosed less mycobacteria at all of the MOI tested although the values did not reach statistical significance (**Figure 3.20**).

Maturation was assessed by determining the expression of the co-receptor CD86 as before. The percentage of DCs infected with different ratios (10, 50, 100) of BCG-GFP in the presence or absence of 100 μ M CP-91149 was determined by flow cytometry after 24 hours of infection. There was decrease in the percentage of DCs expressing CD86 at a ratio of 50 and 100 BCG-GFP with CP-91149 treatment. CP-91149 also reduced the percentage of DCs expressing CD86 induced by LPS. In contrast CD86 MFI was decreased by CP-91149, but this did not reach statistical significance (**Figure 3.21 A&B**).

There was a slight decrease in cell death at MOI 50, and 100 BCG-GFP with CP-91149 treatment but CP-91149 had no effect on cell death induced by MOI 10 BCG-GFP and LPS (**Figure 3.21 C**).

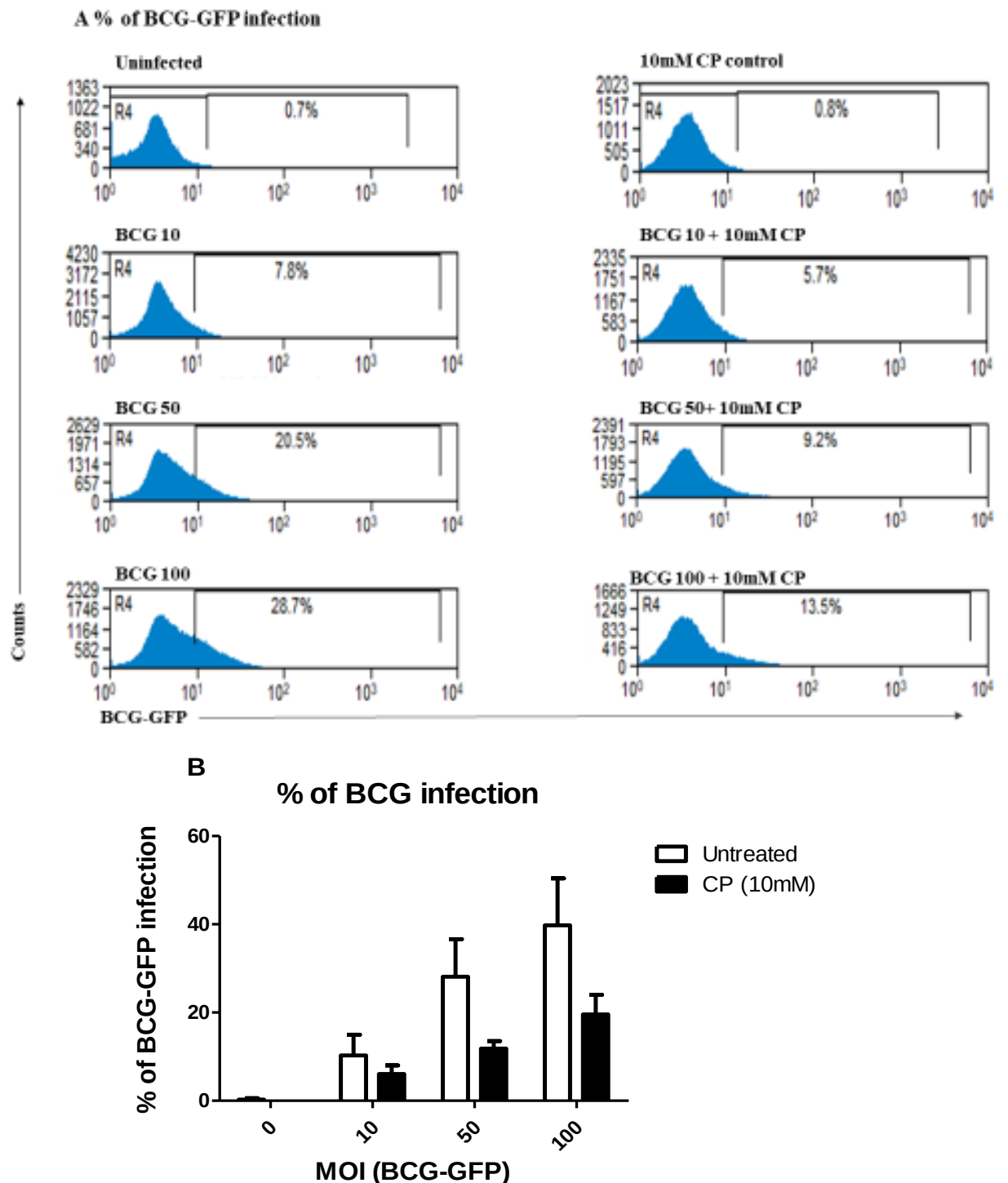


Figure 3.20: Effect of the glycogen phosphorylase (GP) inhibitor CP-91149 (10mM) on BCG-GFP infection (% and MFI). DCs were treated with 10mM CP-91149 5 minutes before infected with MOI 10, 50 and 100 BCG-GFP. Uninfected DCs were treated with lipopolysaccharide (LPS) (1 μ g/ml) as a positive control for maturation and staurosporine (20 μ g/ml) as a positive control for cell death. After 24 hours the level of infection was assessed and analysed by flow cytometry. Zombie Red dye was used to exclude dead cells from the analysis. **A.** Histograms showing the percentage of BCG-GFP infection in uninfected, treated and untreated with 10mM CP-91149 from one donor and are representative of 3 experiments; **B.** illustrate the percentage of BCG-GFP infection. Data represents means ($\pm$ SEM) of 3 different donors.

A Expression of CD86

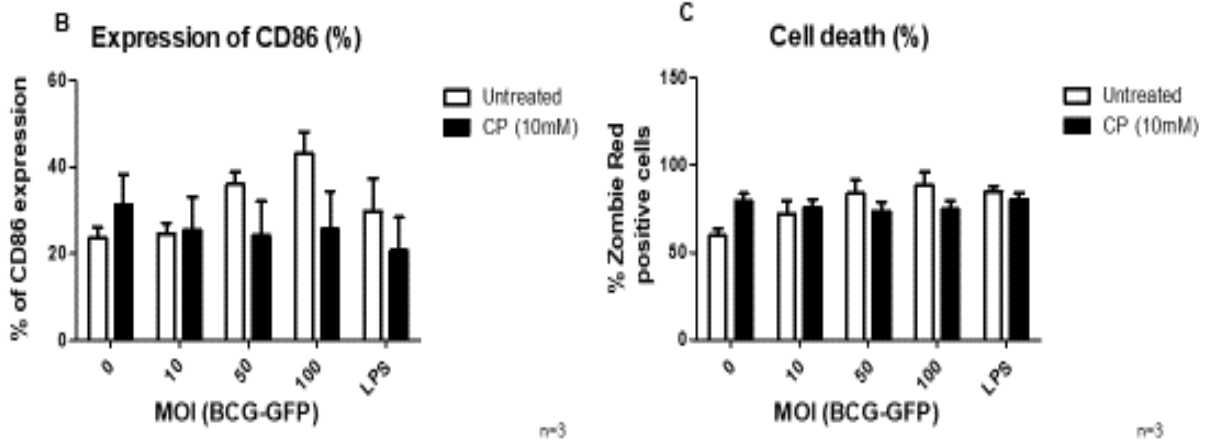
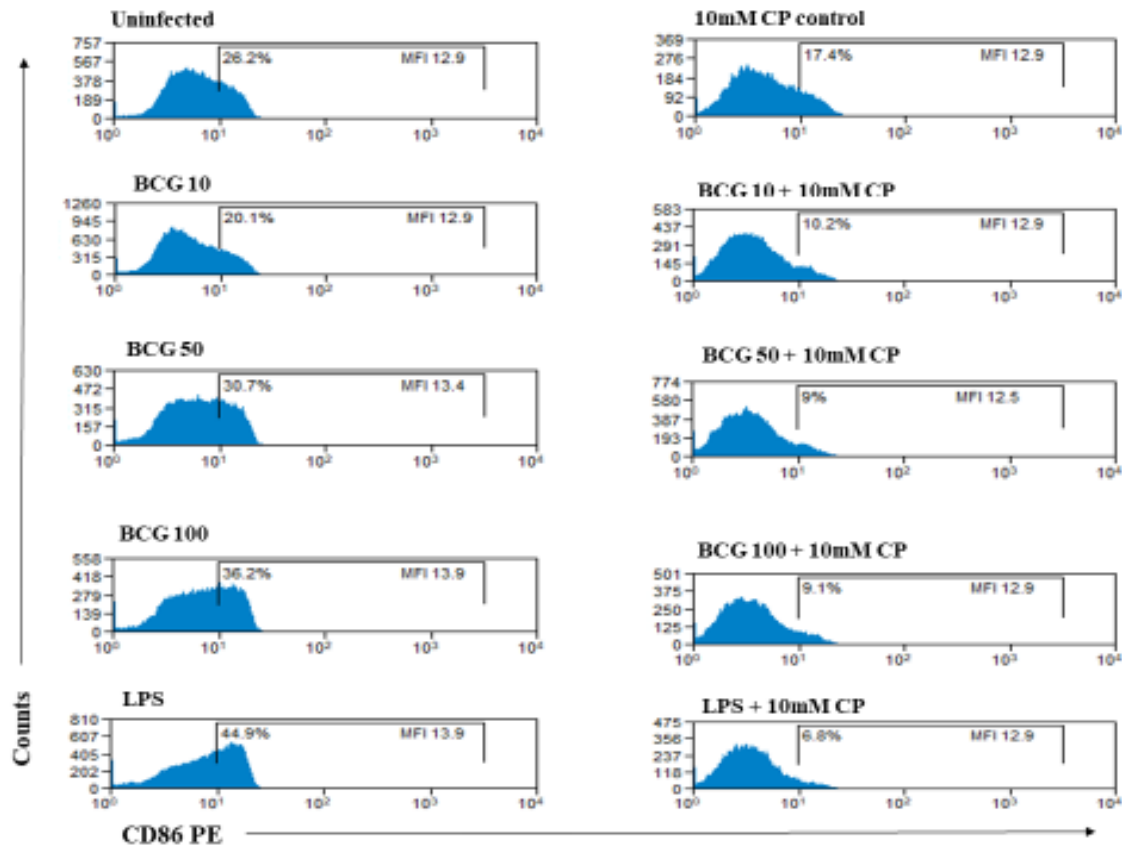


Figure 3.21: Effect of the glycogen phosphorylase (GP) inhibitor CP-91149 (10mM) on CD86 expression. DCs were treated with 10mM CP-91149 5 minutes before infection with MOI 10, 50 and 100 BCG-GFP. The level of infection was assessed at 24 hours and analysed by flow cytometry. DCs were treated with lipopolysaccharide (LPS) (1µg/ml) as a positive control for maturation and staurosporine (20 µg/ml) as a positive control for cell death. **A.** Histograms showing the surface expression of CD86 (% of positive cells and MFI) in uninfected, treated and untreated with 10mM CP-91149 from one donor and are representative of 3 experiments; Zombie Red dye was used to exclude dead cells from the analysis. **B.** illustrate the surface expression of CD86 (%) as assessed by flow cytometry; **C.** show cell death in uninfected, treated and untreated with 10mM CP-91149 (%) was assessed by flow cytometry. Data represents means (± SEM) of 3 different donors.

3.4 Discussion

Exposure of DCs to purified TLR agonists or bacteria leads to an alteration in expression of numerous genes coding for cytokines, chemokine receptors, co-stimulatory molecules, cell death etc., which influence their ability to mature to initiate an adaptive immune response as well as determining the nature of that response i.e. immunogenic or tolerogenic [22], [35], [197], [198]. Much work has been done in areas of TLR driven glycolytic reprogramming in DCs; numerous studies, most of them in mice, have demonstrated that antigen presenting cells (APCs) like macrophages and DCs rely on different metabolic pathways to support their maturation and activation following activation by TLR ligands [104], [120]. However, nothing is known about the role of metabolism in the maturation of DCs infected with mycobacteria. The aim of this chapter was to investigate the role of glycolysis in mo-DC function following infection with the TB vaccine BCG. Firstly, the effect of BCG on DC maturation, migration and viability was analysed. Exposure to BCG lead to the maturation of mo-DCs with increased expression of CD86 and MHC-II, secretion of pro- and anti-inflammatory cytokines and a dose-dependent increase in cell death. Of all of the parameters tested, expression of CD86 and secretion of IL-13 were reduced and cell viability was rescued by 2DG, indicating a role for glycolysis in some aspects of DC maturation but redundancy in others.

Changes in cell surface maturation markers like the co-stimulatory molecule CD86 and MHC-II (Class II MHC) signal the transition from immature to mature DCs. When co-cultured with BCG, flow cytometric analysis showed that there was an increase in the percentage of DCs expressing CD86 and increased fluorescence intensity of the maturation marker CD86 24 hours after infection, which was comparable to that induced by LPS. When 2DG was added, there was a consistent decrease in expression of CD86 compared to untreated controls, which attained statistical significance at the highest MOI tested (250). The majority of uninfected, immature DCs expressed of low levels of HLA-DR in agreement with previous publications [199] but the levels of expression were increased by BCG. However, inhibition of glycolysis had no effect on HLA-DR expression. The differential effects of 2DG on these maturation markers may reflect differences in the way that their expression is regulated. Both proteins are regulated transcriptionally and post-transcriptionally. In immature DCs the MHC-II protein is constitutively ubiquitinated and sequestered in endosomes and lysosomes. Following exposure to a pathogen, MHC-II molecules are loaded with antigens and translocated to the plasma membrane for

presentation to CD4⁺ T-lymphocytes [200]. The CD86 protein is also regulated by ubiquitination but transcription of CD86 gene is also increased by NFκB after LPS stimulation [201]. The different mechanism of regulation of these two co-stimulatory molecules may explain why they responded differently to inhibition of glycolysis by 2DG.

Mature murine DCs depend on ATP generated by glycolysis for their survival; if glycolysis is blocked by 2-DG they will eventually undergo apoptosis due to breakdown in mitochondrial membrane potential [101], [158], [203], [204]. This is due to switching on of the iNOS gene that produces nitric oxide, which in turn inhibits mitochondrial ETC [100]. In the present study, in contrast to this, 2DG treatment was well tolerated by human DCs following both BCG and LPS stimulation. This is consistent with a study by Malinarich et. al. who showed that after 24h incubation of human moDCs in 50mM of 2-DG, only 15-20% of the LPS-stimulated cells lost viability [137]. They found that in human moDCs a complete switch from OXPHOS to glycolysis does not occur, possibly due to epigenetic silencing of the human *NOS2* gene [205]. Immature and tolerogenic moDCs showed high fatty acid oxidation (FAO) index along with mitochondrial FAO genes, unlike mature moDCs (LPS stimulated) that showed low FAO index and expressed FAO genes associated to the peroxisome. This indicated that mitochondrial functions were reduced in mature moDCs [137]. Nevertheless, basal levels of OXPHOS supply about 40% of ATP in human moDCs under glycolytic inhibition, but in murine BMDCs only 10% of ATP is generated under similar conditions [101], [137]. It is likely that the ability of BCG-infected DCs to survive glycolysis inhibition is also due to some level of energetic plasticity that allows them to generate ATP via other metabolic pathways.

Infection with BCG reduced the viability of mo-DCs in a dose-dependent manner. Our group previously showed that human moDCs infected with the H37Ra (avirulent) and H37Rv (virulent) strains of *Mtb*, followed a non-apoptotic mode of cell death as seen by lack of nuclear fragmentation or caspase activity [66]. In this work also necrotic-like cell death was observed in the DC population as evidenced by a lack of increase in staining of dead DCs with the apoptosis marker Annexin V. The levels of cell death after infection varied widely from donor to donor as can be seen by comparing **Figures 3.6B** and **3.13B**. Substantial donor to donor variability is commonly seen in biological responses of human immune cells to physiologically relevant stimuli and may be due to genetic and epigenetic differences between individuals. The basal levels of cell death seen in uninfected moDCs were also quite

variable between the different donors which would account for some of the variability in the response to infection (**Figure 3.21C**). The cells were isolated from buffy coats from blood donors which are stored for several days before being released for use in research. Differences in the length of time between collection of the blood and isolation of cells might account for these differences in viability. The method by which DCs die is also important, because the mechanism of cell death can shape the consequent immune responses [206]. Inflammatory DCs in the lungs of mice infected with Mtb express anti-apoptotic proteins [150] which could inhibit apoptosis of these cells. However, it has been shown that dying murine DCs can release mycobacteria in the lung to disseminate the infection, which may be detrimental to the host during infection with virulent Mtb [81]. Just like in macrophages, wherein products of certain mycobacterial genes (*nuoG*, *pknE* and *secA2*) [207] could steer the cells towards a non-apoptotic form of death, these proteins could also be responsible for the same fate in DCs [66]. These data need to be substantiated by more studies that delve into the details of mechanisms of cell death in DCs infected by Mtb or cultured with BCG vaccine.

It was also seen that on adding 2-DG, the rate of cell death gradually decreased, suggesting glycolysis to be somehow detrimental to DC viability. The reasons why 2DG treatment promotes DC viability are unclear. A large body of evidence suggests that inhibition of glycolysis with 2DG potentiates cell death; for example, after TLR activation of innate immune cells, as mentioned above, and in malignant cells treated with DNA-damaging agents [208]. Nevertheless, in certain circumstances 2DG provides protection from apoptotic and necrotic stimuli. For example, caspase-dependent apoptosis of U937 macrophages treated with etoposide and other DNA-damaging agents [209] and necrosis of rat cardiomyocytes in hypoxic conditions [210]. In addition, when intracellular glucose levels are low, 2DG-6-phosphate, generated via the action of hexokinase, can substitute for glucose-6-phosphate to promote hexokinase association with voltage-dependent anion channel 1 (VDAC) on the outer mitochondrial membrane to prevent mitochondrial cell death pathways [211]. Furthermore, 2DG-6-phosphate can be metabolised via the pentose phosphate pathway (PPP) to increase NADPH levels under low glucose conditions thus protecting against oxidant-induced cell death [212], [213]. Therefore, the relationship between glucose metabolism and cell death is complex and further research will be required to determine the role of glycolysis in mycobacterial-induced cell death. Interestingly, a recent paper implicates mitochondrial dissociation of hexokinase in Mtb-

induced necrosis of macrophages [214]. Although it was proved that blocking glycolysis could decrease cell death, the mechanism behind it needs to be elucidated.

The cytokine profile of activated DCs is an important determinant of T cell responses and depends on modulation of metabolic pathways. BCG-infected DCs secreted increased levels of pro-inflammatory cytokines like TNF α and IL-1 β (which are crucial for controlling Mtb infection) as well as anti-inflammatory IL-10 (which may be important in preventing excessive immunopathology in Mtb infection but can also subvert host immune responses, for example, by inhibiting phagolysosomal maturation [215]. Similar to previous findings from BCG [216] and Mtb-infected moDCs [66], BCG also induced the secretion of IL-6, IL-8 and modest amounts of IL-13, IL-12p70, IL-2 and IFN- γ . Treatment with 2-DG did not significantly alter secretion of most cytokines by moDCs compared to untreated cells, possibly due to donor to donor variability in the cytokine response to infection [66]. However, the level of IL-1 β mRNA was decreased by 2DG treatment following BCG infection as was that of IL-10. 2DG treatment significantly decreased IL-13 secretion by BCG-infected cells at the highest MOI (250) indicating that glycolysis is required for optimal IL-13 secretion. Although this cytokine is most closely associated with allergic disease in the airways and asthma in particular, there is a report of elevated levels of IL-13 in patients with TB, which correlated with the degree of immunopathology in the lung. In addition, mice overexpressing IL-13 and infected with Mtb display uncontrolled bacterial growth leading to centrally necrotising granulomas, which are characteristic of human TB [296], [297]. Taken together with the *in vitro* data from the present study it appears that, by promoting IL-13 secretion, glycolysis might contribute to lung damage in TB. Basal levels of IL-12p70 were significantly reduced by 2DG compared to untreated cells. Activation of Th1 lymphocytes is crucially dependent on secretion of IL-12p70 by DC. Overall though, the levels of IL-12p70 secretion were quite low and, therefore, the significance of this finding is unclear.

Interleukin 1 β is an inflammatory cytokine that is essential for host resistance towards mycobacteria [60]. In Mtb-infected human alveolar macrophages and MDMs, the shift towards aerobic glycolysis enhanced optimal production of IL-1 β and suppressed expression of anti-inflammatory cytokine IL-10 [158]. This cytokine profile helped to control intracellular bacillary replication thereby keeping the infection at bay. Interestingly, in both of those studies however glycolytic block with 2DG did not alter levels of TNF α or IL-12

[158]. In the current study there was a trend towards decreased IL-1 β secretion in 2DG-treated DCs but it was not statistically significant. However, the level of IL-1 β mRNA was significantly decreased by 2DG treatment following BCG infection. Transcription of pro-IL-1 β in macrophages is partially dependent on glycolysis; when 2-DG was used to suppress glycolysis in murine BMDM, IL-1 β expression was lowered [116]. After the induction of glycolysis there is an increase in HIF1 α which targets IL-1 β gene transcription in macrophages rather than directly influencing secretion of the mature cytokine [298] which may explain the significant result seen here with mRNA expression. In addition, BCG does not induce the secretion of large quantities of IL-1 β by macrophages due to the absence of the ESX-1 secretion system (required for optimal inflammasome activity). This may be why the effect of 2-DG on gene transcription is not reflected in the secretion of the mature cytokine which requires several complex post translational steps including activation of the inflammasome and caspase 1 activity [195] which might not be dependent on glycolysis.

In contrast to BCG infection, secretion of IL-1 β by LPS-treated DCs was significantly decreased by 2DG treatment compared to the untreated LPS control indicating a role for glycolysis in TLR4-mediated IL-1 β secretion in human DCs. In macrophages infected with BCG, TLR2 plays a more prominent role in the induction of cytokine secretion [217] than TLR4. Therefore, the lack of a significant role for glycolysis in BCG-induced IL-1 β secretion may also be a reflection of this difference in TLR signalling.

DCs have glycogen stores which may serve as glucose donor for glycolysis right after its activation and also help in subsequent effector immune functions [120]. Glucose-1-phosphate is released from glycogen by glycogen phosphorylase (PYG) followed by conversion to glucose-6-phosphate. The PYG inhibitor, CP91149 (CP), inhibited LPS-induced maturation of BMDCs and the accompanying increase in ECAR, as well as TCA citrate production, indicating that DCs are not completely dependent on extracellular glucose to fuel the TLR-driven glycolytic burst [120]. In our work too, CP-91149 was used to assess the possible role of glycogen in human DC maturation. However, a confounding factor in these experiments was that uptake of BCG by mo-DCs was reduced by pre-treatment with CP-91149. This could be due to a requirement for glycogenolysis during phagocytosis of bacteria. On the other hand, the inhibitor was also somewhat toxic to DCs, killing approximately (59 mean $\pm$ SEM 3.7) of the uninfected cells after 24 hours, which could also account for diminished phagocytosis. There was a trend towards a decrease in

percentage of CD86 positive cells in the presence of the inhibitor compared to untreated cells, but this is likely to be due to the decreased level of BCG infection. Therefore, it was not possible to determine whether glycogen-derived glucose is involved in the maturation of BCG-infected DCs using this approach. A number of other GYP inhibitors have recently been developed as potential therapies for type 2 diabetes [218] and provided they were less toxic than CP-91149, they could be useful in interrogating the role of glycogen metabolism in DC activation.

This is the first study investigating the role of glycolysis in human mo-DC maturation. After stimulating human moDCs with BCG and carrying out various tests using metabolic modulators, we come to the conclusion that there appears to be a limited role for glycolysis in human moDC maturation after BCG infection. Specifically, glycolysis appears to be required for optimal expression of CD86 and for IL-13 secretion. In addition, glycolysis also seems to play an unexpected role in potentiating BCG-induced necrosis. The results also suggest that glycolysis may not be the only pathway for generation of energy in this setting. The work outlined in the following chapters investigates the role of glycolysis in BCG-induced DC activation in more depth and examines the contribution of other metabolic pathways to this process.

CHAPTER 4:

Characterising the metabolic pathways involved in moDC maturation following infection with mycobacteria

4.1 Introduction

4.1.1 Hypothesis: DCs utilize multiple metabolic pathways to provide the building blocks and energy required for maturation following infection with mycobacteria.

As discussed previously, murine BMDCs activated by TLR agonists experience a powerful metabolic switch characterised by an increase in glycolysis and a corresponding dynamic loss of oxidative phosphorylation (OXPHOS) [219]. In murine DCs the switch to glycolysis is driven mainly by nitric oxide, which incapacitates oxidative phosphorylation in the mitochondria [116]. In the previous chapter (**Chapter 3**), inhibition of glycolysis led to reduced cell surface expression of CD86 and improved viability of human moDCs infected with BCG. Nevertheless, other parameters associated with maturation, including expression of HLA-DR, were unchanged when glycolysis was inhibited suggesting that other metabolic pathways might also be involved. This chapter aims to characterise the metabolic pathways associated with human moDC maturation following infection with mycobacteria. To this end, tests were performed to characterise and evaluate the rates of glycolysis and OXPHOS of human moDCs following infection with BCG and compared to a virulent strain of Mtb (H37Rv).

The Seahorse Metabolic Flux Analyser allows the simultaneous measurement of extracellular acidification rate (ECAR) and the oxygen consumption rate (OCR) of live cells. Cells are plated to form a monolayer within the wells of the microplate and the instrument measures the concentration of dissolved oxygen and the concentration of free protons in the media and then calculates the basal OCR and the ECAR. The OCR value represents the mitochondrial oxidative phosphorylation rate, while ECAR represents the glycolytic rate within the cells. The sequential addition of inhibitors to the wells allows the determination of the metabolic potential of the cells of interest and the relative contribution of different metabolic pathways to ECAR and OCR under different conditions.

The Mito Stress Test allows measurement of basal ECAR and OCR and gauges the key parameters of mitochondrial function using a number of metabolic inhibitors. After measuring basal respiration, sequential compound injections allow measurement of ATP production, proton leak, maximal respiration, spare respiratory capacity, and non-mitochondrial respiration. These compounds are modulators of respiration which target components of the electron transport chain (ETC) in the mitochondria to reveal key

parameters of metabolic function. Oligomycin is the first injection and inhibits the ATP synthase (complex V) leading to a decrease in OCR. Then, consequently cells must boost glycolytic rate in order to meet demands for ATP synthesis, reflected by increased ECAR on extracellular flux analysis. FCCP, the second injection, is an uncoupler of oxidative phosphorylation in mitochondria. Therefore, it disrupts ATP synthesis by transporting protons across the mitochondrial membranes. As a result, the electron flow through the ETC is uninhibited, and oxygen is maximally consumed by complex IV leading to increases in both OCR and ECAR, OCR due to uncoupling, and ECAR as the cells try to maintain their energy balance by using glycolysis to generate ATP. The Spare Respiratory Capacity (SRC) is calculated by; the difference between maximal respiration post-FCCP OCR and basal respiration. Spare respiratory capacity (SRC) is a measure of the ability of the cell to increase energy demand *via* OXPHOS under stresses. The third injection is a mix of rotenone, a complex I inhibitor, and antimycin A, a complex III inhibitor. Both inhibitors act to shut down all ETC activity and all mitochondrial oxygen consumption, and therefore reflect the Non-Mitochondrial Oxygen Consumption Rate of the cells. Decrease in OCR due to impaired mitochondrial function, with a concomitant increase in ECAR as the cell shifts to a more glycolytic state in order to maintain its energy balance. See **Figure 2.3** in **Chapter 2**.

The Glycolysis Stress Test Kit measures the extracellular acidification rate (ECAR) of cells. Sequential compound injections measure the following parameters of glycolytic flux: Glycolysis, Glycolytic Capacity, Glycolytic Reserve, and non-glycolytic acidification. The first injection is a saturated concentration of glucose which is added to cells in glucose-free medium. The cells utilize the glucose injection and catabolize it. This results in a rapid increase in ECAR; the rate of glycolysis reported under basal conditions after glucose-induced response. Prior to the glucose injection, the ECAR is a measure of non-glycolytic acidification. The second injection is oligomycin. This inhibits ATP synthase (complex V). Consequently, it shifts the energy production to glycolysis and increases the ECAR revealing the cellular maximum glycolytic capacity. The final injection is 2-deoxy-glucose (2-DG). This is a competitive inhibitor of Hexokinase, the enzyme which catalyzes the first step of the glycolytic pathway. The resulting decrease in ECAR confirms that the ECAR produced in the experiment is due to glycolysis, which is then used to calculate Glycolytic Reserve (GR) via the difference between glycolytic capacity and glycolysis. Glycolytic Reserve is a measure of the ability of the cell to increased glycolytic rate under stresses.

In this study it was not possible to analyse cells infected with live hazard group 2 (BCG) or group 3 (H37Rv) bacteria in the Seahorse instrument as the appropriate containment facilities were not available for the instrument. Therefore, similar to previous studies from our group, killed mycobacteria were used for the metabolic flux analysis [158], [220]. The use of killed mycobacteria ensures that any changes detected in metabolic flux after infection are entirely due to the DCs rather than the metabolism of the infecting mycobacteria. Initial experiments were therefore carried out to determine whether killed BCG could activate moDCs. Next, metabolic flux analysis was performed to measure ECAR and OCR using the Seahorse Metabolic Flux analyser to check the rate of mitochondrial respiration and glycolysis of moDC under different conditions and at different times after infection with killed BCG. Since BCG is an attenuated strain of mycobacteria, for comparison, a virulent strain of Mtb (irradiated H37Rv) was also analysed.

The aims of this chapter were:

1. Establish whether killed BCG activates moDCs
2. Measure metabolic flux (ECAR and OCR) of moDCs infected with killed BCG under basal and stressed conditions.
3. Compare the effects of BCG and Mtb-H37Rv on moDC metabolic flux under basal and stressed conditions.
4. Evaluate the effect of potential modulators of glycolytic reprogramming (IFN- γ and metformin) on the metabolism of BCG and Mtb-infected moDCs.

4.2 Methods

The materials and methods used are summarised here and are more completely described in **Chapter 2**.

4.2.1 Mycobacteria

BCG-GFP was killed by treating a mid-log phase culture with Rifampacin (100 μ g/ml) for two days, and then used to infect moDCs as described in **chapter 2 Section 2.2.2**.

Gamma-irradiated H37Rv (iRv) was obtained from BEI Resources, prepared as described in **Section 2.2.3** and used to infect moDCs at the indicated MOI.

4.2.2 Extracellular flux analysis

Extracellular flux analysis was performed using the Seahorse XFe24 Analyzer (Seahorse Biosciences, Agilent Technologies UK Ltd.), **Chapter 2 Section 2.11**. For all extracellular flux analyses, cells were prepared as outlined in **Chapter 2 Section 2.3** and plated on Seahorse 24-well microplates in the usual culture medium with 4 designated “blank” wells left free of cells, left for at least 2 hours, and then infected as outlined in **Chapter 2 Section 2.2**. The Seahorse XF Cell Mito Stress Test Kit, the Seahorse XF Glycolysis Stress Test Kit and the Seahorse XF Mito Fuel Flex Test Kit were used to interrogate different metabolic pathways - see **Chapter 2 Section 2.11**. Data analysis was carried out using Wave Desktop 2.4 software (Agilent). After the readings were taken, a crystal violet (CV) assay was performed on the microplates as described in **Chapter 2 Section 2.11**. The crystal violet assay is performed to check the cell numbers in each individual well. Dying cells detach from the culture plates therefore cell number is an indication of the cell death. Extracellular flux readings were normalised to the corresponding CV data for each well before analysis using Wave Desktop 2.4 software.

4.2.3 Optimisation of mo-DCs Seeding Density for Extracellular flux analysis

Initially, mo-DCs density optimisation assays were performed. The probes on the XFe 24 Analyser measure ECAR and OCR in an approximately 2µl volume of media approximately 200µm above the monolayer. Therefore, cell numbers must be optimized for the Seahorse metabolic flux assays to ensure that there is a sufficient density of cells on the surface of the plate for the probes to give accurate readings of OCR and ECAR within the linear range of the instrument. On the other hand, if the cells are too crowded then baseline levels of OCR and ECAR will be too high and the dynamic range of the assay will be compromised. In addition, if overcrowded, the response of the cells could be affected by a scarcity of nutrients.

Cells were prepared as described in **Section 2.3.1** and **Section 2.3.2** respectively. On the day of infection, immature moDCs were gently scraped, and plated in duplicate at varying densities - 50,000, 100,000 and 200,000 cells/well - on Seahorse 24-well microplates in RPMI 1640 media supplemented with 10% defined FBS. The surface of the wells had previously been coated with CellTak (**Section 2.3.3**) to allow the DCs to adhere to the plate.

The plate was equilibrated at 37°C and 5% CO₂ for at least 1 hour before the cells were infected with BCG at MOI 100. In addition, as a positive control for maturation, uninfected moDCs were treated with 1 µg/ml LPS. Then, 24 hours after infection, the buffered Seahorse media (containing 10% defined FBS) was supplemented to all wells. The microplate and hydrated Seahorse XFe 24 sensor cartridge were then incubated at 37°C and CO₂-free incubator prior to metabolic flux measurements. All the experiments and a crystal violet (CV) assay were performed as described in **Chapter 2 Section 2.11**.

Basal respiration, proton leak, maximum mitochondrial respiration, non-mitochondrial respiration, and ATP production were linearly correlated with plating density. Proton leak was low indicating that most oxygen consumption was used to synthesize ATP. The recommended range for basal OCR (using the last reading prior to oligomycin injection), according to the manufacturer, is 50 to 200 which was obtained at 100,000 and 200,000 cells per well. Basal ECAR values of around 10-20mPh/min are recommended in order to obtain reproducible data in the linear range of the XFe 24 Analyzer (Agilent Technologies) which was obtained with a cell density of 200,000 cells per well. The moDCs were plated at 200,000 cells per well for further experiments using the Mito stress test kit from Agilent. After measurement of basal ECAR and OCR, oligomycin (1 µM), FCCP (2 µM) and Rotenone and antimycin A (0.5 µM) inhibitors were injected in sequence to assess the response of the moDCs in each group at each cell density to stressed conditions (**Figure 4.1**).

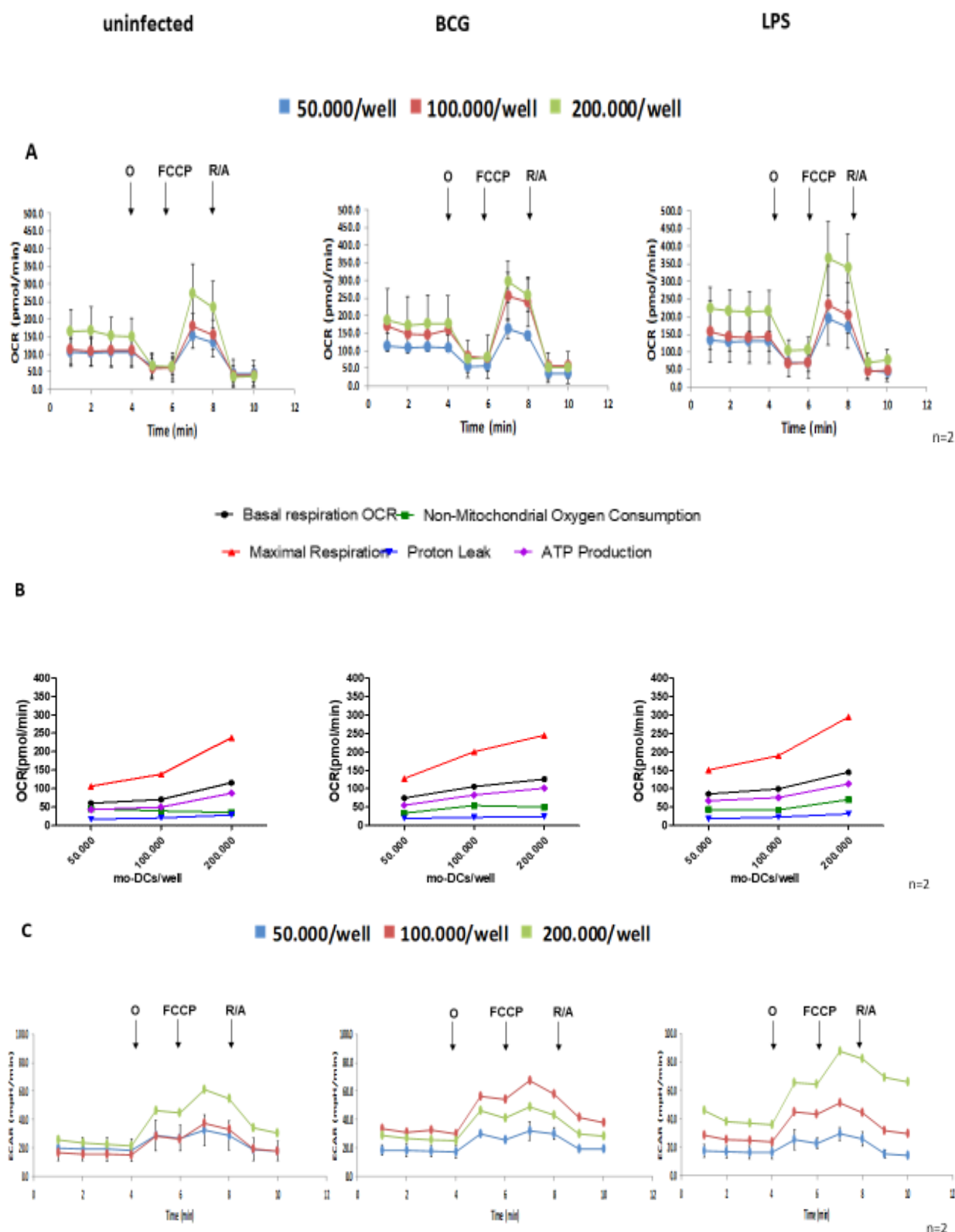


FIGURE 4.1: Optimisation of seeding density of mo-DCs for extracellular flux analysis. moDCs were plated on Seahorse Biosciences 24-well plates at indicated densities, infected with killed BCG (MOI 100), and extracellular flux analyses was performed 24 hours later. Data was normalised using CV assay. Panel **A**, depicts mean $\pm$ SEM OCR of moDCs plated at varying densities. Panel **B**, depicts cell density-dependent changes in the levels of baseline OCR, non-mitochondrial respiration, maximum respiration, oxygen consumption, proton leak and ATP production, in uninfected, BCG infected and LPS treated moDCs. Panel **C**, depicts mean $\pm$ SEM ECAR of moDCs plated at varying densities. O = oligomycin A; FCCP = Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; Rot = Rotenone; A = antimycin A.

4.2.4 Optimisation of Inhibitor Dose for Extracellular flux analysis

For optimisation of all inhibitors, cells were prepared as described in **Section 2.3.1** and **Section 2.3.2** respectively. On day of infection, cells were scraped, and plated on Seahorse Biosciences 24-well plates at 200,000 cell/well (An optimal seeding density of 200,000 cells/well was determined through the optimisation experiments above) in RPMI 1640 media supplemented with 10% defined FBS at least 1 hour before being infected with BCG at MOI 100, uninfected mo-DCs were treated with 1 µg/ml LPS. 24 hours after infection, the Seahorse media was supplemented to all wells. The microplate and hydrated the Seahorse XFe 24 sensor cartridge was then incubated at 37°C and 0% CO₂ prior to interrogation, experiments and a crystal violet (CV) assay was performed as described in **Chapter 2 Section 2.11**. Varying concentrations of inhibitors were made up in supplemented Seahorse medium and loaded into designated ports on the Seahorse Assay Cartridge, with each concentration present in duplicate. The optimal final concentration of oligomycin was determined to be 1 µM, FCCP was 2 µM and Rotenone and antimycin A was 0.5 µM. (**Figure 4.2 A & B**).

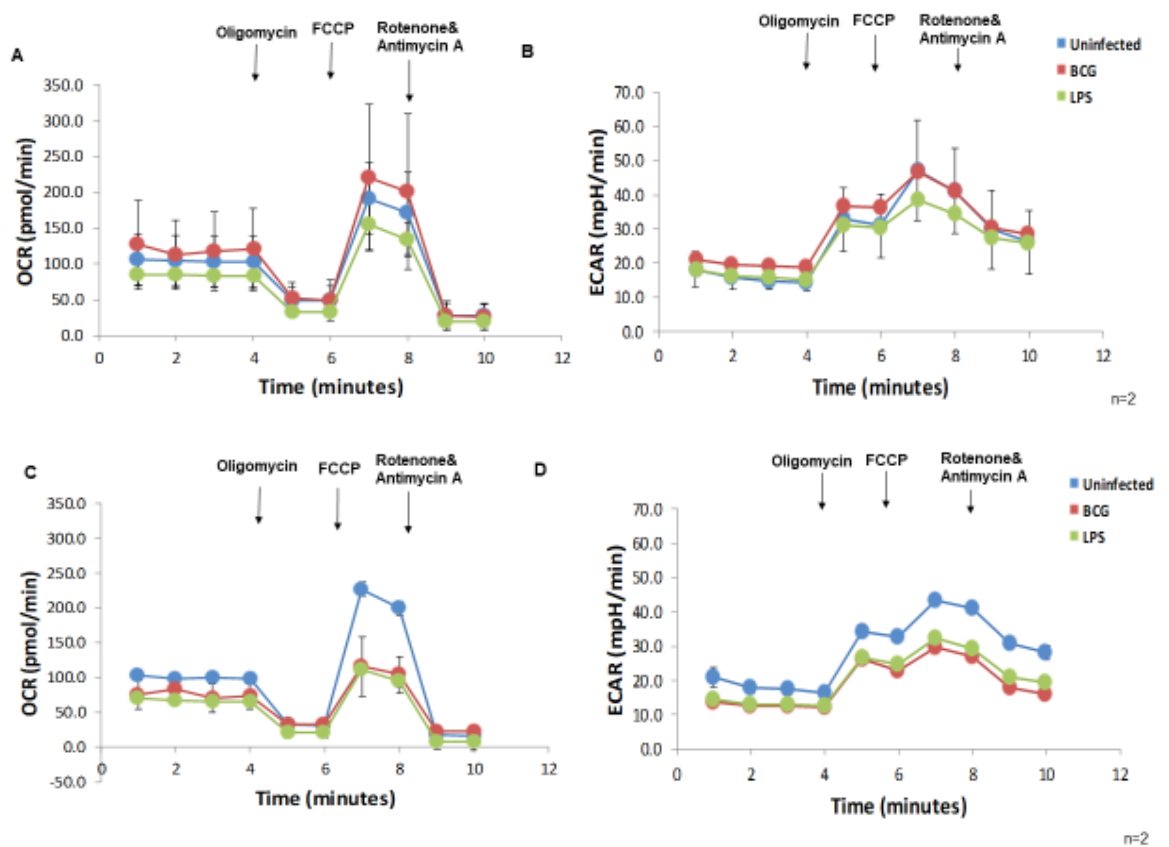


FIGURE 4.2: Optimisation of inhibitor dose of oligomycin, FCCP, Rotenone and antimycin A. Mo-DCs were plated on Seahorse Biosciences 24-well plates at 200,000 cells/well and infected with killed BCG (MOI 100). 24 hours after infection inhibitors of the Mito Stress Test Kit were used at varying concentrations for extracellular flux analyses and data normalised using CV assay. Panels A – B depict OCR and ECAR respectively following addition of inhibitors 1 μ M oligomycin, 2 μ M FCCP and 0.5 μ M Rotenone and antimycin A. Panels C– D depict OCR and ECAR respectively following addition of inhibitors 2 μ M oligomycin, 0.5 μ M FCCP and 1 μ M Rotenone and antimycin A for two individual donors.

4.2.5 Re-optimisation of mo-DC density for the Glycolysis Stress Test Kit

The Glycolysis Stress Test Kit was also used to further investigate glycolytic function in moDCs. Results with the Mito Stress Test (see Section 4.4 of this chapter) showed that there was no significant effect of BCG infection on ECAR, therefore it was hypothesised that increasing number of moDCs above 200,000 cells/well might be necessary to provide a measurable response with this assay. The mo-DCs were prepared as described in **Section 2.3.1** and **Section 2.3.2**, respectively. On the day of infection, cells were scraped, and plated at 200,000, 300,000, 400,000 cells/well on Seahorse 24-well microplates in RPMI 1640 media supplemented with 10% defined FBS and allowed to adhere at least 1 hour before infection at MOI 50 with BCG and iRv. uninfected DCs were also treated with 1µg/ml LPS. 24 hours after infection, the Seahorse media (without glucose and pyruvate) was supplemented to all wells to starve the cells of glucose. The microplate and hydrated the Seahorse XFe 24 sensor cartridge was then incubated at 37°C and 0% CO₂ prior to metabolic flux measurements. ECAR and OCR were measured before and after sequential injections of glucose (to measure basal glycolysis rates), oligomycin A (to measure the maximal glycolytic capacity) and 2-DG (inhibits glycolysis giving a measure of the glycolytic reserve). All the experiments and a crystal violet (CV) assay were performed as described in **Chapter 2 Section 2.11.2**.

Uninfected and BCG-infected moDCs plated at 300,000 cells/well showed an increase in the level of glycolysis and glycolytic capacity compared to uninfected and BCG infected moDCs plated at 400,000 cells/well (Figure 4.3 A and B). Also, in the iRv moDCs group, uninfected and BCG-infected moDCs plated at 300,000 cells/well showed increased levels of glycolysis and glycolytic capacity compared to moDCs plated at 400,000 cells/well (**Figure 4.3 C and D**). Glycolysis was increased in uninfected moDCs and BCG infected moDCs plated at 200,000 and 300,000 cells/well compared to BCG-infected moDCs plated at 400,000 cells/well (Figure 4.3 A and B), while ECAR was not changed in iRv infected moDCs at any of the densities used (Figure 4.3 C and D). These observations indicated that seeding BCG-infected moDCs at 200,000 cells/well to 300,000 cells/well is appropriate to investigate the metabolic response of these cells in the 24 well Seahorse plate, but that they would be plated too densely at 400,000 cells/well to show measurable changes in ECAR with any of the treatments.

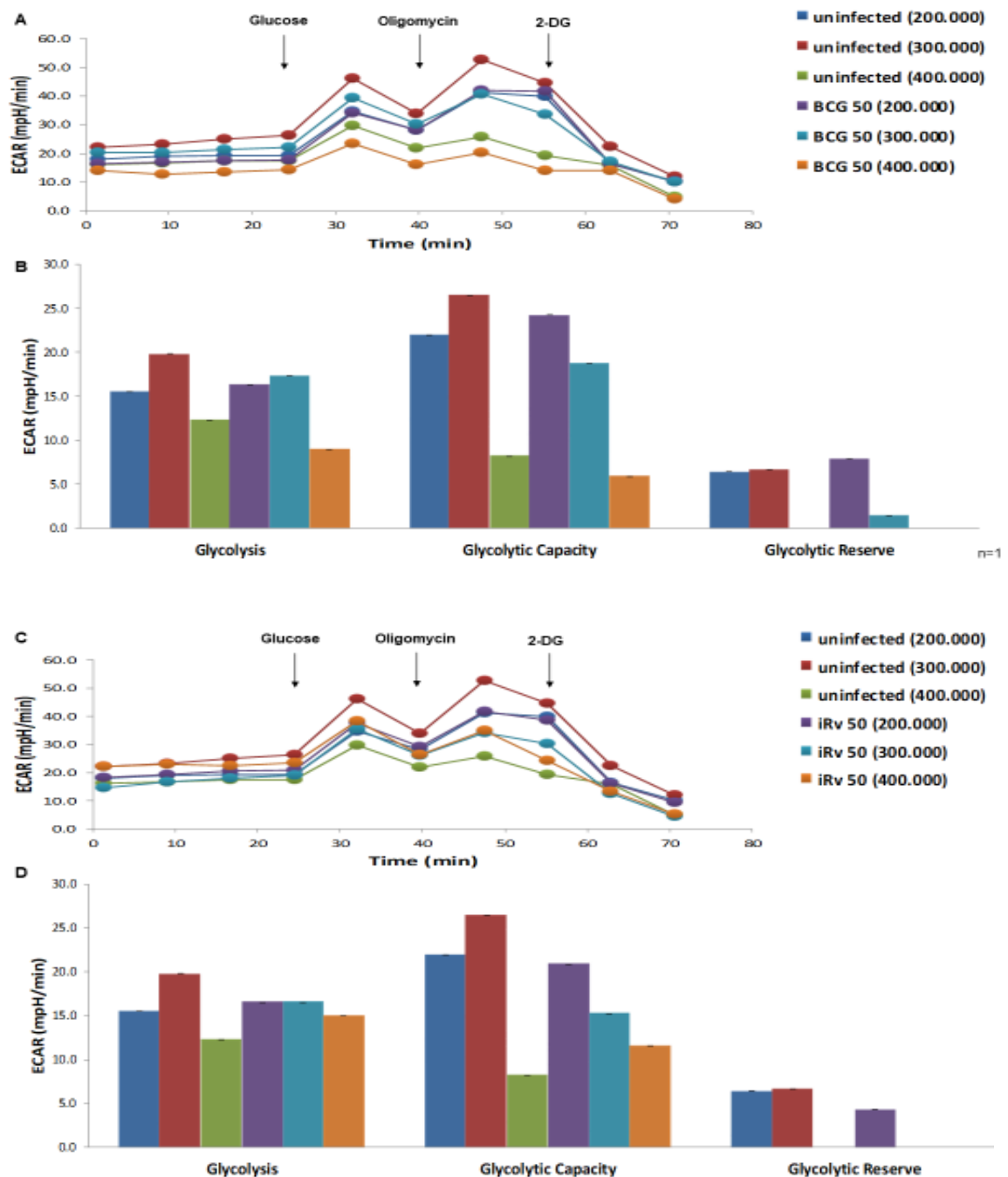


FIGURE 4.3: Re-optimisation of seeding density of killed BCG and iRV infected mo-DCs for the Glycolysis Stress Test moDCs were isolated, cultured for 5 days and plated on Seahorse Biosciences 24-well plates at indicated densities, infected with killed BCG or iRV (MOI 50). 24 hours after infection extracellular flux analysis was performed with sequential addition of glucose (10Mm), oligomycin (1µM) and 2DG (50mM) and data normalised using CV assay. Panels A and C depict ECAR quantification of moDCs plated at varying densities (A) representative kinetic ECAR in uninfected moDCs at density 200.000 cell/well (blue), uninfected moDCs at density 300.000 cell/well (red), uninfected moDCs at density 400.000 cell/well (green), BCG MOI 50 infected moDCs at density 200.000 cell/well (purple), BCG MOI 50 infected moDCs at density 300.000 cell/well (aqua) and BCG MOI 50 infected moDCs at density 200.000 cell/well (orange). (B) individual parameters for glycolysis, glycolytic capacity and glycolytic reserve. (C) representative kinetic ECAR in uninfected moDCs at density 200.000 cell/well (blue), uninfected moDCs at density 300.000 cell/well (red), uninfected moDCs at density 400.000 cell/well (green), iRV MOI 50 infected moDCs at density 200.000 cell/well (purple), iRV MOI 50 infected moDCs at density 300.000 cell/well (aqua) and iRV MOI 50 infected moDCs at density 400.000 cell/well (orange). (D) individual parameters for glycolysis, glycolytic capacity and glycolytic reserve.

4.3 Statistical analysis

Data were analysed using Graphpad Prism 5. A two-way ANOVA with a Bonferrini post-test was used for grouped data. Differences were considered significant when *p*-values were below 0.05. Otherwise, the data were presented as mean +/- standard deviation, with at least three replicates (donors) used for each data point, and repeated measures one-way ANOVA with a Tukey multiple comparison test was used, ****P* < 0.001; ***P* < 0.01, **P* < 0.05.

In some Seahorse Experiments Wave Desktop 2.3 software and Report Generator Version: 1.0.12, (Agilent Technologies) was used for analysis and all data represent mean ± SEM of the independent mean parameter values calculated for each assay group assigned to a Group Collection.

4.4 Results

4.4.1 Comparison of the effect of live and killed BCG-GFP on moDC maturation

The experiments described in Chapter 3 were performed using live BCG. However, it was necessary to challenge the moDCs with killed BCG for the metabolic flux assays on the Seahorse because the containment measures needed for live class II bacteria were not available for this instrument. The use of killed mycobacteria has the advantage that only eukaryotic cell metabolism is measured in co-cultures. It was necessary to first carry out preliminary experiments to determine whether killed BCG caused maturation of moDCs and to assess the effect of 2DG.

To test if killed BCG can induce moDC maturation, the cells were treated with 2DG as outlined in **Chapter 2** and infected with different MOI 50, 100 and 250 - of live and killed BCG. The morphology of the DCs after BCG uptake was assessed by combined phase contrast/fluorescent microscopy. moDCs phagocytosed live and killed BCG and developed dendrites typical of mature DCs (**Figure 4.4**). Percentage of infection, viability of cells and MFI of CD86 expression were measured as before by flow cytometry. For clarity, the data are first shown without 2DG treatment (**Figure 4.5**). Uptake of mycobacteria was lower with killed BCG compared to live BCG at each MOI tested (**Figure 4.5 A**). In addition, while live BCG caused a dose-dependent increase in cell death, killed BCG did not (**Figure 4.5 B**). The percentage of CD86 positive cells was the same with both live and killed BCG indicating that killed BCG also induces moDC maturation (**Figure 4.5 C**), although the MFI was lower in moDCs infected with killed BCG compared to cells infected with live BCG (**Figure 4.5 D**), in keeping with the lower levels of uptake of killed BCG. In figure 4.6 the 2DG data is presented alongside that of the untreated cells. Treatment with 2DG did not affect phagocytosis of BCG and the percentage of cells expressing the maturation marker CD86 was unaffected by 2DG treatment when moDCs were co-cultured with live BCG as observed before (**Chapter 3, Figure 3.10**). There was a slight decrease in the percentage of cells expressing CD86 when moDC were co-cultured with killed BCG and treated with 2DG compared to untreated cells. There was a decrease in the CD86 MFI of moDCs infected with live and killed BCG in the presence of 2DG compared to the corresponding untreated controls (**Figure 4.6 E and F**) indicating that glycolysis is required for optimal expression of CD86 induced by live or killed BCG. Cell death caused by live BCG was inhibited by 2DG treatment as before. Killed BCG did not cause an increase in cell death therefore 2DG had no effect on moDC viability.

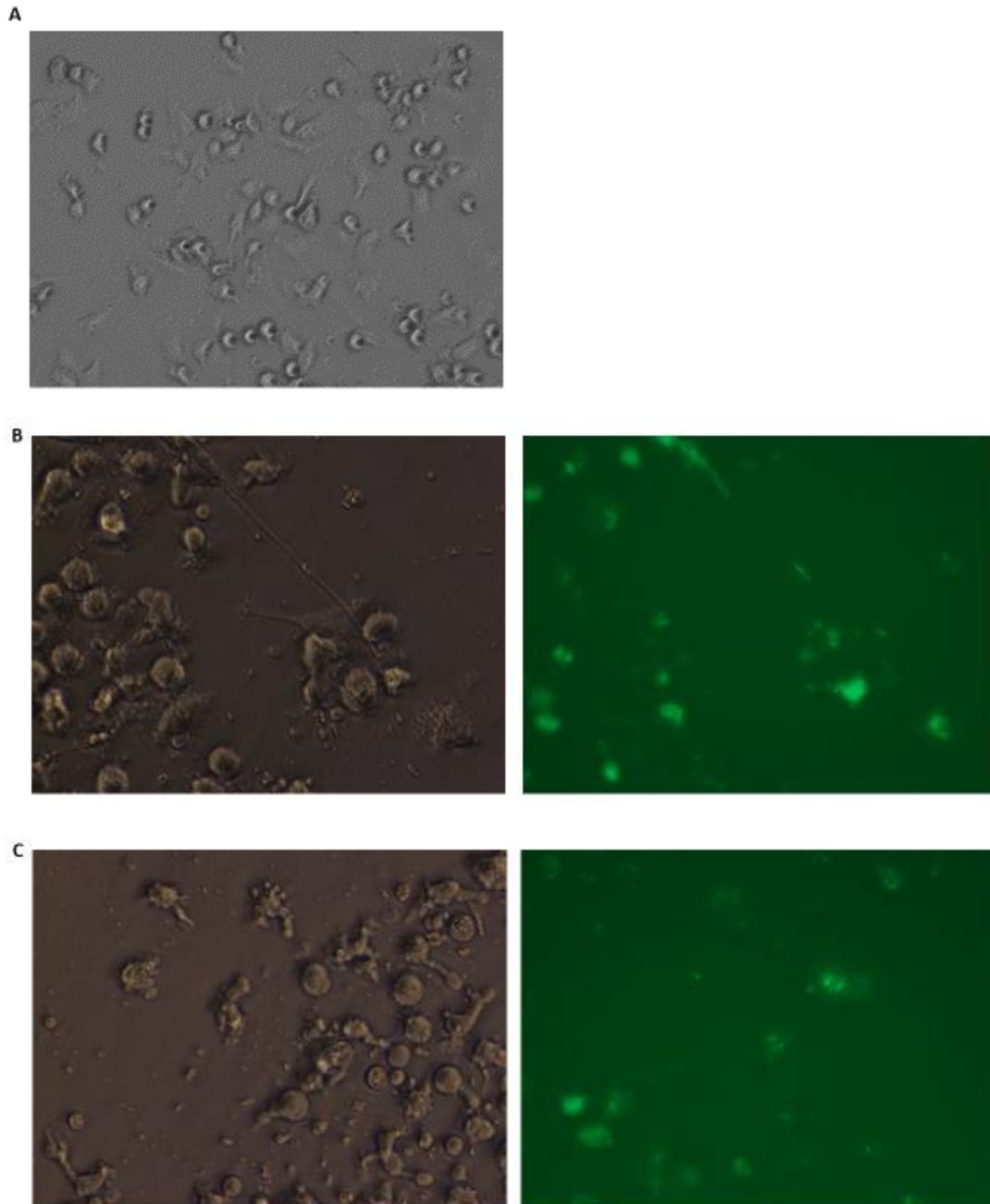


FIGURE 4.4: Live and killed BCG infected moDCs produce dendrites. Human mo-DCs were infected with BCG at an MOI of 100 for 24 hours and were imaged using an inverted microscope fitted with a 40X objective. Panel **A**. Phase contrast micrographs showing uninfected mo-DCs. Panel **B**. showing live BCG at MOI 100. Panel **C**. showing killed BCG at MOI 100. Images show one representative experiment of two.

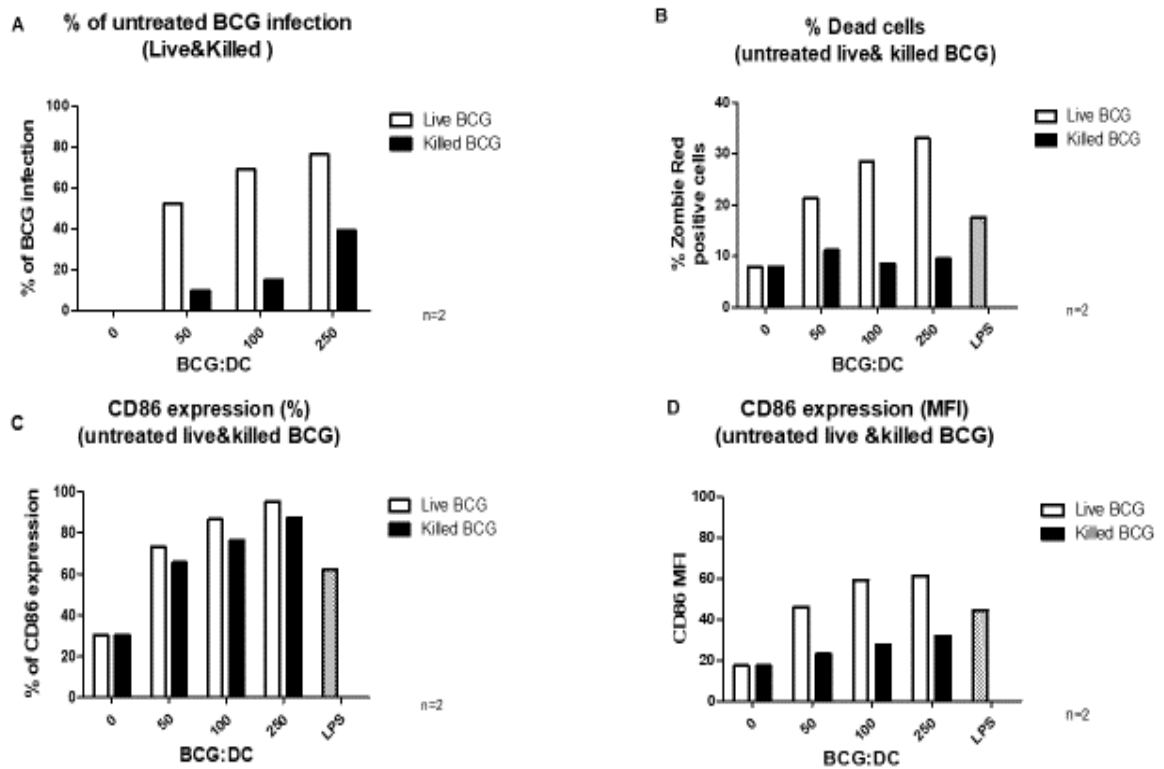


FIGURE 4.5: Comparison of the ability of live and killed BCG to induce mo-DC maturation. Human mo-DCs were infected with live and killed BCG at MOI 50,100 and 250. Uninfected moDCs were treated with lipopolysaccharide (LPS) (1µg/ml) as a positive control for maturation and staurosporine (20 µg/ml) as a positive control for cell death. The level of infection was assessed at 24 hours and analysed by flow cytometry. Zombie Red dye was used to measure the level of cell death and to exclude dead cells from the analysis. **A.** demonstrates the uptake of live and killed BCG; **B.** illustrates live and killed BCG-induced cell death; **C.** and **D.** show surface expression of CD86(MFI and % of positive cells) of live and killed BCG was assessed by flow cytometry. Data represents two different donors.

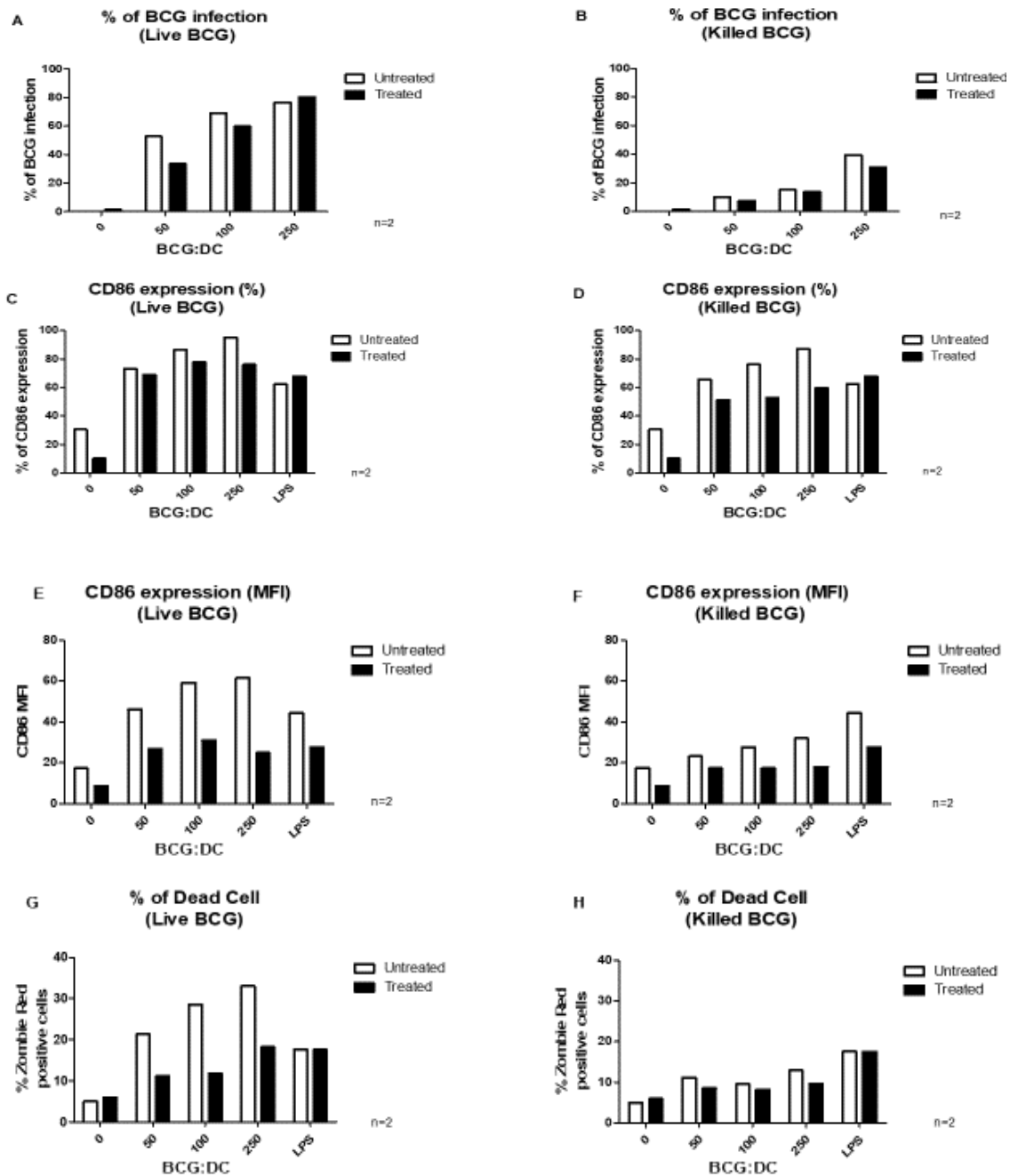


FIGURE 4.6: Effect of 2DG on mo-DCs maturation after challenge with live and killed BCG *continued*..

This figure shows the data from Figure 4.5 with the addition of 2DG treatment. moDCs were untreated or pre-treated with 5mM 2DG for 30 minutes and infected with live and killed BCG at the indicated MOI. The level of infection was assessed at 24 hours and analysed by flow cytometry. Zombie Red dye was used to measure the level of cell death and to exclude dead cells from the analysis. (A) uptake of live BCG with and without 5mM 2DG; (B) uptake of killed BCG (C) surface expression of CD86(MFI) when infected with live BCG; (D) surface expression of CD86(MFI) when infected with killed BCG; (E) surface expression of CD86(%) when infected with live BCG; (F) surface expression of CD86(%) when infected with killed BCG; (G) BCG-induced cell death when infected with live BCG; (H) BCG-induced cell death when infected with killed BCG. Data represents two different donors.

4.4.2 Characterization of ECAR and OCR of moDCs challenged with BCG

MoDCs from 12 donors were prepared as described in **Section 2.3.1** and **Section 2.3.2** respectively. Metabolic flux analysis was carried out as described in **Section 2.11**; moDCs were used in three groups for this experiment. One group contained the uninfected moDCs, the other group contained moDCs which were treated with LPS at the concentration of 1µg/ml for 24 hours and the third group contained moDCs which were infected with killed BCG at an MOI of 100 for 24 hours. The main aim of this test was to identify the ECAR (an indicator of glycolysis) as well as OCR (an indicator of mitochondrial respiration) of moDCs under basal and stressed conditions. Basal ECAR and OCR were measured before the injection of inhibitors. In addition, several measures of mitochondrial respiration, including spare respiratory capacity, maximum respiration, non-mitochondrial oxygen consumption, proton leak, ATP production, coupling efficiency and spare respiratory capacity were obtained by the sequential addition of oligomycin (1µM), FCCP (2µM), and Rotenone and antimycin A (0.5µM) inhibitors to the three groups, as diagrammed in (**Figure 4.7 A & B**). Basal OCR was unchanged in BCG infected moDCs compared to uninfected cells (**Figure 4.7 C & E**). There was a slight increase in ECAR of BCG infected moDCs (14.21 ± 3.013) compared to uninfected (12.43 ± 2.359) and also in LPS (14.15 ± 3.284) stimulated moDCs (**Figure 4.7 D & F**). Consequently, this data was used to calculate the baseline ECAR/OCR ratio; there was no significant difference between all groups (**Figure 4.7 G**). The basal OCR and ECAR data were plotted to obtain a metabolic phenogram for the moDCs under the different conditions tested. The phenogram shows the relative metabolic state of cells under each condition and indicates that there was very little change in metabolic flux with BCG infection or LPS treatment compared to the untreated uninfected cells (**Figure 4.7 H**).

The metabolic flux data for these 12 donors obtained under stressed conditions was further analysed is shown in figure 4.8. There were slight decreases in spare respiratory capacity (**Figure 4.8 A**), maximal respiration (**Figure 4.8 B**), mitochondrial ATP production (**Figure 4.8 C**), and proton leak (**Figure 4.8 E**) in the BCG-infected moDCs. Proton leak represents the flow of protons through lipids or other channels that occurs when protons can no longer be pumped through the ETC. Overall, this data is indicative of a slight reduction in the ability of BCG-infected cells to respond to increased energy demand by boosting mitochondrial respiration and may reflect a bias towards glycolysis in these cells. The coupling efficiency, which is the proportion of mitochondrial respiration that is used for ATP synthesis (**Figure 4.8 G**), and non-mitochondrial oxygen consumption (**Figure 4.8 D**),

were unchanged in BCG-infected moDCs compared to the uninfected controls. So, unexpectedly, these tests indicated that there was no major change in the respiration and glycolysis rates of killed BCG-infected or LPS-treated moDC compared to control cells.

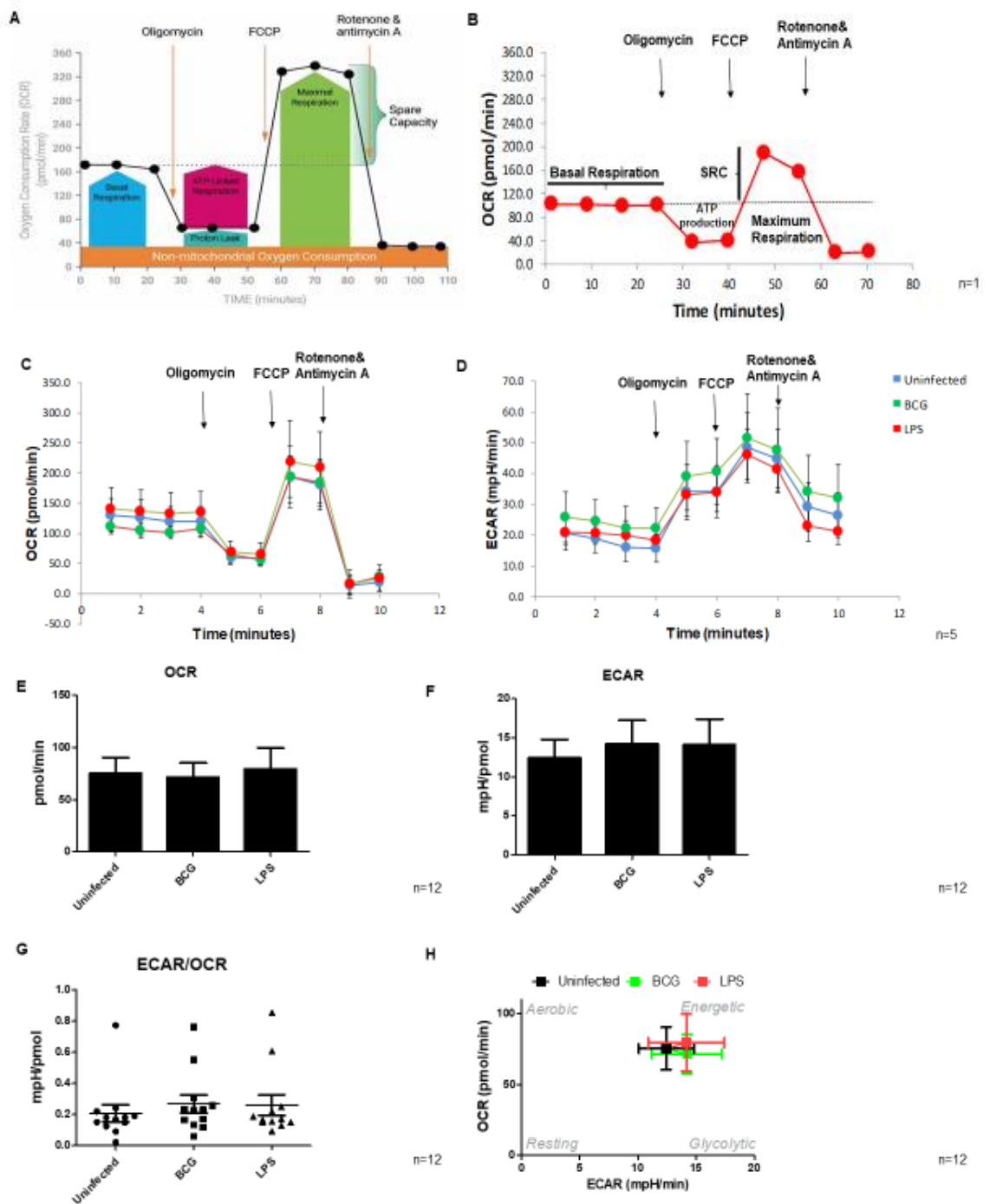


FIGURE 4.7: Extracellular flux analysis of moDCs challenged with BCG. MoDCs were plated on Seahorse Biosciences 24-well plates at 200,000 cells/well. Cells were uninfected, infected with killed BCG at MOI 100 or treated with LPS (1 μ g/ml) for 24 hours and extracellular flux analysis was performed with sequential addition of oligomycin (1 μ M), FCCP (2 μ M) and rotenone/antimycin A (0.5 μ M) and data normalised using CV assay. Panel (A) depicts OCR measurements (in pmol/min) at baseline and following sequential addition of mitochondrial inhibitors, illustrating how different oxygen consumption parameters are calculated. Adapted from Agilent Seahorse XF Cell Mito Stress Test Kit [164]. Panel (B) shows a representative real-time mitochondrial respiration experiment with OCR analysis starting from basal respiration and after the addition of three compounds: OCR (mpH/min) in LPS (red) treated moDCs for one representative donor. Panels C and D depict mean $\pm$ SEM OCR (C), ECAR (D) in uninfected moDCs (blue), BCG infected moDCs (green), and LPS (red) treated moDCs of the independent mean parameter values calculated for five individual donors. Panels E – G depict baseline mean $\pm$ SEM OCR (E), ECAR (F) and ratio of ECAR/OCR (G) for the same 12 individual donors. Statistical analyses were performed using one-way-ANOVA. Panel H depict the metabolic phenogram obtained by plotting the basal ECAR and OCR values from the metabolic flux data above.

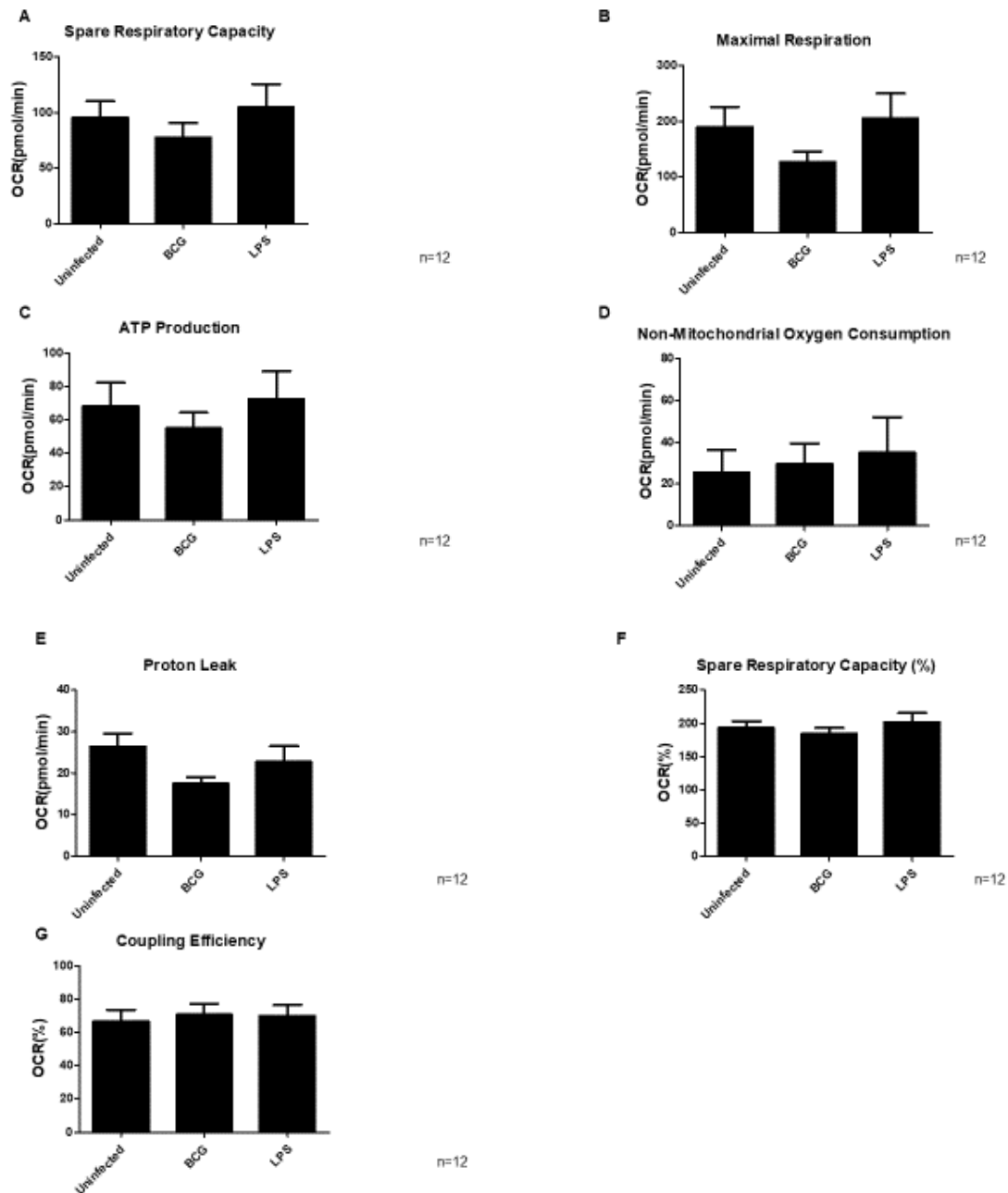


Figure 4.8 Extracellular flux analysis of moDCs challenged with BCG *continued*. Further analysis of the data obtained from the extracellular flux data shown in Figure 4.7. Panels A and B depict OCR quantification of Spare Respiratory Capacity (A), maximum respiration of moDCs (B). Panels C – G depict normalised mean $\pm$ SEM mitochondrial ATP production (C), Non-Mitochondrial oxygen consumption (D), Proton Leak (E), Spare Respiratory Capacity (%) (F) and Coupling Efficiency (%) calculated as ATP production rate/basal respiration $\times 100$ (G) for twelve individual donors. The data were pooled from seven independent experiments, mean $\pm$ SEM, statistical differences were analysed by one-way ANOVA with a Tukey multiple comparison test.

4.4.3 Comparison of the effect of IFN γ and LPS on metabolic flux of moDC

Since LPS did not induce a shift to glycolysis as originally expected, IFN- γ treatment of uninfected cells was included as a potential positive control for metabolic reprogramming [221]. MoDC were prepared as described in **Section 2.3.1** and **Section 2.3.2** respectively. The dendritic cells groups analysed were uninfected, treated with either LPS (1 μ g/ml) or IFN- γ (10ng/ml). On day of infection, cells were plated in at 200,000 cells/well on Seahorse 24-well microplates in RPMI 1640 media supplemented with 10% defined FBS and allowed to adhere for at least 1 hour before infection/treatment. After 24 hours, the Seahorse media was supplemented to all wells and metabolic flux analysis was carried out using the the Seahorse XF Cell Mito Stress Test as before (**Section 2.11**). A crystal violet (CV) assay was performed as described in **Chapter 2 Section 2.11** in order to check the toxicity levels in the culture as well as to check the cell viability.

IFN- γ treatment of uninfected moDCs increased basal OCR and ECAR compared to uninfected untreated moDCs and LPS treated moDCs (**Figure 4.9 C**). Maximum respiration and spare respiratory capacity were not changed in IFN- γ treated moDCs compared to uninfected moDCs and LPS treated moDCs (**Figure 4.10 A&B**) while non-mitochondrial oxygen consumption was slightly higher in IFN- γ treated moDCs compared to uninfected moDCs and LPS treated moDCs (**Figure 4.10 D**). Non-mitochondrial OCR is thought to be due mainly to the production of non-mitochondrial reactive oxygen species (ROS). This increase could be indicative of increased production of ROS by cytoplasmic enzymes such as NADPH oxidases upon IFN- γ treatment. Decreased proton leak was observed in IFN- γ treated moDCs compared to uninfected moDCs and LPS treated moDCs (**Figure 4.10 E**). Thus IFN- γ treatment of moDCs renders them more energetic and may help induce the mechanism of metabolic reprogramming in Mtb infection. This possibility is explored further in Section 4.4.6. The data indicate that even though BCG infection did not have a significant effect on ECAR, moDCs are capable of undergoing an increase in metabolic flux with other stimuli.

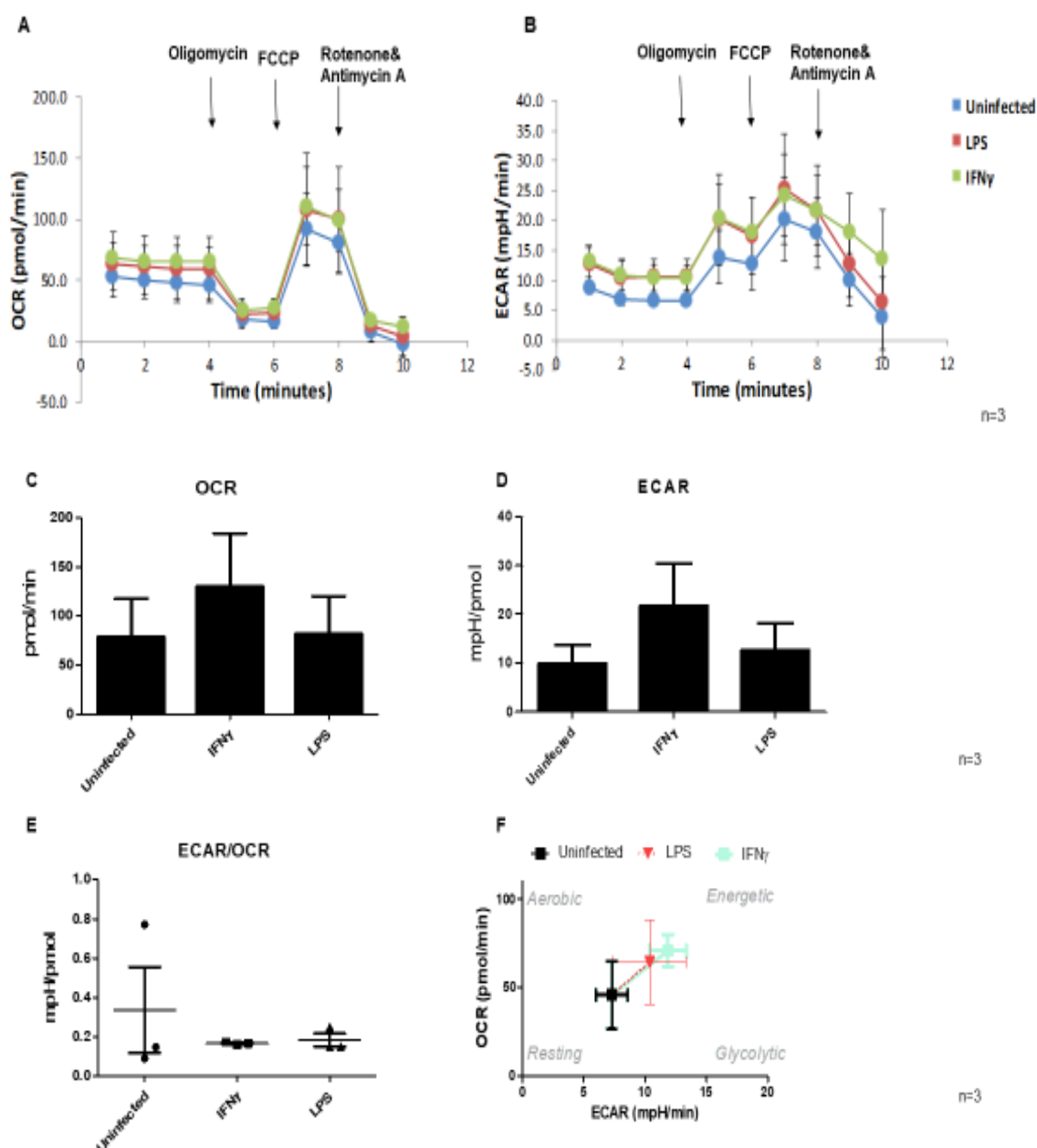


FIGURE 4.9: Comparison of the effect of IFN- γ and LPS on mitochondrial respiration of moDC. moDCs were plated on Seahorse Biosciences 24-well plates at 200,000 cells/well. Cells were treated with 1 μ g/ml LPS and IFN- γ (10ng/ml), extracellular flux analysis was performed with sequential addition of oligomycin (1 μ M), FCCP (2 μ M) and rotenone/antimycin A (0.5 μ M) and data normalised using CV assay. Panels A and B depict mean $\pm$ SEM OCR (A), ECAR (B) of the independent mean parameter values calculated for each assay group assigned to a Group Collection for three individual donors. Panels C – E depict baseline mean $\pm$ SEM OCR (C), ECAR (D) and ratio of ECAR/OCR (E) for the same three individual donors. Statistical analyses were performed using one-way- ANOVA. Panel (F) depict the metabolic phenogram obtained by plotting the basal ECAR and OCR values from the metabolic flux data above.

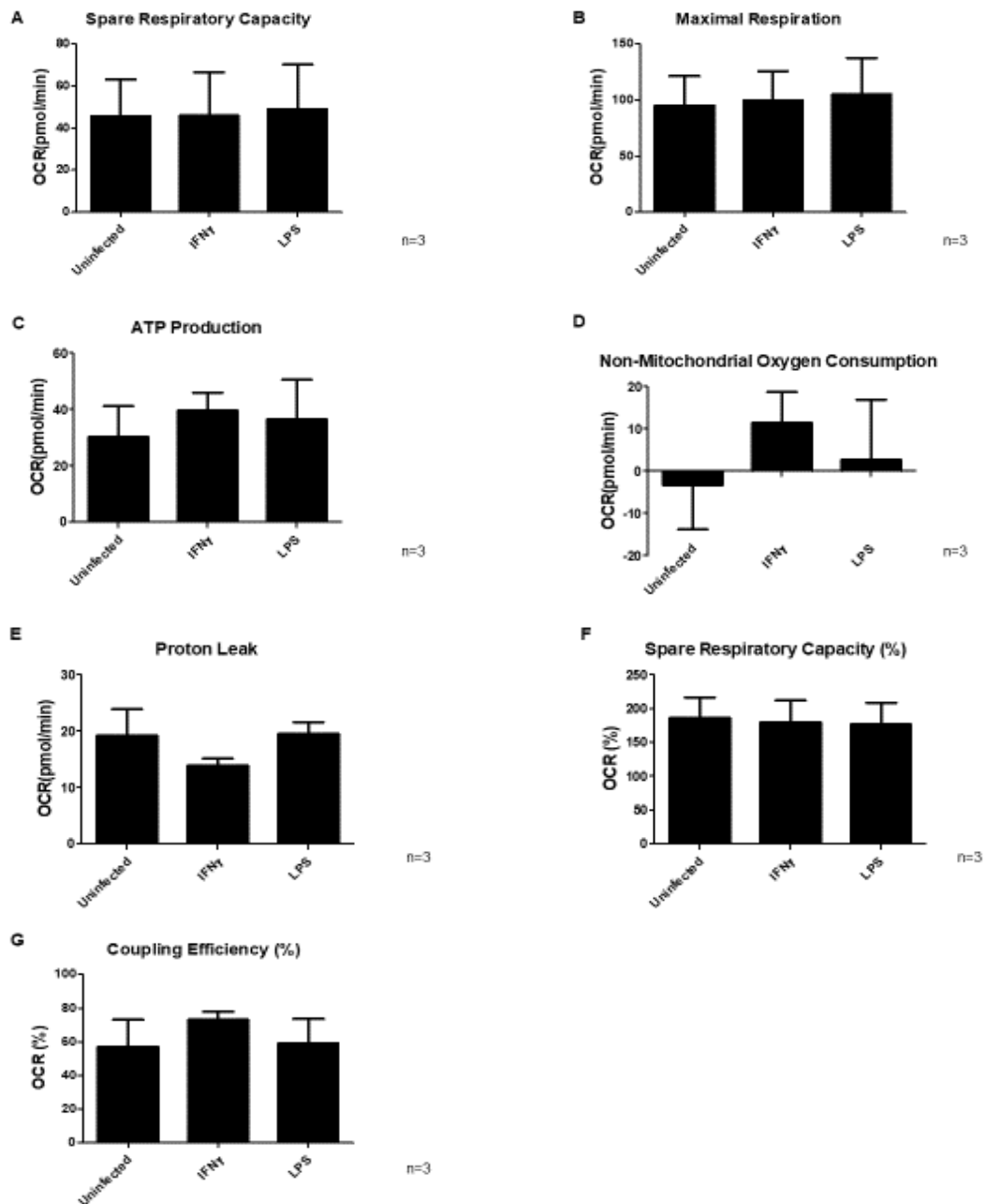


Figure 4.10: Comparison of the effect of IFN- γ and LPS on mitochondrial respiration of moDCs *continued*. Further analysis of the data obtained from the Extracellular flux data shown in Figure 4.9. Panels A and B depict OCR quantification of Spare Respiratory Capacity (A), maximum respiration of moDCs (B). Panels C – G depict normalised mean $\pm$ SEM mitochondrial ATP production (C), Non-Mitochondrial oxygen consumption (D), Proton Leak (E), Spare Respiratory Capacity (%) (F) and Coupling Efficiency (%) (G) for three individual donors. The data were pooled from three independent experiments, mean $\pm$ SEM, statistical differences were analysed by one-way ANOVA with a Tukey multiple comparison test.

4.4.4 Comparison of the effect of BCG and H37Rv on metabolic flux of moDC

BCG is an attenuated strain of *M. bovis* and may not activate moDCs to the same extent as virulent strains of mycobacteria [18]. We wondered whether this difference might be the reason why BCG did not induce a significant metabolic shift towards glycolysis in moDCs. Our group has shown that infection of human macrophages with the virulent Mtb H37Rv strain (both live and killed) induces a shift away from OXPHOS towards glycolysis [158]. Therefore, metabolic flux analysis was carried out on moDCs from seven different donors infected with killed BCG or irradiated H37Rv (iRv) for comparison. MoDC were prepared as described in **Section 2.3.1** and **Section 2.3.2** respectively. The dendritic cells groups analysed were uninfected, infected with killed BCG or iRv (both at MOI 100) or treated with LPS (1µg/ml) or IFN-γ (10ng/ml). On day of infection, cells were plated in at 200,000 cells/well on Seahorse 24-well microplates in RPMI 1640 media supplemented with 10% defined FBS and allowed to adhere for at least 1 hour before infection/treatment. After 24 hours, the Seahorse media was supplemented to all wells and metabolic flux analysis was carried out using the The Seahorse XF Cell Mito Stress Test as before (**Section 2.11**). A crystal violet (CV) assay was performed as described in **Chapter 2 Section 2.11** in order to check the toxicity levels in the culture as well as to check the cell viability.

There was a trend towards increased ECAR in iRv infected moDC compared to uninfected and BCG infected moDCs (**Figure 4.11 B&D**). Mitochondrial ATP production and maximum respiration in iRv infected moDCs was significantly decreased compared with uninfected moDCs (**Figure 4.12 B&C**). The spare respiratory capacity was the same in all of the groups tested (**Figure 4.12 A**). In addition, non-mitochondrial oxygen consumption was unchanged in iRv infected models compared to all other groups (**Figure 4.12 D**). Significantly decreased proton leak was observed in BCG and iRv infected models compared with uninfected moDCs (**Figure 4.12 E**). Kinetic rate data for ECAR was slightly increased in iRv infected moDCs but was not significant (**Figure 4.11 B**). In summary, this test indicated that infection of moDCs with the virulent Mtb H37Rv strain lead to reduced mitochondrial respiration. Furthermore, killed BCG and iRv-infected moDCs showed lower leakage of the protons from the cells, which is an indication of the reduction in the production of energy or ATP. Taken together, these data reflect reduced levels of mitochondrial respiration and may be indicative of damage to the mitochondria or inhibition of mitochondrial function, particularly during virulent Mtb infection.

Cells from three of the donors were treated with IFN- γ , which increased basal levels of both OCR and ECAR (this data is shown in **Figures 4.9**). A phenogram of the basal OCR and basal ECAR for uninfected cells, BCG and iRv-infected cells and IFN- γ is shown in (**Figure 4.11 F**) and shows the contrasting effects of BCG, iRv and IFN- γ on moDC metabolic flux.

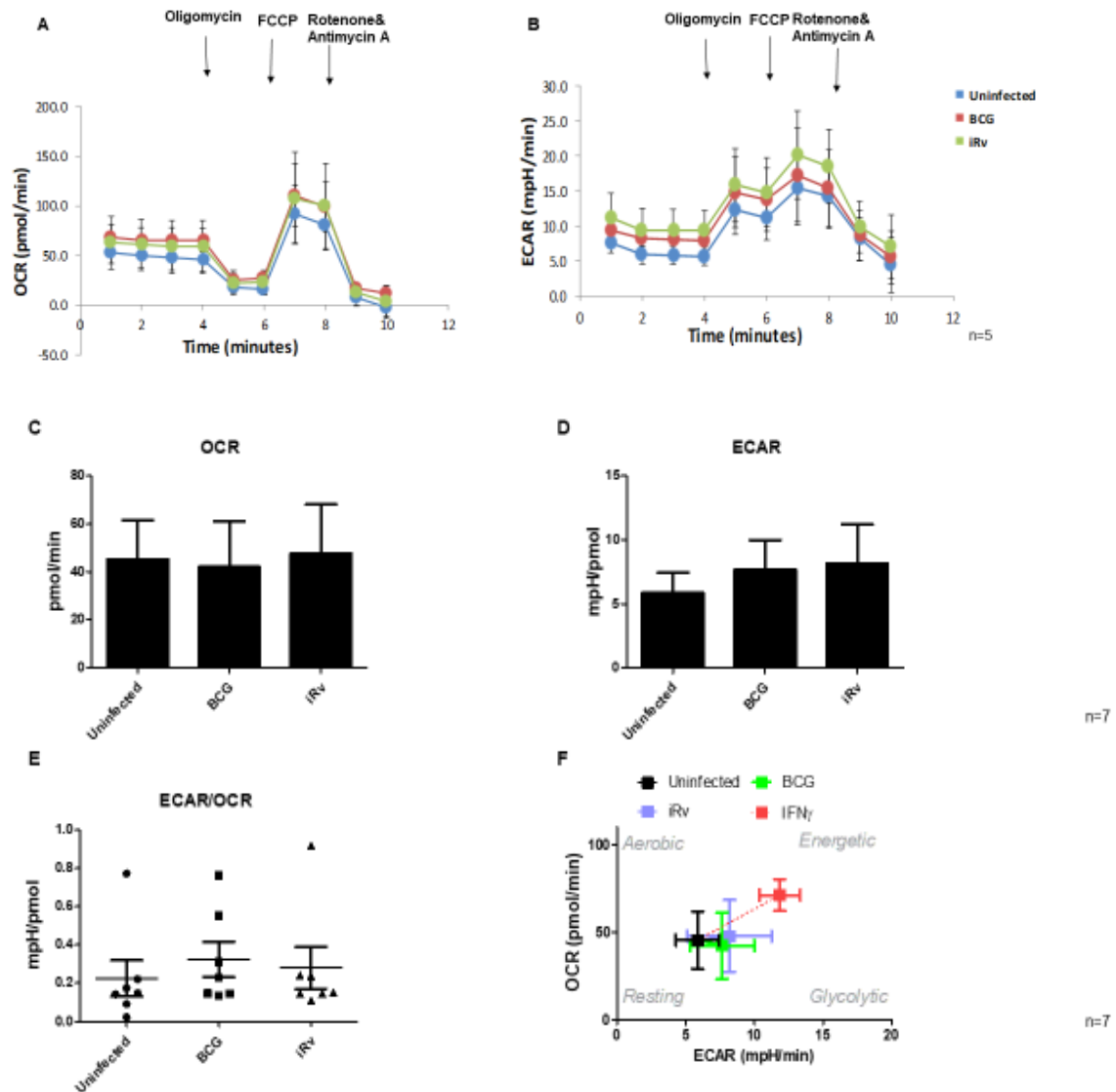


Figure 4.11: Comparison of the effect of BCG and Mtb on mitochondrial respiration of moDCs. MoDCs were plated on Seahorse Biosciences 24-well plates at 200,000 cells/well. Cells were uninfected, infected with killed BCG and iRv at MOI 100 for 24 hours and extracellular flux analysis was performed with sequential addition of oligomycin (1 μ M), FCCP (2 μ M) and rotenone/antimycin A (0.5 μ M) and data normalised using CV assay. Panels A and B depict mean $\pm$ SEM OCR (A), ECAR (B) of the independent mean parameter values calculated for each assay group assigned to a Group Collection for five individual donors. Panels C – E depict baseline mean $\pm$ SEM OCR (C), ECAR (D) and ratio of ECAR/OCR (E) for the same seven individual donors. Statistical analyses were performed using one-way- ANOVA. Panel (F) depict the metabolic phenogram obtained by plotting the basal ECAR and OCR values from the metabolic flux data above.

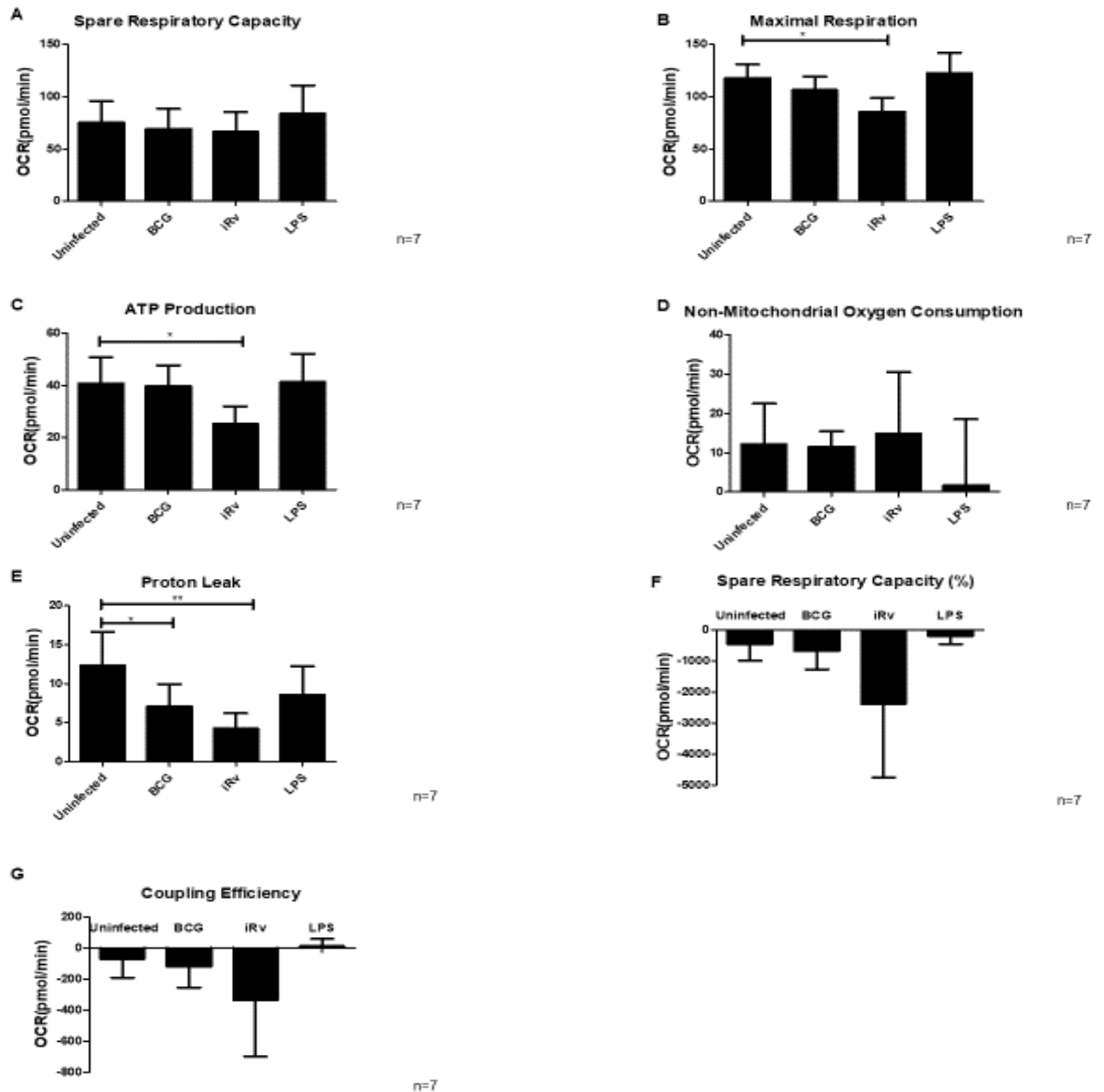


Figure 4.12: Comparison of the effect of BCG and Mtb on mitochondrial respiration of moDCs *continued*. Further analysis of the data obtained from the extracellular flux data shown in Figure 4.9. Panels A and B depict OCR quantification of Spare Respiratory Capacity (A), maximum respiration of moDCs (B). Panels C – G depict normalised mean $\pm$ SEM mitochondrial ATP production (C), Non-Mitochondrial oxygen consumption (D), Proton Leak (E), Spare Respiratory Capacity (%) (F) and Coupling Efficiency (%) (G) for seven individual donors. The data were pooled from seven independent experiments, mean $\pm$ SEM, statistical differences were analysed by one-way ANOVA with a Tukey multiple comparison test.

4.4.5 Dose dependent effect of BCG and Mtb infection on metabolic flux in mo-DCs

Since there was no significant increase in ECAR or OXPHOS in mo-DCs infected with BCG at MOI 100, different multiplicities of infection (MOI) were tested to check whether lower or higher MOIs had any effect on the levels of ECAR or OXPHOS. MoDCs were prepared as described in **Section 2.3.1** and **Section 2.3.2** respectively. On day of infection, cells were plated at 200,000 cells/well on Seahorse 24-well microplates as before. Different MOI of killed BCG and iRv (50,100,250) were added to the wells and the concentrations of 10, 100, 1000 ng LPS were used to test whether there were dose dependent differences in regulation of OXPHOS and ECAR.

Mitochondrial OCR was not altered following BCG infection at any MOI (**Figures 4.13 A & E**). This result indicates that the oxygen consumption levels are not affected at all when moDC were infected with BCG even when different MOI were used. There was a trend towards reduced OCR for cells infected with iRv at MOI 100 and 250 compared to uninfected moDCs (**Figures 4.13 C & G**), also there was a trend towards reduced OCR in LPS treated cells in 10ng/ml and 1000ng/ml compared to 100ng/ml (**Figures 4.14 A & C**).

Studies in human monocytes treated with LPS show concentration-dependent differences in metabolic flux [222]. However, there was no significant difference in OCR or ECAR in moDCs treated with any of the tested doses of LPS (**Figure 4.14**).

The results show that there was no difference in spare respiratory capacity or maximal respiration between all groups (**Figure 4.15**), in keeping with the results obtained in the previous experiment using an MOI of 100 (**Figure 4.12**). Others have shown that non-mitochondrial OCR typically increases in the presence of stressors [223]. In contrast to the previous experiment (**Figure 4.12 D**) non-mitochondrial oxygen consumption in moDCs infected with iRv at MOI 100 was lower compared to the uninfected control and iRv MOI 50 and 250. This could be due to the higher variability between this set of donors for this parameter at MOI 100 (**Figure 4.15 G**) which may be due to the lower number of donors (four compared to seven previously). In keeping with previous results there was a decrease in proton leak with iRv infection at MOI 100 (**Figure 4.15 C**).

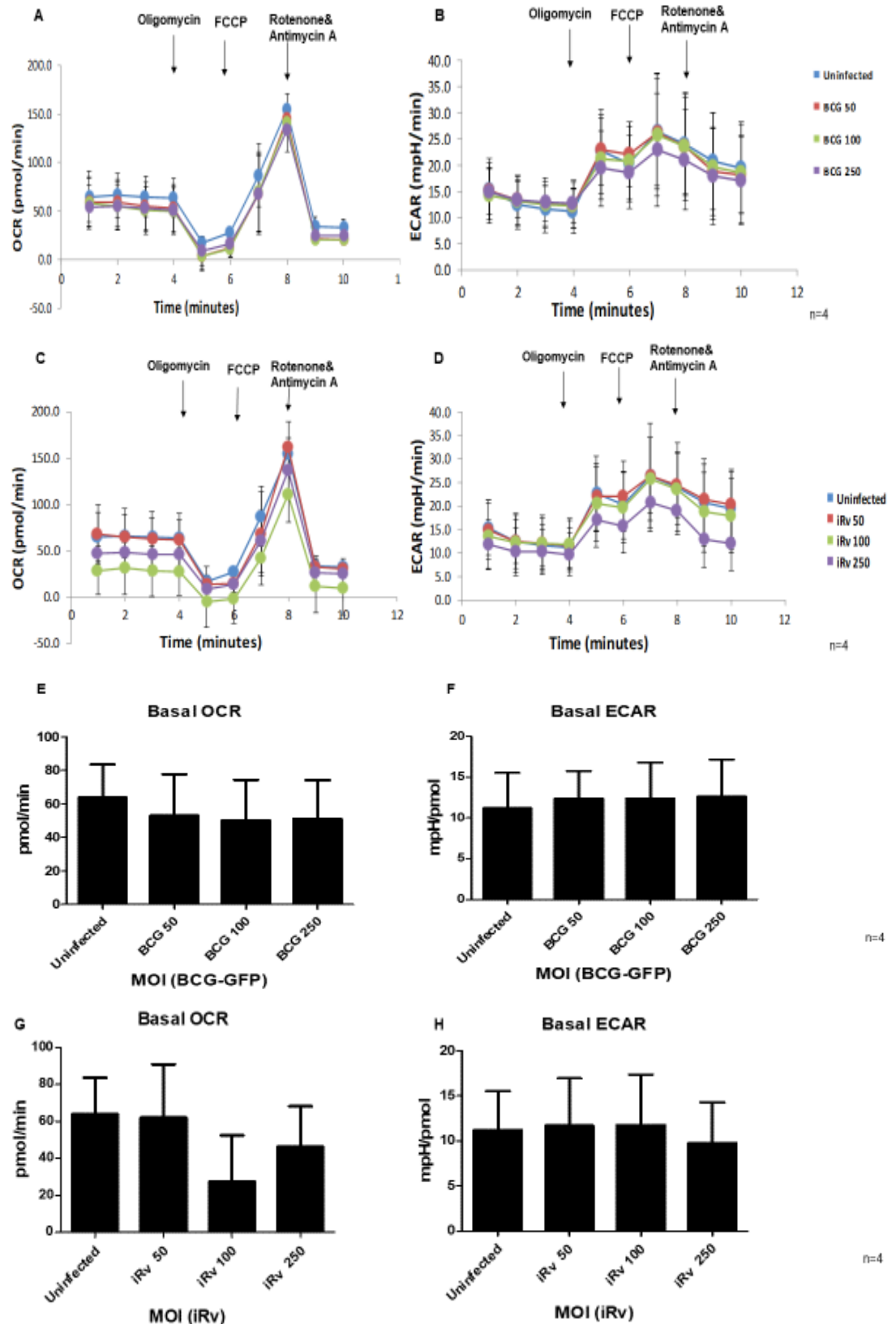


FIGURE 4.13: The effect of BCG, iRv on mitochondrial function varies according to MOI. Mo-DCs were plated on Seahorse Biosciences 24-well plates at 200,000 cells/well. Cells were infected for 24 hours with different MOI of BCG and iRv (50,100,250), different MOI were used to test whether the differences in regulation of OXPHOS were dose dependent. Extracellular flux analysis was performed with sequential addition of oligomycin (1 μ M), FCCP (2 μ M) and rotenone/antimycin A (0.5 μ M) and data normalised using CV assay.

Panels A and B depict mean $\pm$ SEM OCR in BCG (**A**), ECAR in BCG (**B**) of the independent mean parameter values calculated for each assay group assigned to a Group Collection for four individual donors. Panels C and D depict mean $\pm$ SEM OCR in iRv (**C**), ECAR in iRv (**D**) of the independent mean parameter values calculated for each assay group assigned to a Group Collection for four individual donors. Panels E and F depict baseline mean $\pm$ SEM OCR of BCG (**E**), ECAR of BCG (**F**). Panels G and H depict baseline mean $\pm$ SEM OCR of iRv (**G**), ECAR of iRv (**H**) for the same four individual donors. Statistical analyses were performed using one-way- ANOVA with a Tukey multiple comparison test.

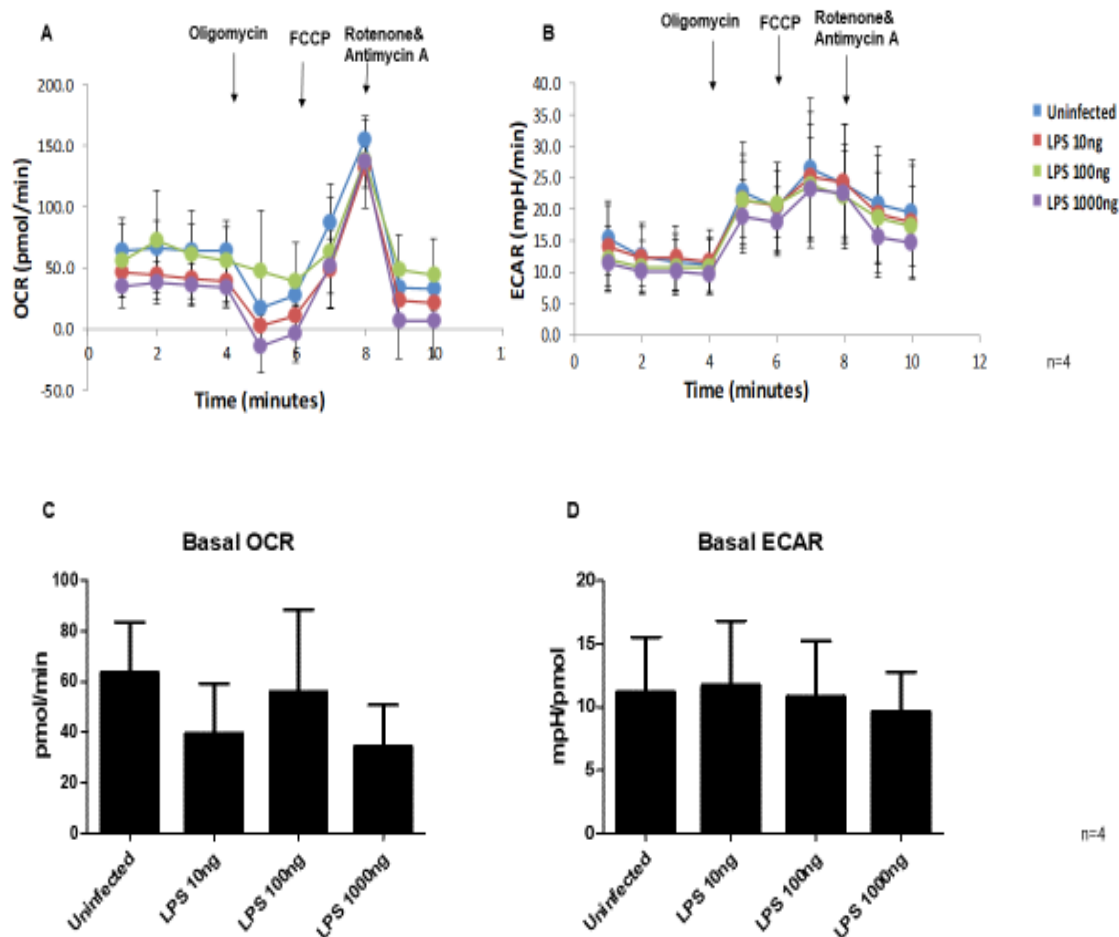


FIGURE 4.14: The effect of LPS on mitochondrial function varies according to concentration. MoDCs were plated on Seahorse Biosciences 24-well plates at 200,000 cells/well. Cells were treated with 1 μ g/ml LPS, extracellular flux analysis was performed with sequential addition of oligomycin (1 μ M), FCCP (2 μ M) and rotenone/antimycin A (0.5 μ M) and data normalised using CV assay. Panels A and B depict mean $\pm$ SEM OCR in LPS (A), ECAR in LPS (B) of the independent mean parameter values calculated for each assay group assigned to a Group Collection for four individual donors. Panels C and D depict baseline mean $\pm$ SEM OCR of LPS (C), ECAR of LPS (D) for the same four individual donors. Statistical analyses were performed using one-way- ANOVA with a Tukey multiple comparison test.

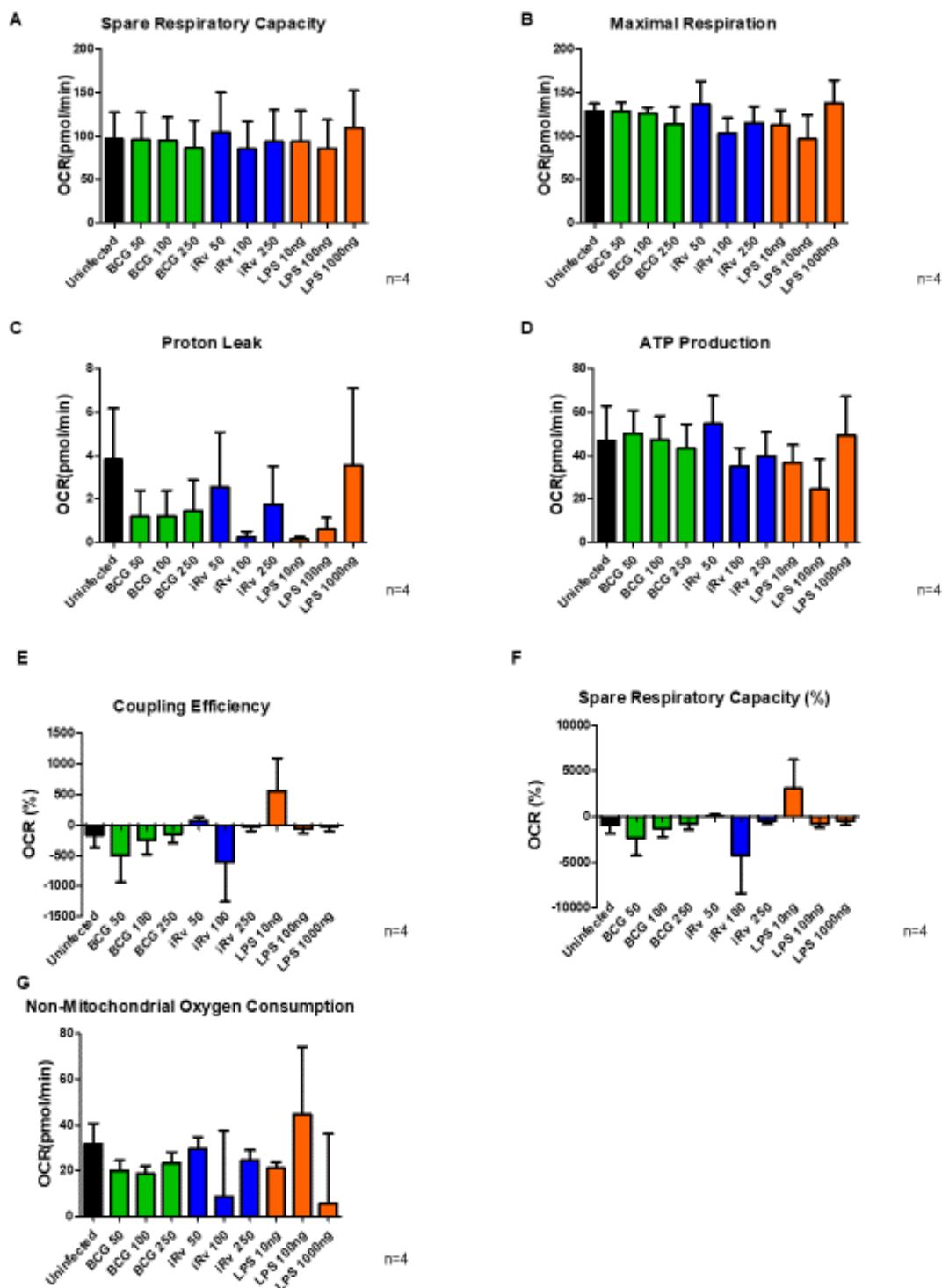


FIGURE 4.15: The effect of BCG, iRv and LPS on mitochondrial function in moDCs varies according to MOI *continued*. Further analysis of the data obtained from the Extracellular flux data shown in Figure 4.13 & Figure 4.14. Panel A depict OCR quantification of Spare Respiratory Capacity. Panels B – G depict normalised mean $\pm$ SEM maximum respiration OCR (B), Proton Leak (C) mitochondrial ATP production (D), Coupling Efficiency (%) (E), Spare Respiratory Capacity (%) (F) and Non-Mitochondrial oxygen consumption (G) for four individual donors. The data were pooled from four independent experiments, mean $\pm$ SEM, statistical differences were analysed by one-way ANOVA with a Tukey multiple comparison test.

4.4.5 Time-dependent glycolytic responses of monocyte-derived DC after challenge with BCG and Mtb

The previous experiments using the Mito Stress test showed no significant change in basal ECAR and OCR in BCG-infected cells relative to uninfected controls 24 hours after infection but it was possible that these changes could be occurring earlier and had been missed. In order to determine whether the dependence of moDCs on glycolysis varies with time post infection, moDCs were prepared as described in **Section 2.3.1** and **Section 2.3.2**, respectively and infected at different times with BCG and H37Rv. On the day of infection, cells were scraped, and plated at 250,000 cells/well on Seahorse 24-well microplates in RPMI 1640 media supplemented with 10% defined FBS at least 1 hour before infection with BCG and iRv at MOI 10, 50, 100. Uninfected moDCs were treated with 1µg/ml LPS. At the following times post infection - 15 minutes, 30 minutes, 1 hour, 3 hours, 6 hours and 24 hours - the cells were washed twice by the addition of 400µl of Seahorse media (without glucose or pyruvate), and then 450µL of fresh Seahorse media (without glucose or pyruvate) was applied to each well. To investigate the glycolytic function in a more precise and detailed way ECAR was measured using the Seahorse XF Glycolysis Stress test. To determine the glycolytic flux profile, ECAR was measured while consecutively injecting glucose, oligomycin, and 2-DG. Afterwards, a crystal violet (CV) assay was performed as described in **Chapter 2 Section 2.11** to check the cell quantity and cell viability and normalise the data.

For the Seahorse XF Glycolysis Stress Test the cells are starved of glucose, therefore non-glycolytic acidification is represented by the last rate measurement prior to glucose injection. The first injection of a saturating concentration of glucose gives a measure of the basal glycolytic rate. The second injection of the ATP synthase inhibitor oligomycin shuts down mitochondrial ATP production causing the cells to switch to glycolysis for energy production and giving a measure of their maximal glycolytic capacity. Finally, 2DG is injected to completely inhibit glycolysis and the difference between basal glycolysis and glycolytic capacity defines the glycolytic reserve of the cells (**Figure 4.16**).

Several pilot time courses were carried out to determine the best time points and MOIs to use. In the first time course, moDCs were infected with BCG or iRv (MOI 10, 50 and 100) or treated with LPS for between 15 minutes to 24 hours and ECAR was measured under basal and stressed conditions (data not shown). There were differences seen in basal ECAR

at some time points in moDCs infected with BCG and iRv at MOI 50 and 100. On the other hand, BCG- and iRv-infected moDCs at MOI 10 showed no difference in ECAR values compared to uninfected cells at any time points (data not shown). As a consequence, the MOI of 10 was not used in any further experiments.

Based on the pilot study above and to further assess the observed differences in these cells, moDCs from three donors were infected with BCG or iRv (at MOI 50 and 100) or treated with LPS for 3, 6 and 24 hours and glycolytic function was interrogated using the glycolysis stress test. ECAR was investigated at 3 hours, 6 hours and 24 hours after infection with BCG or iRv (MOI 50 and 100), similar to the MOIs used by the Steyn group in their studies of macrophage infection with BCG [159]. Glucose addition to moDCs triggered an immediate ECAR increase representing the basal rate of glycolysis of the cells (**Figure 4.16 B**). Interestingly, a significant increase in non-glycolytic acidification in iRv-infected moDCs at MOI 100 was seen after 24 hours (**Figure 4.17 F**). Although there was a trend towards increased ECAR/OCR ratio with BCG MOI 50 after 6 hours and BCG and iRv MOI 100 after 24 hours, there was no significant difference between all groups (**Figure 4.17 I&J**). There was a trend towards increased glycolysis in BCG infected moDCs at MOI 100 moDCs after 6 hours compared to uninfected, and a significant increase after 24 hours (**Figure 4.17 D**), suggesting these cells are more glycolytically active at this time. The data are summarised in metabolic phenograms in **Figure 4.19**. Glycolytic capacity had increased after 3 hours in BCG and iRv infected moDCs at MOI 100 compared to uninfected (**Figure 4.18 A**). Glycolytic reserve was significantly increased after 3 hours in BCG infected moDCs at MOI 100 compared to uninfected while a significantly increased in iRv infected moDCs at MOI 100 after 6 hours (**Figure 4.18 C**). Glycolytic reserve % was significantly decreased in BCG infected moDCs at MOI 100 and LPS treated moDCs after 24 hours (**Figure 4.18 E&F**). Taken together the results show that glycolytic function can be significantly enhanced in BCG infected moDCs and varies depending on the time points examined.

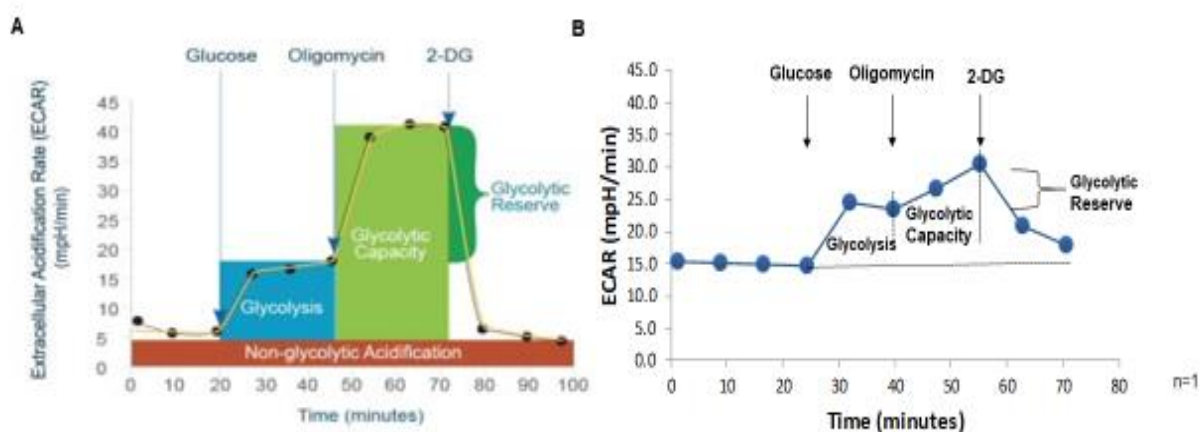


Figure 4.16: Metabolic parameters derived from a XF Extracellular flux assay (Glycolysis stress test). Panel **A** depicts ECAR measurements (in mpH/min) at baseline and following sequential addition of metabolic inhibitors, illustrating how different glycolytic parameters are calculated. Adapted from Agilent Seahorse XF Glycolysis Test Kit [224]. Panel **B** shows a representative real-time glycolysis stress test experiment with ECAR analysis starting from the first 4 readings in glucose-starved cells and after the addition of glucose to measure basal glycolysis, followed by the two inhibitors: ECAR (mpH/min) in uninfected moDCs for one representative donor.

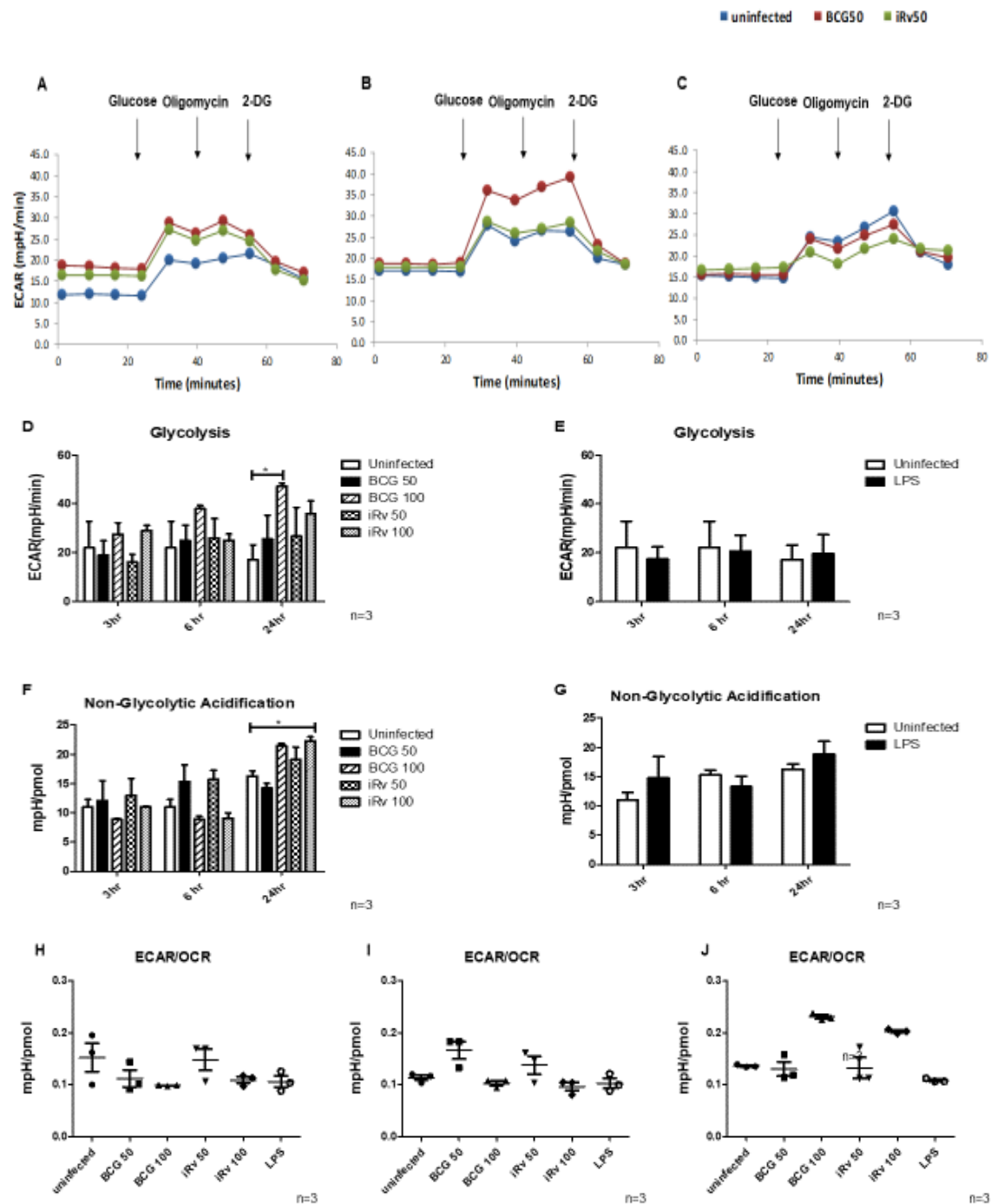


Figure 4.17: The effect of time on the rate of glycolytic flux in moDCs (time post infection 3hr, 6hr and 24hr). Mo-DCs were plated on Seahorse Biosciences 24-well plates at 250,000 cells/well. Cells were infected at indicated time with different MOI of BCG and iRv (50 and 100). Uninfected moDCs were treated with 1μg/ml LPS. Extracellular flux analysis was performed with sequential addition of glucose (10Mm), oligomycin (1μM) and 2DG (50mM) and data normalised using CV assay. Panels A- C depict ECAR quantification, representative kinetic ECAR at time post infection 3hr (A), representative kinetic ECAR at time post infection 6hr (B), representative kinetic ECAR at time post infection 24hr (C) all for one donor. Panels D and E depict ECAR quantification of moDCs of glycolysis after BCG and iRv infected (D), glycolysis after LPS treated (E). Panels F and G depict non glycolytic acidification in BCG and iRv (F) and non glycolytic acidification in LPS treated moDCs (G) for three individual donors. Panels H- J depict the ratio of ECAR/OCR at time post infection 3hr (H), at time post infection 6hr (I) and at time post infection 24hr (J) for the same three individual donors. Statistical analyses were performed using two way- ANOVA with Bonferroni posttests **P < 0.001; **P < 0.01, *P < 0.05 for all data

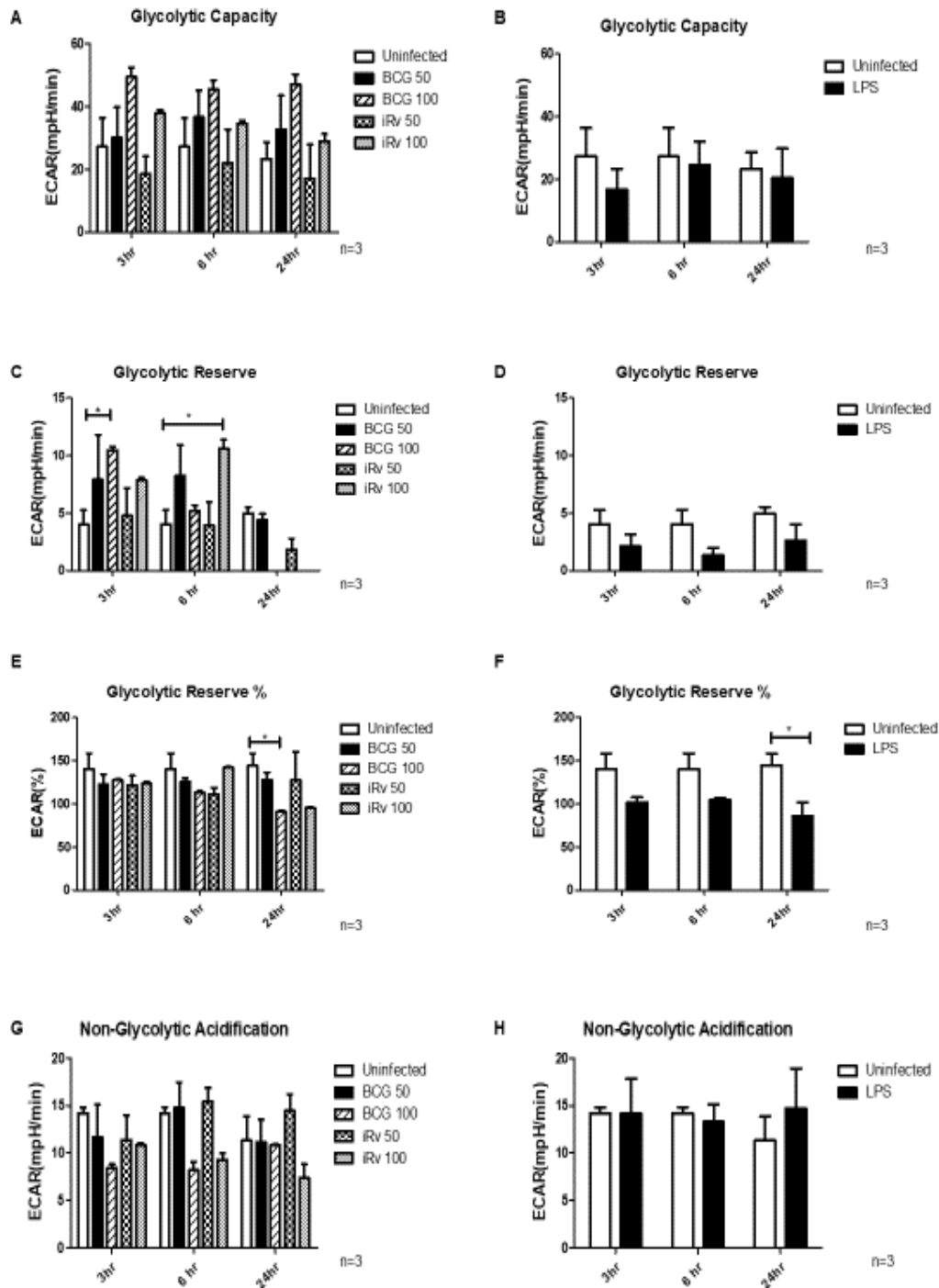


Figure 4.18: The effect of time on the rate of glycolytic flux in moDCs *continued*. Further analysis of the data obtained from the extracellular flux data shown in Figure 4.17. Panels A–H depict ECAR quantification of moDCs of glycolytic capacity after BCG and iRv infected (A), glycolytic capacity after LPS treated (B), glycolytic reserve after BCG and iRv infected (C), glycolytic reserve after LPS treated (D), glycolytic reserve % after BCG and iRv infected (E), glycolytic reserve % after LPS treated (F), non-glycolytic acidification after BCG and iRv infected (G) and non-glycolytic acidification after LPS treated (H) for three individual donors. All data represent mean $\pm$ SEM of the independent mean parameter values calculated for each assay group. Statistical analyses were performed using two way- ANOVA with Bonferroni post-tests $**P < 0.001$; $**P < 0.01$, $*P < 0.05$ for all data.

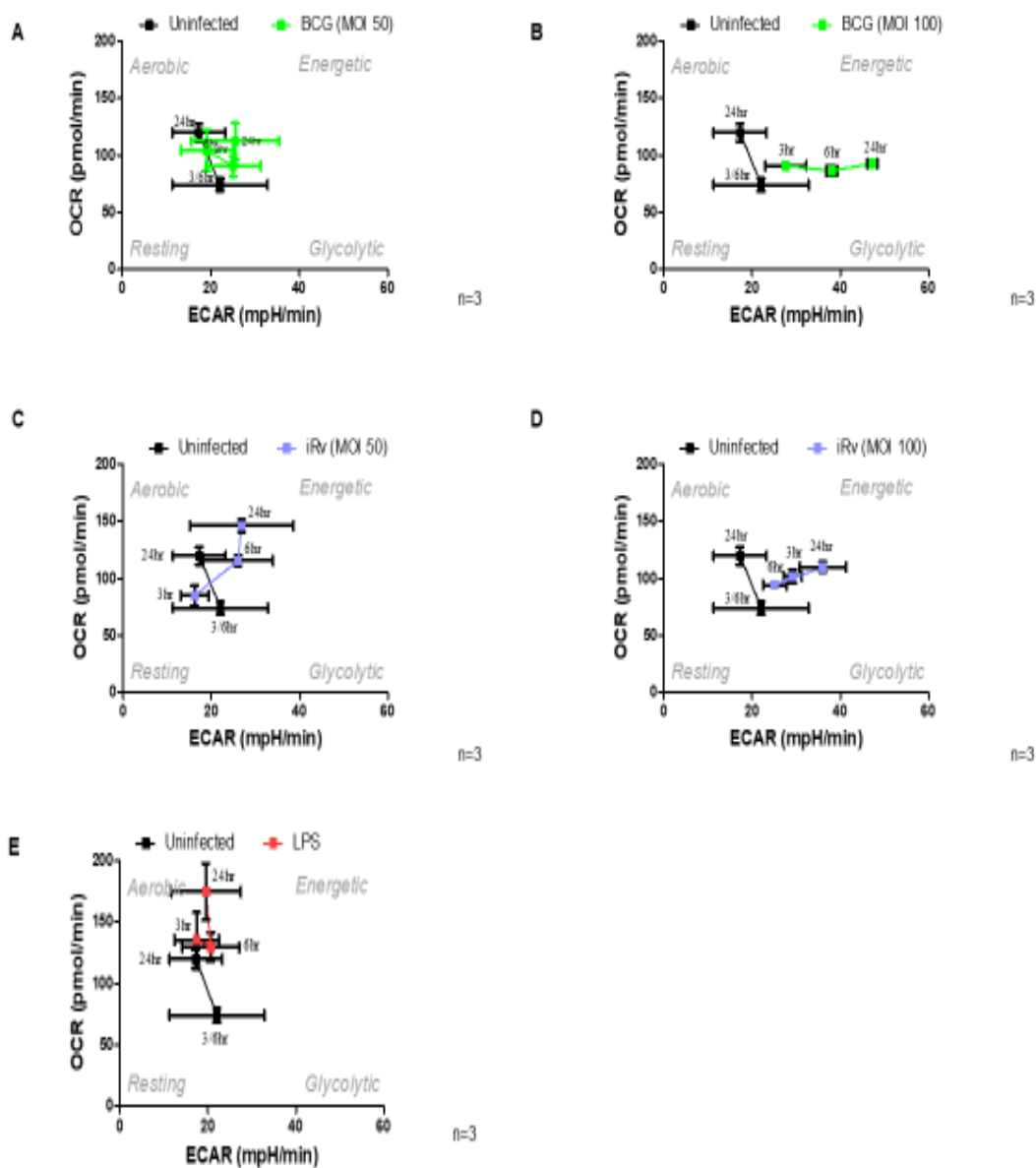


Figure 4.19: The effect of time on the rate of glycolytic flux in moDCs *continued*. Summarise analysis of the data depict the metabolic phenogram obtained by plotting the basal ECAR and OCR values from the metabolic flux data above. Panels A-E depict the metabolic phenogram obtained by plotting the basal ECAR and OCR values from the metabolic flux data above in BCG MOI 50 infected moDCs (**A**), BCG MOI 100 infected moDCs (**B**), iRv MOI 50 infected moDCs (**C**), iRv MOI 100 infected moDCs (**D**) and LPS treated moDCs (**E**)

4.4.6 The impact of Metformin and IFN- γ on glycolytic function of moDCs

The studies outlined so far in this chapter have failed to always detect a strong and consistent shift towards glycolysis and away from OXPHOS in mo-DCs infected with mycobacteria or cells stimulated with LPS. Considering their ability to subvert host immunity it is possible that mycobacteria inhibit this metabolic shift in human mo-DCs to some extent. It was surprising that LPS treatment did not lead to increased glycolytic flux, however it is also possible that human mo-DC responses to LPS differ to those of murine BMDCs. Nevertheless, it would be reassuring to have a good positive control that induces changes in glycolytic flux in these cells to verify that the lack of effect observed so far is not an artefact of the cell culture conditions or some other technical problem with the assays. To this end, mo-DCs were treated with IFN- γ and metformin, both of which have been shown to increase ECAR in other cell types [225–227]. Previous metabolic flux experiments in this chapter (**Section 4.4.3**) carried out using the Seahorse XF Cell Mito Stress Test showed that IFN- γ treatment of moDCs increased both ECAR and OCR compared to untreated or LPS treated cells, therefore it was tested for its effect on mo-DCs. Metformin is a synthetic drug which is commonly known by its market name of Glucophage and is used to treat the diabetes. Metformin is an inhibitor of complex 1 in the mitochondria so it inhibits OXPHOS. Cells treated with metformin led to energetically inefficient mitochondria, resulting in increased aerobic glycolysis to compensate. The sensitivity of cells to metformin-induced cytotoxicity is dependent on their capability to overcome this energetic stress. Inhibition of mitochondrial complex I has been shown to be important for the Warburg effect and the promotion of aerobic glycolysis [228].

The effect of IFN- γ on moDC metabolism

First, the impact of IFN- γ treatment of moDCs metabolism was investigated. Mo-DCs were prepared as described **Section 2.3.1** and **Section 2.3.2** respectively. On day of infection, moDCs were scraped, and plated in at 200,000 cells/well on Seahorse 24-well microplates in RPMI 1640 media supplemented with 10% defined FBS at least 1 hours before BCG and iRv infection at MOI 50 with or without IFN- γ (10ng/ml). Uninfected moDCs were also treated with IFN- γ (10ng/ml). After 24 hours infection, the Seahorse media was supplemented to all wells and metabolic flux was interrogated using the Seahorse XF Glycolysis Stress test. Afterwards, a crystal violet (CV) assay was performed as described

in **Chapter 2 Section 2.11** to check the cell quantity and cell viability and normalise the data.

To confirm that IFN- γ could increase glycolysis as measured by the Glycolytic Stress test moDCs were treated with IFN- γ alone (IFN- γ control) and were also infected with BCG and iRv for comparison. Treatment with IFN- γ alone increased the rate of glycolysis (**Figure 4.20 C**) from 4.82, SD 4.0 to 11.72, SD 7.36 compared to the untreated moDCs. As seen previously, basal OCR was also increased by IFN- γ for untreated cells. In contrast, moDCs infected with either BCG or iRv did not increase glycolysis (**Figure 4.20 C**). BCG and iRv infected moDCs did not alter OCR (**Figure 4.20 B**) but a trend towards increased ECAR/OCR ratio was observed in BCG infected moDCs (**Figure 4.20 D**), though this did not achieve statistical significance. Taken together, the data indicate that IFN- γ alone can drive glycolysis and confirms the data from the Mito Stress test (**Section 4.4.3**) that the moDCs are capable of becoming more metabolically active under certain conditions.

Following on from the above experiments, moDCs were infected and treated with IFN- γ . MoDC were prepared as described in **Section 2.3.1** and **Section 2.3.2** respectively. The dendritic cells groups analysed were uninfected, treated IFN- γ (10ng/ml). On day of infection, cells were plated in at 200,000 cells/well on Seahorse 24-well microplates in RPMI 1640 media supplemented with 10% defined FBS and allowed to adhere for at least 1 hour before infection/treatment. After 24 hours, the Seahorse media was supplemented to all wells. and metabolic flux analysis was carried out the Seahorse XF Glycolysis Stress test. Afterwards, a crystal violet (CV) assay was performed as described in **Chapter 2 Section 2.11** to check the cell quantity and cell viability and normalise the data.

Again, cells treated with IFN- γ alone showed increased glycolysis compared to untreated/uninfected moDCS (**Figure 4.21 C**), and also displayed increased glycolytic capacity compared to untreated cells (**Figure 4.21 H**). In addition, glycolysis was increased in BCG and iRV-infected moDCs and treated IFN- γ compared to untreated/uninfected cells (**Figure 4.21 C**) and the glycolytic capacity of these cells was also increased by IFN- γ (**Figure 4.21 H**). These observations indicate that IFN- γ treatment increased the glycolytic rate of moDCs infected with mycobacteria. IFN- γ treatment also increased the basal OCR of uninfected moDCS (**Figure 4.21 A&B**) as seen in the previous experiment.

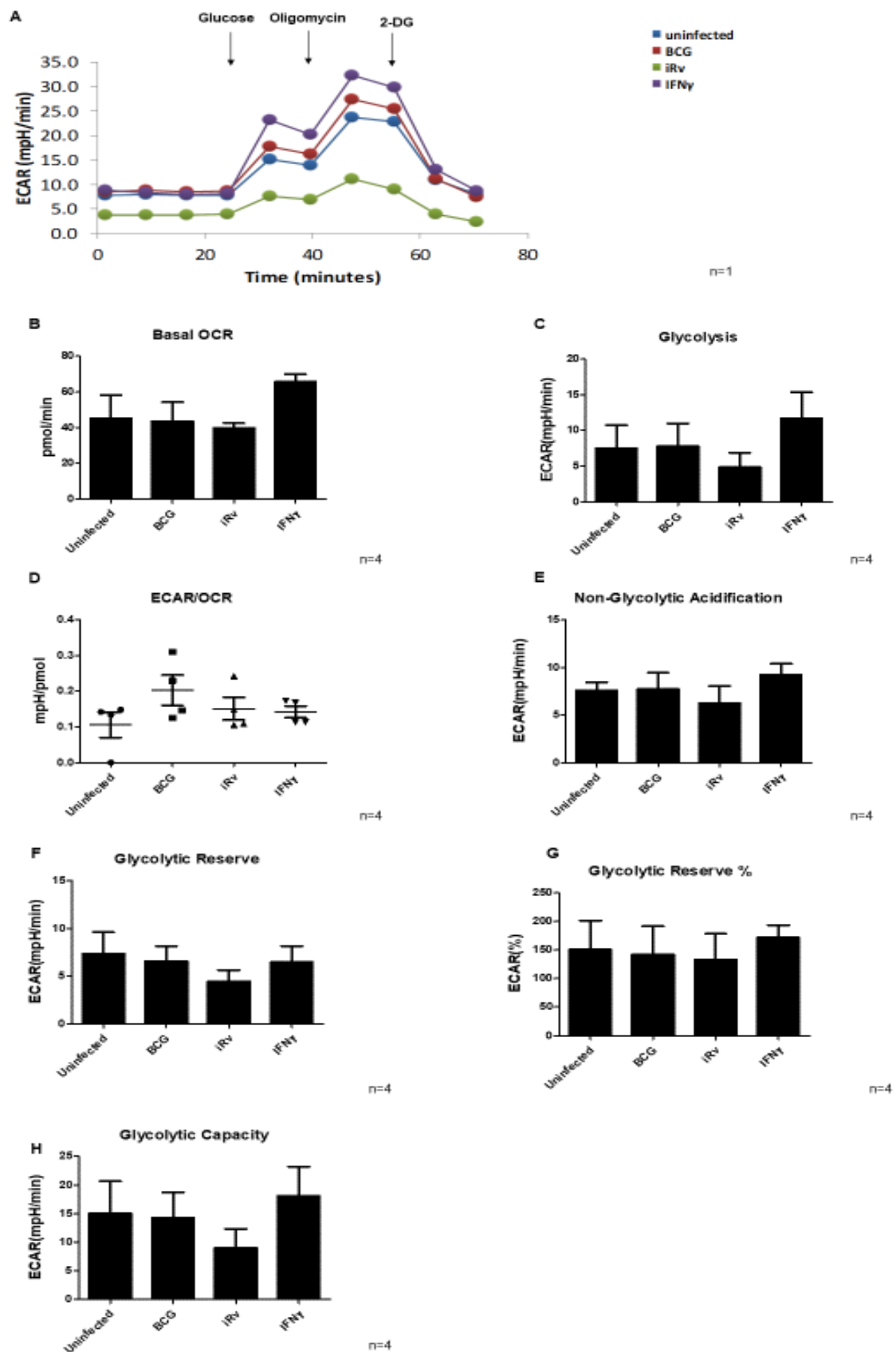


FIGURE 4.20: Effect of IFN- γ (10ng/ml) on glycolytic function of moDC. moDCs were plated on Seahorse Biosciences 24-well plates at 200,000 cells/well. cells were infected for 24 hours with BCG and iRv MOI 50, uninfected moDCs were IFN- γ (10ng/ml) treated. Extracellular flux analysis was performed with sequential addition of glucose (10Mm), oligomycin (1 μ M) and 2DG (50mM) and data normalised using CV assay. Panel

(**A**) depicts representative kinetic ECAR in uninfected moDCs (blue), BCG MOI 50 infected moDCs (red), iRv MOI 50 infected moDCs (green), IFN- γ (10ng/ml) treated moDCs (purple) for one donor. Panels B – D depict baseline mean $\pm$ SEM OCR (**B**), glycolysis (**C**), ratio of ECAR/OCR (**D**). Panels E –H depict ECAR quantification of moDCs of non-glycolytic acidification (**E**), glycolytic reserve (**F**), glycolytic reserve % (**G**) and glycolytic capacity (**H**) for four individual donors. All data represent mean $\pm$ SEM of the independent mean parameter values calculated for each assay group.

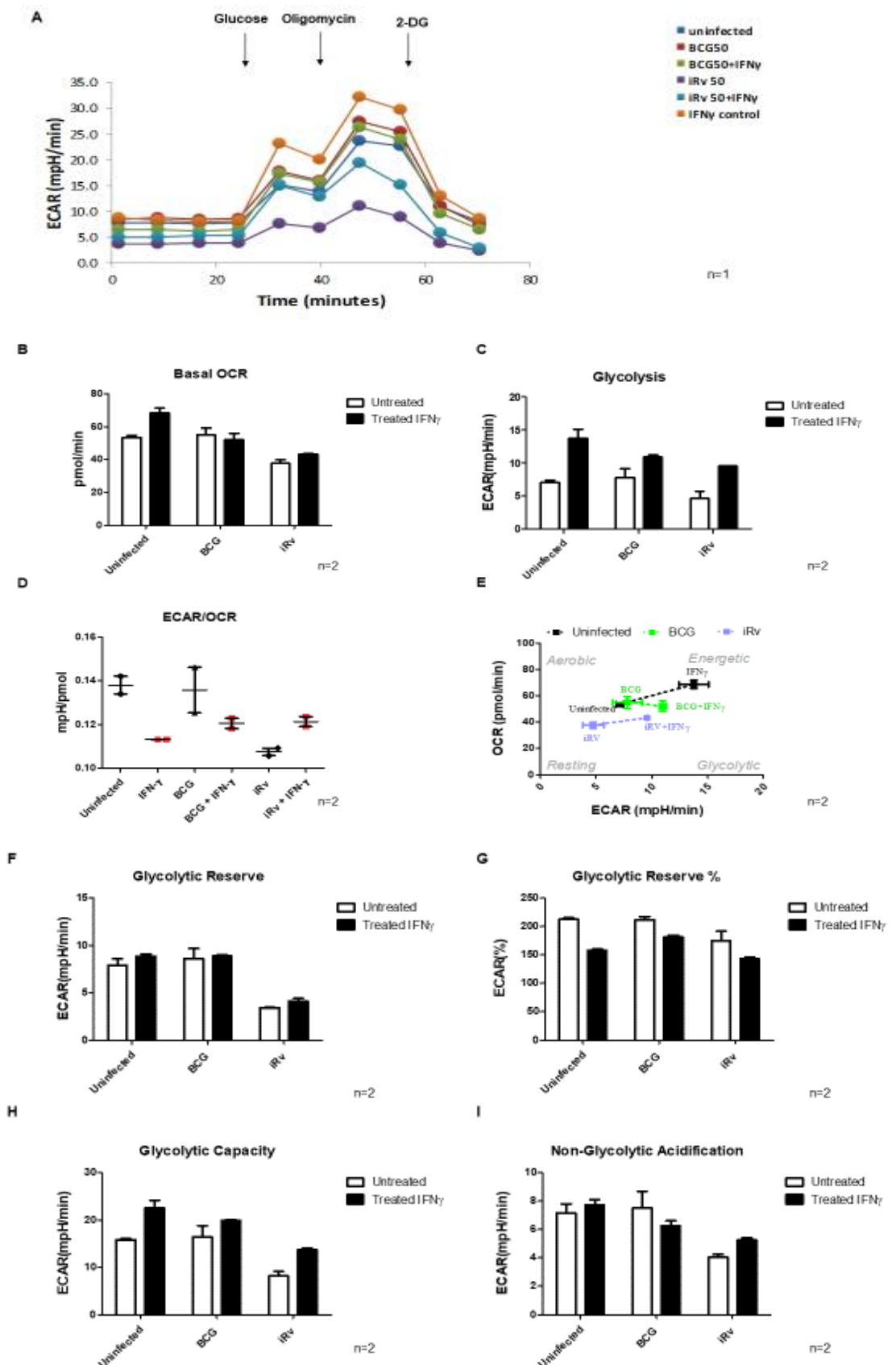


FIGURE 4.21: The impact of IFN- γ (10ng/ml) on glycolytic and mitochondrial function of BCG - infected moDCs. MoDCs were plated on Seahorse Biosciences 24-well plates at 200,000 cells/well. Cells were infected for 24 hours with BCG and iRv MOI 50, IFN- γ (10ng/ml) treated or untreated BCG infected moDCs, uninfected moDCs were IFN- γ (10ng/ml) treated or untreated. Extracellular flux analysis was

performed with sequential addition of glucose (10Mm), oligomycin (1 μ M) and 2DG (50mM) and data normalised using CV assay. Panel **(A)** depict representative kinetic ECAR in uninfected moDCs (blue), untreated BCG MOI 50 infected moDCs (red), IFN- γ (10ng/ml) treated BCG MOI 50 infected moDCs (green), untreated iRv MOI 50 infected moDCs (purple), IFN- γ (10ng/ml) treated iRv MOI 50 infected moDCs (aqua) and IFN- γ (10ng/ml) control (orange) for one donor. Panels B – E depict baseline mean $\pm$ SEM OCR **(B)**, glycolysis **(C)**, ratio of ECAR/OCR **(D)** and a metabolic phenogram showing basal ECAR and OCR levels after glucose addition **(E)**. Panels F –I depict ECAR quantification of moDCs of glycolytic reserve **(F)**, glycolytic reserve % **(G)**, glycolytic capacity **(H)** and non-glycolytic acidification **(I)** for two individual donors. All data represent mean $\pm$ SEM of the independent mean parameter values calculated for each assay group

The effect of metformin on moDC metabolism

Next, to determine the effect of Metformin on glycolytic and mitochondrial function, moDCs were prepared as described in **Section 2.3.1** and **Section 2.3.2** respectively. First, to address if metformin treatment led to cell death, moDCs infected with BCG and iRv (MOI 50 and 100) and treated with 5mM metformin as described. After 24 hours infection, the cells were stained with PI/Hoechst and their viability was determined using the Cytell Analyser (**Chapter 2 Section 2.6.2**). The percentage of PI positive cells in the untreated groups ranged from 12 % to 13.7% and was slightly but consistently increased in metformin-treated groups without or with infection (ranging from 21.1% to 25.1%) (**Figure 4.22**). This data indicates that moDCs are relatively resistant to the cytotoxic effect of metformin

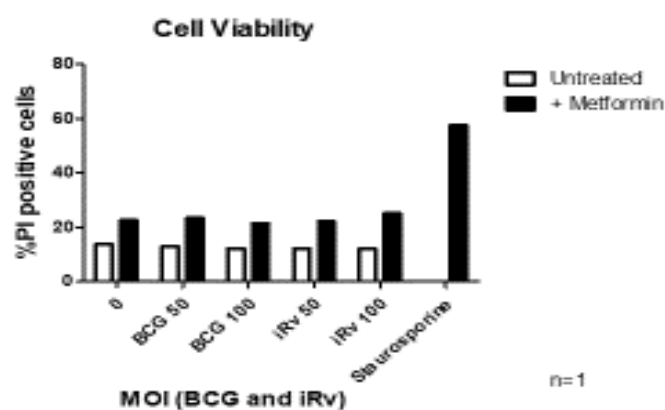


FIGURE 4.22: The impact of Metformin (5mM) on cell viability. moDCs were plated on 48-well, plates at 200,000 cells/well. On day 6 they were infected moDCs with different MOI (50, 100) of BCG and iRv and treated with 5Mm Metformin in duplicate wells for each. Uninfected moDCs were treated with staurosporine (20 µg/ml) as a positive control for cell death. 24 hours after incubation, cells were stained with PI (50µg/ml), Hoechst 33258 (20µg/ml) and Hoechst 33342 (20µg/ml) and analysed using the Cytell Imaging System.

To test the effects of metformin on cellular metabolism, on the day of infection, mo-DCs were scraped, and plated in at 200,000 cells/well on Seahorse 24-well microplates in RPMI 1640 media supplemented with 10% defined FBS at least 1 hours before BCG and iRv infection at MOI 50, with and without metformin (5mM). Uninfected moDCs were also treated with metformin (5mM). 24 hours after infection, the Seahorse media was supplemented to all wells and metabolic flux was interrogated using both the Seahorse XF Glycolysis Stress test. Afterwards, a crystal violet (CV) assay was performed as described in **Chapter 2 Section 2.11** to check the cell quantity and cell viability and normalise the data.

Metformin treatment of uninfected cells led to an increase in glycolysis of moDCs. Furthermore, treatment of BCG and iRv infected moDCs caused a statistically significant increase in ECAR (**Figure 4.23 C**), indicating induction of glycolysis, as predicted. Non-glycolytic acidification was reduced by metformin treatment (**Figure 4.23 I**). There was very little increase in ECAR following the oligomycin injection (i.e. the glycolytic capacity) in iRv infected moDCs treated with metformin, indicating that they are already operating at their maximal glycolytic capacity in the presence of the drug. In keeping with this, the glycolytic reserve of the metformin-treated groups was significantly reduced (**Figure 4.23 F**). Metformin treated groups also showed slightly reduced OCR with iRv infection, consistent with the ability of metformin to inhibit mitochondrial ATP synthase (**Figure 4.23 B**) but this was not statistically significant. Baseline ECAR/OCR ratio in moDCs was higher in iRv infected and treated metformin than that of iRv infected untreated mo-DCs (**Figure 4.23 D**), suggesting these cells have undergone metabolic reprogramming.

Crucially, these results demonstrate that glycolytic reprogramming occurs in response to infection with Mtb in mo-DCs when treated with metformin and that moDCs are relatively resistant to the cytotoxic effect of metformin, probably due to their ability to switch to glycolysis when OXPHOS is inhibited. Thus, further interrogation of the effects of metformin on DC maturation may help elucidate the mechanism of metabolic reprogramming in Mtb infection.

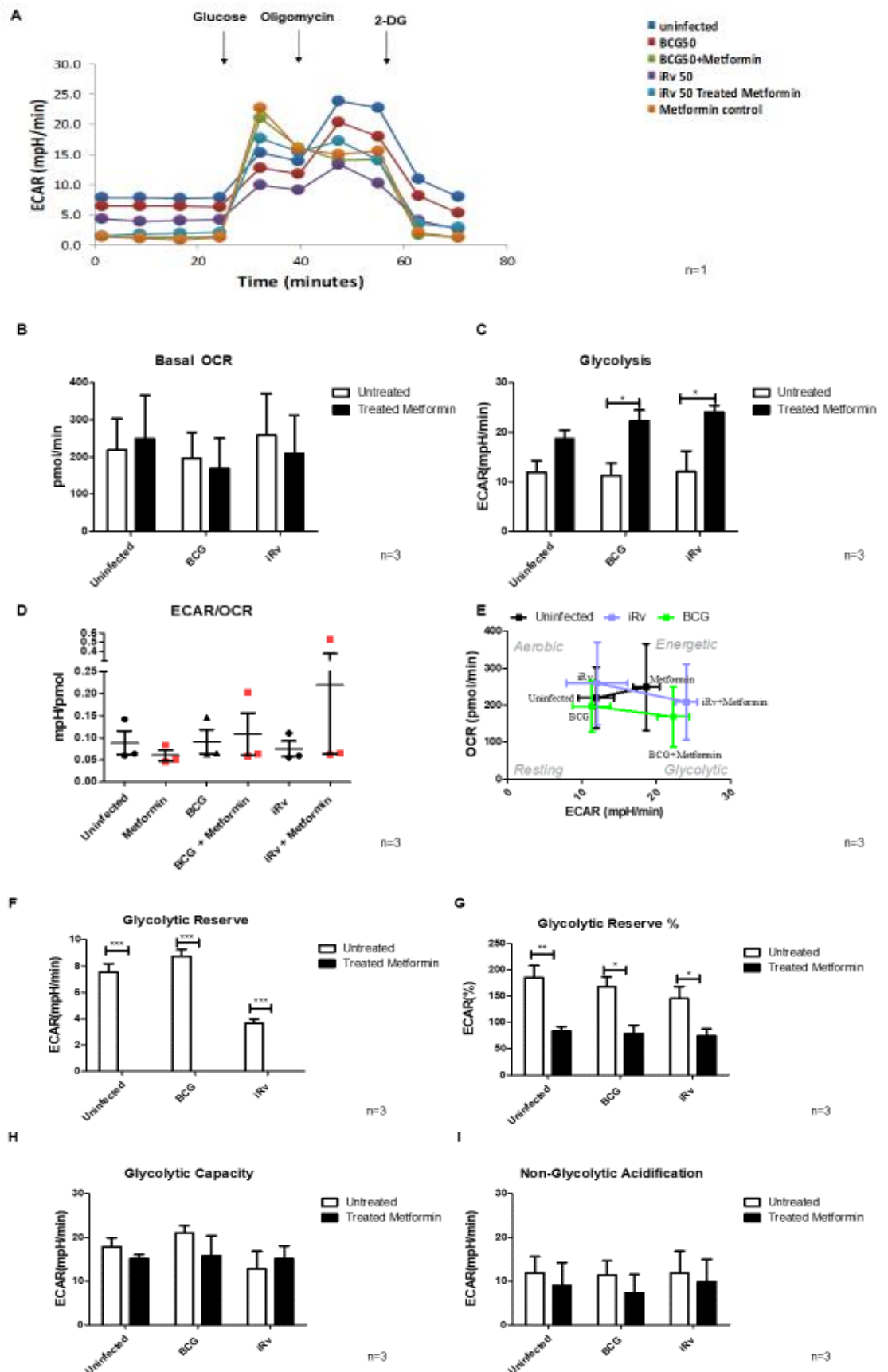


FIGURE 4.23: The impact of Metformin (5mM) on glycolytic and mitochondrial function of BCG and iRv -infected mo-DCs. moDCs were plated on Seahorse Biosciences 24-well plates at 200,000 cells/well. cells were infected for 24 hours with BCG and iRv MOI 50, metformin (5mM) treated or untreated BCG

infected moDCs, uninfected moDCs were metformin (5mM) treated or untreated. Extracellular flux analysis was performed with sequential addition of glucose (10Mm), oligomycin (1 μ M) and 2DG (50mM) and data normalised using CV assay. Panel **A** depict representative kinetic ECAR in uninfected moDCs (blue), untreated BCG MOI 50 infected moDCs (red), metformin (5mM) treated BCG MOI 50 infected moDCs (green), untreated iRv MOI 50 infected moDCs (purple), metformin (5mM) treated iRv MOI 50 infected moDCs (aqua) and metformin control(orange) for one donor. Panels B – E depict baseline mean $\pm$ SEM OCR (**B**), glycolysis (**C**), ratio of ECAR/OCR (**D**) and a metabolic phenogram showing basal ECAR and OCR levels after glucose addition (**E**). Panels F –I depict ECAR quantification of moDCs of glycolytic reserve (**F**), glycolytic reserve % (**G**), glycolytic capacity (**H**) and non-glycolytic acidification (**I**) for three individual donors. Statistical analyses were performed using two way- ANOVA with Bonferroni posttests ****P** < 0.001; ****P** < 0.01, ***P** < 0.05 for all data.

4.5 Discussion

The results presented in this chapter sought to identify metabolic changes occurring during maturation of human mo-DCs infected with BCG. First, immature moDCs were challenged with BCG and the basal oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR) were measured using the Seahorse XFe Analyzer and the Mito Stress test. It was found that BCG or LPS had no statistically significant effect on these parameters. Next, the effect of BCG on moDC metabolic flux was compared to that of the virulent Mtb strain H37Rv (iRv) and it was found that iRv did not significantly affect basal ECAR or OCR. Characterization of mitochondrial function of moDCs was utilised in order to measure OCR under stressed conditions. There was evidence of decreased mitochondrial respiration in moDCs infected with iRv under stressed conditions and a trend towards this in the case of BCG. When the Glycolysis Stress Test Kit was used, the results showed that BCG- and iH37Rv-infected moDCs were capable of increasing their rate of glycolysis under stressed conditions. In addition, treatment of moDCs with IFN- γ or the anti-diabetic drug metformin increased the rates of glycolysis of moDCS infected with either BCG or iRV.

It was somewhat surprising that BCG or LPS stimulation did not induce a significant switch from basal levels of OXPHOS to glycolysis in human moDCs since both stimuli induced maturation of these cells. Maturation of murine BMDCs in response to LPS stimulation is highly dependent on metabolic changes on [30], including an early increase in aerobic glycolysis and OXPHOS followed by a dramatic switch to glycolysis alone. Malinarich and colleagues found that the metabolism in human LPS-matured moDCs is more balanced between OXPHOS and glycolysis. Nevertheless, they found a significant increase in the glycolytic rate of LPS-matured compared to immature moDCs. However, in that study tolerogenic moDCs (generated by treatment with dexamethasone and Vitamin D) also showed increased rate of glycolysis compared to immature moDCs and furthermore had significantly higher glycolytic capacity and reserve indicating that increased glycolytic function does not necessarily directly correlate with increased immunogenicity in human moDCs [137]. Studies in human monocytes treated with LPS show concentration-dependent differences in metabolic flux [222]. The lack of effect of any concentration of LPS on the rate of glycolysis in the present study may be due to differences in cell culture conditions or the source of the LPS. The fact that IFN- γ increased the basal rate of glycolysis in the moDCs indicates that these cells were capable of increasing their ECAR after an appropriate stimulus.

Since BCG is not as immunogenic a stimulus for moDCs as virulent Mtb, the effect of the virulent Mtb H37Rv (iRv) strain was assessed for its effect on basal levels of glycolysis. However, metabolic flux analysis revealed that iRv challenge did not significantly increase basal ECAR above that of uninfected cells or BCG-infected moDCs. On the other hand, iRv infection did lead to some significant changes in mitochondrial function compared to uninfected moDCs, including a decrease in mitochondrial ATP production and reduced proton leak. During OXPHOS protons are pumped out of the mitochondria by complex I, III and IV of the ETC to generate a proton-motive force (Δp). Complex V couples ATP production to the pumping of protons back into the mitochondrial matrix. Any other entry of protons (that persists in the presence of oligomycin) is termed proton leak [229]. Proton leak is regulated by adenine nucleotide translocase (ANT) and uncoupling proteins (UCPs) and can be activated by fatty acids, superoxide or lipid peroxidation products [230]. Mitochondrial ROS production is dependent on Δp and therefore dissipation of Δp by UCPs can have a cytoprotective effect [231] by decreasing superoxide production. Reduced proton leak could therefore reflect some degree of mitochondrial damage induced by ROS during infection with iRv. BCG also reduced proton leak although this was not statistically significant. Cummings and colleagues also observed a reduction in proton leak as well as decreased mitochondrial ATP production in human MDMs infected with live H37Rv and BCG and decreased maximal respiration [159] with live H37Rv but not with BCG. In their hands, killed H37Rv did not have any effect on proton leak or mitochondrial ATP production unlike the results obtained in this study. This may be because of the different cell types (human moDCs versus MDM) or the different methods of generating killed Mtb (heat-killed in their study versus irradiated used here).

In contrast to the results of the Mito Stress test; when the metabolic flux of BCG-matured moDCs was assessed using the Glycolysis Stress test time-dependent and dose-dependent differences in glycolytic function were identified. There was a significant increase in basal ECAR (glycolysis) and increased glycolytic capacity in BCG-infected cells compared to the uninfected control 24 hrs post infection. It is not clear why there was a difference in the results of basal ECAR measurements between the two tests. However, there are differences in the way these tests are performed. In the Glycolysis Stress test the cells are first starved of glucose and then glucose is injected into the medium through the instrument injection port. The subsequent increase in ECAR is considered to reflect the basal glycolytic rate of

the cells. Because glucose is provided during the test increase in ECAR that is measured is specifically due to increased metabolism of glucose which may increase the sensitivity compared to the Mito Stress test. It is possible that the ECAR measured in the Mito Stress test could be generated by substrates other than glucose, for example intracellular glycogen stores, which might alter the levels of ECAR or the time at which ECAR is increased. As well as methodological differences between the two tests, timing and MOI are also factors in the metabolic response of moDCs to mycobacterial infection. Although the Glycolysis Stress test gave significant results at the same time (24hr) and MOI (100) as the Mito Stress test showed no significance in basal ECAR, it is possible that the timing and dose responses vary significantly from donor to donor. Perhaps it is necessary to always test the effects of bacterial infections on metabolic flux at different time points and multiplicities of infection to identify significant changes due to variability in donor responses.

Importantly, IFN- γ and metformin drove increased glycolytic flux in moDCs infected with BCG and iRv. These data show that moDCs infected with BCG are capable of significantly increasing their basal glycolytic rate with the appropriate stimulus. These reagents will be important tools to tease out the possible effects of increased glycolysis on the immunogenicity of the BCG vaccine. IFN- γ plays an essential role in immunity to Mtb. Aerosolized IFN- γ has been used as an adjunct to TB treatment when conventional chemotherapy fails [232]. A recent report showed that a recombinant BCG vaccine expressing IFN- γ stimulated a better cell mediated immune response than the parent BCG strain, providing a better multifunctional Th1 T cell response to Mtb infection [233]. In murine macrophages infected with Mtb, many of the important functions of IFN- γ are mediated by HIF-1 α and iNOS signalling and are dependent on increased glycolytic flux [234]. It would be interesting to determine whether the increase in glycolysis in human moDCs following IFN- γ treatment observed here is regulated by the same pathway. It seems unlikely, as TLR-stimulated human moDCs seem to maintain their metabolic plasticity and increase OXPHOS even while they produce increased levels of nitric oxide in the study conducted by Malinarich [137]. In the present study, IFN- γ did not inhibit OXPHOS and even increased it to some extent, suggesting that functional mitochondrial respiration was maintained.

In patients with type 2 diabetes metformin therapy is associated with reduced incidence of latent TB [235]. In patients with active TB, metformin treatment is associated with improved

control of Mtb infection, reduced lung pathology and an enhanced Th1 response. IFN- γ secreting Th1 T cells provide protection against Mtb, therefore combining BCG vaccination with a drug like metformin might improve its efficacy. It is not clear if the benefits of metformin in TB are related to its effects on glycaemic control. As previously mentioned, metformin inhibits complex I of the ETC which could cause the cells to switch to glycolysis to maintain viability [228- 231]. In addition, metformin also inhibits the activity of AMPK by increasing the ratio of AMP/ATP and inhibits mTOR [236]. Although TB patients had an increased Th1 response to Mtb antigens [235], the drug also has immunosuppressive effect as well. Metformin treatment reduces the secretion of proinflammatory cytokines by human peripheral blood monocytes *ex vivo* and when metformin was administered to healthy volunteers for 5 days before BCG vaccination it temporarily inhibited the induction of trained immunity several months later [236]. It will be important in future to investigate the effects of this drug on DC functions such as upregulation of maturation markers, cytokine secretion and antigen presentation before considering its suitability as an adjunct to the BCG vaccination.

An important limitation of the work shown in this Chapter was that killed BCG was used for the metabolic flux analysis. This was unavoidable as live BCG could not be used for this analysis for reasons explained in **Section 4.4.1**. There were some differences in the response of moDCs to killed BCG including reduced phagocytosis and lack of cell death. However, CD86 expression was increased compared to uninfected indicating, like the effects of live BCG, that maturation had occurred in response to killed BCG. It will be important, however, to compare the effects of live and killed BCG on moDC OCR and ECAR in the future to determine whether they are the same. It is encouraging to note that Cummings found that both live and killed BCG can induce increased ECAR in macrophages [159].

In conclusion, this is the first study investigating metabolic flux in human DCs infected with mycobacteria. Contrary to initial expectations the metabolic changes observed were subtle; there was no dramatic shift from OXPHOS as previously observed in LPS-stimulated murine BMDCs. This may be a feature of human moDC metabolic responses as others have observed a similar complex picture with TLR stimulation in these cells. In keeping with the metabolic flux analysis, the effects of 2DG on DC activation (Chapter 3) were selective for CD86 expression and optimal IL1 gene expression rather than having a global effect on maturation. The glycolytic response of BCG-infected moDCs could be augmented by

treatment with IFN- γ or metformin suggesting the possibility manipulation of DC metabolism may improve immunity to Mtb should be explored further. For this to be feasible, a better understanding of the role of metabolism in DC maturation is required. Consistent with this, in Chapter 5 transcriptional profiling of metabolic and immune genes, is examined to assess the role of diverse metabolic pathways in BCG-infected DCs.

CHAPTER 5:

Transcriptional profiling of metabolic gene expression in moDCs infected with BCG

5.1 Introduction

Chapter 5 aims to identify unique metabolic gene profiles for the first time in human moDCs that are associated with BCG infection. The original hypothesis of this work was that a simple switch from OXPHOS to glycolysis would provide the fuels and ATP required for moDC activation. Over the past few years it has become clear from the literature that the role of metabolism is much more complicated and involves both OXPHOS and glycolysis at different stages in the life cycle of DCs [237]. The results of this thesis also show that maturation of moDCs in response to BCG is not wholly dependent on glycolysis (Chapter 3) and that the changes in metabolic flux are quite subtle following infection with BCG (Chapter 4).

Dendritic cells tend to undergo processes that are catabolic in nature when they are in the resting state. It is suggested to provide them with resources for maintenance of cell and energy expenditure, mainly oxidative phosphorylation. However, once activated, they undergo anabolism to support cell growth, thus switching to fermentation of lactate and glycolysis [139]. As described in detail in Sections 1.4.2, 1.4.3 and 1.4.4, studies have shown these parameters to change both *in vivo* and *in vitro* in murine cultures of DCs and in human moDCs [30]. Specifically, in case of moDCs, it is known that as they differentiate from monocytes to immature DCs in the presence of GM-CSF and IL-4 there is an elevation OXPHOS and in the levels of PPAR γ and cytosolic fatty acid synthesis [41].

It is also becoming clear that different DC subsets have different metabolic requirements and that these changes in metabolism can vary depending on the stimulus used [237]. Following activation of murine BMDM with strong TLR agonists such as LPS there is an early increase in both glycolysis and OXPHOS. Subsequently, there is an increase in mitochondrial metabolism, specifically enzymes like citrate synthase which have an upregulated activity. This is accompanied by production of ROS and accumulation of the Krebs cycle intermediate citrate, that is used in fatty acid synthesis. These fatty acids can be used as building blocks for the Golgi and ER membranes to facilitate cytokine production and secretion or as a substrate for fatty acid oxidation (FAO) (**Figure 5.1**). There can also be increased nitric oxide production which inhibits the electron transport chain, increasing the dependence of DCs on glycolysis to maintain their viability. In contrast to LPS, weaker stimuli of BMDC maturation, for example, house dust mite, stimulate an early increase in

glycolysis but do not downregulate OXPHOS or sustain the induction of glycolysis [119]. In addition, not all DC subsets express nitric oxide synthase (iNOS). Those that do not express iNOS, including the murine cDC1 and cDC2 subsets in lymphoid organs as well as human moDCs, can maintain their metabolic plasticity following activation [99], [101], [205], [237].

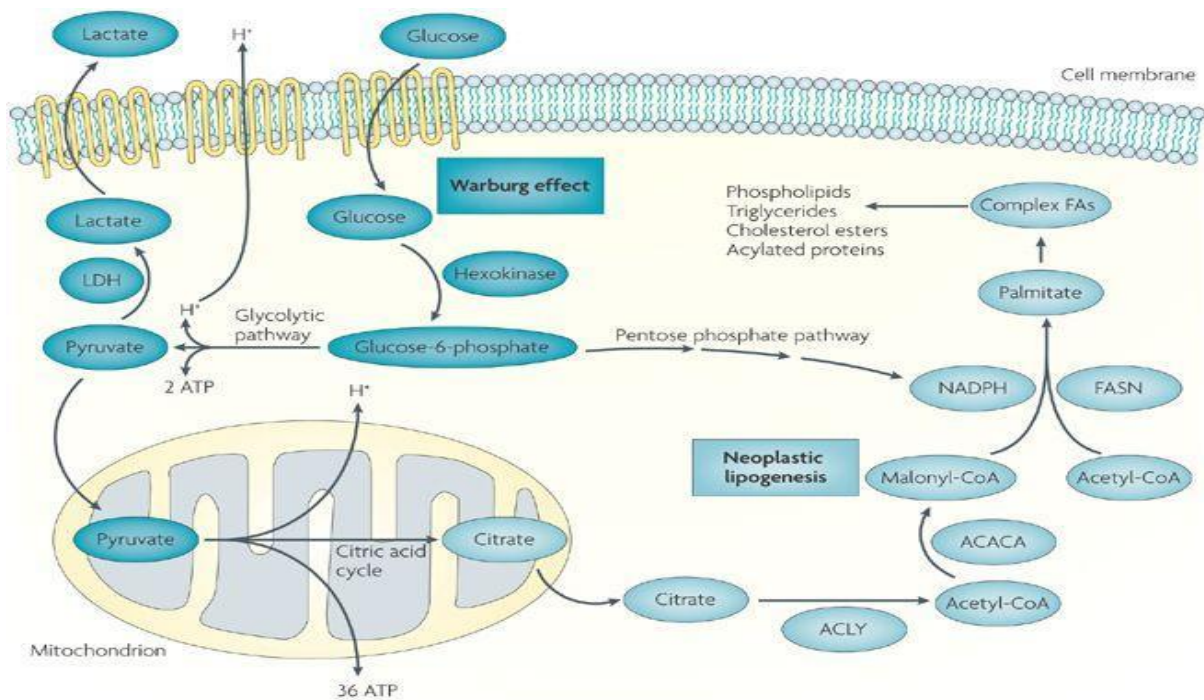


Figure 5.1: Schematic picture of the metabolic pathways from glucose and fatty acid oxidation. About 25 enzymes are involved in the metabolism of glucose to fatty acids (FAs) as shown in figure. Adapted from Menendez & Lupu, 2007 [246].

Signalling pathways like HIF1 α , mTOR and the PI3K-independent Akt pathways are important for regulation of metabolism at different stages of the maturation process. Following LPS stimulation, Akt is responsible for the early glycolytic burst in activated DCs while mTOR and HIF1 α seem to be responsible for maintaining glycolysis in the longer term [237] (see **Section 1.4.3** for details). In the context of the present topic, BCG and Mtb infection has also been considered to be responsible for induction of PPAR γ mediated upregulation of the formation of lipid bodies in mouse cDC1-like BMDCs which imply the linkage of the infection and metabolism in the DCs in order to induce maturation [154]. However, the role of these lipid bodies in the immune response to mycobacteria is unclear at present and the details of these pathways in human DCs have still not been deciphered.

Hence, this study was undertaken in order to understand the details of the metabolic changes happening after BCG infection in moDCs, using a series of molecular assessments and gene expressions while also trying to understand role of specific nutrient molecules like glucose, fatty acids and amino acids like glutamine.

The Nanostring nCounter platform was used to measure the levels of mRNA of 180 genes involved in cellular metabolism in uninfected mo-DCs or those infected with live and killed BCG or in moDCs treated with LPS. Expression of a selection of the most significantly up- and down-regulated genes were validated by Q-PCR. In addition, based on some of the most significant changes in gene expression observed in BCG-infected moDCs, compared to uninfected cells, the dependency of the cells on selected mitochondrial fuels (glucose, glutamine and long chain fatty acids) was tested using the Seahorse analyser.

The Mito Fuel Flex Test Kit involves measuring the rate of oxygen consumption in the presence and absence of inhibitors blocking glucose, glutamine or long chain fatty acid utilization in the organelle. In this process baseline OCR is measured, then one of the pathways to be targeted is first blocked and OCR is measured again. Then the two alternate pathways are also blocked together to check how much the cell depends upon these blocked pathways in order to meet their energy requirement. In this way, it can be determined whether the cell is dependent upon these pathways or not (**Figure 5.1**). Flexibility is defined as the difference between fuel capacity and dependency, that is, the ability of cells to increase oxidation of a specific fuel in order to compensate for inhibition of alternative fuel pathways. In order to calculate the fuel flexibility in the mitochondria, the amount of fuel dependency of organelle is subtracted from the amount of fuel capacity in all the pathways under consideration or the pathways that are involved in providing fuel to the organelle.

$$\text{Dependency\%} = \left[\frac{\text{Baseline OCR} - \text{Target Inhibitor OCR}}{\text{Baseline OCR} - \text{All Inhibitor OCR}} \right] * 100$$

$$\text{Capacity\%} = \left[1 - \frac{\text{Baseline OCR} - \text{Other 2 Inhibitors OCR}}{\text{Baseline OCR} - \text{All Inhibitor OCR}} \right] * 100$$

$$\text{Flexibility\%} = \% \text{Capacity} - \% \text{Dependency}$$

If the result is positive then there is flexibility and this flexibility shows that the mitochondria are not 100 percent dependent on these blocked or inhibited pathways for the generation of energy, but they can use some other pathways to generate energy and the process of respiration will go on. But if the result after subtraction is negative or zero then it indicates

that the only source of respiration for mitochondria were the inhibited pathways and it can not depend on any other pathway to carry out energy generation.

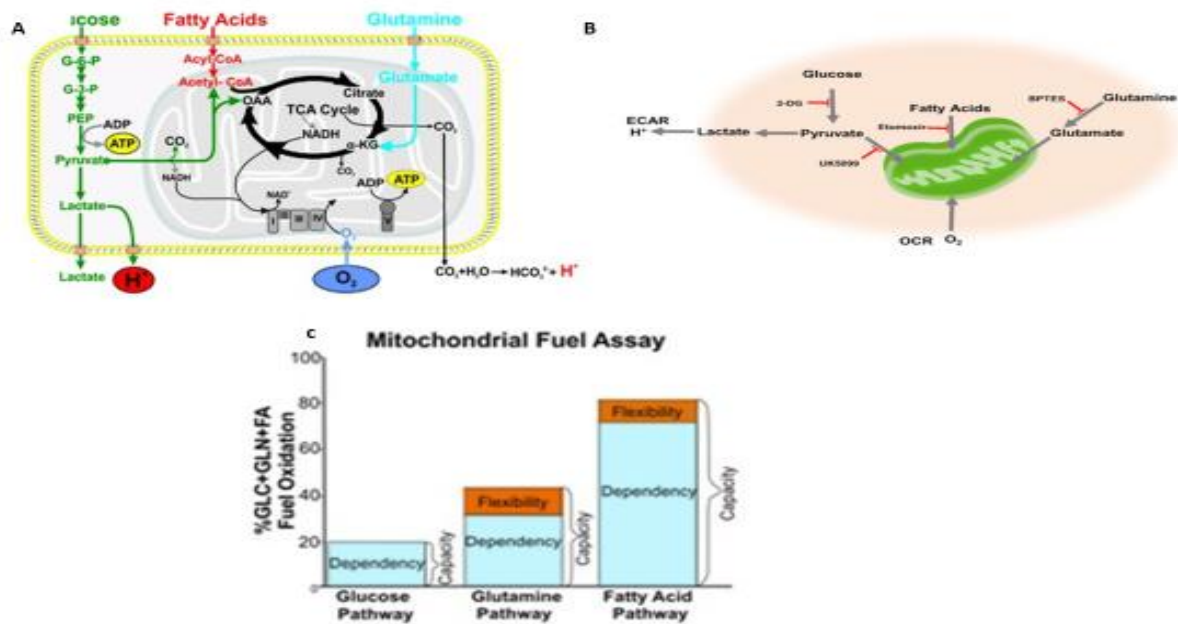


FIGURE 5.2: Schematic illustration of cellular metabolism pathways and mitochondrial fuel test used to analyze metabolic pathways. (A) Seahorse XFe analysis in real time monitors the rate of oxygen consumed by cells (OCR) and the release of protons from the cells into the extracellular medium (ECAR). Adapted from Bridgette M Cumming& Kelvin W Addicott [159]. (B) Schematic of metabolites and inhibitors targets. Adapted from Heather S. Smallwood, Susu Duan et. al. [243]. (C) Shows the dependency, capacity and flexibility used mitochondrial fuel test. Adapted from Bridgette M Cumming& Kelvin W Addicott [159].

5.1.1 Hypothesis: DC activation is a complex process probably involving changes to several metabolic pathways. Transcriptional profiling of moDCs response to BCG infection may help identify the metabolic pathways involved in maturation.

Aims:

1. Carry out differential gene analysis of moDC infected with BCG (live and killed) or stimulated with LPS compared to uninfected cells using the Nanostring nCounter platform.
2. Investigate the role of metabolic pathways identified in Aim 1 above using pharmacological inhibitors and metabolic flux analysis.

5.2 Methods

The results presented in Chapter 4 demonstrate that there is an increase in the glycolytic rate in BCG-infected moDCs under stressed conditions (**Section 4.45**). In the current chapter, transcriptional profiling of metabolic genes was carried out to further investigate the pathways influencing moDCs responses to the bacillus. Metabolic reprogramming in murine DCs is associated with upregulation and downregulation of key glycolytic genes. In addition, other metabolic pathways may be involved in moDC maturation. In order to investigate this in human cells in the setting of BCG infection, human moDCs were isolated and prepared as described in **Section 2.3.1** and **Section 2.3.2** respectively. Cells were cultured and plated at 0.5×10^6 /mL on 24 well plates. Side-by-side infections with killed BCG and live BCG at MOI 50 and 100 were performed as described in **Chapter 2, Section 2.2**. At 24 hours post infection, cells were lysed, and RNA extracted as outlined in **Section 2.7**

Realtime PCR for cytokine expression

Expression of cytokine genes (IL-1 β , TNF α , IL-10) was used (measured by real-time PCR) as a first step to determine that the moDCs were responding appropriately to infection. Extracted RNA was used to generate cDNA, and real-time PCR was performed in triplicate using Taqman probes, with normalisation of data to GAPDH rRNA. This method is described in detail in **Chapter 2 (Section 2.7.4)**.

Assessment of RNA integrity

First, the concentration and purity of the isolated RNA was determined using the Qubit™ RNA HS Assay Kit (Invitrogen). Then the quality and size of extracted RNA were

determined using agarose gel electrophoresis (Agilent RNA 6000 Nano Kit, Agilent Technologies, Lithuania.) followed by quantification and QC with the Agilent 2100 Bioanalyzer as described in **Chapter 2 (Section 2.8.6)**.

Nanostring assay

Real time PCR requires extensive labour and time; hence, it is not ideal for studying large numbers of genes. NanoString is a medium-throughput technology that can measure the expression of up to 800 target genes in a more user-friendly way [238]. The technology reduces labour and manual error by automating sample preparation and requiring very little sample quantity. In addition, it utilises mRNA without the need to carry out cDNA synthesis or RT-PCR, both of which can lead to inaccuracies in gene expression assays.

After evaluating the cytokine levels to test for a response to BCG infection (see above) RNA from three donors was run on the Nanostring system (NanoString Technologies) using nCounter® Vantage 3D™ Cancer Metabolism Panel (Human) VRXC-CM1-12. Calculation of gene expression was performed with the nSolver Analysis Software 4.0, which allows for normalization of log-transformed data, normalization is the most important step in NanoString data analysis, normalization methods specific to NanoString have been developed and include background correction using negative controls and normalizing counts relative to internal and external positive controls.

Mito Fuel Flex Test

For all extracellular flux analyses, cells were prepared as outlined in **Chapter 2 Section 2.3** and plated on Seahorse 24-well microplates in the usual culture medium with 4 designated “blank” wells left free of cells, left for at least 2 hours, and then infected as outlined in **Chapter 2 Section 2.2**. The Seahorse XF Mito Fuel Flex Test Kit were used to interrogate different metabolic pathways - see **Chapter 2 Section 2.11**. Data analysis was carried out using Wave Desktop 2.4 software (Agilent). After the readings were taken, a crystal violet (CV) assay was performed on the microplates as described in **Chapter 2 Section 2.11**. The crystal violet assay is performed to check the cell numbers in each individual well. Dying cells detach from the culture plates therefore cell number is an indication of the cell death. Extracellular flux readings were normalised to the corresponding CV data for each well before analysis using Wave Desktop 2.4 software.

5.3 Results

5.3.1 BCG-GFP-induced cytokine gene expression in human monocyte-derived DCs

This work seeks to identify metabolic pathways that are important in moDC maturation using transcriptional profiling of 180 metabolic genes using the Nanostring platform. To make sure that the samples being analysed using this expensive assay had responded appropriately to BCG infection an initial quality control test was carried out on the RNA extracted from uninfected, BCG-infected and LPS-treated cells to confirm that the moDCs were responding as expected to infection before moving on to the Nanostring analysis. TNF α (Tumor necrosis factors), IL-1 α and IL-1 β play a vital role in TB control [239], [240] and we have found that increased expression of these cytokines occurs reliably when moDCs are infected with BCG (**Chapter 3, Section 3.3.8**) and therefore induction of these genes was used to confirm a successful response.

To assess gene expression, we investigated IL-1 β , TNF α , IL-10 expression using GAPDH as the reference gene (**Figure 5.3**). As expected from the results in (**Chapter 3, Section 3.3.8**), infection of moDCs with live BCG resulted in increased expression of IL 1 β , IL-10 and TNF α and there was no difference between the two MOIs. Although levels of both IL1 β and TNF α mRNA were considerably lower in cells infected with killed BCG, there was a dose-dependent increase in their expression but IL-10 was not induced. The samples infected with live and killed BCG at MOI 100 were then taken forward for Nanostring Analysis.

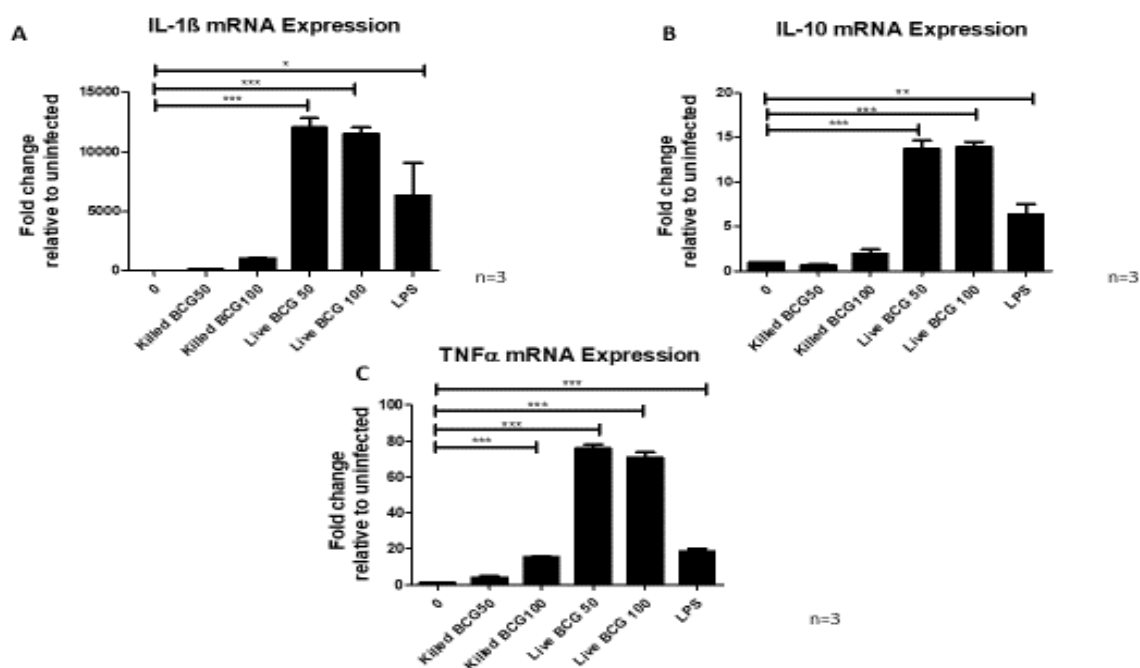
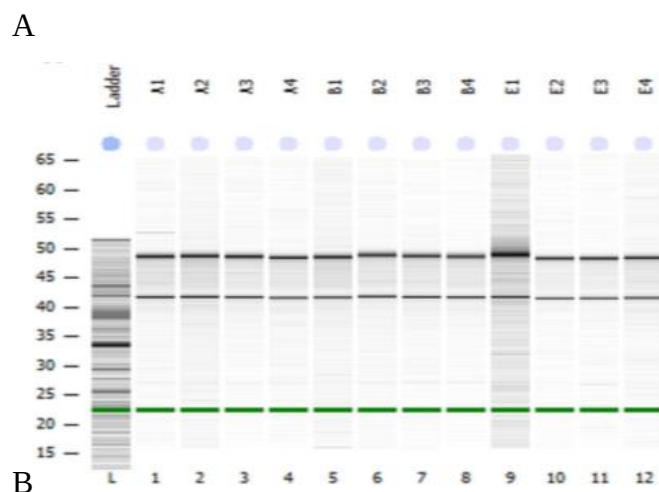


FIGURE 5.3: Upregulation of cytokine gene expression in live and killed BCG infected moDCs at different MOI. Mo-DCs were infected with live and killed BCG at MOI 50, 100 for 24 hours. Uninfected moDCs were treated with lipopolysaccharide (LPS) (1µg/ml) as a positive control or left uninfected (control). At 24 hours' post-infection, supernatants were removed. Cells were lysed in RLT buffer, RNA extracted and cDNA generated, and qPCR was performed. Data was normalised to GAPDH and mRNA expression fold-change relative to uninfected calculated using the $2^{-\Delta\Delta C_t}$ method. Panels A -C depict relative mRNA expression of IL-1β (A), IL-10 (B) and TNFα (C). Statistical differences were analysed by one-way ANOVA with a Tukey multiple comparison test. ***P < 0.001; **P < 0.01, *P < 0.05 for all data.

5.3.2. Assessment of RNA integrity

The results of agarose electrophoresis suggested that the RNA Integrity Number (RIN) was in all samples >7.5 which indicates good RNA quality (**Figure 5.4**).



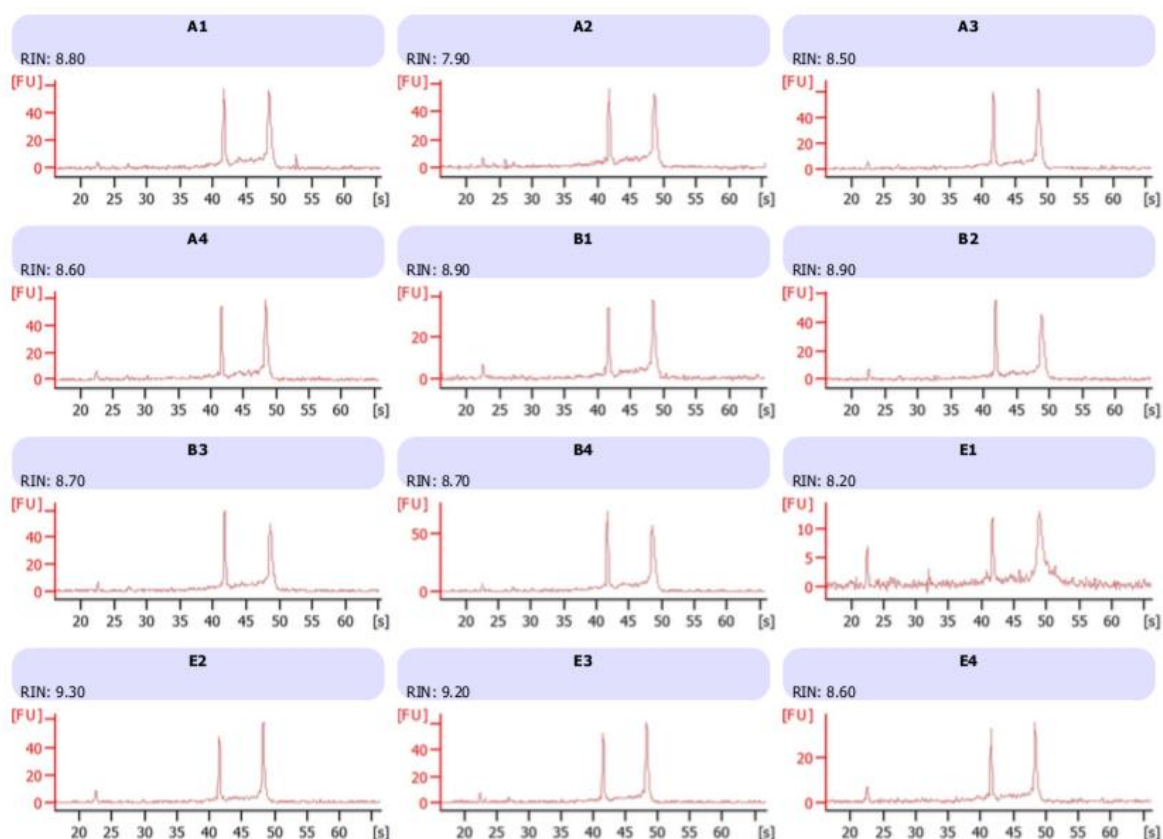


FIGURE 5.4: Analysis of RNA integrity. moDCs were infected with live and killed BCG at MOI 100 for 24 hours. Uninfected moDCs were treated with lipopolysaccharide (LPS) (1 μ g/ml) as a positive control or left uninfected (control). At 24 hours post-infection, supernatants were removed, cells were lysed and RNA extracted. RNA was analysed using the Agilent 2100 Bioanalyzer. **A.** Virtual gel image generated showing various degradation levels of the RNA samples. **B** The quantity of RNA is approximated by taking the area under the 28S and 18S rRNA peaks of the electropherogram. The quality is reflected in the 28S/18S ratio and RIN (RNA integrity number), the 28S/ 18S ratios are automatically generated by the software application in Bioanalyzer 2100. Results are from three independent donors, donor 1; uninfected (A1); Killed MOI 100 (A2), Live 100 (A3); LPS (A4), donor 2; uninfected (B1); killed 100 (B2), live 100 (B3); LPS (B4), donor 3; uninfected (E1); killed 100 (E2), live 100 (E3); LPS (E4).

5.3.3 Gene profiling analysis using the Nanostring nCounter platform

Next, we sought to investigate gene expression of BCG-GFP infected moDCs, killed BCG infected moDCs, LPS treated moDCs and uninfected moDCs using NanoString nCounter technology. Expression data were normalized with nSolver™ Analysis Software (NanoString), using housekeeping genes (**Table 5.1**). All samples show a low MSE of reference profile and the high quality of normalization (**Figure 5.5 & 5.6**). All samples passed the quality control criteria evaluated by the nSolver software.

Table 5.1: HK genes selected by geNorm", "SD after normalization

Gene Name	Used or Discarded
SAP130-mRNA	Used
ZC3H14-mRNA	Used
PRPF38A-mRNA	Used
COG7-mRNA	Used
GPATCH3-mRNA	Used
EDC3-mRNA	Used
NUBP1-mRNA	Discarded
HDAC3-mRNA	Discarded
TLK2-mRNA	Discarded
MTMR14-mRNA	Discarded
CC2D1B-mRNA	Discarded
SF3A3-mRNA	Discarded

Principal component analysis

To assess the signatures induced by live BCG infected moDCs, killed BCG infected moDCs and LPS treated moDCs, we analysed the expression data of a total of 180 genes in the 3 donors, using unsupervised principal component analysis (PCA). Shown in **Figure 5.7** are PCA analysis of the four groups, live BC, killed BCG, LPS and uninfected. Global differences in expression are indicated by clustering of the sample groups on the PC1/PC2 plot. Uninfected and live BCG-infected moDCs showed the most distinct separation indicative of the degree of differential gene expression. Cells infected with killed BCG or LPS were intermediate on this plot.

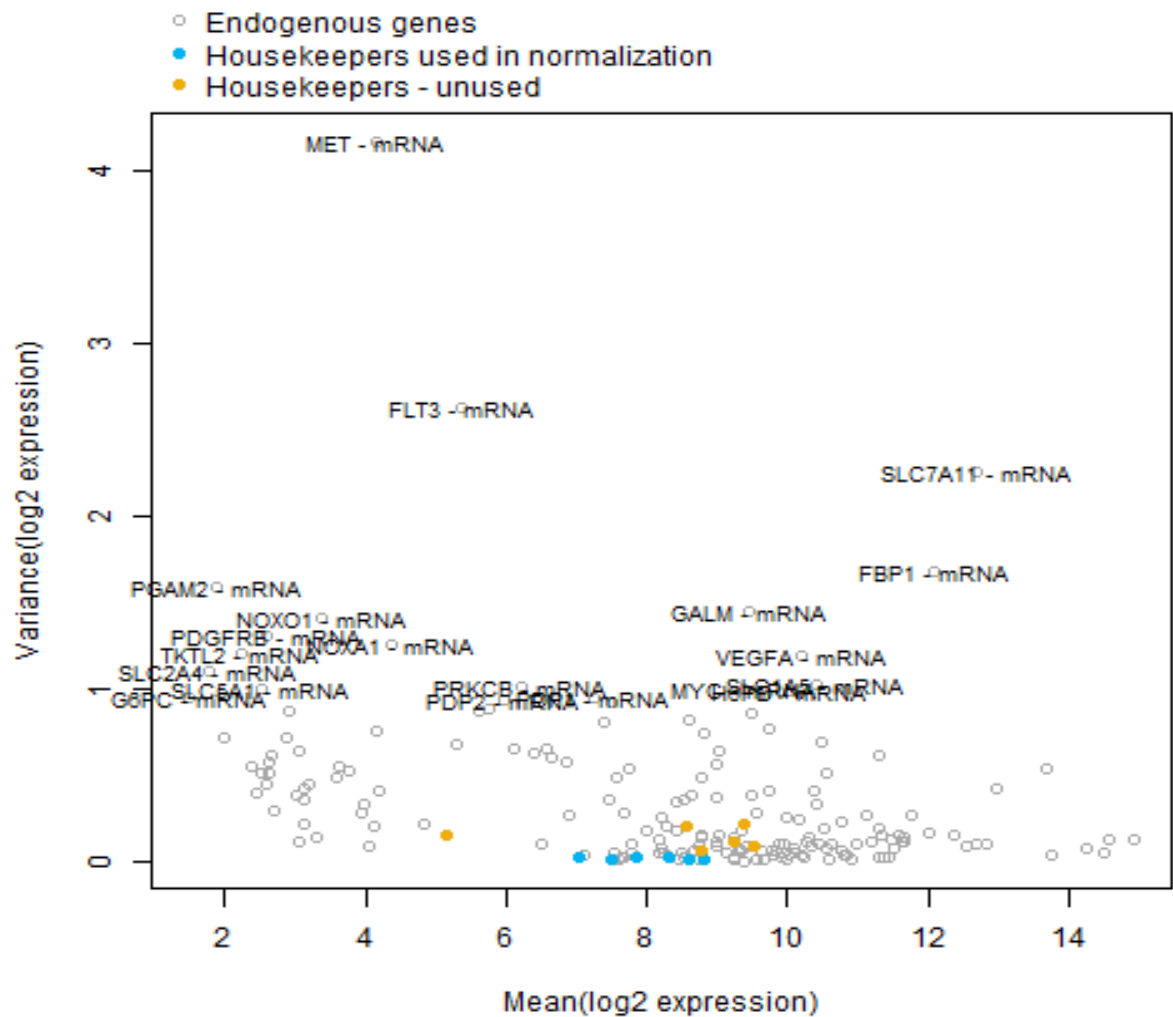


FIGURE 5.5: Variance vs. Mean normalized signal plot across all targets/probes. Human monocytes were separated from buffy coats using negative selection and differentiated to moDCs as before. On day 6 moDCs were infected with live BCG and killed BCG at MOI 100 for 24 hours. Uninfected moDCs were treated with lipopolysaccharide (LPS) (1 μ g/ml) as a positive control or left uninfected (control). At 24 hours' post-infection, supernatants were removed. Cells were lysed in RLT buffer, RNA was extracted and analyzed with nCounter instrument (nanoString) using pan-cancer immune panel detecting expression of 180 metabolism associated genes. Each gene's variance in the log-scaled, normalized data is plotted against its mean value across all samples combined. Highly variable genes are indicated by gene name. Generally, good housekeeping genes should have high mean expression and low variance. Uncoloured circles are target genes, yellow circles are housekeeping genes unused in normalization because of high variance (i.e. their expression changed after treatment) and blue circles are housekeeping genes with low variance (SAP130-mRNA, ZC3H14-mRNA, PRPF38A-mRNA, COG7-mRNA, GPATCH3-mRNA, EDC3-mRNA) used in normalization, which is a best fit loess curve through the data.

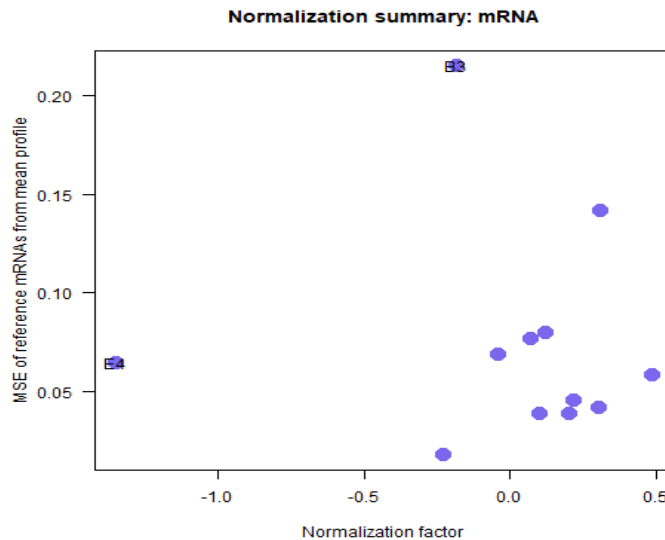


FIGURE 5.6: Normalization Summary: mRNA. The plot shows the Mean Squared Error (MSE) of reference genes included in the panel versus the normalisation factor. The lower the MSE (y axis) of the samples the higher quality of normalization. Sample B3 (Donor 2, live BCG) shows the lowest quality of normalisation. The more positive the normalization factor (i.e. when data points are located on the right hand side of the X axis), the higher the counts registered for the sample and the lower the standard error. Samples B4 (donor 2, LPS-treated moDCs) has relatively lower counts than the other samples and a relatively higher standard error.

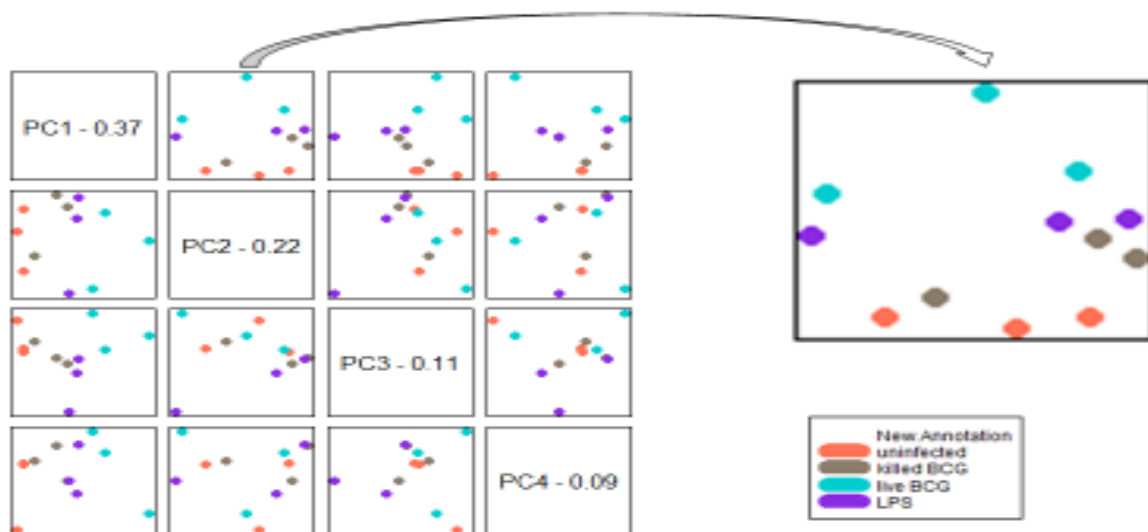


FIGURE 5.7: Principal Components Analysis of All Data. Principal component (PC) analysis of the Nanostring gene expression data mapping the high-dimensional dataset onto a smaller number of highly informative dimensions. Here, the first four principal components of the gene expression data are plotted against each other and coloured by the values of the covariates: uninfected (orange), live BCG (aqua), killed BCG (brown) and LPS (purple). This analysis transformed the datasets into a set of summary indices called principal components which are plotted to illustrate clusters in the data and to potentially identify replicate samples with high variability. In the moDC dataset most of the variability is accounted for in the first two dimensions - by PC1 (37%) and PC2 (22%). The samples from live BCG-treated cells (blue) show the most distinct separation from uninfected cells (orange) indicating that the most significant changes in gene expression occur following live BCG infection.

Heat map analysis

Heat maps of the raw (data not shown) and the data normalised to the house keeping genes (data not shown) were analysed. Some genes were scored as absent by the Nanostring software due to low counts (<50), in all 12 genes were excluded from the analysis for this reason. Some samples with the same treatment did not cluster together using unsupervised hierarchical clustering, probably due to the relatively low number of genes analysed (compared to whole genome sequencing). However, when the normalised samples from each donor are placed side by side it can be seen that there are broad similarities between donors under each condition and differential expression between treatments and a discriminative pattern in genes expression between the different conditions (**Figure 5.8**).

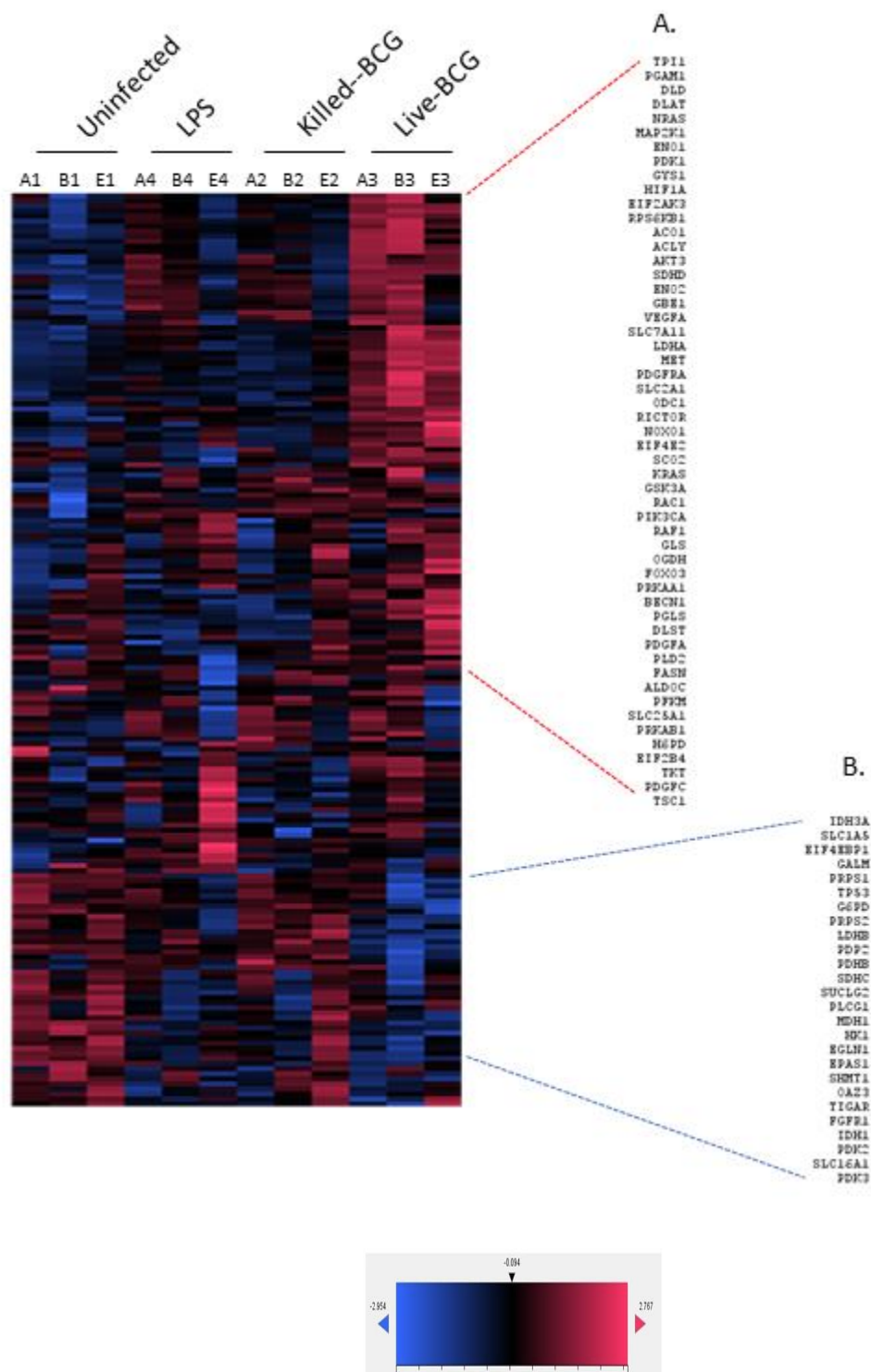


Figure 5.8: Heatmap of normalised data for detectable genes for the grouped samples. Hierarchical clustering of all detectable (168 out of 180 genes analysed), normalised genes analysed using nSolver software 4. 0. Data is shown as fluorescent counts for each gene normalised to housekeeping genes. Red indicates high gene expression; blue indicates low expression for different treatments. The heatmap gives an overview of the gene expression data. Individual gene labels are too small to be read and so are omitted, however the names of several broad clusters of highly expressed (A) and downregulated (B) genes have been magnified so that they are visible.

Differential Gene Expression Analysis

When the data was normalised to the uninfected control to calculate fold change, statistically significant differences in gene expression were found, with the most strongly differentially expressed gene expression pattern seen for live BCG. For live BCG infected moDCs 80 genes were scored as being upregulated (fold change ≥ 1) versus 81 for killed BCG infected cells. Of these Genes, 59 were common to both live and killed BCG and 21 and 22 were unique to live and killed BCG infected cells respectively. Comparing live and killed BCG to LPS treatment, they had 58 upregulated genes in common with 1 gene (Hras) unique to LPS, 12 to killed BCG and 7 to live BCG (**Figure 5.9 A&B**). For downregulated genes (fold change < 1) the number of detectable genes was 74 in live BCG infected moDCs and 73 in killed BCG moDCs verses uninfected moDCs with 52 genes in common. Comparing live and killed BCG to LPS treatment verses uninfected moDCs: of the 71 genes detectable in LPS-treated cells 51 were also detected in BCG infected cells and 1 was unique to LPS (PKFM, phosphofructokinase) respectively (**Figure 5.9 C&D**).

Volcano plots of the data for each condition are shown in **Figure 5. 10** and separately in **Figure 5.11, 5.12 & 5.13**. The Nanostring software plots the data as the fold difference on the X-axis (log2) versus the adjusted p value using the Benjamini-Hochberg correction which is generally used as a correction to avoid false positives when conducting multiple comparisons of very large datasets, for example in whole genome analysis. The actual p values are given here and in the tables as they are more appropriate based on the number of donors and relatively low numbers of genes analysed in the assay. The 40 most statistically significant differentially expressed genes are labelled automatically in the volcano plots. The 20 most highly upregulated and downregulated genes are shown in **Table 5.2 - 5.3**. The String database (<https://string-db.org/>) integrates gene expression analysis studies and public databases to predict functional interactions between groups of proteins [299]. String analysis of the Nanostring data shows the functional relationship between the top 20 genes, up (**Figure 5.12**) and down regulated (shown in **Figure 5.13**) by live BCG. This illustrates the modest upregulation (fold change 1.9-2.7) in glycolytic genes including ALDOC (aldolase, fructose-bisphosphate C), ENO3 (enolase 3) and PKFM (phosphofructokinase). Other genes involved in glucose metabolism are also upregulated including H6PD (hexose-6-phosphate dehydrogenase/glucose 1-dehydrogenase) and GYS (glycogen synthase 1). Of the genes downregulated in live BCG-infected moDCs, several of the most highly and significantly downregulated were also involved in glycolysis including GALM (Galactose mutarotase) -

fold change was 0.2, p-value 0.000149, PDK2 (pyruvate dehydrogenase kinase 2) - fold change was 0.2, p-value 0.00287 and IDH1 (isocitrate dehydrogenase 1) fold change was 0.3, p-value 0.00174. The proto oncogene MYC, involved in regulating glutamine metabolism was also significantly downregulated. Of note also, hexose-6-phosphate dehydrogenase (H6PD) was also significantly up regulated.

Also upregulated by live BCG were the angiogenic factors VEGFA (**Figure 5.12**), PDGFA and PDGFRA (the PDGF receptor). As well as being pro-angiogenic both VEGFA and PDGFA can suppress the maturation of DCs. VEGF treatment reduces the ability of murine BMDCs to stimulate and antigen-specific T cell response [241]. PDGF-treated DCs do not fully mature and stimulate the development of IL-10 producing T regulatory cells [242]. Interestingly, vaccination of mice with BCG also gives rise to a Treg response *in vivo* [15]. Increased expression of these genes by moDCs could contribute to the inadequate protection given by the BCG vaccine.

Figure 5.14 shows a volcano plot of gene expression in killed BCG infected moDCs versus uninfected. String analysis showing the functional network relationship between top 20 up regulated and (**Figure 5.15**) and down regulated (**Figure 5.16**) genes. Compared to live BCG, killed BCG showed lower levels of gene expression and less significance (**Figure 5.15 & Table 5.4**). Nevertheless, many of the same genes were also upregulated including the glycolytic genes ENO2 and LDHA, although the p values were greater than 0.05. Similar to live BCG-infected cells, SLC7A11 was increased 6.2-fold above the uninfected control, $p=0.002$. As seen in the String analysis, a number of other SLC also showed increased expression as SLC2A3 (solute carrier family 2 member 3), PPARGC1A (PPARG coactivator 1 alpha) and PLD2 was the only other gene whose expression was significantly increased compared to control (1.7 fold, $p=0.001$). MET was the most highly expressed gene, similar to moDCs infected with live BCG (up 7.52) but the p value was not quite significant (0.071). Similarly, with VEGFA; its expression was increased 2.16 fold, $p=0.087$. FASN-mRNA expression in live BCG infected moDCs was increased 4 fold, $p=0.0002$ when compared with uninfected moDCs. While the increases were 1.1 fold, $p=0.6$ and 1.4 fold, $p=0.1$ in killed BCG infected moDCs and LPS treated moDCs respectively. We found that SLC7A11-mRNA overexpressing in live BCG infected moDCs was increased 9.4 fold, $p=0.00067$ when compared with uninfected moDCs. While the increases were 6.2 fold, $p=0.00232$ and 6.4 fold, $p=0.00214$ in killed BCG infected moDCs and LPS

treated moDCs respectively. With killed BCG infection the most significantly downregulated genes were Serine Hydroxymethyltransferase 1 (SHMT1), Endothelial PAS domain protein 1 (EPAS1), Pyruvate dehydrogenase kinase 3 (PDK3), Succinate-CoA ligase GDP-forming beta subunit (SUCLG2) and (Aryl Hydrocarbon Receptor Nuclear Translocator) ARNT.

Gene expression in LPS-treated cells bore some similarities to that of BCG-infected cells in that MET, SLC7A11, VEGFA, PDGFRA were upregulated. In addition, some of the glycolysis genes were also modestly upregulated including ALDO, SDHD and ENO2, the most highly downregulated gene was SHMT1(0.3 fold, $p=0.00264$), IDH1 and MYC were also downregulated but did not quite reach significance ($p=0.0596$). There were also some similarities between LPS and killed BCG including increased expression of EGF (epidermal growth factor) and PPARGC1A which were absent from the top 20 upregulated genes in live BCG-infected cells.

Table 5.2 and 5.3 show the top 20 genes found significantly elevated and lowered in live BCG infected moDCs. Also **Table 5.4 and 5.5** show the top 20 genes found elevated and lowered in killed BCG infected moDCs respectively. **Table 5.6 and 5.7** show the top 20 genes found elevated and lowered in LPS treated moDCs respectively. Expression levels of all genes for each condition are shown in the supplemental data table in the Appendix. The top 20 upregulated and down regulated genes also sorted by live BCG (**Figure 5.20 & 5.21**).

Directed Global Significance Scores (**Figure 5.22**) for each treatment confirmed that live BCG had the most significant effect on gene expression with Glycolysis, Carbon Metabolism, Choline Metabolism and “other metabolic pathway” showing an increase over uninfected cells and Cancer Metabolism Drivers being the most strongly down regulated.

Finally, we chose a number of genes to validate in live and killed BCG infected moDCs verses uninfected moDCs. In live BCG infected moDCs Solute carrier family 7 member (SLC7A11), phospholipase D2 (PLD2), the tyrosine kinase receptor MET, fatty acid synthase (FASN) and AKT3 were among the most highly upregulated. AKT3-mRNA expression in live BCG infected moDCs was increased 3.2 fold and 1.3 fold in killed BCG infected moDCs when compared with uninfected moDCs, while the increases was 2.1 fold in LPS treated moDCs, also PLD2-mRNA overexpressing in live BCG infected moDCs was

increased 2.7 fold and 1.7 fold in killed BCG infected moDCs when compared with uninfected moDCs, while the increases were 2.4 fold in and LPS treated moDCs. Importantly, we found that MET-mRNA upregulation in live BCG infected moDCs was increased 51.2 fold when compared with uninfected moDCs, while the increase was 7.5 fold and 16.2 in killed BCG infected moDCs and LPS treated moDCs respectively. Interestingly, GALM-mRNA expression in live BCG infected moDCs was decreased 0.2 fold when compared with uninfected moDCs, while the decreases was 0.9 fold and 0.7 in killed BCG infected moDCs and LPS treated moDCs respectively. Expression of GALM, FASN, PLD2, AKT3, SLC7A11 and MET was further validated in those BCG infected moDCs using qPCR.

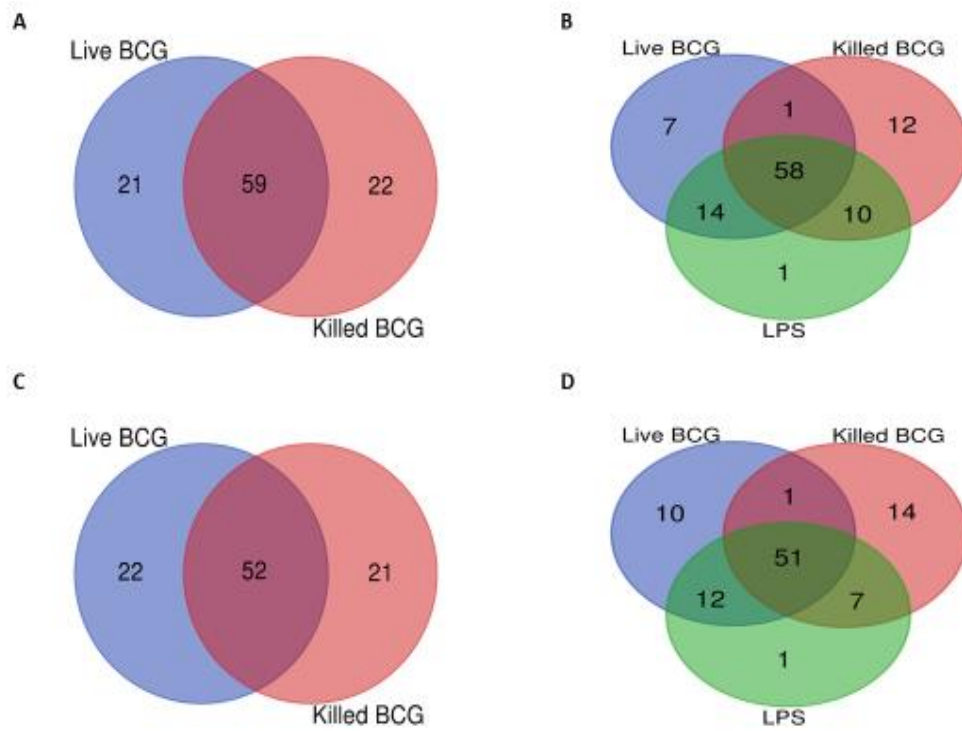


Figure 5.9: Venn diagram of upregulated (A, B) and down regulated (C, D) genes for live BCG and killed BCG (fold change ≥ 1) infected moDCs. The total numbers of genes detected for each infection are shown in live BCG (blue) and killed BCG (orange). The union of all unique detected genes under all conditions is also shown for both

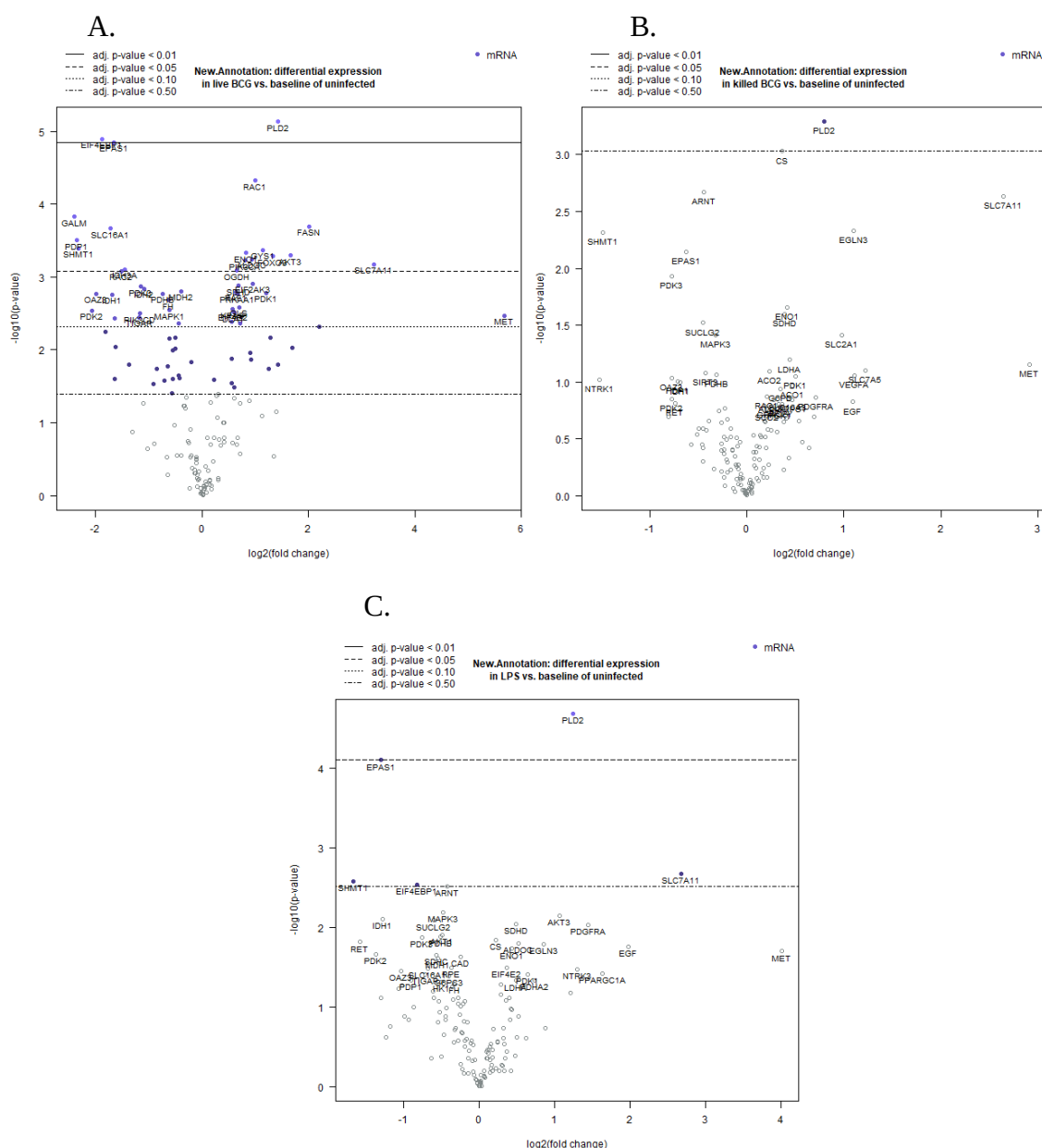


FIGURE 5.10: Volcano plots of the gene expression data for each condition. Human moDCs were infected with live and killed BCG at MOI 100 for 24 hours. Uninfected moDCs were treated with lipopolysaccharide (LPS) (1 μ g/ml) as a positive control or left uninfected (control). At 24 hours post-infection cells were lysed, RNA was extracted and analyzed with nCounter station (nanoString) using pan-cancer metabolism panel detecting expression of 180 metabolic genes. NanoString software generated volcano plots displaying the log (to the base 10) for each each gene $-\log_{10}(\text{p-value})$ and the differential gene expression as $\log_2$ transformation of the fold change (compared to uninfected cells) after live BCG (**A**), killed BCG (**B**) and LPS (**C**), treatment. Highly statistically significant genes ($P < 0.001$) fall at the top of the plot, and highly differentially expressed genes fall to either side – down-regulated genes are to the left of the origin (0) and up-regulated genes to the right. Horizontal lines indicate thresholds for $p = 0.01$ and $p = 0.001$. A total of 40 most statistically significant genes ($P < 0.001$) are labelled in the plot. These volcano plots are shown again individually in figures 5.11, 5.14 and 5.17.

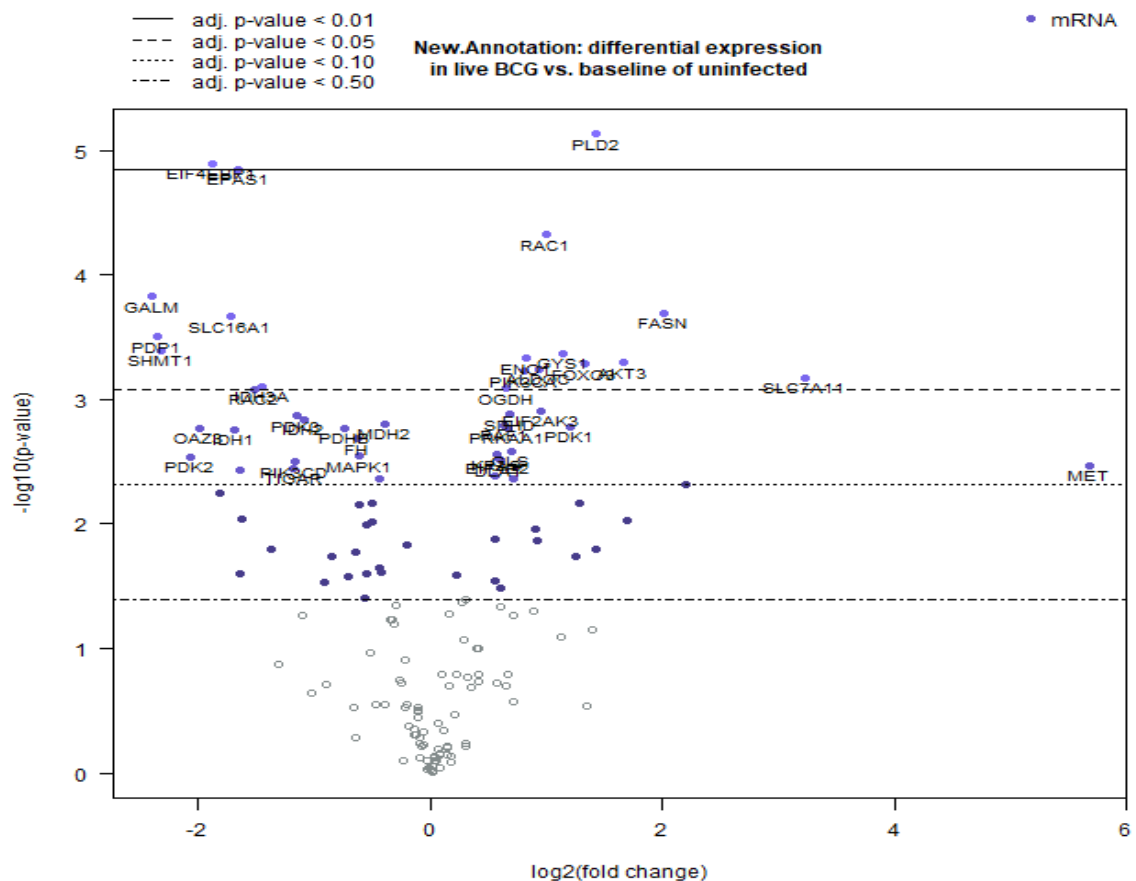


FIGURE 5.11: Differentially expressed genes in live BCG infected moDCs. Human monocytes were separated from buffy coats using negative selection and differentiated to moDCs. On day 6 moDCs were infected with live BCG at MOI 100 for 24 hours or left uninfected (control). At 24 hours post-infection, supernatants were removed. Cells were lysed in RLT buffer, RNA was extracted and analyzed on the nCounter station (nanoString) using pan-cancer immune panel detecting expression of 180 metabolic genes. NanoString analysis was carried out using nCounter 4.0 to calculate differential analysis between uninfected moDCs with live BCG infection. Volcano plot displaying each gene's $-\log_{10}$ (p-value) and $\log_2$ fold change with the selected covariate. Highly statistically significant genes ($P < 0.001$) fall at the top of the plot, and highly differentially expressed genes fall to either side. Horizontal lines indicate thresholds for $p = 0.01$ and $p = 0.001$. A total 40 most statistically significant genes ($P < 0.001$) are labelled in the plot.

Table 5.2: List of the top 20 most highly upregulated genes in moDCs after infection with live BCG. Fold changes of mRNA counts between live BCG infected moDCs and uninfected moDCs samples using the nanoString nCounter. Results are shown for mRNAs which showed a significant difference.

Gene	Log2 fold change	Fold change	P-value	Gene.sets
MET	5.7	51.2	0.00339	Carbon Metabolism
SLC7A11	3.2	9.4	0.00067	Carbon Metabolism
VEGFA	2.2	4.6	0.00477	MTOR Pathway
FASN	2.0	4.0	0.0002	Other Metabolic Pathway
H6PD	1.7	3.3	0.00938	Glucose Metabolism
AKT3	1.7	3.2	0.00051	Carbon Metabolism, Choline Metabolism, MTOR Pathway
PLD2	1.4	2.7	7.39E-06	Choline Metabolism
PFKM	1.4	2.7	0.0158	Carbon Metabolism, KEGG Glycolysis
ENO3	1.4	2.6	0.0701	Glucose Metabolism, KEGG Glycolysis
FLT3	1.4	2.6	0.29	Carbon Metabolism
FOXO3	1.3	2.5	0.00052	Other Metabolic Pathway
PDGFA	1.3	2.4	0.00687	Carbon Metabolism, Choline Metabolism
PDGFRA	1.3	2.4	0.0182	Carbon Metabolism, Choline Metabolism
PDK1	1.2	2.3	0.00166	Carbon Metabolism, Glucose Metabolism, Hypoxia Signaling
GYS1	1.2	2.2	0.00043	Glucose Metabolism
ENO2	1.1	2.2	0.0803	Glucose Metabolism, KEGG Glycolysis
RAC1	1.0	2.0	4.73E-05	Carbon Metabolism, Choline Metabolism
EIF2AK3	0.9	1.9	0.00123	Carbon Metabolism, Hypoxia Signaling
ALDOC	0.9	1.9	0.00057	Glucose Metabolism, KEGG Glycolysis
SLC25A1	0.9	1.9	0.0136	Cancer Metabolism Drivers, Carbon Metabolism, Choline Metabolism, Hypoxia Signaling, MTOR Pathway

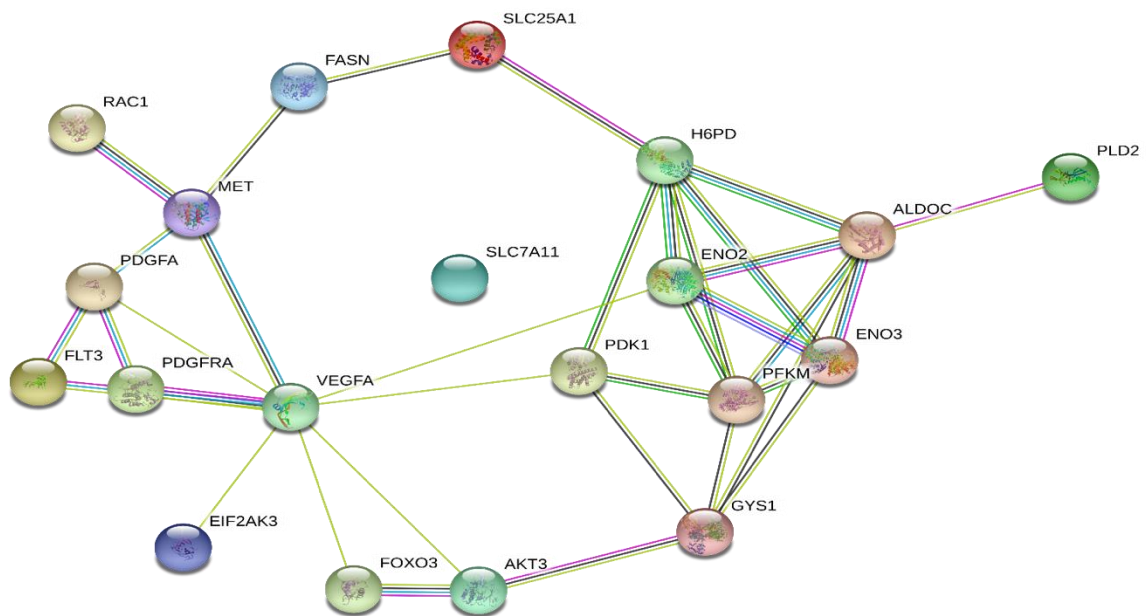


FIGURE 5.12: String analysis of the first 20 most highly upregulated 20 genes in live BCG infected moDCs vs uninfected moDCs. Following Nanostring analysis the top 20 genes most highly upregulated genes in live BCG infected moDCs compared to uninfected cells) were further analysed using the String database (<https://string-db.org/>). String analysis showing the functional network relationship between the proteins encoded for by these genes.

Table 5.3: List of the top 20 genes that are significantly down regulated in moDCs after infection with live BCG. Fold changes of mRNA counts between live BCG infected moDCs and uninfected moDCs samples using the nanoString nCounter. Results are shown for mRNAs which showed a significant difference.

Gene	Log2 fold change	Fold change	P-value	Gene.sets
PDK3	-1.2	0.4	0.00136	Glucose Metabolism
PIK3CD	-1.2	0.4	0.00318	Carbon Metabolism, Choline Metabolism, MTOR Pathway
TIGAR	-1.2	0.4	0.00358	Other Metabolic Pathway
FBP1	-1.3	0.4	0.133	Glucose Metabolism, KEGG Glycolysis
HK3	-1.4	0.4	0.016	Carbon Metabolism, Glucose Metabolism, KEGG Glycolysis
IDH3A	-1.5	0.4	0.000781	Glucose Metabolism
RAC2	-1.5	0.3	0.000823	Carbon Metabolism, Choline Metabolism
FGFR1	-1.6	0.3	0.009	Carbon Metabolism
SLC1A5	-1.7	0.3	0.00369	Carbon Metabolism
PRKCB	-1.7	0.3	0.0248	Carbon Metabolism, Choline Metabolism
EPAS1	-1.7	0.3	1.41E-05	Cancer Metabolism Drivers
IDH1	-1.7	0.3	0.00174	Cancer Metabolism Drivers, Glucose Metabolism
SLC16A1	-1.7	0.3	0.000216	Carbon Metabolism
MYC	-1.8	0.3	0.00567	Cancer Metabolism Drivers, Carbon Metabolism
EIF4EBP1	-1.9	0.3	1.27E-05	Carbon Metabolism, Choline Metabolism, Hypoxia Signaling, MTOR Pathway
OAZ3	-2.0	0.3	0.00169	Other Metabolic Pathway
PDK2	-2.1	0.2	0.00287	Glucose Metabolism
SHMT1	-2.3	0.2	0.000405	Other Metabolic Pathway
PDP1	-2.4	0.2	0.000312	Hypoxia Signaling
GALM	-2.4	0.2	0.000149	Glucose Metabolism, KEGG Glycolysis

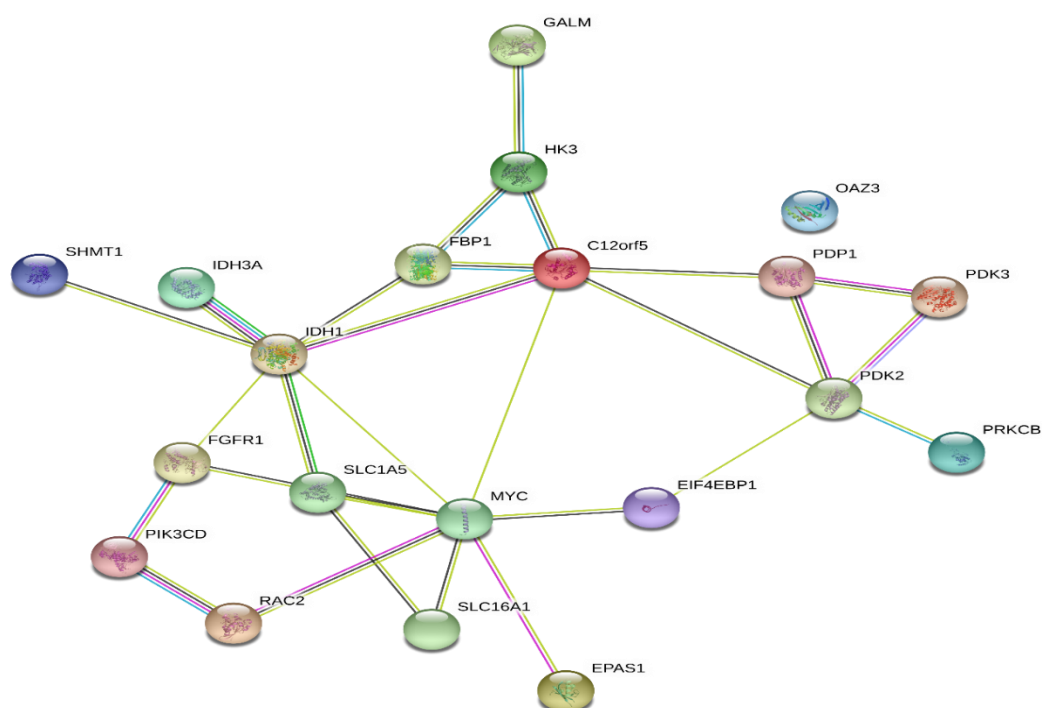


FIGURE 5.13: String analysis of the top 20 genes (down regulated) in live BCG infected moDCs vs uninfected moDCs. Following Nanostring analysis the top 20 genes most strongly down regulated genes in BCG-infected moDCs (compared to uninfected cells) were analysed using the String database. String analysis showing the functional network relationship between the proteins encoded for by these genes.

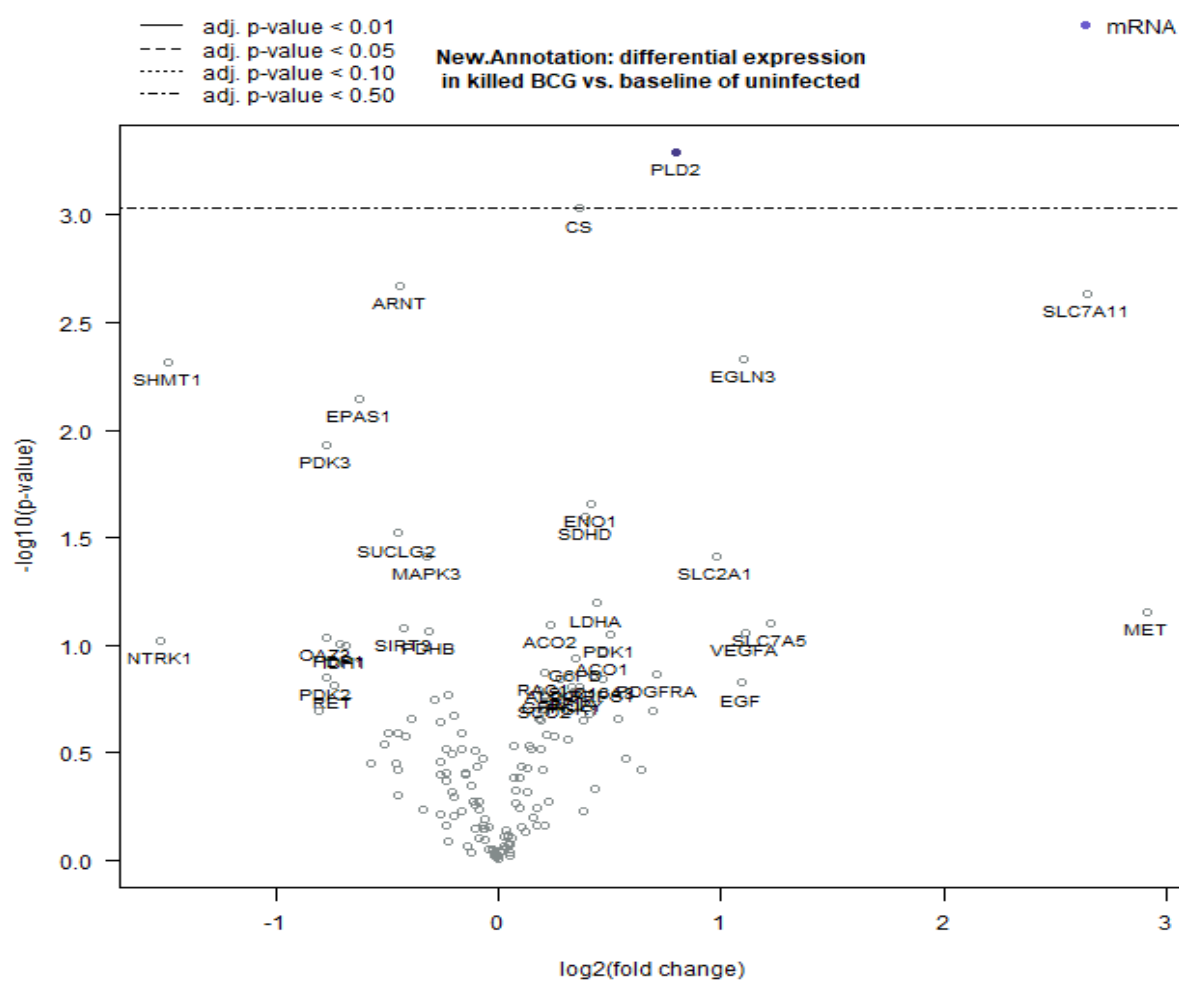


FIGURE 5.14: Differentially expressed genes in killed BCG infected moDCs. Volcano plot displaying each gene's $-\log_{10}(\text{p-value})$ and $\log_2$ fold change compared to uninfected cells. Highly statistically significant genes ($P < 0.001$) fall at the top of the plot, and highly differentially expressed genes fall to either side. Horizontal lines indicate thresholds for $p = 0.01$ and $p = 0.001$. The 40 most statistically significant genes ($P < 0.001$) are labelled in the plot.

Table 5.4: List of the top 20 genes that are highly upregulated in moDCs after infection with killed BCG.
Fold changes of mRNA counts between killed BCG infected moDCs and uninfected moDCs samples using the nanoString nCounter.

Gene	Log2 fold change	Fold change	P-value	Gene.sets
MET	2.9	7.5	0.07	Carbon Metabolism
SLC7A11	2.6	6.2	0.00	Carbon Metabolism
SLC7A5	1.2	2.3	0.08	Carbon Metabolism
VEGFA	1.1	2.2	0.09	MTOR Pathway
EGLN3	1.1	2.1	0.00	Carbon Metabolism, Hypoxia Signaling
EGF	1.1	2.1	0.15	Carbon Metabolism, Choline Metabolism
SLC2A1	1.0	2.0	0.04	Carbon Metabolism
PLD2	0.8	1.7	0.00	Choline Metabolism
PDGFRA	0.7	1.6	0.14	Carbon Metabolism, Choline Metabolism
SLC2A3	0.7	1.6	0.20	Other Metabolic Pathway
PPARGC1A	0.6	1.6	0.38	Other Metabolic Pathway
ENO2	0.6	1.5	0.34	Glucose Metabolism, KEGG Glycolysis
SLC1A5	0.5	1.5	0.22	Carbon Metabolism
PDK1	0.5	1.4	0.09	Carbon Metabolism, Glucose Metabolism, Hypoxia Signaling
ACO1	0.5	1.4	0.11	Glucose Metabolism
PRPS1	0.5	1.4	0.14	Glucose Metabolism
LDHA	0.4	1.4	0.06	Hypoxia Signaling, KEGG Glycolysis
HK2	0.4	1.4	0.47	Carbon Metabolism, Glucose Metabolism, KEGG Glycolysis
SLC16A3	0.4	1.3	0.14	Carbon Metabolism
AKT3	0.4	1.3	0.02	Carbon Metabolism, Glucose Metabolism, KEGG Glycolysis

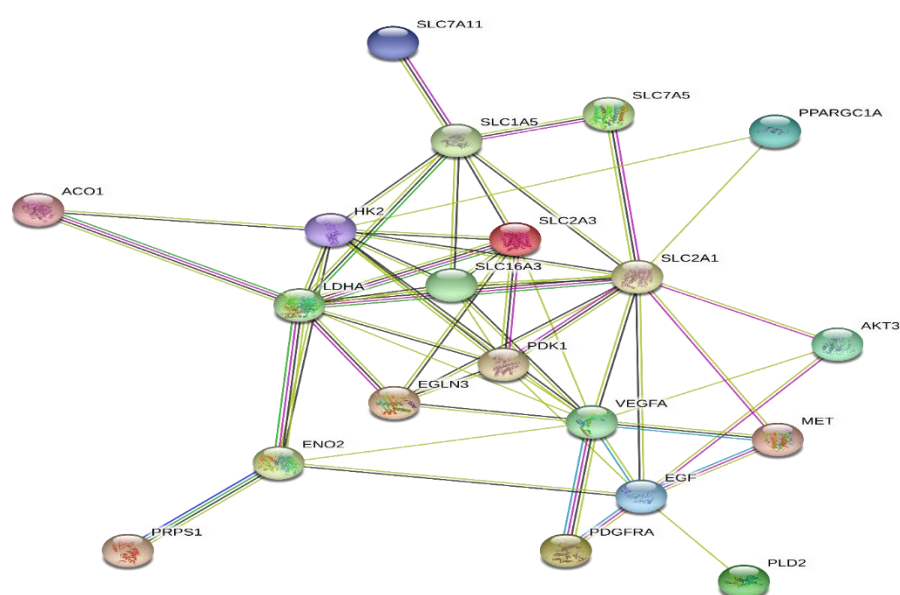


FIGURE 5.15: String analysis of the first 20 genes (upregulated) in killed BCG infected moDCs vs uninfected moDCs. Following Nanostring analysis the top 20 genes most highly upregulated genes in killed BCG infected moDCs compared to uninfected cells) were further analysed using the String database (<https://string-db.org/>). String analysis showing the functional network relationship between the proteins encoded for by these genes.

Table 5.5: List of 20 genes that are most down regulated in moDCs after infection with killed BCG. Fold changes of mRNA counts between killed BCG infected moDCs and uninfected moDCs samples using the nanoString nCounter.

Gene	Log2 fold change	Fold change	P-value	Gene.sets
SIRT3	-0.4	0.7	0.08	Carbon Metabolism
ARNT	-0.4	0.7	0.00	Hypoxia Signaling
SUCLG2	-0.4	0.7	0.03	Glucose Metabolism
PDP2	-0.4	0.7	0.50	Glucose Metabolism
PDGFA	-0.4	0.7	0.26	Carbon Metabolism, Choline Metabolism
MYC	-0.5	0.7	0.38	Cancer Metabolism Drivers, Carbon Metabolism
FGFR1	-0.5	0.7	0.36	Carbon Metabolism
ERBB2	-0.5	0.7	0.26	Carbon Metabolism
HK3	-0.5	0.7	0.29	Carbon Metabolism, Glucose Metabolism, KEGG Glycolysis
PLCG1	-0.6	0.7	0.36	Carbon Metabolism, Choline Metabolism
EPAS1	-0.6	0.6	0.01	Cancer Metabolism Drivers
IDH1	-0.7	0.6	0.10	Cancer Metabolism Drivers, Glucose Metabolism
PDP1	-0.7	0.6	0.10	Hypoxia Signaling
RET	-0.7	0.6	0.16	Carbon Metabolism
OAZ3	-0.8	0.6	0.09	Other Metabolic Pathway
PDK2	-0.8	0.6	0.14	Glucose Metabolism
PDK3	-0.8	0.6	0.01	Glucose Metabolism
PLD1	-0.8	0.6	0.20	Choline Metabolism
SHMT1	-1.5	0.4	0.00	Other Metabolic Pathway
NTRK1	-1.5	0.3	0.10	Carbon Metabolism

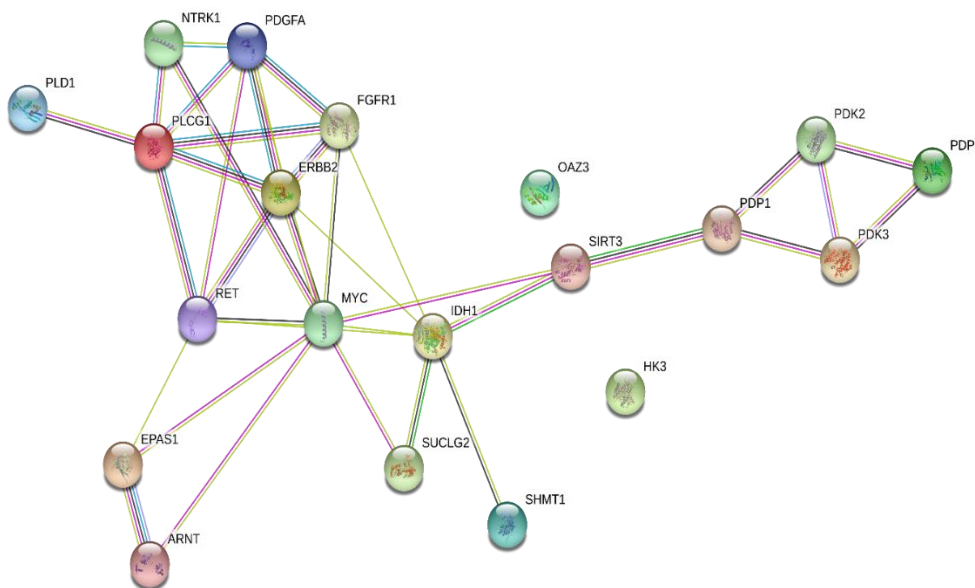


FIGURE 5.16: String analysis of top 20 genes (down regulated) in killed BCG infected moDCs vs uninfected moDCs. Following Nanostring analysis the top 20 genes most strongly down regulated genes in killed BCG infected moDCs compared to uninfected cells) were further analysed using the String database (<https://string-db.org/>). String analysis showing the functional network relationship between the proteins encoded for by these genes.

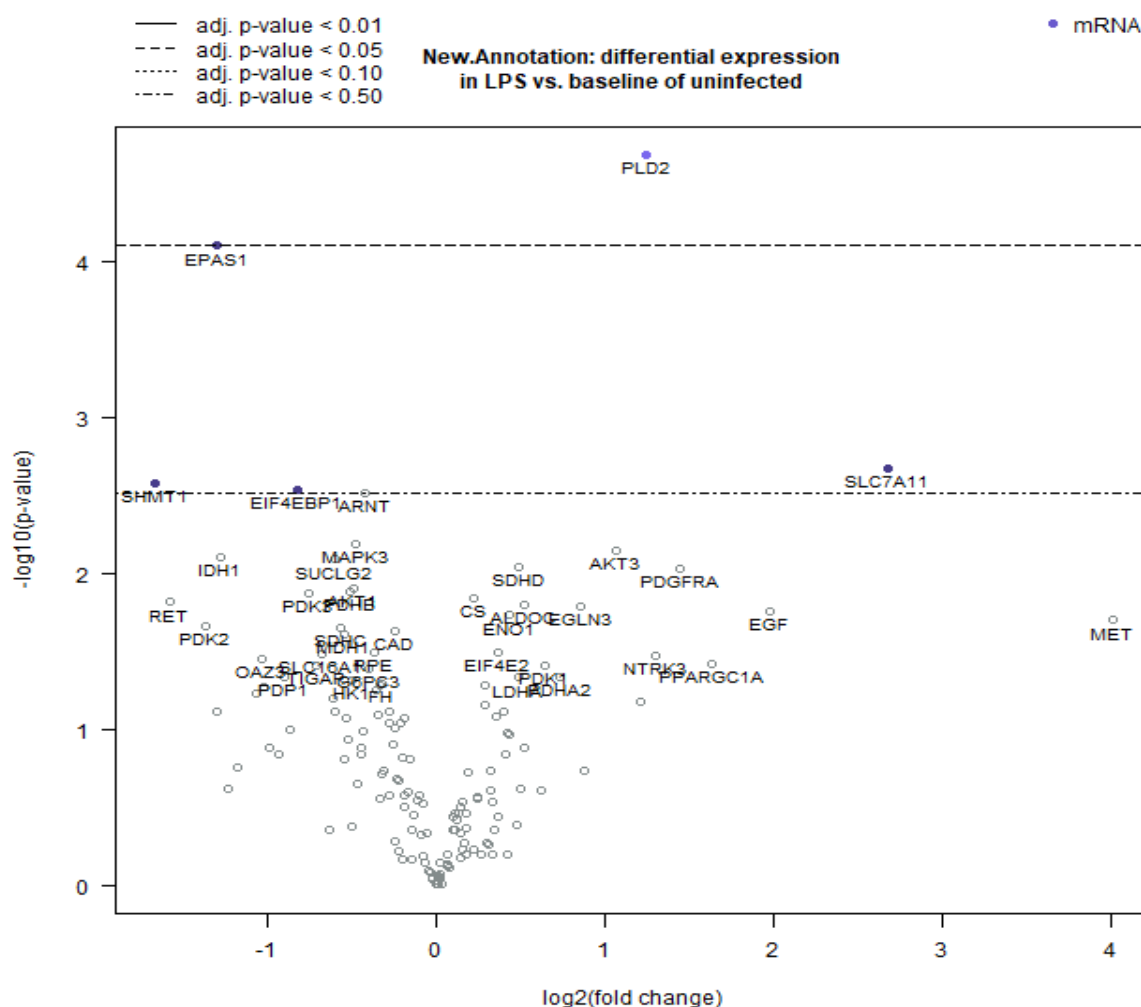


FIGURE 5.17: Differentially expressed genes in LPS treated moDCs. Volcano plot displaying each gene's $-\log_{10}(\text{p-value})$ and $\log_2$ fold change for LPS-treated moDCs compared to the uninfected/untreated control. Highly statistically significant genes ($P < 0.001$) fall at the top of the plot, and highly differentially expressed genes fall to either side. Horizontal lines indicate thresholds for $p = 0.01$ and $p = 0.001$. The 40 most statistically significant genes are labelled in the plot (blue).

Table 5.6: List of 20 genes that are most highly upregulated in moDCs after treatment with LPS. Fold changes of mRNA counts between LPS treated moDCs and uninfected moDCs samples for the top 20 most highly upregulated genes using the nanoString nCounter.

Gene	Log2 fold change	Linear fold change	P-value	Gene.sets
MET	4.0	16.2	0.02	Carbon Metabolism
SLC7A11	2.7	6.4	0.00214	Carbon Metabolism
EGF	2.0	3.9	0.0174	Carbon Metabolism, Choline Metabolism
PPARGC	1.6	3.1	0.0377	Other Metabolic Pathway
PDGFRA	1.5	2.7	0.00927	Carbon Metabolism, Choline Metabolism
NTRK3	1.3	2.5	0.0334	Carbon Metabolism
PLD2	1.3	2.4	2.08E-05	Choline Metabolism
VEGFA	1.2	2.3	0.0669	MTOR Pathway
AKT3	1.1	2.1	0.00709	Carbon Metabolism, Choline Metabolism, MTOR Pathway
SLC7A5	0.9	1.8	0.184	Carbon Metabolism
EGLN3	0.9	1.8	0.0163	Carbon Metabolism, Hypoxia Signaling
PDHA2	0.7	1.7	0.0464	Carbon Metabolism, KEGG Glycolysis
PDK1	0.6	1.6	0.0391	Carbon Metabolism, Glucose Metabolism, Hypoxia Signaling
H6PD	0.6	1.6	0.244	Glucose Metabolism
FASN	0.5	1.4	0.13	Other Metabolic Pathway
ALDOC	0.5	1.4	0.016	Glucose Metabolism, KEGG Glycolysis
SLC2A1	0.5	1.4	0.243	Carbon Metabolism
SDHD	0.5	1.4	0.009	Glucose Metabolism
LDHA	0.5	1.4	0.0468	Hypoxia Signaling, KEGG Glycolysis
ENO2	0.5	1.4	0.415	Glucose Metabolism, KEGG Glycolysis

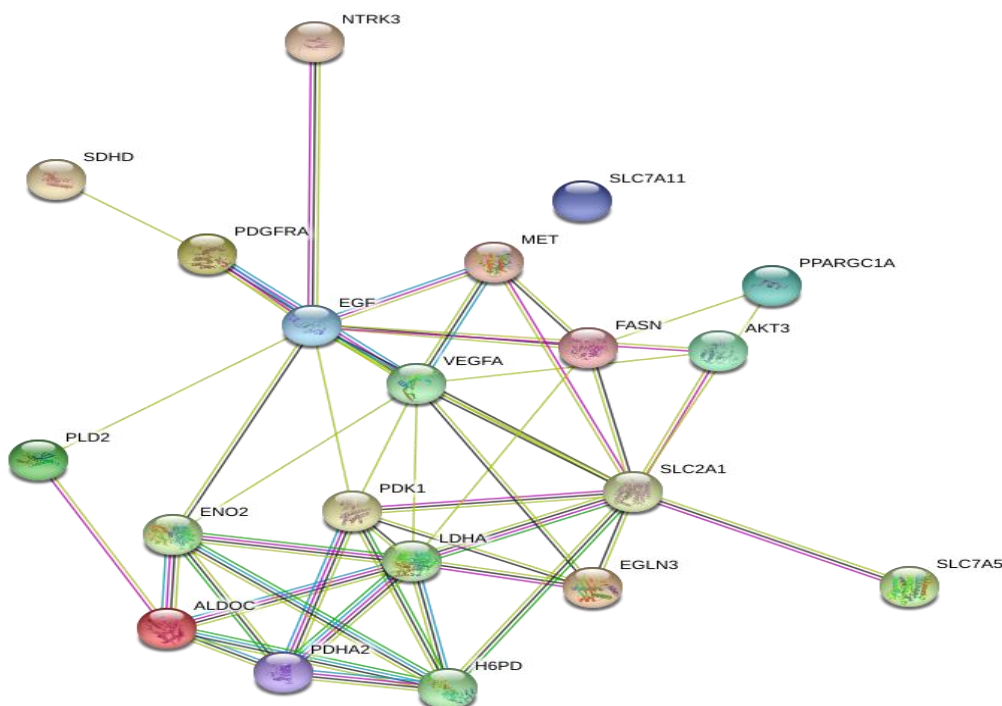


FIGURE 5.18: String analysis of the first 20 genes (upregulated) in LPS treated moDCs vs uninfected moDCs. Following Nanostring analysis the top 20 genes most highly upregulated genes in LPS-treated moDCs compared to uninfected cells) were further analysed using the String database (<https://string-db.org/>). String analysis showing the functional network relationship between the proteins encoded for by these genes.

Table 5.7: List of 20 genes that are most highly down regulated in moDCs after treatment with LPS.
Fold changes of mRNA counts between LPS treated moDCs and uninfected moDCs samples using the nanoString nCounter.

Gene	Log2 fold change	Linear fold change	P-value	Gene.sets
FBP1	-0.6	0.6	0.445	Glucose Metabolism, KEGG Glycolysis
SLC16A1	-0.7	0.6	0.0329	Carbon Metabolism
TIGAR	-0.7	0.6	0.0388	Other Metabolic Pathway
PDK3	-0.8	0.6	0.0133	Glucose Metabolism
EIF4EBP1	-0.8	0.6	0.00288	Carbon Metabolism, Choline Metabolism, Hypoxia Signaling, MTOR Pathwa
FGFR1	-0.9	0.5	0.101	Carbon Metabolism
PDP1	-0.9	0.5	0.0466	Hypoxia Signaling
PLD1	-0.9	0.5	0.144	Choline Metabolism
PLCG1	-1.0	0.5	0.13	Carbon Metabolism, Choline Metabolism
HK3	-1.0	0.5	0.0534	Carbon Metabolism, Glucose Metabolism, KEGG Glycolysis
OAZ3	-1.0	0.5	0.035	Other Metabolic Pathway
MYC	-1.1	0.5	0.0596	Cancer Metabolism Drivers, Carbon Metabolism
NTRK1	-1.2	0.4	0.177	Carbon Metabolism
NOXA1	-1.2	0.4	0.242	Hypoxia Signaling
IDH1	-1.3	0.4	0.00791	Cancer Metabolism Drivers, Glucose Metabolism
EPAS1	-1.3	0.4	7.93E-05	Cancer Metabolism Drivers
PDP2	-1.3	0.4	0.077	Glucose Metabolism
PDK2	-1.4	0.4	0.0217	Glucose Metabolism
RET	-1.6	0.3	0.0153	Carbon Metabolism
SHMT1	-1.7	0.3	0.00264	Other Metabolic Pathway

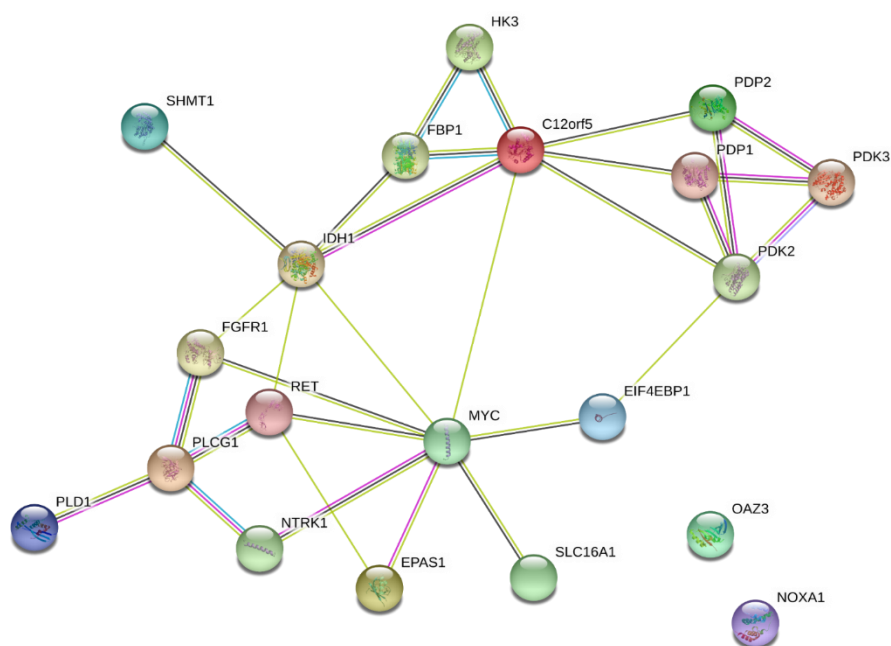


FIGURE 5.19: String analysis of top 20 genes (down regulated) in LPS treated moDCs vs uninfected moDCs. Following Nanostring analysis the top 20 genes most strongly down regulated genes in LPS-treated moDCs compared to uninfected cells) were further analysed using the String database (<https://string-db.org/>). String analysis showing the functional network relationship between the proteins encoded for by these genes.

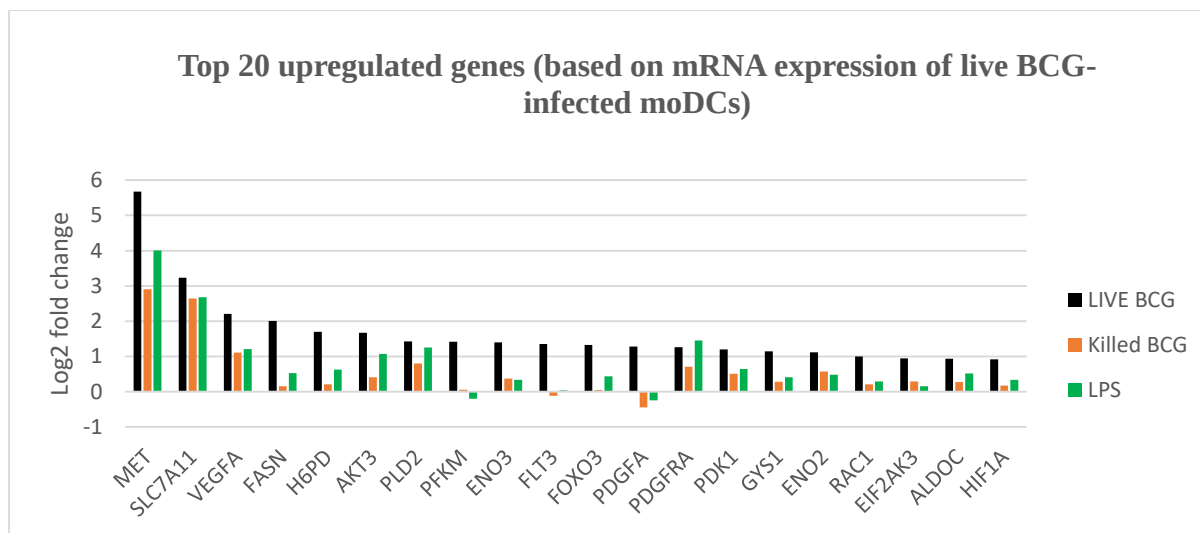


Figure 5.20: Significantly upregulated mRNA in live BCG, killed BCG and LPS infected moDCs (sort by live BCG). Fold changes of mRNA counts in live BCG infected moDCs, killed infected moDCs and LPS treated moDC compared to uninfected cells using the nanoString nCounter. Fold changes were calculated using the nSolver software from nanoString Technologies. The genes are plotted according to the top 20 most highly expressed genes in live BCG-infected cells.

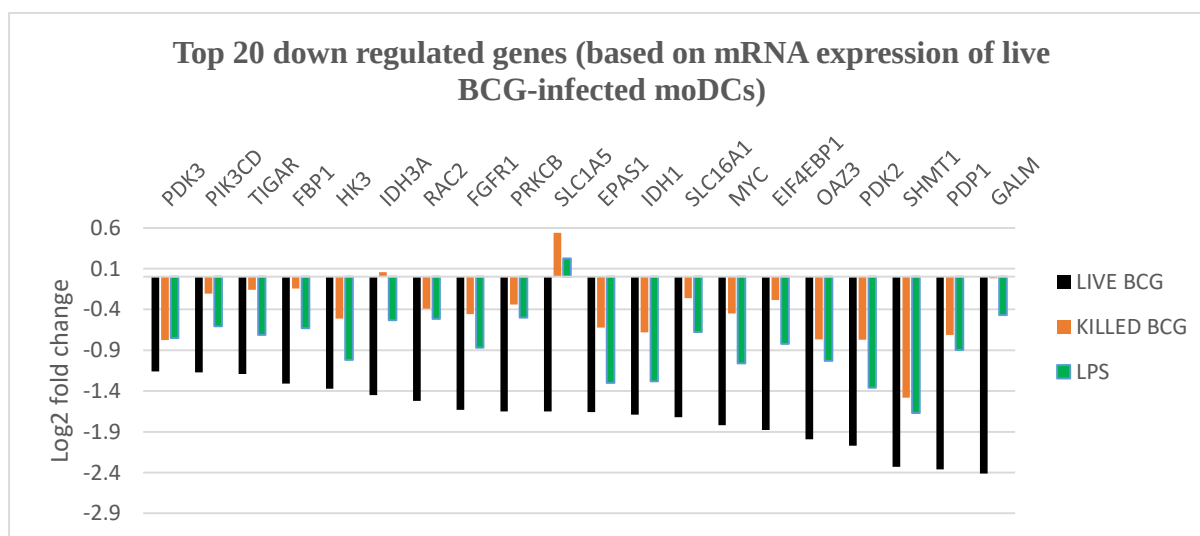


Figure 5.21: Significantly down regulated mRNA in live BCG, killed BCG and LPS infected moDCs (sort by live BCG). Fold changes of mRNA counts in live BCG infected moDCs, killed infected moDCs and LPS treated moDCs using the nanoString nCounter. Fold changes were calculated using the nSolver software from nanoString Technologies. The genes are plotted according to the top 20 most highly down regulated genes in live BCG-infected cells.

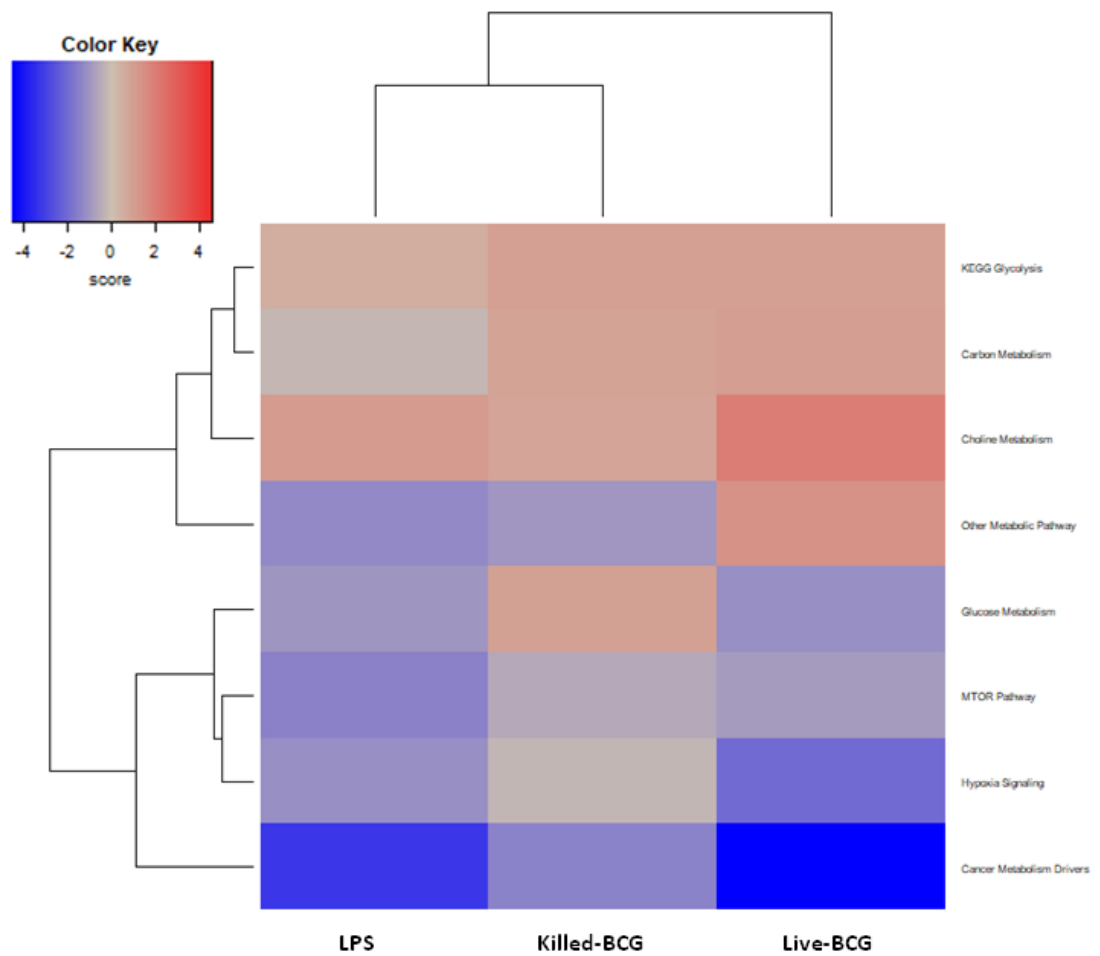


FIGURE 5.22: Directed Global Significance Scores. Heatmap displaying each sample's directed global significance scores for all of the gene sets tested. The metabolic gene sets are grouped based on the Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway database. Global significance statistics measure the extent of differential expression of a gene set's genes with the control (uninfected) samples for that particular gene set. Red denotes gene sets whose genes exhibit extensive over-expression with uninfected cells while blue indicates genes with extensive downregulation compared to uninfected cells.

5.3.4 Comparison of gene expression measured by the Nanostring method and by qPCR for six selected genes

As mentioned above, in order to confirm gene expression data from Nanostring analysis by another method, six genes were selected from the genes with the highest degree of upregulation and one with lowest differential expression between BCG infected DCs and uninfected DCs by quantitative RT-PCR.

As before, human monocyte-derived dendritic cells were cultured and plated at 0.5×10^6 /mL on 24 well plates as outlined in **Chapter 2 Section 2.3**, and infected as outlined in **Chapter 2 Section 2.2**. After 24 hours, RNA isolation was carried out as described in **Section 2.7.1** Supernatants were removed and cells lysed in RLT buffer to facilitate RNA extraction, cDNA generation as described in **Section 2.7.3**. Seven donors (including the 3 donors run in the Nanostring assay presented above) were run on the qPCR as outlined in **Section 2.7.5**. First, primer efficiency was assessed using the standard curve method (1:4 dilution over 7 points), all primers were shown to have efficiencies of between 90% and 110%. And then optimal reference target selection was carried out using 8 potential reference genes including ACTB, GAPDH, H3F3A, PPIA, RPL19, YWHAZ, RNF11 and SDHA as outlined in **Chapter 2, Section 2.7.5**. YWHAZ and PPIA were used as reference targets gene. Data was analysed used qbase+ software by Biogazelle. A significant difference in gene expression was analysed by one-way ANOVA ($p < 0.05$).

In the qPCR assay FASN-mRNA was upregulated following BCG infected moDCs (live and dead) and LPS treated moDCs. We found that FASN-mRNA was significantly upregulated in live BCG infected moDCs when compared with uninfected moDCs. The increases were non-significant in killed-BCG infected moDCs and LPS-treated moDCs respectively (**Figure 5.23 A**), suggesting that activated moDCs accelerate FASN rates in live BCG infected moDCs and this agrees with results that found in Nanostring, FASN-mRNA expression in live BCG infected moDCs was increased 4 fold, $p = 0.0002$ when compared with uninfected moDCs. While the increases were 1.1 fold, $p = 0.6$ and 1.4 fold, $p = 0.1$ in killed BCG infected moDCs and LPS treated moDCs respectively.

The Nanostring study demonstrated upregulation of SLC7A11 in BCG infected moDCs (live and killed) as well as LPS treated moDCs. By qPCR we found that SLC7A11-mRNA expression in live BCG infected moDCs was significantly increased when compared with

uninfected moDCs. While SLC7A11-mRNA expression was also increased in killed BCG infected moDCs and LPS treated moDCs the result was not statistically significant by qPCR (**Figure 5.23 B**). The significance of this increase in SLC7A11 expression for moDCs infected with BCG is unclear. It could protect infected cells from undergoing oxidant-induced cell death through the formation of GSH. However, it may also influence the dependency and flexibility of moDCs on glucose and glutamine, which is tested in **Section 5.3.5**.

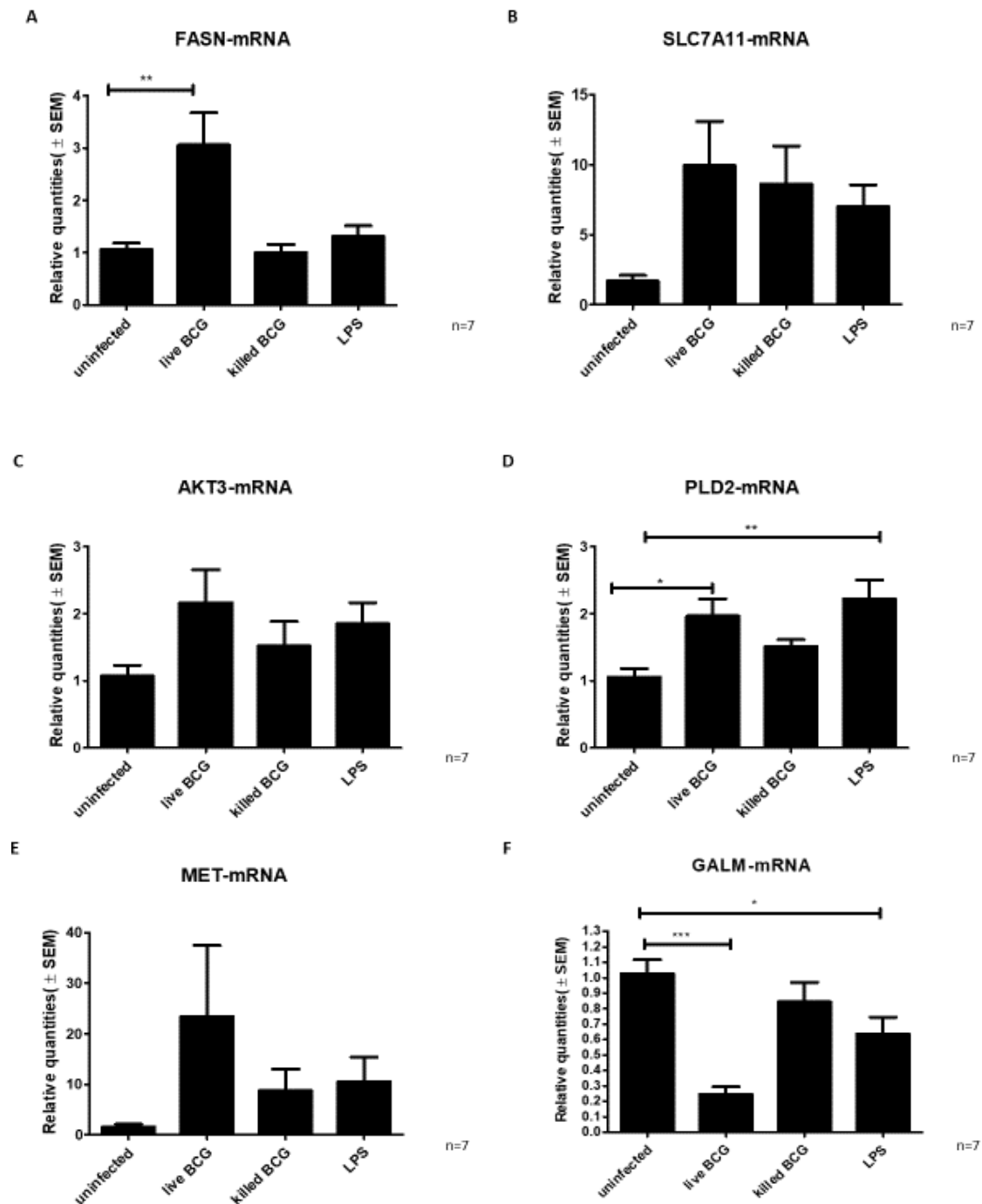
By Nanostring analysis gene expression levels of AKT3 were increased in infected BCG and treated LPS groups comparing them to uninfected cells. By qPCR AKT3-mRNA expression in live BCG infected moDCs was a significantly increased while there was a non-significant increase in killed BCG infected moDCs and LPS treated moDCs (**Figure 5.23 C**).

In line with previous results observed in moDCs, following infection with BCG (live and killed) and LPS treated moDCs, were significant upregulation of PLD2. We found that PLD2-mRNA expression in live BCG infected moDCs and LPS treated moDCs was significantly increased while there was a non-significant increase in killed BCG infected moDCs when compared with uninfected moDCs (**Figure 5.23 D**) suggesting a previously unrecognised role for this gene during mycobacterial infection.

In human moDCs the Nanostring study revealed upregulation of MET- mRNA following BCG infection (live and dead) and LPS treatment. In fact, it was the most highly expressed gene in both live and killed BCG-infected cells (**Figure 5.11 and Figure 5.14, Table 5.2 and Table 5.4**). By qPCR in the 7 donors tested MET-mRNA expression was highly variable (**Figure 5.23 E**). When the donors were segregated into high and low expressors there was a significant increase in expression in live BCG infected moDCs, killed BCG infected moDCs and LPS treated moDCs in the 3 high expressors (data not shown) which was similar to the results obtained with the Nanostring method.

Interestingly, gene expression levels of GALM were significantly decreased in live BCG infected moDCs and LPS treated moDCs comparing them to uninfected by both the Nanostring and qPCR methods (**Figure 5.23 F**).

Overall, results from the qPCR (**Figure 5.23**) largely confirmed the findings of the nanostring analysis. **Table 5.8** shows the results of 6 genes interest obtained from Nanostring, qPCR results indicate that AKT3, FASN, MET, PLD2, SLC7A11 are upregulated and GALM is downregulated similar to the Nanostring results.



5.23: Gene Expression in live and killed BCG infected moDCs. Human monocytes were separated from buffy coats using negative selection and differentiated to moDCs. The moDCs were infected with live or killed BCG at MOI 100 for 24 hours. Uninfected moDCs were treated with lipopolysaccharide (LPS) (1µg/ml) as a positive control or left uninfected (control). At 24 hours post-infection, supernatants were removed. Cells were lysed in RLT buffer, RNA extracted and cDNA generated, and qPCR was performed. Data analysed used qbase+ software by Biogazelle. A significant difference in gene expression was analyzed by one-way ANOVA ($p < 0.05$). Panels **A –F** depict relative mRNA expression of FASN (**A**), SLC7A11 (**B**), AKT3 (**C**), PLD2 (**D**), MET (**E**) and GALM (**F**) for seven donors.

Table 5.8: The results of 6 genes of interest obtained from Nanostring and qPCR (log2 fold change compared to control). NanoString (black value) and qPCR (red value)

Gene	Killed vs uninfected		LPS vs uninfected		Live vs uninfected	
AKT3	1.29	1.52	2.02	1.85	3.6	2.16
FASN	1.2	0.99	1.6	1.30	4.78	3.06
GALM	1	0.84	-1.41	0.63	-5.12	0.24
MET	4.21	8.72	8.14	10.54	16.52	23.45
PLD2	1.78	1.51	2.46	2.21	3.11	1.96
SLC7A11	5.37	3.3	5.79	2.3	9.7	3.9

5.3.5 Evaluating the mitochondrial fuel flexibility of moDCs after BCG infection

Our Nanostring data indicates that several energy pathways may be used by moDCs for maturation under normal and stressed conditions. Accordingly, to further investigate and understand the interaction of the cellular metabolic pathways and changes during infection with mycobacteria the role of several metabolic fuels (glucose, glutamine and long chain fatty acids) was tested using the Seahorse Analyser.

MoDCs were prepared as described in **Section 2.3.1** and **Section 2.3.2** respectively. On the day of infection, cells were scraped, and plated in at 250.000 cells/well on Seahorse 24-well microplates in RPMI 1640 media supplemented with 10% defined FBS at least 1 hours before BCG infected at MOI 100, uninfected moDCs were treated with 1µg/ml LPS. On the day of interrogation, the Seahorse media was supplemented with 1 mM pyruvate, 2 mM glutamine, and 10 mM glucose, warmed to 37°C and filtered through 0.2µm sterile syringe filters, thereafter 200 µl media was removed from all wells of the Seahorse microplate, the cells washed twice by the supplementary addition of 400µl Seahorse media, and 450µL of fresh Seahorse media applied to each well (including blank wells by added 500µl of fresh Seahorse media). The microplate was then incubated at 37°C and 0% CO₂ prior to interrogation using the Seahorse XFe24 Analyser.

Suspecting that moDCs used metabolic pathways in addition to glycolysis during maturation, we explored the relative flexibility of moDCs in oxidizing glucose, endogenous FAs, and glutamine for mitochondrial respiration. First, OCR was unchanged in uninfected moDCs and BCG infected moDCs after treatment with UK5099 (an inhibitor of the

mitochondrial pyruvate carrier that transports pyruvate into the mitochondria and thus an inhibitor of glucose oxidation). However, there is decreased OCR when FA and glutamine oxidation are inhibited with etomoxir (inhibits carnitine palmitoyl-transferase 1A which transports long-chain fatty acids from the cytosol into the mitochondria) and BPTES (an inhibitor of inhibitor of glutaminase that blocks glutamine oxidation) respectively (**Figure 5.23 A&B i, ii**). UK5099 did not change the basal OCR indicating uninfected moDCs and BCG infected moDCs are not dependent on glucose oxidation. The difference between the full capacity and dependency is equivalent to moDCs flexibility in using glucose oxidation, all groups were completely flexible in their ability to use glucose for mitochondrial oxidation. Their capacity to use glucose for mitochondrial respiration was unchanged by BCG infection (**Figure 5.24 B**). Next, moDCs flexibility in oxidizing glutamine was measured without infection and after BCG infection. OCR was unchanged after inhibition of glutamine oxidation by BPTES. However, there was decreased OCR when FA and glucose oxidation were inhibited (**Figure 5.23 C&D i, ii**). The capacity of moDCs to utilise glutamine in the mitochondria was increased by BCG infection although this increase was not statistically significant. Uninfected moDCs and BCG infected moDCs were completely flexible in using glutamine for mitochondrial oxidation. In contrast, LPS treated moDCs showed slightly increased dependency on glutamine (**Figure 5.24 C**).

Next, moDCs flexibility in oxidizing endogenous FAs was measured in uninfected, BCG infected and LPS-treated cells. Inhibition of FA oxidation by etomoxir reduced OCR in all groups, data shown is one representative of three donors (**Figure 5.23 E&F i, ii**). There was no difference in the capacity of uninfected and BCG infected moDCs to use long chain fatty acids for mitochondrial respiration. Flexibility in FA oxidation was lowest in BCG infected moDCs as compared with uninfected moDCs but this did not reach statistical significance (**Figure 5.24 A**). The data indicate a striking dependence of immature (uninfected) and mature (BCG infected moDCs or LPS treated) human moDCs on fatty acid oxidation to fuel mitochondrial respiration.

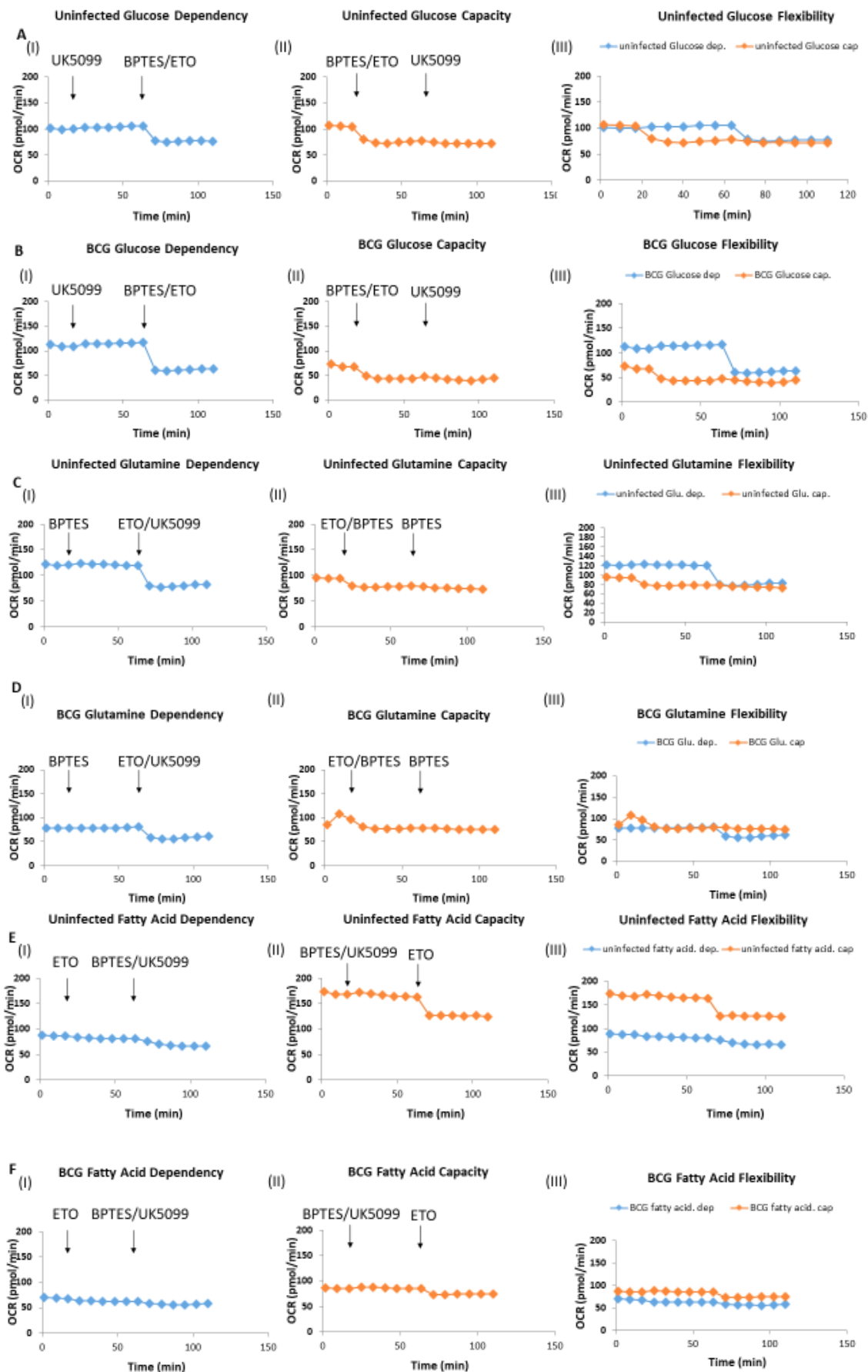


FIGURE 5.24: MoDCs mitochondrial fuel flexibility. moDCs were plated on Seahorse Biosciences 24-well plates at 250,000 cells/well. Cells were infected for 24 hours with 100 MOI of killed BCG. Uninfected moDCs were also treated with 1 μ g/ml LPS. Extracellular flux analysis was performed and moDC mitochondrial flexibility in glucose oxidation was measured using the XFe mitochondrial fuel flexibility test, with sequential

addition of inhibitors and data normalised using CV assay. Panels A and B depict OCR in uninfected moDCs and BCG infected moDCs, extracellular flux analysis was performed with sequential addition of UK5099 (2 μ M), BPTES (3 μ M) and Etomoxir (4 μ M). (i) The oxygen consumption rate (OCR) was plotted over time to determine the dependence of moDCs on glucose oxidation. The basal OCR was established before the sequential injection of UK5099 and the combination of BPTES and etomoxir. (ii) The average OCR was plotted over time for moDC capacity to oxidize glucose. The basal OCR was established before the sequential injection of the combination of etomoxir and BPTES followed by UK5099 and (iii) flexibility (dependency and capacity together) in uninfected moDCs and BCG infected moDCs. Panels C and D depict OCR in uninfected moDCs and BCG infected moDCs, moDC mitochondrial flexibility in glutamine oxidation was measured with sequential addition of BPTES (3 μ M), Etomoxir (4 μ M) and UK5099 (2 μ M). The basal OCR was established before the sequential injection of BPTES and the combination of etomoxir and UK5099. (ii) The average OCR was plotted over time for moDC capacity to oxidize glutamine. The basal OCR was established before the sequential injection of the combination of etomoxir and UK5099 followed by BPTES and (iii) flexibility (dependency and capacity together) in uninfected moDCs and BCG infected moDCs. Panels E and F depict OCR in uninfected moDCs and BCG infected moDCs. To assess mitochondrial flexibility of moDC in fatty acid (FA) oxidation extracellular flux analysis was performed with sequential addition of Etomoxir (4 μ M), BPTES (3 μ M) and UK5099 (2 μ M). The basal OCR was established before the sequential injection of Etomoxir and the combination of BPTES and UK5099. (ii) The average OCR was plotted over time for moDC capacity to oxidize glutamine. The basal OCR was established before the sequential injection of the combination of BPTES and UK5099 followed by Etomoxir and (iii) flexibility (dependency and capacity together) in uninfected moDCs and BCG infected moDCs for three donors. Data shown is for one representative donor.

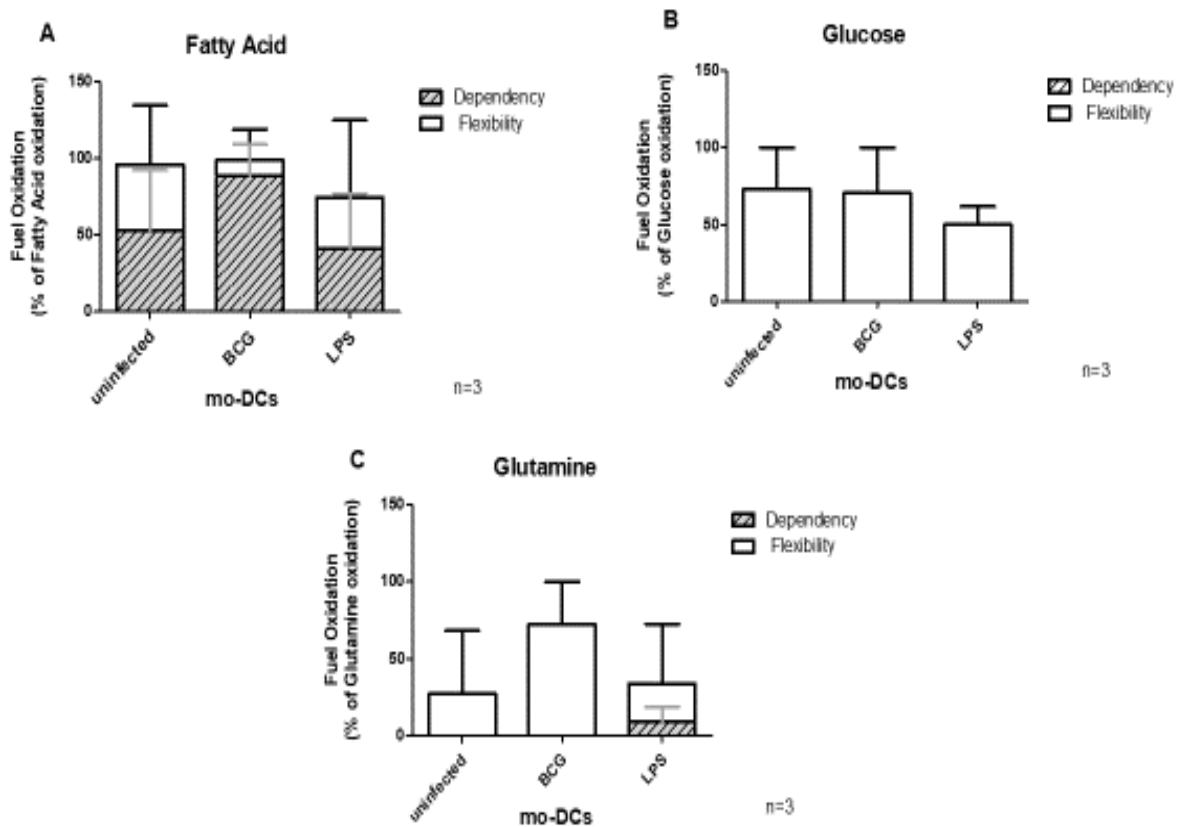


FIGURE 5.25: BCG infected moDCs is associated with alterations in mitochondrial fuel flexibility. The data depicted in **Figure 5.25** was combined and analysed for all three donors. **Panel A.** Percentage moDCs mitochondrial dependency on, capacity and flexibility in fatty acid (FA) oxidation. **Panel B.** Percentage moDCs mitochondrial dependency on, capacity and flexibility in glucose oxidation. **Panel C.** Percentage moDCs mitochondrial dependency on, capacity and flexibility in glutamine oxidation for three donors

5.4 Discussion

Nanostring analysis revealed that infection with either live BCG or killed BCG induced the upregulation and downregulation of numerous metabolic genes in moDCs compared to uninfected cells. There was modest upregulation of glycolytic genes by both live and killed BCG which may account for the limited effects of the glycolysis inhibitor 2DG on DC maturation (Chapter 3) and metabolism (Chapter 4). Some of the most highly upregulated genes were involved in metabolic pathways other than glycolysis, including SLC7A11 (glutamine metabolism) and FASN (fatty acid synthesis) suggesting a high degree of metabolic plasticity of BCG-infected moDCs. Extracellular flux analysis confirmed the metabolic flexibility of BCG-infected moDCs with regard to glucose and glutamine use for OXPHOS but showed that infected human moDCs are dependent on FAO for this process when glucose and glutamine are lacking. Directed Global Significance Analysis (GSA) was carried out by the nCounter software for each treatment compared to uninfected cells. This statistical analysis gives an overview of the extent to which each metabolic pathway varies compared to the control. GSA indicated that live BCG had the most significant effect on differential gene expression; Glycolysis, Carbon Metabolism, Choline Metabolism and “Other Metabolic Pathway” showing an increase over uninfected cells and Cancer Metabolism Drivers were the most strongly down regulated.

The Choline pathway was the most upregulated in BCG-infected moDCs due to significantly increased expression of AKT3, PLD2, PDGFA, PDGFRA and RAC1. Choline metabolism refers to the metabolism of all choline-containing molecules and is particularly important in phospholipid metabolism. PLD2 catalyses the breakdown of the phospholipid phosphatidylcholine, the products of this reaction are phosphatidic acid and choline. PLD1 and PLD2 play coordinate roles in macrophage phagocytosis [261]. Research conducted on identifying the role of PLD gene indicated that this gene has a significant role in cancer and its expression is greatly increases in tumour cells. This gene is especially involved in important cellular processes such as signal transduction during proliferation as well as preventing apoptosis. It was shown that in patients with breast cancer, upregulation of this gene is associated with a decrease in the rate of apoptosis and hence increased tumour formation [262]. PLD2 expression was up regulated in gastric cancer also [263], [264]. Oncogene activity is also enhanced due to overexpression of PLD gene [265]. The choline pathway is regulated by AKT and RAS signalling (RAC1 belongs to the RAS pathway)

which are activated downstream of growth factor signalling. The AKT family consists of AKT1, AKT2 and AKT3 serine-threonine protein kinases and are deregulated in certain types of cancers [256–259]. When a growth factor binds to its receptor phosphatidylinositol 3-kinase (PI3K) is recruited to the receptor resulting in phosphorylation of AKT. AKT then goes on to phosphorylate a wide range of other proteins. Although the AKT isoforms are encoded by different genes they have very similar amino acid sequences. Nevertheless, the proteins seem to have distinct functions. AKT1 appears to be important in regulating cell proliferation while AKT2 regulates cell signalling in response to insulin and growth factors such as PDGF and also regulates glycogen synthesis and glucose uptake [260]. The role of AKT3 has mainly been studied in relation to its involvement in brain development and cancer metastasis, its role in cellular metabolism is unknown. Interestingly PDGF and VEGF expression were also upregulated in moDCs infected with live and killed BCG compared to control cells (**Figures 5.12. 5.15**). Therefore, infected moDCs might have increased AKT3 activity in response to these growth factors. While the involvement of AKT2 in cellular metabolism is well established [260] it remains to be seen whether or not AKT3 influences moDC metabolism.

Lipid accumulation is associated with DC dysfunction in cancer but can support an immunogenic immune response and promote cross-presentation of antigens to CD8⁺ T cells [237]. “Other Metabolic Pathway” was the second most upregulated gene set in live BCG-infected. The only member of this gene set in the top 20 upregulated genes in BCG-infected moDCs was FASN. A lot of studies have been conducted on this gene which suggest that it is an oncogene and is crucial for the metabolic progression of tumours. Cancer cells use the de novo synthesis pathway to generate fatty acids as a fuel for beta-oxidation in the mitochondria to generate ATP as well as for lipid modification of proteins. Some of the types of cancers in which this gene is overexpressed are the prostate [245], thyroid, ovarian, breast, lungs as well as gastric carcinomas [246]. FASN is crucial to de novo long-chain fatty acid synthesis (FAS), needed to meet cancer cells' increased demands for membrane, energy, and protein production. During FAS, malonyl-CoA is combined with acetyl-CoA by FASN generating long-chain fatty acids which are incorporated into lipids that are used for the synthesis of membranes or posttranslational modifications of proteins [244]. Recently, it was suggested that TLR-driven activation and cytokine production in DCs is dependent on de novo FAS, which serves as a source of lipids for the expansion of the ER and Golgi apparatus [100]. TLR-activated DCs are known to also accumulate triglycerides

in lipid droplets (LDs); the increased expression of FASN is in keeping with this as long chain fatty acids synthesised by this enzyme can be incorporated into triglycerides. Mycobacterial infection also drives LD formation in macrophages and DCs *in vivo* and it is not yet clear whether this benefits the host or the pathogen; there is evidence for both outcomes [152], [154]. Further investigation of BCG-infected DC maturation in the presence of modulators of lipid metabolism will be needed to delineate the influence of these pathways.

As well as supplying fatty-acids for membrane synthesis, FASN can also contribute to energy production by synthesising fatty acids for β -oxidation in the mitochondria. The increased dependence of BCG-infected moDCs on endogenous FAO seen in the present study suggests that FASN may provide fuel for mitochondrial respiration during mycobacterial infection. Increased FAO is also seen in murine BMDCs in the early stages of TLR-mediated activation, along with the increase in glycolytic flux [137], although these cells lose their metabolic plasticity later. Acetyl-CoA carboxylase (ACC) 1 and 2 (ACC1 and ACC2) are the rate-limiting enzymes for FAS and fatty acid oxidation (FAO) respectively. Both enzymes catalyse the carboxylation of acetyl-CoA to malonyl-CoA but are present in different locations. Rewiring of the TCA cycle during DC maturation leads to the accumulation of citrate which can be shunted out of the mitochondria to the cytosol where it is converted to acetyl Co-A by ATP citrate lyase. ACC1 converts the acetyl Co-A to malonyl Co-A in the cytosol for synthesis of long chain fatty acids and its inhibition reduces DC differentiation. ACC2 controls FAO by regulating the transport of long chain fatty acids into the mitochondria by carnitine palmitoyltransferase 1 (CPT1) [99]. Recent work by Philipp Stüve et al suggests that ACC1 and ACC2 expression in DCs and macrophages is unnecessary for mycobacterial control in a mouse model of TB implying that neither FAO nor FAS are required to mount a successful immune response. However, the role of FASN in human DC responses to mycobacterial infection has not been studied. Glucose levels available to activated DCs *in vivo* could be limited in the presence of other immune cells, for example proliferating T cells [247]. Therefore, increased FASN may be required to allow moDCs to utilise long chain fatty acids rather than glucose as a fuel for OXPHOS under glucose-limiting conditions [237], [248], thereby benefiting the host [153]. On the other hand, host-derived fatty acids can be utilised as nutrients by mycobacteria in macrophages [152], therefore, the increase in FASN may be a way that the pathogen exploits moDC metabolism for its own purposes. The long-term goal of this research is to determine

whether manipulation of metabolic pathways can influence the immunogenicity of the BCG vaccine. In order to address this issue, it would be interesting to assess the role of fatty acid metabolism – including FAO and FASN - in moDC maturation and survival during infection with mycobacteria using pharmacological inhibitors (Etomoxir and C75 respectively) of both pathways.

The third most upregulated pathway in live and killed BCG-infected cells according to GSA was KEGG Glycolysis. The most significantly upregulated genes in this pathway were PFKM, ENO2, ENO3 and HIF1 α . HIF1 α is a master-regulator of glycolysis in immune cells and the promoters of many glycolytic genes contain HIF1 α response elements (HRE) [100]. Interestingly the HIF gene set as a whole was significantly downregulated due to highly significantly decreased expression of genes involved in the hypoxia signalling pathway such as PDP1 and EIF4EBP1. PFKM, the muscle form of phosphofructokinase, is one of those HIF-inducible genes which catalyzes the phosphorylation of fructose-6-phosphate to fructose-1,6-bisphosphate, the rate limiting step in glycolysis. Enolase also contains a HRE in its promoter region [170] and catalyzes the second last step in glycolysis i.e., converting 2-phosphoglycerate to phosphoenolpyruvate. Upregulation of ENO2 has recently been observed in the human cDC2 myeloid DC subset following TLR 7/8 stimulation [300] and ENO2 inhibition inhibited cDC2 maturation and activation. This was associated with metabolic reprogramming and mitophagy in these cells. ENO1, which was also significantly upregulated but was this less than 2 fold (x1.78), has previously been shown to be involved in mitochondrial dysfunction in BMDC [301]. Although classified as belonging to the “Glucose Metabolism” pathway another gene that was highly significantly upregulated by BCG was H6PD. The H6PD enzyme catalyses the first steps of the pentose phosphate pathway, diverting glucose-6-phosphate from glycolysis to generate NADPH (a cofactor for FAS) and pentoses as well as ribose 5-phosphate (which may be required for the synthesis of mRNA for increased expression of metabolic and cytokine genes during moDC maturation).

“Carbon Metabolism” pathway was also upregulated according to the Nanostring analysis. The two most highly upregulated genes across live and killed BCG and LPS-treated cells were MET and SLC7A11 and both belong to this pathway. The glutamate/cystine antiporter solute carrier family 7 member 11 (SLC7A11) gene encodes the cystine/glutamate transporter xCT which imports cystine into the cell while exporting glutamate. This gene

was found to be significantly upregulated in live and killed BCG-infected moDCs by both Nanostring and RT-PCR. It has been shown that cysteine-glutamate transporter is overexpressed in many types of cancer including brain tumours (glioma) and has the capability to act as a screening biomarker for epilepsy [252] as well as being overexpressed in hepatic cancer [253]. Recently SLC7A11 was found to be upregulated in the peripheral blood monocytes of TB patients, in mouse lung [254] and murine macrophages infected with Mtb [249]. Macrophages from SLC7A11 knockout mice had increased mycobactericidal activity and the mice had reduced lung pathology compared to the wild-type animals [255]. The cystine imported by SLC7A11 is converted to cysteine and is used in the synthesis of the antioxidant tripeptide glutathione (GSH) which is important for redox balance and protects cells from oxidative stress-induced cell death [249]. On the other hand, SLC7A11 expression also renders cancer cells more dependent on glucose, sensitizing the cells to glucose starvation-induced cell death due, in part, to a reduction in glutamate levels. Many cancer cells are dependent on glutamine as a mitochondrial fuel and SLC7A11 expression limits their metabolic flexibility making them more reliant on glucose for survival [250], [251]. In the present study moDCs did not alter their dependence on glutamine or glucose as fuels for OXPHOS following BCG infection. LPS treatment also induced an increase in SLC7A11. In contrast to mycobacterial infection, there was increased dependence of LPS-treated moDCs on glutamine but not FAO for mitochondrial respiration indicating a marked difference in the metabolic requirements of these cells in response to different stimuli.

Met, the most highly upregulated gene in BCG and LPS stimulated moDCs, encodes the tyrosine kinase receptor for hepatocyte growth factor (HGF) and is a well-known proto-oncogene. It is involved in invasive growth of tumours and as well as in metastasis and expression of Met gene was upregulated in colorectal cancer [266] liver cancer [267] gastric adenocarcinoma [268] and breast cancer [269]. *Listeria monocytogenes* exploits the MET receptor to enter the host epithelial cells. MET expression is increased in antigen-presenting cells of the immune system in response to pro-inflammatory cytokines. HGF signalling through MET is essential for DC migration from the skin to draining lymph nodes [270], but also promotes a tolerogenic immune response with increased induction of Tregs and decreased Th1 and Th17 cells [271], [272] without upregulating IL-10 or TGF β and may have a role in protecting against autoimmunity. The significance of MET signalling in the immune response to pathogens is not well studied and its involvement in DC metabolism

has not yet been investigated, however, this pathway may contribute to the lack of efficacy of the BCG vaccine by reducing the immunogenic potential of DCs.

The most significantly downregulated pathway in all of the conditions tested was Cancer Metabolism Drivers and this gene set includes EPAS1, IDH1, IDH2 and MYC. The IDH1 protein is the NADP(+)-dependent isocitrate dehydrogenase found in the cytoplasm and peroxisomes and catalyses the conversion of alpha-ketoglutarate to D-2-hydroxyglutarate. IDH2 is an NADP(+)-dependent isocitrate dehydrogenase found in the mitochondria. Downregulation of this pathway occurs in murine BMDCs and macrophages stimulated with LPS/IFN- γ and leads to accumulation of isocitrate and a “broken” Krebs cycle. The Nanostring results suggest that there may also be a breakpoint in the Krebs cycle during BCG infection of human moDCs. The resulting citrate could be exported to the cytosol as acetyl CoA where it could be used for FAS or for histone acetylation to alter the expression of metabolic and proinflammatory cytokine genes [124], [125].

The most significantly downregulated individual gene in BCG and LPS-infected moDC was galactose mutarotase (GALM) which was included in the Glucose Metabolism, KEGG Glycolysis gene sets. This gene codes for an enzyme that is involved in catalysing the epimerization of six carbon sugar molecules e.g. glucose and galactose, with a preference for galactose. Galactose can enter glycolysis by its conversion to glucose-6-phosphate via the Leloir pathway in which GALM carries out the first step [273]. Hexoses that are absorbed from diet can get metabolized by controlling the increased basal degree of GALM gene activity in the liver. The sudden rise in the expression of GALM gene occurs by the virtue of retinoic acid-mediated signalling in macrophages [274]. GALM mRNA expression is increased in nutrient uptake of human myeloid cells. So, down regulation of GALM leads to inactivation of immune modulation and it is involved in pathogenesis of cancers like leukaemia and breast cancer [274]. It is a prognostic marker in renal cancer and in urothelial cancer [275]. Little is known about the role of GALM in the immune response. Since it is involved in the Leloir pathway and provides glucose for glycolysis it is tempting to speculate that a reduction in GALM expression by BCG infection may reduce the rate of glycolysis in moDCs thereby reducing the immunogenic potential of the vaccine. This metabolic pathway will need more exploration to determine its role in DC maturation and TB disease.

When considering the GSA results it must be borne in mind that, although 180 genes were analysed, the gene set for each pathway consisted of relatively small numbers of genes. Therefore, the analysis could be skewed by small changes in several genes in a pathway or by large changes in one gene. The Nanostring results for six of the most highly upregulated and downregulated were validated by RT-PCR indicating that the differential gene analysis carried out on this platform was robust and reproducible. Overall, in the Nanostring analysis, there was stronger and more significant up and down regulation of genes with live BCG infection compared to killed BCG, although many of the same genes trended in the same direction. Although both killed and live BCG treatment lead to maturation of the moDCs, phagocytosis of the mycobacteria was lower with killed BCG compared to live BCG. The more robust effect of live BCG on differential gene expression may be due to the decreased uptake of killed BCG compared to live BCG. Killed BCG was used for the metabolic flux assays, but these data indicate that the metabolic response of moDCs to live BCG might be also different from that of the dead BCG. Therefore, the use of killed BCG to carry out metabolic flux analysis, although it was unavoidable, is a major limitation of this study. Metabolic flux analysis should be repeated with moDCs infected with live BCG when a Seahorse Instrument becomes available for use with Class 2 bacteria.

In conclusion, the results of this chapter show for the first time that infection of human moDC with BCG leads to significant changes in the expression of genes involved in glycolysis, long chain fatty acid synthesis, glutamate metabolism and the pentose phosphate pathway. Metabolic flux analysis confirmed that BCG-infected moDC are dependent on long chain fatty acids for mitochondrial respiration when other fuels are unavailable. These data indicate that, following infection with mycobacteria, the metabolic requirements of moDC are complex than previously thought. Further in-depth investigation of the role of these metabolic pathways in DC maturation and function will be required to translate the promise of immunometabolism for better protection against TB.

CHAPTER 6:

General Discussion

TB is one of the deadliest diseases worldwide, mainly affecting young adults in low-income countries for whom the health and financial burdens can be catastrophic. The rise in the incidence of MDR-TB and the lack of efficacy of the BCG vaccine mean that it is more important than ever to understand host-bacillus interactions in order to develop advanced therapies and design a better vaccine [276]. Understanding the DC's response to mycobacterial infection is crucial for the development of vaccines [277]. The long-term goal of this research is to determine whether manipulation of metabolic pathways can influence the immunogenicity of the BCG vaccine to stimulate a better more protective T cell response. This is the first study to begin to investigate the role of immunometabolism in that response in human DCs. Recent advances reveal that metabolic reprogramming is required for adequate antibacterial responses of DCs that possess the capacity to initiate innate and adaptive immune responses [278]. Although recent years have seen an exponential increase in immunometabolism research, this work is the first to investigate the role of glycolysis in the maturation of DCs infected with mycobacteria.

After stimulating human moDCs with BCG and carrying out various tests using metabolic modulators, several lines of evidence suggest that glycolysis plays an important but limited role in BCG-induced maturation of human moDC. Specifically, glycolysis appears to be required for optimal cell surface expression of CD86, for IL1 β and IL-10 mRNA expression and for IL-13 secretion by moDCs. In addition, glycolysis also seems to play an unexpected role in potentiating BCG-induced necrosis. Interestingly, there is evidence that inflammatory DCs found in the lungs of mice infected with Mtb express anti-apoptotic proteins [150]. This would not prevent necrosis and it has been shown that dying murine DCs can release mycobacteria in the lung to disseminate the infection, which may be detrimental to the host during infection with virulent Mtb [81]. But in the context of vaccination with BCG, host cell death could be beneficial in promoting an immunogenic response by exposing DAMPs to stimulate other innate immune cells and amplify inflammation. Promoting glycolysis during vaccination might boost this immunogenic response. On the other hand, inhibiting glycolysis, and thus cell death, may increase the time that moDCs are able to interact with T cells thereby promoting expansion of antigen-specific cells [189].

HIF1 α is a master-regulator of glycolysis in immune cells and the promoters of many glycolytic genes contain HIF1 α response elements [100]. Transcriptional profiling carried out using the NanoString nCounter platform showed modest up-regulation (approximately 2-fold) in the expression of some glycolytic genes (PKFM, ALDOC and ENO2) but other genes in the pathway were downregulated (HK1, HK3). HIF1 α itself was upregulated by less than 2-fold although post translational modifications are more important in its regulation [114]. There was evidence of significant upregulation of other HIF1 α target genes including IL-1 β and VEGFA at the mRNA level. This was in line with the increased levels of IL-1 β mRNA detected by RT-PCR in moDCs infected with BCG.

Certain important aspects of DC maturation seemed to be unaltered by glycolysis. Secreted levels of IL-10, IL-1 β and other pro-inflammatory cytokines, as well as cell surface expression of HLA-DR, were unaltered by the glycolysis inhibitor 2DG. Increased glycolytic flux was detected in BCG-challenged cells upon the addition of high levels of glucose to starved cells but not under basal cell culture conditions. The main function of mature DCs is to initiate adaptive immunity by activating T cells. Future work should include determining the effect of inhibiting moDC glycolysis with 2DG on antigen specific CD4⁺ and CD8⁺ T cell proliferation and polarisation in PBMCs from BCG-vaccinated individuals to determine whether the glycolytic pathway is required for optimal T cell function.

The NanoString analysis suggests that glycolysis may not be the only pathway for the generation of energy in this setting. There was evidence in BCG-infected DCs for changes in lipid metabolism including upregulation of FASN, involved in fatty acid synthesis, Citrate, formed by the oxidation of pyruvate (itself generated from glucose by glycolysis) in the TCA cycle, can be used to provide acetyl-CoA for FAS. Alternatively, acetyl-CoA can be formed by β -oxidation of long chain fatty acids and, consistent with this, increased dependence of BCG-infected moDC on FAO for mitochondrial respiration was observed in this study. Increased FAO is also seen in murine DCs in the early stages of TLR-mediated activation, along with an increase in glycolytic flux, although these cells lose their metabolic plasticity later [99], [154]. The data presented here shows that human moDC maintained their metabolic plasticity in so far as they seemed to utilise both OXPHOS and glycolysis to meet their biosynthetic and bioenergetics needs for up to 24 hours post infection or treatment with LPS. TLR-activated DCs are known to also accumulate triglycerides in lipid droplets

(LDs); the increased expression of FASN is in keeping with this. Mycobacteria also drive LD formation and it is not yet clear whether this benefits the host or the pathogen; there is evidence for both [152], [153]. A recent study showed that neither a functional FAO nor a FASN pathway were required in DCs for immune responses to BCG in a mouse model of infection [154]. Further investigation of BCG-infected DC maturation in the presence of modulators of lipid metabolism (i.e. inhibitors of FAO and FASN) will be needed to delineate the influence of these pathways in human cells.

There are several limitations to this work. Firstly, and probably most important, is that the metabolic flux analysis was carried out using killed BCG. This was unavoidable but it should be kept in mind that live BCG may have different effects on ECAR and OCR compared to killed BCG. It is interesting to note that a previously published study of human monocyte-derived macrophages showed that live BCG induces glycolysis and increases the dependency of macrophages on FAO [159] like the results found in the present work. Nevertheless, it will be important to assess the effects of BCG viability on moDC metabolic flux to address this issue. Secondly, the role of metabolism in moDC maturation was extensively studied in this work but the effects of DC metabolism on T cell responses to BCG was not addressed. Comparing *in vitro* T cell proliferation in the presence of untreated BCG-infected moDCs to that of infected moDCs treated with 2DG will be necessary to answer this question.

The results of this study suggest that simply promoting glycolysis to improve vaccine efficacy might be too simplistic an approach to take. Considering all of the data, there seems to be a role for glycolysis in certain aspects of human moDC maturation after BCG infection but not in others. Glycolysis was required for optimal maturation of moDCs infected with BCG and thus could contribute to the immunogenicity of the vaccine. On the other hand, it also seemed to promote certain processes that may be unhelpful to the immune response to Mtb, for example, secretion of IL-13. This Th2 cytokine is associated with excessive immunopathology in the lungs of TB patients [302]. A Th2 response in general is thought to undermine the Th1 response required to fight Mtb infection. Many individuals in developing countries have a heightened Th2 immune response at baseline due to exposure to helminth infection [282]. These are the very places with a high incidence of TB and where a good vaccine is needed the most. Nevertheless, maintaining a balance between Th1 and

Th2 responses seems to be important in controlling Mtb infection so there may be some benefit to the host from a mixed cytokine response from DCs [303].

The BCG vaccine protects young children from disseminated TB, particularly TB-meningitis. However, the vaccine is variable in the degree of protection that it gives to adolescents and adults. This work shows that the metabolism of BCG-infected moDC can be manipulated using already approved therapeutics, namely metformin and IFN- γ . This raises the hope that the host response to BCG could be improved upon by pharmacological manipulation or immunotherapy at the time of vaccination to provide better immunity to Mtb. In this study metformin treatment induced a metabolic switch towards glycolysis in BCG-infected cells. However, although metformin improves the Th1 response to Mtb antigens in TB patients [235], the drug also has immunosuppressive effects. Metformin treatment reduced proinflammatory cytokine secretion by LPS-stimulated peripheral blood monocytes *ex vivo* and, when administered to healthy volunteers before BCG vaccination, temporarily inhibited the induction of trained immunity several months later [236]. Taken together with the mixed effects of glycolysis on moDC maturation mentioned above, these data suggest that metformin treatment might not be a good adjunct to BCG vaccination.

In contrast to metformin, IFN- γ treatment also increased the rate of mitochondrial respiration in BCG-infected moDCs as well as increasing the glycolytic rate. A recombinant BCG vaccine that expressed IFN- γ was recently tested in mice and stimulated a better cell mediated immune response than the parent BCG strain, providing a better multifunctional Th1 T cell response to Mtb infection [233]. Maintaining a balance between glycolysis and OXPHOS may be more beneficial to the host than completely skewing metabolism towards glycolysis. While glycolysis might be helpful in the early stages of maturation and migration, maintaining metabolic flexibility may be important for DCs when they arrive in the lymph nodes and are competing for extracellular glucose with proliferating T cells [247]. Therefore, IFN- γ or another molecule that increases both glycolysis and mitochondrial respiration might be more successful at boosting the protective effects of BCG. The interplay between glycolysis and mitochondrial metabolism in DC maturation will need to be delineated fully before the results of this work could be translated into a treatment that benefits patients.

In fact, much remains to be understood about the link between cellular metabolism and DC activation in response to mycobacteria. Further investigation of BCG-infected DC maturation and function in the presence of modulators of metabolism will be needed to delineate the influence of these pathways. In order to address this issue, it would be necessary to determine the effect on T cell function of inhibiting glycolysis and OXPHOS in BCG-infected DC. In addition, it will also be important to assess the role of fatty acid metabolism in DC maturation and survival during infection with mycobacteria using pharmacological inhibitors, as alluded to above. It will also be vital to determine whether metformin and IFN- γ influence DC maturation in response to mycobacteria in order to begin to assess the feasibility of using approved drugs as adjuncts to the vaccine. Furthermore, there was evidence from the Nanostring analysis for alterations in expression of genes involved in glutamine/glutamate metabolism and the pentose phosphate pathway in moDC maturation. Much remains to be understood about the link between metabolic pathways and DC activation. To gain further insight into the metabolic networks involved in DC maturation the remainder of the Nanostring data generated for this project could also be mined and validated to determine the potential role of other metabolic genes in DC maturation.

Clearly, dendritic cells are an important entity needed for the adaptive immune response to Mtb through activation of T-cells and subsequent pathogen killing. *In vivo* experimental infections have shown that, when DCs infected with BCG *ex vivo* were administered intratracheally in mice, strong T-cell responses were recorded, along with higher production of IFN- γ , that later protected the mice against Mtb aerosol infection. Therefore, vaccination strategies using such DCs could be a step towards controlling TB in humans also, after proper clinical studies are carried out [83]. It is possible that DC vaccination could be considered to treat the most intractable of MDR-TB patients. However, it is likely that this approach would not be practical in most cases, especially in low income countries where TB incidence is at its highest and the healthcare infrastructure is poor. More likely, improving the existing vaccine or designing new vaccines that would target DCs *in vivo* would be a better option. Hence, this research into the metabolic events within DCs after BCG infection sheds new light on the host immune cells crosstalk with pathogens and may eventually aid in the rational design of new vaccines and immunization protocols.

CHAPTER 7:
References

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Appendix: Gene expression profile in moDCs

Table 1: Over expression genes for live BCG and killed BCG

Names	Total	Elements
Live BCG, Killed BCG	59	ENO3 GSK3A KRAS LDHA ENO1 BECN1 PPARGC1A PDGFRA AKT3 H6PD DLAT EIF4E2 SLC2A3 HIF1A RAC1 SCO2 OGDH BPGM ALDOC FOXO3 EIF2AK3 RPIA EGLN3 ENO2 TALDO1 SDHD SLC7A5 GYS1 HK2 VEGFA PFKM RAF1 NRAS GPI SLC7A11 PDK1 PGAM1 SIRT6 SLC2A1 MAP2K1 IKBKB EGF RICTOR MET GBE1 DLD PLD2 ACO2 ACO1 ODC1 TPI1 PGLS PKM OAZ2 SLC16A3 PGK1 CS ACLY FASN
Live BCG	21	PDGFA PLD1 TKT PDHA2 GLS TSC1 PRKAA1 PRKAB1 PRKAB2 PDGFC AKT2 DLST EIF2B4 NTRK3 MAP2K2 PIK3CA FLT3 SLC25A1 RPS6KB1 RBKS SLC44A4
Killed BCG	22	PFKL FGFR3 RPTOR ALDOA FH MLST8 PDHA1 MAPK1 CAD SLC1A5 TP53 PTEN IDH2 MDH2 G6PD PRPS1 mTOR JUN IDH3A IDH3G SUCLG1 RPS6KB2

Table 2: Over expression genes for live BCG, killed BCG and LPS

Names	Total	Elements
Live BCG, LPS, Killed BCG	58	ENO3 GSK3A KRAS LDHA ENO1 BECN1 PPARGC1A PDGFRA AKT3 H6PD DLAT EIF4E2 SLC2A3 HIF1A RAC1 SCO2 OGDH BPGM ALDOC FOXO3 EIF2AK3 RPIA EGLN3 ENO2 TALDO1 SDHD SLC7A5 GYS1 HK2 VEGFA RAF1 NRAS GPI SLC7A11 PDK1 PGAM1 SIRT6 SLC2A1 MAP2K1 IKBKB EGF RICTOR MET GBE1 DLD PLD2 ACO2 ACO1 ODC1 TPI1 PGLS PKM OAZ2 SLC16A3 PGK1 CS ACLY FASN
Live BCG, Killed BCG	1	PFKM
LPS, Live BCG	14	PDHA2 GLS PRKAA1 PRKAB1 PRKAB2 EIF2B4 NTRK3 MAP2K2 PIK3CA FLT3 SLC25A1 RPS6KB1 RBKS SLC44A4
Killed BCG, LPS	10	PFKL FGFR3 ALDOA SLC1A5 TP53 PTEN G6PD PRPS1 IDH3G RPS6KB2
Live BCG	7	PDGFA PLD1 TKT TSC1 PDGFC AKT2 DLST
Killed BCG	12	RPTOR FH MLST8 PDHA1 MAPK1 CAD IDH2 MDH2 mTOR JUN IDH3A SUCLG1
LPS	1	HRAS

Table 3: Down expression genes for live BCG and killed BCG

Names	Total	Elements
Live BCG, Killed BCG	52	ARNT PDPR PDP1 SDHC EGLN1 AKT1 PRKCB TIGAR EIF4EBP1 PDP2 PFKP G6PC3 PDK2 SUCLA2 SLC38A2 SUCLG2 IDH3B FBP1 EPAS1 SLC16A1 SHMT1 HRAS RAC2 NTRK1 PIK3CB IDH1 RET EGLN2 NOXA1 PLCG1 PRPS2 SDHA PIK3CD VHL RPE PDK3 MDH1 SP1 FGFR1 HK1 MYC HK3 LDHB PDHB SDHB MAPKAP1 MAPK3 GALM TSC2 ERBB2 SIRT3 OAZ3
Live BCG	22	PFKL FGFR3 RPTOR ALDOA FH MTOR MLST8 PDHA1 MAPK1 CAD SLC1A5 TP53 PTEN IDH2 MDH2 G6PD PRPS1 JUN IDH3A IDH3G SUCLG1 RPS6KB2
Killed BCG	21	PDGFA PLD1 TKT PDHA2 GLS TSC1 PRKAA1 PRKAB1 PRKAB2 PDGFC AKT2 DLST EIF2B4 NTRK3 PIK3CA MAP2K2 FLT3 SLC25A1 RPS6KB1 SLC44A4 RBKS

Table 4: Down expression genes for live BCG, killed BCG and LPS

Names	Total	Elements
Live BCG, LPS, Killed BCG	51	ARNT PDPR PDP1 SDHC EGLN1 AKT1 PRKCB TIGAR EIF4EBP1 PDP2 PFKP G6PC3 PDK2 SUCLA2 SLC38A2 SUCLG2 IDH3B FBP1 EPAS1 SLC16A1 SHMT1 RAC2 NTRK1 PIK3CB IDH1 RET EGLN2 NOXA1 PLCG1 PRPS2 SDHA PIK3CD VHL RPE PDK3 MDH1 SP1 FGFR1 HK1 MYC HK3 LDHB PDHB SDHB MAPKAP1 MAPK3 GALM TSC2 ERBB2 SIRT3 OAZ3
Live BCG, Killed BCG	1	HRAS
LPS, Live BCG	12	RPTOR FH MTOR MLST8 PDHA1 MAPK1 CAD IDH2 MDH2 JUN IDH3A SUCLG1
Killed BCG, LPS	7	PDGFA PLD1 TKT TSC1 PDGFC AKT2 DLST
Live BCG	10	PFKL FGFR3 ALDOA SLC1A5 TP53 PTEN G6PD PRPS1 IDH3G RPS6KB2
Killed BCG	14	PDHA2 GLS PRKAA1 PRKAB1 PRKAB2 EIF2B4 NTRK3 PIK3CA MAP2K2 FLT3 SLC25A1 RPS6KB1 SLC44A4 RBKS
LPS	1	PFKM

Table 5: List of all genes that are significantly over or down expressed in moDCs after infection with live BCG. Fold changes of mRNA counts between live BCG infected moDCs and uninfected moDCs samples using the nanoString nCounter. Results are shown for mRNAs which showed a significant difference.

Gene	Fold change	Expression	P-value	Gene.sets					
MET	51.2	up	0.00339	Carbon Metabolism					
SLC7A11	9.4	up	0.000666	Carbon Metabolism					
VEGFA	4.6	up	0.00477	MTOR Pathway					
FASN	4	up	0.000201	Other Metabolic Pathway					
H6PD	3.3	up	0.00938	Glucose Metabolism					
AKT3	3.2	up	0.000505	Carbon Metabolism, Choline Metabolism, MTOR Pathway					
PLD2	2.7	up	7.39E-06	Choline Metabolism					
PFKM	2.7	up	0.0158	Carbon Metabolism, KEGG Glycolysis					
ENO3	2.63	up	0.0701	Glucose Metabolism, KEGG Glycolysis					
FLT3	2.55	up	0.29	Carbon Metabolism					
FOXO3	2.5	up	0.000519	Other Metabolic Pathway					
PDGFA	2.4	up	0.00687	Carbon Metabolism, Choline Metabolism					
PDGFRA	2.4	up	0.0182	Carbon Metabolism, Choline Metabolism					
PKD1	2.3	up	0.00166	Carbon Metabolism, Glucose Metabolism, Hypoxia Signaling					
GYS1	2.2	up	0.000426	Glucose Metabolism					
ENO2	2.17	up	0.0803	Glucose Metabolism, KEGG Glycolysis					
RAC1	2	up	4.73E-05	Carbon Metabolism, Choline Metabolism					
ALDOC	1.9	up	0.000574	Glucose Metabolism, KEGG Glycolysis					
EIF2AK3	1.9	up	0.00123	Carbon Metabolism, Hypoxia Signaling					
SLC25A1	1.9	up	0.0109	Carbon Metabolism					
HIF1A	1.9	up	0.0136	Cancer Metabolism Drivers, , Choline Metabolism, Hypoxia Signaling,					
RBKS	1.86	up	0.05	Glucose Metabolism					
ENO1	1.8	up	0.000466	Carbon Metabolism, Glucose Metabolism, KEGG Glycolysis					
PIK3CA	1.8	up	0.000597	Carbon Metabolism, Choline Metabolism, MTOR Pathway					
PRKAB1	1.64	up	0.0539	Other Metabolic Pathway					
SLC7A5	1.64	up	0.27	Carbon Metabolism					
OGDH	1.6	up	0.000817	Glucose Metabolism					
SDHD	1.6	up	0.00131	Glucose Metabolism					
RAF1	1.6	up	0.0016	Carbon Metabolism, Choline Metabolism					
PRKAA1	1.6	up	0.00169	MTOR Pathway					
GLS	1.6	up	0.00259	Carbon Metabolism					
MAP2K1	1.6	up	0.00428	Carbon Metabolism, Choline Metabolism					
GBE1	1.58	up	0.163	Glucose Metabolism					
SLC44A4	1.57	up	0.198	Carbon Metabolism, Choline Metabolism					
KRAS	1.5	up	0.00277	Carbon Metabolism, Choline Metabolism					
EIF4E2	1.5	up	0.00298	Carbon Metabolism, Hypoxia Signaling, MTOR Pathway					
DLAT	1.5	up	0.00308	Glucose Metabolism, KEGG Glycolysis					
SCO2	1.5	up	0.00413	Other Metabolic Pathway					
NRAS	1.5	up	0.0131	Carbon Metabolism, Choline Metabolism					
LDHA	1.5	up	0.0283	Hypoxia Signaling, KEGG Glycolysis					
ACLY	1.5	up	0.0327	Glucose Metabolism					
ACO1	1.5	up	0.0458	Glucose Metabolism					
SLC2A1	1.49	up	0.187	Carbon Metabolism					
PDGFC	1.34	up	0.163	Carbon Metabolism, Choline Metabolism					

ODC1	1.34 up	0.182	Other Metabolic Pathway			
PGK1	1.33 up	0.0984	Carbon Metabolism, Glucose Metabolism, KEGG Glycolysis			
PGAM1	1.32 up	0.1	Carbon Metabolism, KEGG Glycolysis			
RICTOR	1.31 up	0.1	MTOR Pathway			
SLC16A3	1.28 up	0.204	Carbon Metabolism			
BPGM	1.24 up	0.17	Glucose Metabolism, KEGG Glycolysis			
NTRK3	1.24 up	0.583	Carbon Metabolism			
HK2	1.23 up	0.609	Carbon Metabolism, Glucose Metabolism, KEGG Glycolysis			
TPI1	1.22 up	0.0847	Glucose Metabolism, KEGG Glycolysis			
BECN1	1.2 up	0.0258	Hypoxia Signaling			
DLD	1.2 up	0.0404	Glucose Metabolism, KEGG Glycolysis			
GSK3A	1.2 up	0.0431	Glucose Metabolism			
PRKAB2	1.17 up	0.162	Other Metabolic Pathway			
IKBKB	1.16 up	0.339	MTOR Pathway			
SLC2A3	1.13 up	0.729	Other Metabolic Pathway			
CS	1.12 up	0.0533	Glucose Metabolism			
DLST	1.12 up	0.201	Glucose Metabolism			
EGF	1.12 up	0.82	Carbon Metabolism, Choline Metabolism			
TALDO1	1.11 up	0.602	Glucose Metabolism			
EGLN3	1.1 up	0.63	Carbon Metabolism, Hypoxia Signaling			
OAZ2	1.1 up	0.709	Other Metabolic Pathway			
EIF2B4	1.09 up	0.448	Hypoxia Signaling			
PGLS	1.07 up	0.161	Glucose Metabolism			
RPIA	1.06 up	0.703	Glucose Metabolism			
TKT	1.06 up	0.715	Glucose Metabolism			
PPARGC1A	1.06 up	0.906	Other Metabolic Pathway			
RPS6KB1	1.04 up	0.401	Carbon Metabolism, Choline Metabolism, MTOR Pathway			
GPI	1.04 up	0.634	Glucose Metabolism, KEGG Glycolysis			
SIRT6	1.03 up	0.756	Carbon Metabolism			
MAP2K2	1.03 up	0.788	Carbon Metabolism, Choline Metabolism			
TSC1	1.03 up	0.79	Choline Metabolism, MTOR Pathway			
AKT2	1.02 up	0.734	Carbon Metabolism, Choline Metabolism, MTOR Pathway			
PDHA2	1.02 up	0.948	Carbon Metabolism, KEGG Glycolysis			
ACO2	1.01 up	0.887	Glucose Metabolism			
PKM	1.01 up	0.942	Carbon Metabolism			
PLD1	1.01 up	0.974	Choline Metabolism			
EGLN2	0.99 down	0.914	Carbon Metabolism, Hypoxia Signaling			
PDPR	0.979 down	0.92	Glucose Metabolism			
MTOR	0.975 down	0.785	Carbon Metabolism, Choline Metabolism, MTOR Pathway			
MLST8	0.963 down	0.585	MTOR Pathway			
CAD	0.955 down	0.471	Other Metabolic Pathway			
MAPKAP1	0.95 down	0.604	MTOR Pathway			
PFKL	0.942 down	0.75	Carbon Metabolism, Glucose Metabolism, KEGG Glycolysis			
PDHA1	0.937 down	0.519	Carbon Metabolism, Glucose Metabolism, KEGG Glycolysis			
RPTOR	0.934 down	0.57	MTOR Pathway			
RPS6KB2	0.931 down	0.353	Carbon Metabolism, Choline Metabolism, MTOR Pathway			
VHL	0.93 down	0.312	Hypoxia Signaling			
ARNT	0.93 down	0.321	Hypoxia Signaling			
IDH3G	0.926 down	0.299	Glucose Metabolism			
HRAS	0.919 down	0.491	Carbon Metabolism, Choline Metabolism			
SP1	0.91 down	0.446	Carbon Metabolism, Choline Metabolism			

G6PD	0.907	down	0.497	Carbon Metabolism, Glucose Metabolism				
PTEN	0.9	down	0.0146	Carbon Metabolism				
SIRT3	0.883	down	0.423	Carbon Metabolism				
SDHB	0.867	down	0.281	Glucose Metabolism, Hypoxia Signaling				
MDH1	0.861	down	0.299	Glucose Metabolism				
MAPK3	0.857	down	0.123	Carbon Metabolism, Choline Metabolism, MTOR Pathway				
FGFR3	0.846	down	0.789	Carbon Metabolism				
PIK3CB	0.836	down	0.19	Carbon Metabolism, Choline Metabolism, MTOR Pathway				
ALDOA	0.835	down	0.177	Carbon Metabolism, Glucose Metabolism, KEGG Glycolysis				
SUCLA2	0.808	down	0.0629	Glucose Metabolism				
MDH2	0.8	down	0.00156	Glucose Metabolism				
IDH3B	0.8	down	0.0452	Glucose Metabolism				
TSC2	0.8	down	0.0589	Choline Metabolism, MTOR Pathway				
SDHA	0.78	down	0.0588	Glucose Metabolism				
JUN	0.765	down	0.283	Cancer Metabolism Drivers, Carbon Metabolism, Choline Metabolism				
ERBB2	0.72	down	0.28	Carbon Metabolism				
MAPK1	0.7	down	0.00286	Carbon Metabolism, Choline Metabolism, MTOR Pathway				
SUCLG1	0.7	down	0.0043	Glucose Metabolism				
RPE	0.7	down	0.00675	Glucose Metabolism				
SUCLG2	0.7	down	0.00705	Glucose Metabolism				
AKT1	0.7	down	0.00963	Carbon Metabolism, Choline Metabolism, MTOR Pathway				
G6PC3	0.7	down	0.0102	Glucose Metabolism				
TP53	0.7	down	0.0225	Cancer Metabolism Drivers, Carbon Metabolism				
EGLN1	0.7	down	0.0242	Hypoxia Signaling				
SDHC	0.7	down	0.0251	Glucose Metabolism				
PFKP	0.7	down	0.0397	Carbon Metabolism, KEGG Glycolysis				
LDHB	0.696	down	0.109	Hypoxia Signaling, KEGG Glycolysis				
NOXA1	0.64	down	0.523	Hypoxia Signaling				
PLCG1	0.634	down	0.294	Carbon Metabolism, Choline Metabolism				
PDHB	0.6	down	0.00169	Carbon Metabolism, Glucose Metabolism, KEGG Glycolysis				
FH	0.6	down	0.00206	Glucose Metabolism, Hypoxia Signaling				
HK1	0.6	down	0.0169	Carbon Metabolism, KEGG Glycolysis				
PRPS1	0.6	down	0.0184	Glucose Metabolism				
SLC38A2	0.6	down	0.0266	Carbon Metabolism				
PDP2	0.535	down	0.194	Glucose Metabolism				
IDH2	0.5	down	0.00145	Cancer Metabolism Drivers, Glucose Metabolism				
PRPS2	0.5	down	0.0298	Glucose Metabolism				
NTRK1	0.488	down	0.229	Carbon Metabolism				
RET	0.47	down	0.0537	Carbon Metabolism				
FBP1	0.403	down	0.133	Glucose Metabolism, KEGG Glycolysis				
IDH3A	0.4	down	0.000781	Glucose Metabolism				
PDK3	0.4	down	0.00136	Glucose Metabolism				
PIK3CD	0.4	down	0.00318	Carbon Metabolism, Choline Metabolism, MTOR Pathway				
TIGAR	0.4	down	0.00358	Other Metabolic Pathway				
HK3	0.4	down	0.016	Carbon Metabolism, Glucose Metabolism, KEGG Glycolysis				
EIF4EBP1	0.3	down	1.27E-05	Carbon Metabolism, Choline Metabolism, Hypoxia Signaling, mTOR Pathway				
EPAS1	0.3	down	1.41E-05	Cancer Metabolism Drivers				
SLC16A1	0.3	down	0.000216	Carbon Metabolism				
RAC2	0.3	down	0.000823	Carbon Metabolism, Choline Metabolism				
OAZ3	0.3	down	0.00169	Other Metabolic Pathway				
IDH1	0.3	down	0.00174	Cancer Metabolism Drivers, Glucose Metabolism				

SLC1A5	0.3	down	0.00369	Carbon Metabolism			
MYC	0.3	down	0.00567	Cancer Metabolism Drivers, Carbon Metabolism			
FGFR1	0.3	down	0.009	Carbon Metabolism			
PRKCB	0.3	down	0.0248	Carbon Metabolism, Choline Metabolism			
GALM	0.2	down	0.000149	Glucose Metabolism, KEGG Glycolysis			
PDP1	0.2	down	0.000312	Hypoxia Signaling			
SHMT1	0.2	down	0.000405	Other Metabolic Pathway			
PDK2	0.2	down	0.00287	Glucose Metabolism			

Table 6: List of all genes that are significantly over or down expressed in moDCs after infection with killed BCG. Fold changes of mRNA counts between killed BCG infected moDCs and uninfected moDCs samples using the nanoString nCounter. Results are shown for mRNAs which showed a significant difference.

Gene	Fold change	P-value	Gene.sets				
MET	7.52 up	0.071	Carbon Metabolism				
SLC7A11	6.2 up	0.002	Carbon Metabolism				
SLC7A5	2.33 up	0.079	Carbon Metabolism				
VEGFA	2.16 up	0.087	MTOR Pathway				
EGF	2.13 up	0.15	Carbon Metabolism, Choline Metabolism				
EGLN3	2.1 up	0.005	Carbon Metabolism, Hypoxia Signaling				
SLC2A1	2 up	0.039	Carbon Metabolism				
PLD2	1.7 up	0.001	Choline Metabolism				
PDGFRA	1.63 up	0.136	Carbon Metabolism, Choline Metabolism				
SLC2A3	1.62 up	0.203	Other Metabolic Pathway				
PPARGC1A	1.56 up	0.379	Other Metabolic Pathway				
ENO2	1.48 up	0.338	Glucose Metabolism, KEGG Glycolysis				
SLC1A5	1.45 up	0.22	Carbon Metabolism				
PDK1	1.42 up	0.089	Carbon Metabolism, Glucose Metabolism, Hypoxia Signaling				
ACO1	1.38 up	0.108	Glucose Metabolism				
PRPS1	1.38 up	0.144	Glucose Metabolism				
LDHA	1.36 up	0.064	Hypoxia Signaling, KEGG Glycolysis				
HK2	1.35 up	0.466	Carbon Metabolism, Glucose Metabolism, KEGG Glycolysis				
SLC16A3	1.34 up	0.138	Carbon Metabolism				
AKT3	1.33 up	0.211	Carbon Metabolism, Choline Metabolism, MTOR Pathway				
ENO1	1.3 up	0.022	Carbon Metabolism, Glucose Metabolism, KEGG Glycolysis				
SDHD	1.3 up	0.025	Glucose Metabolism				
CS	1.3 up	0.001	Glucose Metabolism				
ODC1	1.3 up	0.223	Other Metabolic Pathway				
ENO3	1.3 up	0.588	Glucose Metabolism, KEGG Glycolysis				
ACLY	1.28 up	0.158	Glucose Metabolism				
G6PD	1.27 up	0.116	Carbon Metabolism, Glucose Metabolism				
PGK1	1.27 up	0.164	Carbon Metabolism, Glucose Metabolism, KEGG Glycolysis				
RPIA	1.26 up	0.157	Glucose Metabolism				
TALDO1	1.24 up	0.273	Glucose Metabolism				
EIF2AK3	1.22 up	0.18	Carbon Metabolism, Hypoxia Signaling				
GYS1	1.22 up	0.192	Glucose Metabolism				
ALDOC	1.21 up	0.143	Glucose Metabolism, KEGG Glycolysis				
BPGM	1.19 up	0.268	Glucose Metabolism, KEGG Glycolysis				
ACO2	1.18 up	0.08	Glucose Metabolism				
OAZ2	1.17 up	0.538	Other Metabolic Pathway				
RAC1	1.16 up	0.134	Carbon Metabolism, Choline Metabolism				
SCO2	1.16 up	0.17	Other Metabolic Pathway				
TP53	1.16 up	0.2	Cancer Metabolism Drivers, Carbon Metabolism				
MAP2K1	1.16 up	0.26	Carbon Metabolism, Choline Metabolism				
PKM	1.15 up	0.173	Carbon Metabolism				
DLAT	1.15 up	0.199	Glucose Metabolism, KEGG Glycolysis				
RICTOR	1.15 up	0.38	MTOR Pathway				
H6PD	1.15 up	0.69	Glucose Metabolism				

GPI	1.14	up	0.163	Glucose Metabolism, KEGG Glycolysis		
EIF4E2	1.14	up	0.22	Carbon Metabolism, Hypoxia Signaling, MTOR Pathway		
TP11	1.14	up	0.225	Glucose Metabolism, KEGG Glycolysis		
NRAS	1.14	up	0.305	Carbon Metabolism, Choline Metabolism		
HIF1A	1.13	up	0.574	Cancer Metabolism Drivers, Carbon Metabolism, Choline Metabolism		
GBE1	1.13	up	0.691	Glucose Metabolism		
KRAS	1.11	up	0.307	Carbon Metabolism, Choline Metabolism		
FASN	1.11	up	0.632	Other Metabolic Pathway		
DLD	1.1	up	0.297	Glucose Metabolism, KEGG Glycolysis		
PDHA1	1.1	up	0.371	Carbon Metabolism, Glucose Metabolism, KEGG Glycolysis		
ALDOA	1.09	up	0.486	Carbon Metabolism, Glucose Metabolism, KEGG Glycolysis		
JUN	1.09	up	0.735	Cancer Metabolism Drivers, Carbon Metabolism, Choline Metabolism		
RPS6KB2	1.07	up	0.365	Carbon Metabolism, Choline Metabolism, MTOR Pathway		
GSK3A	1.07	up	0.411	Glucose Metabolism		
RPTOR	1.07	up	0.575	MTOR Pathway		
PFKL	1.07	up	0.708	Carbon Metabolism, Glucose Metabolism, KEGG Glycolysis		
OGDH	1.06	up	0.548	Glucose Metabolism		
PTEN	1.05	up	0.296	Carbon Metabolism		
MDH2	1.05	up	0.413	Glucose Metabolism		
IDH3G	1.05	up	0.472	Glucose Metabolism		
PGAM1	1.04	up	0.789	Carbon Metabolism, KEGG Glycolysis		
IDH2	1.04	up	0.794	Cancer Metabolism Drivers, Glucose Metabolism		
IDH3A	1.04	up	0.841	Glucose Metabolism		
PFKM	1.04	up	0.915	Carbon Metabolism, KEGG Glycolysis		
FGFR3	1.04	up	0.951	Carbon Metabolism		
RAF1	1.03	up	0.764	Carbon Metabolism, Choline Metabolism		
FH	1.03	up	0.775	Glucose Metabolism, Hypoxia Signaling		
FOXO3	1.03	up	0.847	Other Metabolic Pathway		
CAD	1.02	up	0.725	Other Metabolic Pathway		
MLST8	1.02	up	0.784	MTOR Pathway		
MAPK1	1.02	up	0.859	Carbon Metabolism, Choline Metabolism, MTOR Pathway		
IKBKB	1.02	up	0.871	MTOR Pathway		
PGLS	1.01	up	0.912	Glucose Metabolism		
BECN1	1.01	up	0.928	Hypoxia Signaling		
mTOR	1	up	0.975	Carbon Metabolism, Choline Metabolism, MTOR Pathway		
SIRT6	1	up	0.986	Carbon Metabolism		
SUCLG1	1	up	0.993	Glucose Metabolism		
RPE	0.99	down	0.923	Glucose Metabolism		
EIF2B4	0.99	down	0.944	Hypoxia Signaling		
GLS	0.99	down	0.95	Carbon Metabolism		
GALM	0.99	down	0.959	Glucose Metabolism, KEGG Glycolysis		
MAP2K2	0.99	down	0.96	Carbon Metabolism, Choline Metabolism		
SDHB	0.98	down	0.892	Glucose Metabolism, Hypoxia Signaling		
PFKP	0.98	down	0.914	Carbon Metabolism, KEGG Glycolysis		
DLST	0.97	down	0.708	Glucose Metabolism		
PDHA2	0.97	down	0.895	Carbon Metabolism, KEGG Glycolysis		
IDH3B	0.96	down	0.647	Glucose Metabolism		
EGLN1	0.96	down	0.709	Hypoxia Signaling		
PIK3CA	0.96	down	0.711	Carbon Metabolism, Choline Metabolism, MTOR Pathway		
TKT	0.96	down	0.806	Glucose Metabolism		
RPS6KB1	0.95	down	0.34	Carbon Metabolism, Choline Metabolism, MTOR Pathway		

SP1	0.95	down	0.69	Carbon Metabolism, Choline Metabolism				
PIK3CB	0.95	down	0.72	Carbon Metabolism, Choline Metabolism, MTOR Pathway				
AKT2	0.94	down	0.366	Carbon Metabolism, Choline Metabolism, MTOR Pathway				
MAPKAP1	0.94	down	0.533	MTOR Pathway				
SUCLA2	0.94	down	0.58	Glucose Metabolism				
PRKAB1	0.94	down	0.79	Other Metabolic Pathway				
VHL	0.93	down	0.311	Hypoxia Signaling				
HRAS	0.93	down	0.539	Carbon Metabolism, Choline Metabolism				
TSC1	0.93	down	0.549	Choline Metabolism, MTOR Pathway				
SLC25A1	0.93	down	0.714	Carbon Metabolism				
TSC2	0.92	down	0.45	Choline Metabolism, MTOR Pathway				
FLT3	0.92	down	0.923	Carbon Metabolism				
SDHA	0.91	down	0.394	Glucose Metabolism				
FBP1	0.91	down	0.861	Glucose Metabolism, KEGG Glycolysis				
G6PC3	0.9	down	0.397	Glucose Metabolism				
TIGAR	0.9	down	0.59	Other Metabolic Pathway				
EGLN2	0.89	down	0.258	Carbon Metabolism, Hypoxia Signaling				
PRKAB2	0.89	down	0.306	Other Metabolic Pathway				
PRKAA1	0.87	down	0.212	MTOR Pathway				
PIK3CD	0.87	down	0.488	Carbon Metabolism, Choline Metabolism, MTOR Pathway				
LDHB	0.87	down	0.508	Hypoxia Signaling, KEGG Glycolysis				
RBKS	0.87	down	0.619	Glucose Metabolism				
SDHC	0.86	down	0.32	Glucose Metabolism				
AKT1	0.85	down	0.17	Carbon Metabolism, Choline Metabolism, MTOR Pathway				
HK1	0.85	down	0.307	Carbon Metabolism, KEGG Glycolysis				
SLC38A2	0.85	down	0.396	Carbon Metabolism				
PDGFC	0.85	down	0.427	Carbon Metabolism, Choline Metabolism				
NTRK3	0.85	down	0.689	Carbon Metabolism				
NOXA1	0.85	down	0.818	Hypoxia Signaling				
MDH1	0.84	down	0.227	Glucose Metabolism				
SLC16A1	0.83	down	0.347	Carbon Metabolism				
PDPR	0.83	down	0.399	Glucose Metabolism				
SLC44A4	0.83	down	0.614	Carbon Metabolism, Choline Metabolism				
EIF4EBP1	0.82	down	0.179	Carbon Metabolism, Choline Metabolism, Hypoxia Signaling, mTOR Pathway				
PDHB	0.81	down	0.087	Carbon Metabolism, Glucose Metabolism, KEGG Glycolysis				
MAPK3	0.8	down	0.039	Carbon Metabolism, Choline Metabolism, MTOR Pathway				
PRKCB	0.79	down	0.581	Carbon Metabolism, Choline Metabolism				
RAC2	0.76	down	0.219	Carbon Metabolism, Choline Metabolism				
PRPS2	0.75	down	0.267	Glucose Metabolism				
SIRT3	0.74	down	0.088	Carbon Metabolism				
PDGFA	0.73	down	0.257	Carbon Metabolism, Choline Metabolism				
FGFR1	0.73	down	0.356	Carbon Metabolism				
MYC	0.73	down	0.381	Cancer Metabolism Drivers, Carbon Metabolism				
PDP2	0.73	down	0.501	Glucose Metabolism				
ERBB2	0.71	down	0.259	Carbon Metabolism				
ARNT	0.7	down	0.002	Hypoxia Signaling				
SUCLG2	0.7	down	0.08	Glucose Metabolism				
EPAS1	0.7	down	0.007	Cancer Metabolism Drivers				
HK3	0.7	down	0.288	Carbon Metabolism, Glucose Metabolism, KEGG Glycolysis				
PLCG1	0.67	down	0.356	Carbon Metabolism, Choline Metabolism				
IDH1	0.62	down	0.1	Cancer Metabolism Drivers, Glucose Metabolism				

IDH1	0.62	down	0.1	Cancer Metabolism Drivers, Glucose Metabolism			
PDP1	0.61	down	0.098	Hypoxia Signaling			
PDK3	0.6	down	0.012	Glucose Metabolism			
RET	0.6	down	0.155	Carbon Metabolism			
OAZ3	0.59	down	0.092	Other Metabolic Pathway			
PDK2	0.59	down	0.142	Glucose Metabolism			
PLD1	0.57	down	0.203	Choline Metabolism			
SHMT1	0.4	down	0.005	Other Metabolic Pathway			
NTRK1	0.35	down	0.096	Carbon Metabolism			

Table 7: List of all genes that are significantly over or down expressed in moDCs after treated with LPS. Fold changes of mRNA counts between LPS treated moDCs and uninfected moDCs samples using the nanoString nCounter. Results are shown for mRNAs which showed a significant difference.

Gene	Fold change	Expression	P-value	Gene.sets					
MET	16.2	up	0.02	Carbon Metabolism					
SLC7A11	6.4	up	0.00214	Carbon Metabolism					
EGF	3.9	up	0.0174	Carbon Metabolism, Choline Metabolism					
PPARGC1A	3.1	up	0.0377	Other Metabolic Pathway					
PDGFRA	2.7	up	0.00927	Carbon Metabolism, Choline Metabolism					
NTRK3	2.5	up	0.0334	Carbon Metabolism					
PLD2	2.4	up	2.08E-05	Choline Metabolism					
VEGFA	2.3	up	0.0669	MTOR Pathway					
AKT3	2.1	up	0.00709	Carbon Metabolism, Choline Metabolism, MTOR Pathway					
EGLN3	1.8	up	0.0163	Carbon Metabolism, Hypoxia Signaling					
SLC7A5	1.8	up	0.184	Carbon Metabolism					
PDHA2	1.7	up	0.0464	Carbon Metabolism, KEGG Glycolysis					
PDK1	1.6	up	0.0391	Carbon Metabolism, Glucose Metabolism, Hypoxia Signaling					
H6PD	1.6	up	0.244	Glucose Metabolism					
ALDOC	1.4	up	0.016	Glucose Metabolism, KEGG Glycolysis					
SDHD	1.4	up	0.009	Glucose Metabolism					
LDHA	1.4	up	0.0468	Hypoxia Signaling, KEGG Glycolysis					
ENO1	1.4	up	0.0186	Carbon Metabolism, Glucose Metabolism, KEGG Glycolysis					
FOXO3	1.4	up	0.109	Other Metabolic Pathway					
FASN	1.4	up	0.13	Other Metabolic Pathway					
SLC2A1	1.4	up	0.243	Carbon Metabolism					
ENO2	1.4	up	0.415	Glucose Metabolism, KEGG Glycolysis					
FGFR3	1.4	up	0.628	Carbon Metabolism					
EIF4E2	1.3	up	0.0322	Carbon Metabolism, Hypoxia Signaling, MTOR Pathway					
GYS1	1.3	up	0.0769	Glucose Metabolism					
MAP2K1	1.3	up	0.0829	Carbon Metabolism, Choline Metabolism					
ACLY	1.3	up	0.105	Glucose Metabolism					
ACO1	1.3	up	0.146	Glucose Metabolism					
PGK1	1.3	up	0.185	Carbon Metabolism, Glucose Metabolism, KEGG Glycolysis					
SLC16A3	1.3	up	0.247	Carbon Metabolism					
HIF1A	1.3	up	0.289	Cancer Metabolism Drivers, Carbon Metabolism, Choline Metabolism, Hypoxia					
RBKS	1.3	up	0.367	Glucose Metabolism					
GBE1	1.3	up	0.446	Glucose Metabolism					
ENO3	1.3	up	0.632	Glucose Metabolism, KEGG Glycolysis					
CS	1.2	up	0.0145	Glucose Metabolism					
RAC1	1.2	up	0.0517	Carbon Metabolism, Choline Metabolism					
SCO2	1.2	up	0.07	Other Metabolic Pathway					
RAF1	1.2	up	0.187	Carbon Metabolism, Choline Metabolism					
BPGM	1.2	up	0.271	Glucose Metabolism, KEGG Glycolysis					
RICTOR	1.2	up	0.276	MTOR Pathway					
SLC44A4	1.2	up	0.541	Carbon Metabolism, Choline Metabolism					
SLC2A3	1.2	up	0.551	Other Metabolic Pathway					
SLC1A5	1.2	up	0.594	Carbon Metabolism					
HK2	1.2	up	0.64	Carbon Metabolism, Glucose Metabolism, KEGG Glycolysis					

DLAT	1.1 up	0.295	Glucose Metabolism, KEGG Glycolysis		
KRAS	1.1 up	0.318	Carbon Metabolism, Choline Metabolism		
GSK3A	1.1 up	0.345	Glucose Metabolism		
SIRT6	1.1 up	0.345	Carbon Metabolism		
NRAS	1.1 up	0.348	Carbon Metabolism, Choline Metabolism		
IDH3G	1.1 up	0.362	Glucose Metabolism		
PKM	1.1 up	0.385	Carbon Metabolism		
RPIA	1.1 up	0.426	Glucose Metabolism		
DLD	1.1 up	0.441	Glucose Metabolism, KEGG Glycolysis		
TP11	1.1 up	0.441	Glucose Metabolism, KEGG Glycolysis		
EIF2AK3	1.1 up	0.464	Carbon Metabolism, Hypoxia Signaling		
TALDO1	1.1 up	0.534	Glucose Metabolism		
PRPS1	1.1 up	0.589	Glucose Metabolism		
OAZ2	1.1 up	0.628	Other Metabolic Pathway		
PIK3CA	1.1 up	0.641	Carbon Metabolism, Choline Metabolism, MTOR Pathway		
PRKAA1	1.1 up	0.642	MTOR Pathway		
PRKAB1	1.1 up	0.662	Other Metabolic Pathway		
G6PD	1.1 up	0.739	Carbon Metabolism, Glucose Metabolism		
IKBKB	1.1 up	0.759	MTOR Pathway		
SLC25A1	1.1 up	0.77	Carbon Metabolism		
PTEN	1 up	0.297	Carbon Metabolism		
RPS6KB1	1 up	0.464	Carbon Metabolism, Choline Metabolism, MTOR Pathway		
PRKAB2	1 up	0.644	Other Metabolic Pathway		
PGLS	1 up	0.714	Glucose Metabolism		
GLS	1 up	0.717	Carbon Metabolism		
ALDOA	1 up	0.801	Carbon Metabolism, Glucose Metabolism, KEGG Glycolysis		
HRAS	1 up	0.817	Carbon Metabolism, Choline Metabolism		
MAP2K2	1 up	0.831	Carbon Metabolism, Choline Metabolism		
GPI	1 up	0.852	Glucose Metabolism, KEGG Glycolysis		
BECN1	1 up	0.868	Hypoxia Signaling		
TP53	1 up	0.901	Cancer Metabolism Drivers, Carbon Metabolism		
RPS6KB2	1 up	0.901	Carbon Metabolism, Choline Metabolism, MTOR Pathway		
OGDH	1 up	0.907	Glucose Metabolism		
PFKL	1 up	0.924	Carbon Metabolism, Glucose Metabolism, KEGG Glycolysis		
ACO2	1 up	0.942	Glucose Metabolism		
FLT3	1 up	0.973	Carbon Metabolism		
EIF2B4	1 up	0.977	Hypoxia Signaling		
ODC1	1 up	0.979	Other Metabolic Pathway		
PGAM1	1 up	0.989	Carbon Metabolism, KEGG Glycolysis		
MLST8	0.9 down	0.085	MTOR Pathway		
SUCLG1	0.9 down	0.0917	Glucose Metabolism		
IDH3B	0.9 down	0.152	Glucose Metabolism		
AKT2	0.9 down	0.155	Carbon Metabolism, Choline Metabolism, MTOR Pathway		
RPTOR	0.9 down	0.209	MTOR Pathway		
SDHA	0.9 down	0.211	Glucose Metabolism		
EGLN2	0.9 down	0.253	Carbon Metabolism, Hypoxia Signaling		
MDH2	0.9 down	0.262	Glucose Metabolism		
EGLN1	0.9 down	0.265	Hypoxia Signaling		
VHL	0.9 down	0.286	Hypoxia Signaling		
PIK3CB	0.9 down	0.318	Carbon Metabolism, Choline Metabolism, MTOR Pathway		
PDHA1	0.9 down	0.358	Carbon Metabolism, Glucose Metabolism, KEGG Glycolysis		

SDHB	0.9	down	0.446	Glucose Metabolism, Hypoxia Signaling			
DLST	0.9	down	0.471	Glucose Metabolism			
ERBB2	0.9	down	0.601	Carbon Metabolism			
JUN	0.9	down	0.682	Cancer Metabolism Drivers, Carbon Metabolism, Choline Metabolism			
PFKM	0.9	down	0.691	Carbon Metabolism, KEGG Glycolysis			
CAD	0.8	down	0.0236	Other Metabolic Pathway			
RPE	0.8	down	0.0318	Glucose Metabolism			
G6PC3	0.8	down	0.0406	Glucose Metabolism			
ARNT	0.8	down	0.00306	Hypoxia Signaling			
FH	0.8	down	0.051	Glucose Metabolism, Hypoxia Signaling			
MAPK1	0.8	down	0.052	Carbon Metabolism, Choline Metabolism, MTOR Pathway			
TSC1	0.8	down	0.0565	Choline Metabolism, MTOR Pathway			
MAPKAP1	0.8	down	0.0778	MTOR Pathway			
SP1	0.8	down	0.0799	Carbon Metabolism, Choline Metabolism			
SUCLA2	0.8	down	0.0909	Glucose Metabolism			
MTOR	0.8	down	0.0989	Carbon Metabolism, Choline Metabolism, MTOR Pathway			
TSC2	0.8	down	0.124	Choline Metabolism, MTOR Pathway			
SIRT3	0.8	down	0.183	Carbon Metabolism			
TKT	0.8	down	0.192	Glucose Metabolism			
IDH2	0.8	down	0.267	Cancer Metabolism Drivers, Glucose Metabolism			
LDHB	0.8	down	0.28	Hypoxia Signaling, KEGG Glycolysis			
PDGFA	0.8	down	0.518	Carbon Metabolism, Choline Metabolism			
MAPK3	0.7	down	0.00649	Carbon Metabolism, Choline Metabolism, MTOR Pathway			
AKT1	0.7	down	0.0125	Carbon Metabolism, Choline Metabolism, MTOR Pathway			
HK1	0.7	down	0.0485	Carbon Metabolism, KEGG Glycolysis			
PDHB	0.7	down	0.0132	Carbon Metabolism, Glucose Metabolism, KEGG Glycolysis			
MDH1	0.7	down	0.0246	Glucose Metabolism			
SDHC	0.7	down	0.0222	Glucose Metabolism			
SUCLG2	0.7	down	0.00815	Glucose Metabolism			
PIK3CD	0.7	down	0.063	Carbon Metabolism, Choline Metabolism, MTOR Pathway			
PDPR	0.7	down	0.0778	Glucose Metabolism			
IDH3A	0.7	down	0.0849	Glucose Metabolism			
PFKP	0.7	down	0.102	Carbon Metabolism, KEGG Glycolysis			
RAC2	0.7	down	0.115	Carbon Metabolism, Choline Metabolism			
SLC38A2	0.7	down	0.131	Carbon Metabolism			
PDGFC	0.7	down	0.145	Carbon Metabolism, Choline Metabolism			
PRPS2	0.7	down	0.154	Glucose Metabolism			
GALM	0.7	down	0.222	Glucose Metabolism, KEGG Glycolysis			
PRKCB	0.7	down	0.423	Carbon Metabolism, Choline Metabolism			
SLC16A1	0.6	down	0.0329	Carbon Metabolism			
TIGAR	0.6	down	0.0388	Other Metabolic Pathway			
PDK3	0.6	down	0.0133	Glucose Metabolism			
EIF4EBP1	0.6	down	0.00288	Carbon Metabolism, Choline Metabolism, Hypoxia Signaling,			
FBP1	0.6	down	0.445	Glucose Metabolism, KEGG Glycolysis			
PDP1	0.5	down	0.0466	Hypoxia Signaling			
OAZ3	0.5	down	0.035	Other Metabolic Pathway			
HK3	0.5	down	0.0534	Carbon Metabolism, Glucose Metabolism, KEGG Glycolysis			
MYC	0.5	down	0.0596	Cancer Metabolism Drivers, Carbon Metabolism			
FGFR1	0.5	down	0.101	Carbon Metabolism			
PLCG1	0.5	down	0.13	Carbon Metabolism, Choline Metabolism			
PLD1	0.5	down	0.144	Choline Metabolism			

IDH1	0.4	down	0.00791	Cancer Metabolism Drivers, Glucose Metabolism		
EPAS1	0.4	down	7.93E-05	Cancer Metabolism Drivers		
PDK2	0.4	down	0.0217	Glucose Metabolism		
PDP2	0.4	down	0.077	Glucose Metabolism		
NTRK1	0.4	down	0.177	Carbon Metabolism		
NOXA1	0.4	down	0.242	Hypoxia Signaling		
RET	0.3	down	0.0153	Carbon Metabolism		
SHMT1	0.3	down	0.00264	Other Metabolic Pathway		