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Nutrient availability regulates Dendritic cell metabolism and function to modulate T cell responses

Thesis submitted to University of Dublin for the degree of Doctor of Philosophy

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For my Mam and Dad

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Abstract

An emerging view in the field of immunometabolism is that cellular metabolism has a role that extends beyond simply allowing the cell to meet energy and biosynthetic demands, and represents a fundamental physiological mechanism to control immune responses. This is certainly true in CTLs, NK cells and macrophages(Finlay et al., 2012, Donnelly et al., 2014, Tannahill et al., 2013). Work presented in this PhD investigated metabolism in activated BMDCs and how this was related to DC effector function. This work reveals a complex signaling circuit involving mTORC1/HIF-1α/iNOS that determines DC metabolism characterized by a metabolic shift towards elevated glycolysis and decreased OxPhos. The data describes a complex interplay between HIF-1α and iNOS in activated DCs; each is required for the full activity of the other; both are required for LPS induced metabolic changes. Recent evidence has showed that nutrients in the microenvironment can impact on immune cell metabolism and effector function(Chang et al., 2015).Work presented in this PhD shows that amino acids and glucose deprivation in the cellular microenvironment blocks mTORC1 signaling in DCs. Replacing glucose with galactose blocked the metabolic shift in activated DCs independently of mTORC1. This was explained by the novel observation that O-linked glycosylation was required for HIF-1α activity and expression in activated DCs. HIF-1α is usually associated with a pro-inflammatory role in the field of immunometabolism. However we present the novel finding that deleting or inhibiting HIF-1α enhances the pro-inflammatory functions of activated BMDCs. These conditions promote increased IL-12 expression, maintain elevated CD80 and CD86 expression and sustain a DCs ability to induce CD8+ T cell responses. This research demonstrates that T cells can deplete nutrients in the DC microenvironment during T cell DC interactions. Nutrients depletion inactivates mTORC1/HIF-1α signaling, enhances DCs pro-inflammatory state and serve as a feed forward mechanism to enhance T cell responses. The research presented in this thesis demonstrates that nutrient availability is a key signal that determines DC function and impacts on DC responses. This is an important paradigm shift that adds a previously unappreciated level of complexity to our understanding of T cell responses.

Summary

Immunometabolism is the study of the two fields of immunology and metabolism and how they are related. There has been growing interest in this field due to the rise of obesity in the western world and the understanding that the immune system plays an important role in the pathology of diseases associated with this condition such as type 2 diabetes (Mathis and Shoelson, 2011). However this PhD deals with the second area of study in the field of immunometabolism, that is the study of how metabolism is integrally linked to immune cell function. Evidence has emerged that many pro-inflammatory immune cells such as T cells, NK cells and macrophages undergo a metabolic switch towards elevated levels of glycolysis (Pearce and Pearce, 2013, Donnelly et al., 2014, Tannahill et al., 2013). Increased glycolysis is thought to provide the biosynthetic precursors needed for proliferation and growth. However recently evidence has emerged that directly links metabolism to immune cell effector function (Finlay et al., 2012, Chang et al., 2013, Ho et al., 2015). DCs also undergo a metabolic shift towards increased glycolysis with activation (Krawczyk et al., 2010). However the metabolic regulators involved in this process and the relationship between DC metabolism and function remain to be fully elucidated.

Data presented here finds that both mTORC1 and HIF-1α are required for increased glycolysis observed in BMDCs after 24 hours LPS activation. This is due to metabolic reprogramming and the increase in expression of key glycolytic enzymes and glucose transporters. mTORC1 is required for both HIF-1α and iNOS expression in DCs. iNOS had previously been linked to the LPS induced metabolic shift in DCs (Everts et al., 2012). We make the novel discovery that the expression and activity of HIF-1α and iNOS are integrally linked with the activity of both required for the metabolic shift in DCs. Recent research has begun to highlight an important role for nutrients for normal immune cell function (Ho et al., 2015). Our data shows that mTORC1/HIF-α signaling in DCs are acutely sensitive to nutrients in the cellular microenvironment such as glucose and amino acids. Using galactose as an alternative carbon source to glucose we show that glucose is required for HIF-1α activity independently of mTORC1 signaling. HIF-1α activity in DCs requires

glucose for O-linked glycosylation and inhibition of OGT, the enzyme that catalyzes the addition of an O-GlcNAc moiety onto a protein, blocks HIF-1α activity.

The expression of HIF-1α is predominantly associated with a pro-inflammatory phenotype in immune cells such as in CTLs and macrophages (Finlay et al., 2012, Tannahill et al., 2013). However work presented in this PhD makes the novel finding that deleting HIF-1α in BMDCs enhances pro-inflammatory DC functions with increased expression of IL-12 and decreased IL-10, the maintained expression of CD80 and CD86 co-stimulatory molecules and the sustained ability to induce T cell responses. Following the discovery that glucose is required for HIF-1α, a role for glucose for DC functions was investigated by replacing with the alternative fuel galactose. Glucose deprivation resulted in an increase in IL-12 expression, prolonged expression of CD80 and CD86 and the sustained ability to induce CD8+ T cell proliferation and IFNγ production. To our knowledge this is the first time that manipulation of nutrients in the microenvironment has been shown to affect DC effector function.

mTORC1/HIF1α and glycolysis promote pro-inflammatory T cell responses but have anti-inflammatory functions in activated DC. We developed a model to try to account for this apparent discrepancy in metabolic state and inflammatory function. We predicted that activated T cells interacting directly with DCs can modulate the nutrient availability in the DC microenvironment. In turn, nutrients act as a signal to control the inflammatory outputs of DC by regulating DC signaling pathways (including mTORC1), DC metabolism and ultimately DC function. Indeed the data shows that increasing the numbers of T cells interacting with DC inhibits mTORC1 signaling in the DC and enhances T cell IFNγ production. To support this model we also demonstrated that T cells can modulate DC mTORC1 activity in vivo in draining lymph nodes. Therefore, through a metabolically controlled feed forward mechanism, activated T cells can enhance DC pro-inflammatory function to shape the T cell response. This is an important new paradigm for the regulation of T cell immunity that demonstrates a previously unappreciated level of complexity where nutrient availability impacts upon DC stimulated T cell responses.

Abbreviations

2DG	2-deoxy-D-glucose
4E-BP1	Eukaryotic translation initiation factor binding protein
AMPK	AMP-activated protein kinase
APC	Antigen presenting cell
BMDC	Bone marrow derived dendritic cell
cDC	Classical/conventional dendritic cell
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DEPTOR	DEP-containing mTOR interacting protein
ECAR	Extracellular acidification rate
ELISA	Enzyme linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
FACS	Fluorescent-activated cell sorting
FIH	Factor inhibiting HIF
FKBP12	FK506 binding protein 12
Flt3	Fms-like tyrosine kinase 3
GAP	GTPase activating protein
GAPDH	Glyceraldehyde 3-phospahate dehydrogenase
GM-CSF	Granulocyte macrophage colony-stimulating factor
GSK3	Glycogen synthase kinase 3
HIF	Hypoxic inducible factor
HK2	Hexokinase 2
HRE	Hypoxia response element
IKKε	Inhibitor of NF-κB subunit epsilon
iNOS	Inducible nitrogen oxide synthase
LAT1	L-type amino acid transporter 1
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MDSC	Myeloid derived suppressor cell
MHC	Major histocompatibility complex
mTORC1	Mammalian target of rapamycin complex 1

NF-κB	Nuclear factor kappa enhancer of activated B cells
NK	Natural killer
NO	Nitric oxide
OCR	Oxygen consumption rate
OxPhos	Oxidative phosphorylation
PAMP	Pathogen associated molecular pattern
pDC	Plasmacytoid dendritic cell
PDK-1	Phosphoinositide-dependent kinase 1
PFK	Phosphofructokinase
PHD	Prolyl hydroxylase
PI3K	Phosphoinositide 3-kinase
PKB/AKT	Protein kinase B
PKM	Pyruvate kinase muscle
PMA	Phorbol 12-myristate 13-actetate
Poly I:C	Polyinosinic:polycytidylic acid
PRR	Pathogen recognition receptor
RAPTOR	Regulatory associated protein of mTOR
RHEB	Ras homolog enriched in brain
RICTOR	Rapamycin insensitive companion of mTOR
ROS	Reactive oxygen species
S6	Ribosomal protein S6
S6K	P70-S6 kinase
SMC1	Structural maintenance of chromosomes 1
SREBP	Sterol regulatory element-binding protein
TBK1	TANK-binding kinase 1
TCA	Citric acid cycle
TCR	T cell receptor
T_H	T helper cell
TLR	Toll like receptor
TSC	Tuberous sclerosis
VHL	Von Hippel-Lindau

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Chapter 1

Introduction

1.1 Dendritic Cells

Dendritic cells (DC's) were discovered by Ralph Steinman in 1973 as unidentified white blood cells present in the mouse spleen. Today they are recognized as "sentinels of the immune system", having a key role to play in linking the innate and adaptive immune systems (Steinman, 2012).

Dendritic cells are professional antigen presenting cells (APC's). APC's function by presenting antigen from pathogens to T cells which leads to priming of these naïve T cells to differentiate into effector T cells. DC's develop from a common myeloid precursor cell in the bone marrow that is dependent on fms-like tyrosine kinase 3 (Flt3) (Waskow et al., 2008). In the mouse the main subsets of DC's are plasmacytoid DC's (pDC's) and classical/conventional DC's (cDC's) (Schlitzer and Ginhoux, 2014). pDC's circulate in the blood and can secrete large amounts of interferon's (Lande and Gilliet, 2010). cDC's in mouse are split further into two main subsets, CD8α+ and CD8α- DC's and are resident in lymphoid organs (Segura and Amigorena, 2013). An ability to migrate from lymphoid organs to draining lymph nodes and tissues is a distinguishing characteristic between DC's and macrophages. This migration increases under inflammatory conditions(Randolph et al., 2008). A distinct subset of DC's only develops in response to inflammation. These are called inflammatory DC's that develop from tissue infiltrating monocytes. They are characterized by high expression of iNOS and TNFα (Serbina et al., 2003). Bone marrow derived dendritic cells (BMDC's) grown in the presence of the growth factor granulocyte macrophage colony stimulating factor (GM-CSF) are thought of as a model of these inflammatory DC's. The DC subsets in humans are much more complicated but there are mouse homologues for most identified human subsets and mice are thought to be a good model to study dendritic cell biology (Schlitzer and Ginhoux, 2014).

Naïve DC's are very active in taking up antigens and sensing their microenvironment for the presence of pathogens. The receptors they use to sense the microenvironment are called toll like receptors (TLRs). TLR's are among the pathogen recognition receptors (PRR's) that recognize pathogen-associated molecular pattern (PAMP's) (Creagh and O'Neill, 2006). PAMP's are structurally

conserved pathogen structures such as lipopolysaccharide (LPS) or viral single stranded RNA (Kumar et al., 2009). TLR stimulation by PAMPs leads to activation of DC's. Activation of DC's leads to a complete change in the expression profile within the cell. DC's cease taking up antigen and instead present the processed antigen on its surface on major histocompatibility complex class I (MHC I) and II (MHC II) molecules (Chow et al., 2002). MHC I molecules interact with the T cell receptor (TCR) on CD8+ T cells while MHC II molecules interact with CD4+ T cells (Mellman and Steinman, 2001). Presentation of antigen in itself is not enough to activate T cells, there must be other stimulatory signals given. DC's express co-stimulatory molecules CD80 and CD86 that interact with their counter receptor CD28 on T cells. Activated DC's increase their expression of co-stimulatory molecules. TCR interaction in the absence of co-stimulatory molecules drives T cell anergy, a process of T cell non-reactivity to TCR-antigen recognition (Slavik et al., 1999).

Activated DC's also secrete a cocktail of cytokines that can drive different T cell responses. DC's secrete pro-inflammatory cytokines including IL-6, IL-12, IL-18, TNFα, IL-1α and IL-1β (Blanco et al., 2008). These drive tissue inflammation, can mediate B cell activation and importantly contribute to the type of T cell response that develops upon TCR activation. DC's also play an important part in the development of T cell anergy and immune tolerance. DC's that are presenting self antigens or non pathogenic antigens can induce regulatory T cells or make T cells unresponsive to the presented antigen. The cytokines IL-10 and TGF-β can drive this process and DC's produce both (de Saint-Vis et al., 1998).

Recently evidence has emerged that activation of DC's, as well as other pro-inflammatory immune cells, is linked with a change in the metabolism of these cells. It has been demonstrated that activation induces a switch from oxidative phosphorylation to glycolysis. This has led to the emergence of a new field of study called immunometabolism.

1.2 Immunometabolism

Immunometabolism is a developing field that combines the established areas of immunology and the biochemical study of metabolism (Mathis and Shoelson, 2011). This field can be further broken down into two distinct fields of research, the first being the investigation into the link between metabolic diseases such as type 2 diabetes, cardiovascular disease and cirrhosis and the underlying inflammation associated with these diseases (Han and Levings, 2013). A surge in interest in this area in recent years has been caused by the ever-increasing problem of obesity in the Western World. The rise in obesity is correlated with an increase in many types of disease such as the ones mentioned and understanding these links and developing therapies to target the underlying inflammation is the goal of this area of research.

The other area of research in immunometabolism is the investigation into the metabolism of immune cells. It was found that certain immune cells massively increase their rate of glycolysis when they are activated (Pearce and Pearce, 2013). The form of glycolysis they use is called aerobic glycolysis and is similar to the "Warburg metabolism" first described in cancer cells (Zu and Guppy, 2004). This involves the breakdown of glucose through the glycolytic pathway to make pyruvate. Normally in oxygen rich environments this pyruvate enters the citric acid cycle (tricarboxylic acid cycle TCA) and is converted to CO_2 and water with lots of ATP produced. However in aerobic glycolysis a cell chooses to convert pyruvate to lactate even in the presence of oxygen. This process is much less efficient in making ATP. However cells relying on glycolysis maintain similar levels of ATP to those relying on OxPhos by increasing the rate of glycolysis (Palsson-McDermott and O'Neill, 2013).

An advantage of aerobic glycolysis to both cancer cells and immune cells is that the glycolytic pathway provides the biosynthetic intermediates needed to make new proteins, lipids, nucleotides and ultimately for cell proliferation (Lunt and Vander Heiden, 2011). Evidence is emerging that effector cells that drive an immune response undergo a metabolic switch towards glycolysis while naïve, regulatory and memory cells rely on oxidative phosphorylation to meet their metabolic demands. Although glucose is the preferred substrate for glycolysis galactose can also be

metabolized through this pathway. Galactose can enter the body through the consumption of dairy products and sugary foods. The main pathway for its metabolism is the Leloir pathway. This is an enzymatic pathway that starts with galactose and its product is UDP-glucose. This UDP-glucose can then enter the glycolytic pathway below the hexokinase-controlled step. Although galactose is eventually metabolized through the glycolytic pathway the rate is severely reduced and cells cannot sustain elevated glycolysis such as observed in cancer cells (Sellick et al., 2008).

1.2.1 T cell metabolism

T cells are immune cell subtypes that are involved in a variety of essential roles, from helping to fight infection to clearing cancerous cells. T cells are defined by the T cell receptor that they express with the vast majority expressing the alpha beta TCR (αβ) and a small subset expressing the gamma delta TCR (γδ) (Kreslavsky et al., 2008). Not much is known about γδ T cell metabolism whereas αβ T cell metabolism has been investigated extensively. αβ T cells are broadly split into two distinct subsets, CD4+ and CD8+ T cells (Ellmeier et al., 2013). CD4+ T cells have TCR's that only interact with major histocompatibility complex class II (MHC I) surface proteins. In contrast CD8+ T cells have TCR's that only interact with MHC I surface proteins. Another distinction between CD4+ and CD8+ T cells is the distinction between helper T cells and cytotoxic T cells. CD4+ helper T cells are able to enhance the immune response, mainly by the cytokines that they secrete (Mosmann et al., 1986). CD8+ T cells or cytotoxic T lymphocytes (CTL's) can directly kill cancerous or infected cells by releasing perforin or granzyme, or by binding to the cell surface of these damaged cells and inducing signaling events which tell the cell to die (Andersen et al., 2006).

Upon recognition of its unique antigen with costimulation, T cells quickly enter a process of rapid proliferation called clonal expansion. This allows the rapid development of a large number of T cells responsive to a specific antigen, necessary to mount a robust immune response. This proliferation requires large amounts of energy and also biosynthetic precursors necessary to provide the building blocks for

a new cell. The kinetics of this rapid proliferation is akin to cancerous cells dividing. Perhaps not surprisingly, proliferating T cells switch their metabolism to aerobic glycolysis or Warburg metabolism (Waickman and Powell, 2012). This metabolic switch has been demonstrated in all effector T cell subtypes: Th1, Th2, Th17 and cytotoxic T lymphocytes (CTL's) (Michalek et al., 2011, Maciver et al., 2008, Shi et al., 2011, Finlay et al., 2012). All these studies showed that inhibiting the switch to glycolysis inhibited the differentiation of these T cell subtypes showing that glycolysis plays an important part in this process. Interestingly inhibiting glycolysis in CTL's, which blocked their differentiation and function, did not inhibit their proliferation showing that increased glycolysis serves other purposes than just meeting proliferative demands (Slavik et al., 2001, Finlay et al., 2012).

Activated effector T cells have high oxidative phosphorylation as well as increased glycolysis. The majority of their energy demands are met through glycolysis so the purpose for maintaining high OxPhos remained unresolved. Recently it was shown that reactive oxygen species (ROS) produced during OxPhos was shown to be required for IL 2 production (Sena et al., 2013), an important cytokine that maintains activation. This suggests that OxPhos as well as a shift towards glycolysis is required for correct T cell effector function. Activated T cells also need glutamine metabolism, called glutaminolysis, as this amino acid can enter the TCA cycle and be converted to make new lipids (Carr et al., 2010). In contrast it has been shown that naïve, regulatory and memory T cells rely on oxidative phosphorylation to meet their energy demands (Delgoffe et al., 2009, Araki et al., 2009, van der Windt and Pearce, 2012).

1.2.2 Dendritic cell and macrophage metabolism

Similarly to DC's, macrophages act as APC's but also have other important roles in the immune system. They are resident in tissues throughout the body or can differentiate from monocytes during inflammation. Macrophages can be divided into two subsets. M1 macrophages are considered pro-inflammatory cells and function by promoting Th1 T cell responses through antigen presentation and

cytokine production and directly killing pathogens, as well as dead or cancerous cells, by the process of phagocytosis (Molawi and Sieweke, 2013, Locati et al., 2013). M1 macrophages express TLRs and are activated in a similar way to DC's. M1 macrophages can also be activated by IFN-γ (Lyamina et al., 2012). Activated DC's and M1 macrophages have an increased rate of glycolysis but, in contrast to effector T cells, they completely inhibit oxidative phosphorylation (Rodriguez-Prados et al., 2010, Krawczyk et al., 2010).

Inhibiting the switch to glycolysis in both these cell types inhibits their correct activation. The switch to glycolysis was demonstrated to maintain the levels of ATP with OxPhos inhibited (Everts et al., 2012). A recent paper looked at the initial metabolic events after TLR stimulation in DC's. This study found that glycolysis is increased minutes after stimulation. This required the kinases tank-binding kinase 1 (TBK1), IκB kinase ε (IKKε) (Everts et al., 2014). These kinases induced the association of the glycolytic enzyme hexokinase 2 (HK2) with the mitochondria allowing increased glycolytic flux and efficiency. This initial increased glycolysis is required to meet the anabolic demands of DC activation and cytokine production. Blocking this initial increase again inhibited DC activation. As stated above activated DC's have a substantial increase in glycolysis while decreasing their rate of oxidative phosphorylation. The decrease in oxidative phosphorylation was shown to be dependent on inducible nitric oxide (iNOS), which is expressed upon TLR stimulation, in BMDC's (Everts et al., 2012). Deleting this gene prevents this decrease while also limiting the shift to glycolysis. The mechanism proposed involves nitric oxide (NO) inhibiting cytochrome c oxidase, a complex in the electron transport chain, and thus inhibiting OxPhos. With OxPhos inhibited there is an increase in flux of glucose through the glycolytic pathway and thus an increased glycolytic rate.

In contrast M2 macrophages are associated with tissue repair and immunoregulation displaying a more anti-inflammatory phenotype. They are polarized "alternatively" by Th2 cytokines such as IL 4. These cells are very important in wound healing and the removal of dead cells. In contrast to M1 macrophages they display an increased rate of oxidative phosphorylation and minimal glycolysis. Blocking OxPhos impairs the polarization of M2 macrophages showing that committing to OxPhos is required to meet effector function (Shapiro et al., 2011).

1.3 mTORC1 is a key metabolic regulator

Rapamycin was discovered in the 1970's as an antifungal in *Streptomyces hygroscopicus* by blocking fungal cell cycle in the G1 phase. This new compound was found to have remarkably specific inhibitory effects on a protein called TOR (target of rapamycin) in mammal's mTOR (mammalian TOR). mTOR is a 289 kDa serine/threonine protein kinase that is highly conserved in nature. It is a member of the phosphoinsitide 3-kinase (PI3K) related family of proteins which include ATM, ATR and DNA-PKcs which all share a structurally similar C-terminus kinase domain. mTOR was found to associate with a number of proteins and formed into two distinct complexes, mTORC1 (mammalian target of rapamycin complex 1) and mTORC2 (Heitman et al., 1991, Sarbassov et al., 2006). Rapamycin was found to be a dose dependent inhibitor of mTORC1 and has enabled substantial investigation into its function since this discovery (Thoreen and Sabatini, 2009). The complex mTORC1 is made up of a number of proteins. The central protein mTOR, regulatory-associated protein of mTOR (RAPTOR) that is involved in mTOR substrate recruitment(Hara et al., 2002) and mTOR associated protein LST8 homolog (mLST8 also called GβL) that may be involved in the assembly of the complex.

There are proteins that directly bind to the complex and negatively regulate its function which include proline-rich Akt substrate of 40 kDa (PRAS40) that has a regulatory role as dissociation of this protein from the complex is required for full mTORC1 activity and DEP domain-containing mTOR interacting protein (DEPTOR) that is broken down on mTORC1 activation (Sancak et al., 2007) (Peterson et al., 2009). Rapamycin inhibits mTORC1 by binding to the protein 12-kDa FK506-binding protein (FKBP12). This complex then binds to mTORC1 and inhibits the catalytic cleft of this kinase(Yang et al., 2013). The complex mTORC2 shares the mTORC1 proteins mTOR and mLST8 with the additional proteins rapamycin insensitive companion of mTOR (RICTOR) and mammalian stress-activated protein kinase interacting protein 1 (mSIN1) (Cybulski and Hall, 2009).

The complex mTORC1 is an acute sensor of the cellular microenvironment and is able to respond to the extracellular levels of nutrients such as amino acids and

glucose, oxygen levels and growth factors through complex signaling pathways upstream of mTORC1. Growth factors signal to and activate mTORC1 by various signaling pathways. Growth factors, signaling through the PI3K/AKT pathway, bind to their receptors that result in phosphorylation of AKT by phosphoinositide-dependent kinase 1 (PDK-1), which requires PI3K. AKT in turn phosphorylates the mTORC1 regulator tuberous sclerosis complex 2 (TSC), which consists of TSC1 and TSC2, blocking the formation of this dimer and hence its ability to inhibit mTOR (Inoki et al., 2002). AKT can also directly phosphorylate PRAS40 thereby releasing it from the mTORC1 complex and allowing full activation (Vander Haar et al., 2007). The MAPK(mitogen activated protein kinase)/ERK(extracellular signal-regulated kinases) signaling pathway also results in phosphorylation of TSC by the protein 90kDa ribosomal S6 kinase (RSK) (Anjum and Blenis, 2008). The TSC complex is an important regulator of mTORC1 as many extracellular nutrient signals converge on TSC. TSC is a GTPase-activating protein (GAP) and phosphorylation by AKT inhibits this process(Inoki et al., 2002). TSC stimulates GTPase activity in the protein ras homolog enriched in brain (RHEB) that in its GTP bound form directly interacts and activates mTORC1 (Long et al., 2005).

Energy levels in the cell result in varying levels of AMP (adenosine mono phosphate) and ATP (adenosine tri-phosphate). Low energy levels are detected by the protein AMP-activated protein (AMPK), which ultimately leads to phosphorylation of TSC that stabilizes the complex thereby inhibiting mTORC1 (Inoki et al., 2003). mTORC1 can also be inhibited in TSC2 knockout mice. The mechanism for this involves AMPK directly phosphorylating the mTORC1 complex protein Raptor that blocks mTORC1 function(Gwinn et al., 2008). Genotoxic stress can also activate AMPK and inhibit mTORC1. The kinase glycogen synthase kinase 3(GSK3) is an important negative regulator of mTORC1 signaling in a number of processes including glycogen synthase, cytokine expression and the migration capacity of some cells (Beurel et al., 2010). GSK3 phosphorylates TSC that activates the complex and inhibits mTORC1.

Amino acid depletion is a strong inhibitor of mTORC1 but for a long time how this was signaled to mTORC1 was not understood other than it was shown to be a TSC independent process. A breakthrough was made with the discovery that a group of

four small GTPases, the Rags, are essential for amino acid signaling to mTORC1 (Sancak et al., 2008). Sufficient availability of amino acids causes the Rags to switch to an active form, bind to mTORC1 and recruits it so that mTORC1 can interact with its activator RHEB. The location of this activation has recently been shown to take place at the lysosomal surface (Demetriades et al., 2014).

mTORC1, acting through diverse downstream targets, is involved in many processes within the cell and is seen as a master regulator of cell growth and metabolism (Fig 1.2). It has many downstream targets that are required for it to carry out its cellular function. Phosphorylation of the protein 4E-binding protein 1 (4E-BP1) and p70 ribosomal S6 (S6K) kinase, which phosphorylates its target ribosomal protein S6 (S6) result in increased protein synthesis (Wang and Proud, 2011). Activated mTORC1 promotes lipid synthesis through regulating the activity of sterol regulatory element binding protein 1 (SREBP1) (Porstmann et al., 2008). mTORC1 has also been linked to mitochondrial biogenesis and the rate of mitophagy but the exact mechanisms remain unclear (Chen et al., 2008, Schieke et al., 2006).

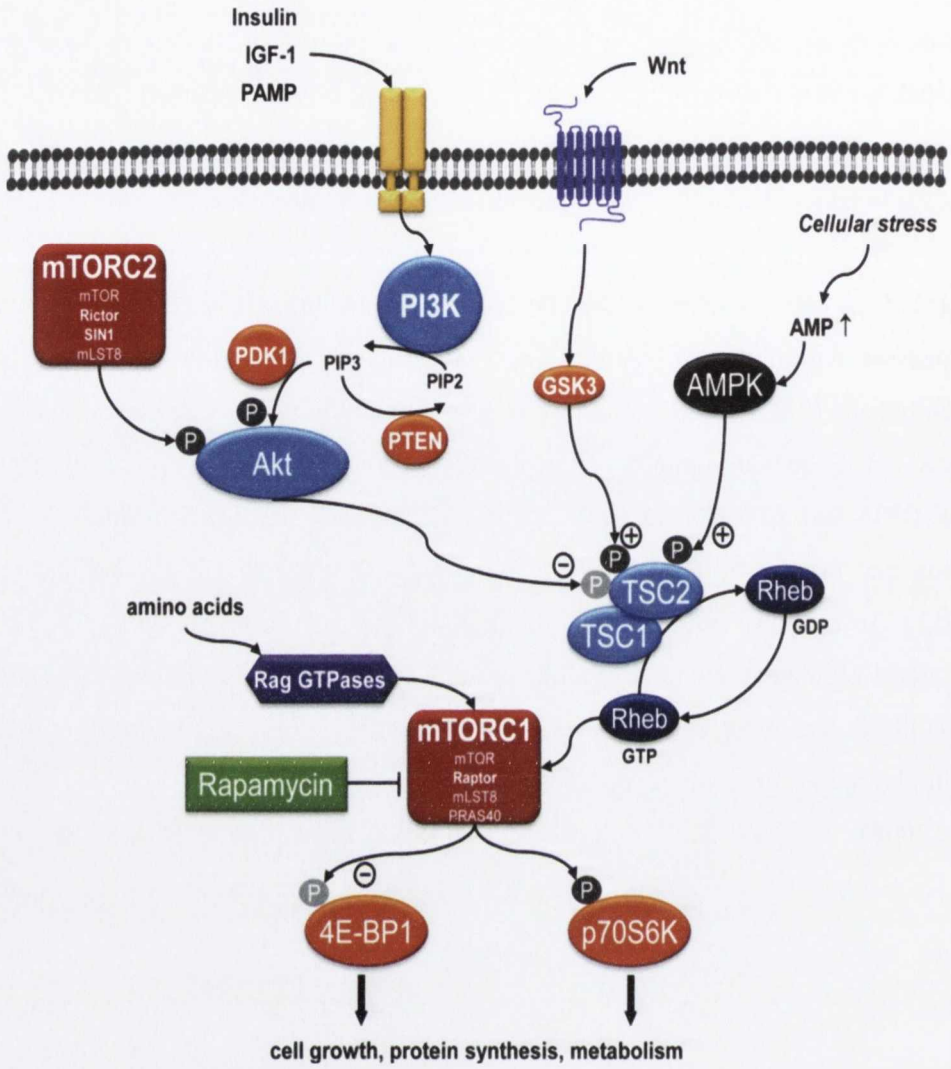


Fig 1.1. A schematic of the upstream signaling events that regulate mTORC1 signaling. mTORC1 is a key sensor of the cellular microenvironment that receives signals from upstream regulators. (Katholnig et al., 2013b).

mTORC1 is a key regulator of aerobic glycolysis in cancer cells and many oncogenes affect this pathway (Dienstmann et al., 2011). By its involvement in aerobic glycolysis it also promotes nucleotide and amino acid synthesis, as aerobic glycolysis can provide the biosynthetic precursors needed for their production. The mTORC1 signaling pathway leads to the expression of important transcription factors for the above anabolic processes (Dibble and Manning, 2013). As mTORC1 is a key regulator of cell growth it is no surprise that it is essential for the control of autophagy (Dunlop and Tee, 2013). Autophagy is a highly regulated cellular catabolic process that leads to the degradation of cellular components for recycling and is crucial for homeostasis. When mTORC1 is turned on in the presence of abundant nutrients mTORC1 phosphorylates unc-51-like kinase 1 (ULK1) and autophagy-related gene 13 (ATG13) thereby blocking the autophagy pathway (Ganley et al., 2009). Recently evidence has emerged that mTORC1 is a key regulator of the immune response and of metabolism in immune cells. In effector immune cells there is an increase in glycolysis that has been demonstrated to be mTORC1 dependent. In some of these cells hypoxia inducible factor alpha (HIF-1α), downstream of mTORC1, has been shown to be responsible for this metabolic switch. How mTORC1/HIF-1α regulates glycolysis will be discussed in greater detail below.

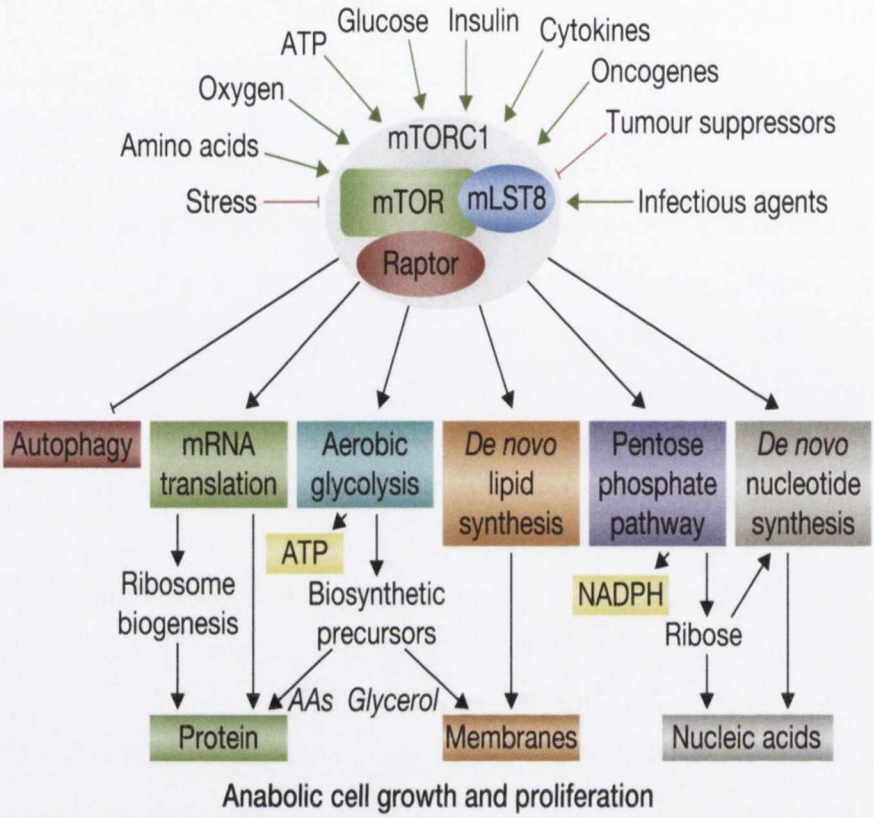


Fig.1.2 mTORC1 is an acute sensor of signals in the cellular microenvironment and converts these signals to biosynthetic outputs. The complex mTORC1 is shown here in its active form, as its negative regulators DEPTOR and PRAS40 are unbound. mTORC1 converts a multitude of extracellular and intracellular signals into the production of new proteins, lipids and nucleic acids. In this way mTORC1 is viewed as a master regulator of cell growth. When mTORC1 is active autophagy is inhibited(Dibble and Manning, 2013).

1.4 mTORC1 regulates cellular glucose metabolism

In a majority of cancers there is deregulation of the mTORC1-signaling pathway. Recently a key contribution of this pathway to the metabolic cancer hallmarks of increased glucose uptake and rate of glycolysis has been described (Yecies and Manning, 2011). Glycolysis is the metabolic reaction that converts glucose into pyruvate and in the process generates a net positive of two adenosine tri-phosphate (ATP) molecules, represented by the following equation

$$Glucose + 2ATP + 2[NAD+] \longrightarrow 2pyruvate + 4ATP + 2NADH$$

In mammalian tissues where the oxygen supply isn't limiting, glycolysis is usually considered the first step of cellular respiration as pyruvate enters the TCA cycle to produce many more molecules of ATP. When oxygen levels are low and the TCA cycle is switched off glycolysis can be used to maintain a supply of ATP. Glycolysis is a 10-step process starting with the addition of a phosphate group by the enzyme hexokinase to produce glucose-6-phosphate and ending in the conversion of phosphoenolpyruvic acid to pyruvic acid by the enzyme pyruvate kinase, in the process adding a phosphate to ADP. From the equation above it can be noted that glycolysis needs a supply of NAD+ to proceed. This molecule can be produced from pyruvate itself by its conversion to lactate by the enzyme lactate dehydrogenase. The rate-limiting step of glycolysis was long thought to be the step involving phosphofructokinase, as it requires a large input of energy and is essentially irreversible (Mor et al., 2011). Now it is appreciated that hexokinase and pyruvate kinase can also be considered rate limiting. The concentration of substrate and allosteric effects on enzymes can also control the rate of glycolysis.

In mammalian cells glycolysis is usually used as the first step in cellular respiration. However in certain circumstances cells can switch their metabolism from oxidative phosphorylation to glycolysis, with glycolysis used to meet the cells energy demands even in an oxygen rich environment. This phenomenon was first described by Otto Warburg in cancer cells (Mathupala et al., 2010). Nearly all cancer cells have a massively increased rate of glycolysis, in some cases over 200 times more than the glycolytic rate of resting mammalian cells (Jang et al., 2013). This form of

glycolysis is called aerobic glycolysis or Warburg metabolism as it takes place in the presence of oxygen.

Cancer cells up-regulate the transcription factor HIF-1α, which is important for the increase in glycolysis observed in these cells (Rohwer et al., 2013). In cancer cells, and more recently some immune cells, the expression of the transcription factor HIF-1α has been shown to require mTORC1 signaling (Finlay et al., 2012, Sakamoto et al., 2014, Miyazawa et al., 2009). HIF-1α is a key transcription factor for many glycolytic enzymes and glucose transporters, and its expression can lead to increased glycolytic rates (Semenza et al., 1994). HIF was originally discovered as a transcription factor that promoted the erythropoietin gene in hypoxic conditions (Semenza and Wang, 1992). Hypoxia is a condition where oxygen supplies becomes limiting in the microenvironment. Normoxia is the name given to normal oxygen levels in the body. HIF exists as a heterodimer with one subunit being the constitutively expressed and stable β-subunit, originally called ARNT, and the other being the highly regulated HIF-1α subunit (Semenza et al., 1997).

The expression and stability of HIF-1α is tightly regulated under normoxia. HIF-1α protein is targeted for ubiquitin-proteasomal degradation by the von Hippel Lindau protein (pVHL) pathway (Fig 1.3) (Jaakkola et al., 2001, Shay and Celeste Simon, 2012). In this pathway there are four oxygen sensing hydroxylases, prolyl-hydroxylase domain containing protein 1 (PHD1), PHD2, PHD3 and factor inhibiting HIF (FIH). These hydroxylases require Fe^{2+}, 2-oxogluterate (a Krebs cycle intermediate) and molecular O2 for them to function (Scholz and Taylor, 2013, Kaelin and Ratcliffe, 2008). In normoxia these proteins hydroxylate specific residues on HIF-1α, leading to binding of pVHL and ultimately ubiquitylation of the protein for degradation. These hydroxylases can be inhibited by different mechanisms other than just low oxygen supply. As mentioned the hydroxylation of HIF-1α requires 2-oxogluterate as a co-factor. 2-oxogluterate is an intermediate in the Krebs (TCA) cycle and suggests a link between this process and HIF-1α protein accumulation. Indeed the TCA intermediates succinate and fumerate have both been shown induce HIF-1α protein expression, presumably by limiting 2-oxogluterate for the hydroxylation reaction (Selak et al., 2005, Hewitson et al., 2007). Reactive oxygen species and the gas nitric oxide (NO) can also inhibit hydroxylases, even in

a normoxic environment (Sandau et al., 2001, Bonello et al., 2007). The TLR4 agonist LPS has also been shown to increase HIF-1α in normoxia. This has been shown *in vitro* using macrophages and dendritic cells(Blouin et al., 2004, Spirig et al., 2010). LPS induces HIF-1α in a nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) dependent manner in macrophages (Frede et al., 2006). The PI3K signaling pathway has also been shown to be involved in this LPS induced response in macrophages (Westra et al., 2010).

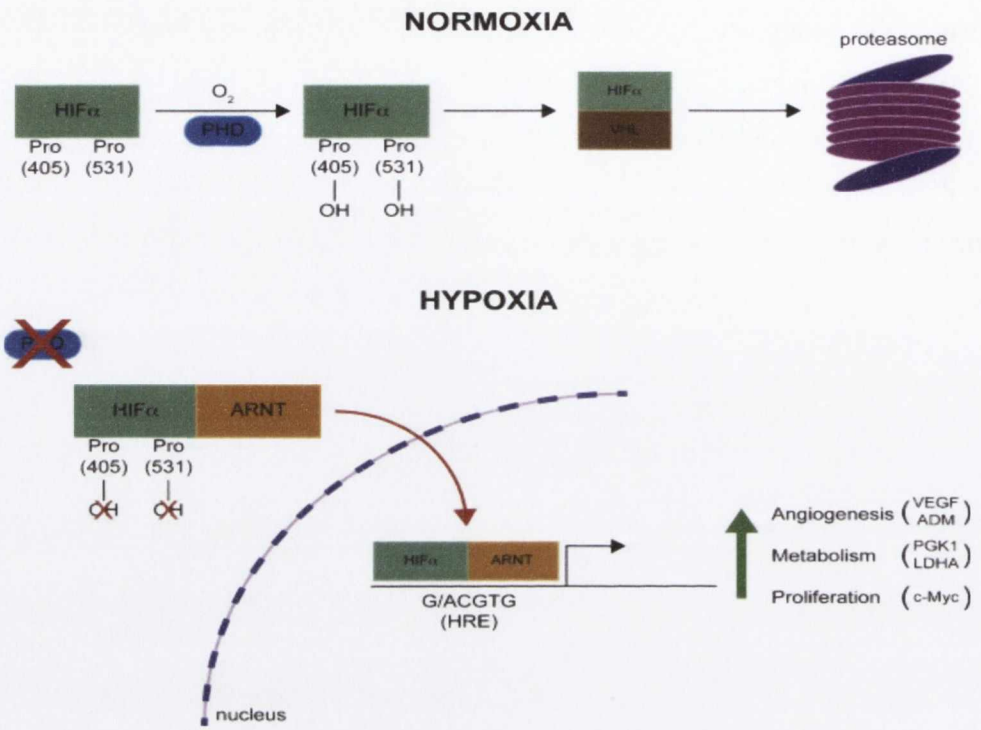


Fig.1.3 HIF-1α is degraded in normoxia by the Von Hippel Lindau protein pathway. HIF-1α in normoxia is targeted for hydroxylation on conserved sites by the prolyl hyroxylases (PHD's) and factor inhibiting Hif (FIH). This leads to binding of HIF-1α by VHL that ultimately leads to ubiquitylation and degradation by proteasomes. In hypoxia, or conditions that promote HIF-1α accumulation, the PHD's are inhibited. This allows HIF-1α to bind to the constitutively expressed HIF-1β subunit or ARNT. This allows this complex to enter the nucleus and bind to hypoxia response elements (HRE) at the start of genes involved in angiogenesis, metabolism and proliferation (Shay and Celeste Simon, 2012).

1.5 mTORC1 integrates the control of T cell metabolism and function

T cells treated with rapamycin results in these cells failing to up regulate glycolysis and impaired their differentiation. Rapamycin inhibits mTORC1 but to absolutely prove the mTORC1 requirement for the shift to glycolysis in these cells gene knock out studies were performed. T cells in which mTOR, the central kinase in both mTORC1 and mTORC2, has been deleted in a T cell specific manner fail to differentiate into Th1, Th2 and Th17 effector cells and indeed numbers of regulatory T cells increase (Delgoffe et al., 2009). In T cells were RHEB had been deleted Th1 and Th17 cells failed to differentiate but Th2 cell differentiated normally (Delgoffe et al., 2011). Interestingly in T cells where the mTORC2 specific component RICTOR had been deleted, Th1 and Th17 cells differentiated normally but Th2 cells did not (Lee et al., 2010, Delgoffe et al., 2011). Rapamycin treatment of CTL's blocks the shift to glycolysis and effector functions but does not stop proliferation (Slavik et al., 2001, Finlay et al., 2012).

Signaling through mTORC1 also links nutrient availability in the cellular microenvironment to T cell activation. Deleting the large neutral amino acid transporter (LAT1) results in impaired CTL clonal expansion and effector differentiation (Sinclair et al., 2013). It is clear that metabolism/mTORC1 is required for correct effector T cell differentiation but the exact role remains unclear. Recently there has been some evidence directly linking metabolism to immune cell function. The enzyme GAPDH is important in the glycolytic pathway. However it also appears to have other properties one of which is the ability to bind mRNA. A study in T cells found that GAPDH could bind to IFNγ mRNA and inhibit its translation. When T cells are activated they increase their rate of glycolysis meaning GAPDH is required for the glycolytic pathway. GAPDHs function in the glycolytic pathway and as an mRNA binding protein is multi-exclusive meaning that when GAPDH is engaged in glycolysis more IFNγ can be translated (Chang et al., 2013).

In CTLs, mTORC1 has been shown to be required for the expression of HIF-1α (Finlay et al., 2012). This protein has been shown to be important for the increase in

glycolysis in effector immune cells. Deleting HIF-1α in CD4+ T cells prevented Th17 differentiation and promoted Treg development (Shi et al., 2011, Dang et al., 2011). This showed that HIF-1α is the key regulator downstream of mTORC1 that promotes Th17 differentiation. Interestingly it has been shown that HIF-1α is not required for the initial shift to glycolysis in CD8+ T cells. In CD8+ T cells the initial increase in glycolysis was shown to be c-myc dependent (Wang et al., 2011). C-myc is a transcription factor that promotes the expression of many genes involved in cell proliferation and cell growth. Importantly it promotes the expression of many genes in the glycolytic pathway. Deleting c-myc blocked the shift to glycolysis and glutaminolysis in activated CD8+ T cells. HIF-1α is not involved in the initial shift to glycolysis in activated CD8+ T cells, however recent evidence has shown that HIF-1α is required to maintain elevated glycolysis with IL 2 stimulation (Finlay et al., 2012). HIF-1α deficient CTL's had decreased expression of perforin and granzymes but IFN-γ expression was not affected. This suggests that HIF-1α is required for certain CTL effector function but is not a global regulator. HIF-1α also affected CTL migration, as deleting this gene caused CTL's to have increased expression of CD62L and CCR7. Activated CTL's decrease expression of these molecules to allow trafficking towards sites of inflammation. Interestingly the mTORC1/HIF-1α signaling axis in CTL's was shown to be PI3K/AKT independent, even though it is usually considered a linear signaling pathway. Instead mTORC1 activation was shown to be phosphoinositide-dependent kinase 1 (PDK1) dependent, although how this signaled directly to mTORC1 was not determined.

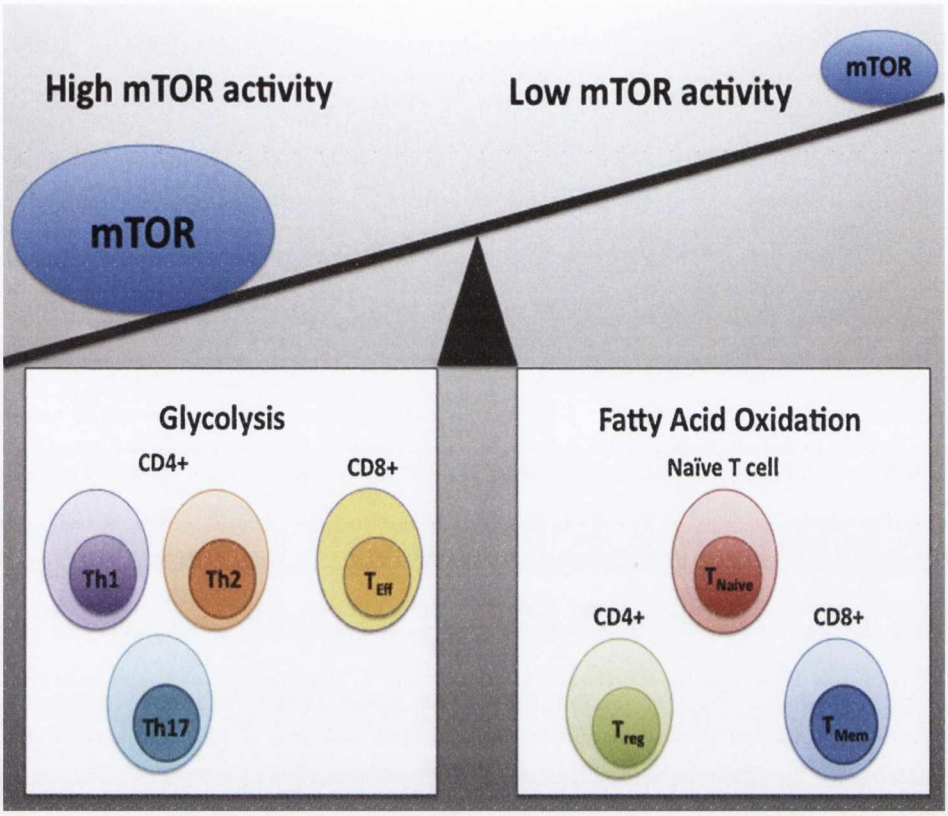


Fig.1.4 A graphical representation of the emerging view in the field of T cell metabolism. T cells that are not very metabolically active and are slow proliferators, such as naïve, regulatory and memory T cells, have low mTOR activity and rely on fatty acid oxidation to meet their metabolic demands. In contrast activated effector T cells that are rapidly dividing, such as Th1, Th2, Th17 and CD8+ T cells, have high mTOR activity and switch their metabolism towards aerobic glycolysis (Heikamp and Powell, 2012).

1.6 mTORC1 controls DC function

DCs are known to increase their rate of glycolysis upon activation. Indeed inhibiting glycolysis by adding 2DG blocks DC activation (Krawczyk et al., 2010). Rapamycin treatment also blocks the shift to glycolysis in DC's (Amiel et al., 2012), however the production of pro-inflammatory cytokines is increased, such as IL-12, IL-6, IL-23 and TNFα, and the expression of the anti-inflammatory cytokine IL-10 is reduced (Ohtani et al., 2008, Weichhart et al., 2008, Haidinger et al., 2010). mTORC1 signaling promotes IL 10 expression and negatively regulates IL 12 expression. This was initially found to be a PI3K/AKT controlled signaling event but recently it has been found that there is both a PI3K/AKT dependent and independent pathway, with the independent pathway signaling to mTORC1 by MAPK protein p38α (Fukao et al., 2002, Katholnig et al., 2013a). This is illustrated in Fig.1.5. Control of the NF-κB signaling pathway by mTORC1 has been linked to its involvement in pro-inflammatory cytokine expression, while mTORC1 promotion of STAT3 signaling links it to IL 10 expression (Weichhart et al., 2008). This increase in pro-inflammatory cytokines is also seen in macrophages (Schmitz et al., 2008).

Rapamycin treated DC's also have increased expression of co-stimulatory molecules and increased ability to stimulate T cells (Amiel et al., 2012, Haidinger et al., 2010). This suggests that mTORC1 plays an anti-inflammatory role in DC's and macrophages. This is backed up from clinical studies of patients treated with rapamycin following kidney transplantation. Patients have increased risk of some inflammatory conditions and show elevated levels of pro-inflammatory cytokines in their serum (Saemann et al., 2009). As described in section 1.5 a shift to glycolysis, regulated by mTORC1, is directly linked to the pro-inflammatory effector functions of CTLs. However in DC's and macrophages mTORC1 signaling appears to be anti-inflammatory. A reason for this discrepancy may have been explained in a recent paper looking at the initial metabolic events after TLR stimulation in DC's. This study found that glycolysis is increased minutes after stimulation. This required the kinases tank-binding kinase 1 (TBK1), IκB kinase ε (IKKε) and AKT but was independent of mTORC1 (Everts et al., 2014). A study looking at DC metabolism after 24 hours found that rapamycin inhibited the shift to glycolysis

(Amiel et al., 2012). This suggests that an initial increase in glycolysis is required for normal DC activation independent of mTORC1 whereas mTORC1 becomes important later after activation. In macrophages a shift to glycolysis has been shown to be HIF-1α dependent. In these cells deleting HIF-1α specifically in myeloid cells blocked increased glucose consumption and lactate production while also inhibiting cytokine production (Cramer et al., 2003).

TLR activation promotes HIF-1α expression in macrophages that has shown to be NF-κB dependent (Ramanathan et al., 2009). Recently the shift to glycolysis in DC's has also been shown to be HIF-1α dependent following activation by type I IFNs (Pantel et al., 2014). TLR stimulation has been shown to promote HIF-1α protein expression in DC's (Spirig et al., 2010). Knockdown of HIF-1α by siRNA inhibits DC activation while deleting this gene leads to decreased expression of type I interferons (Wobben et al., 2013). A recent study has shown that the initial increase in glycolysis following TLR stimulation is HIF-1α independent (Everts et al., 2014). Recently a direct link between metabolism, through regulation of HIF-1α, and macrophage effector function was suggested. The TCA cycle intermediate succinate has been shown to be an inflammatory signal. Succinate builds up in activated macrophages, stabilizes HIF-1α that then promotes the expression of IL-1β (Tannahill et al., 2013). However how mTORC1 controlled DC metabolism is linked to the immunoregulatory function for mTORC1 in DC's has not been directly investigated.

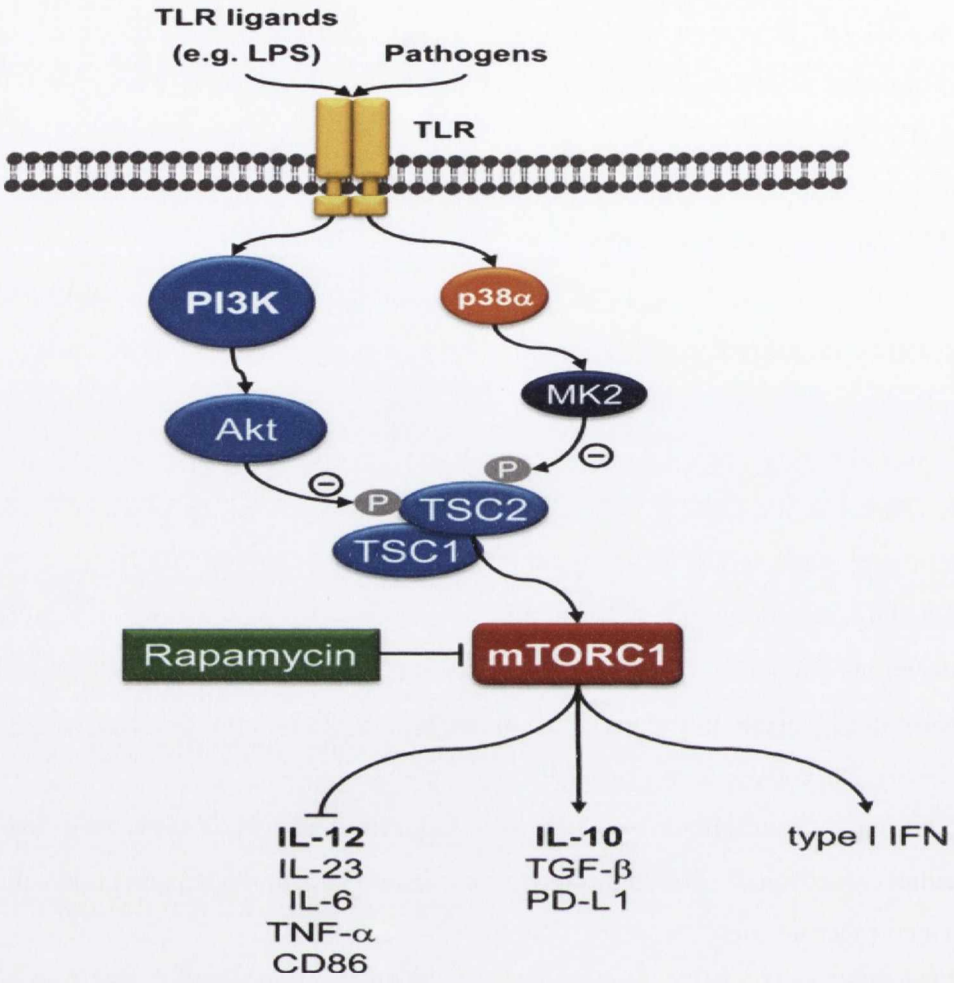


Fig.1.5 mTORC1 activation in dendritic cells by TLR stimulation is an anti-inflammatory signaling event. TLR stimulation can signal to mTORC1 in two ways. It can signal by the established PI3K/AKT signaling axis that phosphorylates TSC2 and promotes mTORC1 activation. An alternative pathway exists that relies on MAPK p38α. This kinase phosphorylates MAPK-activated protein kinase 2 (MK2) that in turn phosphorylates TSC2 and activates mTORC1. These pathways concurrently modulate the expression of IL 10 and IL 12 downstream of mTORC1. IL 10, TGF-β, programmed death ligand 1 (PD-L1) and type I interferons are positively regulated by mTORC1. IL 12, IL 23, IL 6, TNF-α and CD86 are negatively regulated by mTORC1 (Katholnig et al., 2013b).

1.7 Aims and objectives

It is known that DCs undergo a metabolic shift upon activation (Krawczyk et al., 2010). However the precise metabolic regulators involved in this process have not been determined. Given recent data linking metabolism directly to immune cell function it is important to investigate if there is a direct link between metabolism and function in DCs (Chang et al., 2013, Finlay et al., 2012). Nutrient availability is starting to be appreciated as a critical determinant for cellular function particularly in T cells. However very little is known about how nutrient availability impacts on DC metabolism and if it plays a role in DC effector function.

- Investigate the key metabolic regulators involved in DC metabolism
- Does nutrient availability have an effect on DC metabolism and function?
- Investigate if there is a direct link between DC metabolism and effector function?

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Chemicals and Equipment

LPS from E. *coli*, Serotype EH100 (Enzo lifesciences), Rapamycin (Fisher Scientific), Dimethyloxaloyglycine (DMOG) (VWR), S-Nitroso-N-Acetyl-D,L-Penicillamine (SNAP) (Sigma), S-ethyl Isothiourea (SEITU) (Cayman Chemicals), GM-CSF (Peprotech) were compounds used on cells in cell culture studies. QSCRIPT cDNA Supermix was from Quanta Biosciences. RNAeasy kit was from Qiagen. Taq-man gene expression assay kit (Applied Biosystems) and Perfect CTa SYBR Green FastMix medium ROX reagent were the materials used for real-time PCR reaction. Ammonium persulphate (Sigma-Aldrich), Glycine (Fisher Scientific), Tris (Acros Organics), Bovine serum albumin (BSA – Sigma-Aldrich), Phosphate buffered saline (PBS – Sigma-Aldrich), Ammonium persulphate (Sigma-Aldrich), 30% Acrylamide A3699 (Sigma-Aldrich), TEMED (Sigma-Aldrich), Tween (Croda International), Sodium dodecyl-sulphate (Sigma-Aldrich) were chemicals used for western blot. Electrophoresis running system was from Atto and transfer system was the XCELL II Blot Module from Invitrogen. ECL blotting reagent was from Pierce. X-ray film for blot development was from Fujifilm. XF24 Extracellular Flux Analyzer from Seahorse Bioscience was used for metabolic studies. 2-Deoxy-D-Glucose, FCCP, Oligomycin, Antimycin A and Rotenone were from Sigma. Cell culture medium was from Gibco. Fetal calf serum was from Biosera. Cell culture dishes and flasks were from Nunc.

2.1.2 Antibodies

2.1.2.1 Western Blot

S6 ribosomal protein, phospho-S6 S235/236, phospho-S6K Thr 389, ribosomal S6K, phospho-4E-BP1 were all purchased from Cell signaling. NOS2 (iNOS) antibody was from Santa Cruz Biotechnology Inc., HIF-1α was from Novus Biologicals. SMC1 was purchased from Bethyl Laboratories Inc.

Antibody	Source	Dilution	2^{O} Ab
S6 ribosomal protein	Cell Signalling	1/5000 5% BSA/PBST	Rabbit
phospho-S6 S235/236	Cell Signalling	1/5000 5% BSA/PBST	Rabbit
phospho-p70 S6K Thr 389	Cell Signalling	1/5000 5% BSA/PBST	Rabbit
S6K	Cell Signalling	1/5000 5% BSA/PBST	Rabbit
phospho-4E-BP1	Cell Signalling	1/5000 5% BSA/PBST	Rabbit
NOS2(iNOS)	Santa Cruz Biotechnology Inc.	1/200 5% BSA/PBST	Mouse
HIF-1α	Novus Biologicals	1/5000 5% Milk	Rabbit
SMC1	Bethyl Laboratories Inc.	1/5000 5% Milk	Rabbit
PKB	Cell Signaling	1/5000 5% BSA/PBST	Rabbit

Table 2.1 Western blot antibodies

2.1.2.2 FACS antibodies

Primary phospho-S6 antibody used was same antibody used for western blot (Cell Signaling). R-Phycoerythrin-conjugated Anti-Rabbit IgG was purchased from Jackson ImmunoResearch Laboratories Inc. Anti-mouse CD3 FITC, Anit-mouse CD40 PerCP-eFluor 710, Anti-mouse CD19 PE-Cy 7, ~Anti-mouse TCRβ APC were all from eBiosciences. Anti-mouse CD11c APC, Anti-mouse CD11c BV421, Anti-mouse CD80 FITC and Anti-mouse CD86 PE were all purchased from BD biosciences

Antibody	Flurophore	Source	Dilution
CD3	FITC	eBiosciences	1/200
TCRβ	APC	eBiosciences	1/200
CD19	PE-Cy7	eBiosciences	1/200
CD40	PerCP-eFluor 710	eBiosciences	1/420
CD11c	APC	BD biosciences	1/420
CD11c	BV421	BD biosciences	1/420
CD80	FITC	BD biosciences	1/700
CD86	PE	BD biosciences	1/700
MHCII	A780	eBioscience	1/420
Anit-Rabbit IgG	PE	Jackson ImuunoResearch Laboratories Inc.	1/100
phospho-S6 S235/236	Primary	Cell Signaling	1/100

Table 2.2 FACS antibodies

2.1.3 Buffers

Buffer	Composition
Running Buffer	25mM Tris, 192mM Glycine, 0.1% SDS
Transfer Buffer	25mM Tris, 192mM Glycine, 10% methanol
Lysis Buffer	10mM Tris pH 7.05, 50nM NaCl, 30mM Na pyrophosphate, 50mM NaF, 5μM ZnCl2, 10% glycerol, 0.5% Triton X100
Stripping Buffer	0.7% β-mercaptoethanol, 2% SDS, 62.5mM Tris
4X SDS-PAGE Sample Buffer	240mM Tris/HCl pH 6.8, 40% Glycerol, 8% SDS, 5% β-Mercaptoethanol, 0.04% bromophenol blue

Table 2.3 Buffers

2.1.4 Animals

C57BL/6J mice were purchased from Harlan (Bicester, U.K.) and maintained in compliance with Irish Department of Health and Children regulations and with the approval of the University of Dublin's ethical review board. A competent and licensed researcher carried out all animal-handling procedures.

2.2 Methods

2.2.1 Cell Culture

Cells were maintained in RPMI 1640 medium with L-Glutamine, supplemented with 10% foetal calf serum (FCS) and 1% Penicillin/Streptomycin (Pen/Step) antibiotic solution (hereon referred to as culture medium). For glucose deprivation or galactose experiments RPMI 1640 glucose free medium with L-Glutamine was used, supplemented with 10% dialysed FCS, 1% Pen/Strep, 1% Vitamin Cocktail and 1% Insulin/Selenium/Transferrin with glucose/galactose added to required concentrations. For amino acid manipulation experiments Hank's Balanced Salt Solution (HBSS) media was used supplemented with 10% dialysed FCS, 1% Vitamin Cocktail and 1% Insulin/Selenium/Transferrin and 75mM HEPES were appropriate with amino acids added to required concentrations.

All cell culture media were warmed to 37°C before use.

2.2.2 Bone Marrow derived Dendritic Cells (BMDC's)

BMDC's were isolated as previously described (Lutz et al., 1999). Briefly Tibiae and Femurs from sacrificed C57/BL6 mice were flushed through with culture medium through a 70μm nylon mesh cell strainer (Fisher). Isolated cells were centrifuged for 3 minutes at 300g and the supernatant discarded. The pellet was suspended for 1 minute in 1ml ammonium chloride (1.5%) to selectively lyse red blood cells. The suspension was made to 30ml with culture medium to terminate osmotic stress and centrifuged at 300g for 3 minutes. Pelleted cells were re-suspended in 10ml culture medium and counted. 30-38ml of cells in culture

medium were seeded in culture flasks at $4x10^5$/ml with 20ng/ml GMCSF. Flasks were incubated in a 5% CO_2 maintained incubator at 37°C.

Cells were fed on days 3 and 8 with 30ml culture media containing GMCSF (20ng/ml) added directly to culture flask. On day 6 all media was removed from the flask leaving adherent cells, and 30ml culture medium with GMCSF (20ng/ml) was added. On day 10 culture flasks were agitated and the contents collected to harvest cells. The suspension was centrifuged at 1400rpm for 3 minutes and pelleted cells suspended in 10ml culture medium for counting. Mature BMDCs were then plated according to experimental parameters. This procedure resulted in $CD11c^+$ DCs at consistently >90% purity.

2.2.3 Stimulation of cells

Mature BMDCs were plated at $1x10^6$/ml in culture media supplemented with 1ng/ml GMCSF and incubated in a 5% CO_2 maintained incubator at 37°C. Cells were allowed to settle for 4 hours for same day stimulation or 24 hours for next day stimulation. On stimulation half the culture medium was extracted from the plates and replaced with culture medium containing the reagents of interest and GMCSF at 1ng/ml. LPS was used at 100ng/ml, rapamycin at 20nM, DMOG at 200μM, SNAP at 250μM, SEITU at 500μM.

2.2.4 Nutrient Deprivation of Bone Marrow derived Dendritic Cells

Glucose deprivation experiments were carried out in glucose free media containing additives as decribed in section 2.2.1. BMDC's plated at required concentrations were stimulated as normal for 8 hours in culture media. Following this stimulation media was removed and cells were washed twice with glucose free media. Cells were then left in glucose free media with desired glucose/galactose concentrations added for time points up to 3 days after initial DC stimulation with 1ng/ml GM-CSF. For amino acid manipulation experiments HBSS media was used. Freshly isolated BMDC's at $2x10^6$/ml were added to an eppendorf tube. These were spun down at 300g for 3 min at 4^OC and washed twice with HBSS. These cells were then resuspended in HBBS with supplements and desired amino acids at required

concentrations, L-Arginine at 1.15mM, L-IsoLeucine at 0.382mM, L-Leucine at 0.382mM, L-Valine at 0.171mM and L-Glutamine at 2.05mM.

2.2.5 Protein Extraction

Following stimulation for indicated time points media was removed from wells and spun down at 300g for 3 mins. 1X SDS-PAGE sample buffer (made up with lysis buffer) was added to wells ($10x10^6$ cells/ml sample buffer) and cells were removed from well by scraping using a cell scraper into an eppendorf. Media that had been spun down was poured off and half of sample buffer was added to cell pellet. This was then combined with sample buffer from wells to give complete cell lysis. Samples were then heated to 90°C for 15 minutes before freezing for subsequent use.

2.2.6 Sodium Dodecyl-Sulphate Poly-Acrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE is a technique resulting in the separation of proteins based in their molecular weight. SDS is an anionic detergent that wraps around a polypeptide backbone after heating, at a constant weight ratio. This yields a constant mass/charge ratio for all proteins, resulting in a logarithmic relationship between the speed of migration through polyacrylamide gel and the molecular weight of the protein. Cell lysates prepared in SDS sample buffer (1× final concentration) were used. The gels were run using the AE6450 system from Atto Corporation. The recipe for 10% gels is shown in table, 8% and 14% gels were also used depending on protein size.

	Reagent	Composition
Running Gel	Tris (pH 8.8)	0.375M
	SDS	0.10%
	Acrylamide	10%
	APS	0.075%
	TEMED	0.10%
Stacking Gel	Tris (pH 6.8)	0.125M
	1% SDS	0.10%
	30% acrylamide	4%
	1.5% APS	0.075%
	TEMED	0.10%

Table 2.4 SDS page gel recipes

2.2.7 Western Blot

To transfer protein to nitrocellulose membrane the gels were removed from the plates and assembled into a gel-membrane sandwich in the transfer apparatus as dictated in the manufacturer's protocol. Nylon sponge pads, Whatmann 3-MM chromatography paper and Hybond-C extra Nitrocellulose membrane (Amersham Biosciences) was soaked in transfer buffer containing 10% methanol. The sandwich was loaded into the transfer apparatus with the nitrocellulose membrane on the side of the anode. Proteins were transferred at 45V for 2 hours or 15V overnight. To determine quality of transfer, membranes were stained with Ponceau S solution for 30 seconds and rinsed with water. Remaining Ponceau S was removed during the blocking step prior to primary antibody addition. Membranes were blocked in a 5% milk/PBST solution for 40 minutes at room temperature then washed twice in PBST for 5 minutes. The membrane was subsequently incubated in primary antibody (concentration and solution given in table) for 4 hours at room temperature, washed (3 washes of 5 minutes in PBST) and transferred to appropriate secondary antibody (1/5000 in 5% milk/PBST) for 2 hours at room temperature. Blots for HIF-1α were

incubated with antibodies and washed at 4°C. After washing (3 washes of 5 minutes in PBST) Pierce ECL 2 Western Blotting Substrate was applied to the membrane in a developing plate and luminescence assessed by photographic film.

2.2.8 RNA Extraction

Following stimulation media from each well was removed and centrifuged at 300g for 5 minutes at 4°C. RLT buffer (with 1% β-Mercaptoethanol) from Qiagen RNAeasy kit was added (600µl up to $1x10^7$ cells) to samples in wells. Half lysed cellular mix was added to spun cell pellet and pooled with original sample. Equal part 70% ethanol was added and RNA purification was performed according to manufacturer's instructions. The final RNA preparation was snap frozen using dry ice in methanol and stored at -80°C.

2.2.9 Measurement of RNA concentration

RNA preparations were measured for purity and RNA concentration using the Nanodrop spectrophotometer (ND-1000). 2µl sample were added onto the podium after a blank reading using RNAse free water and concentration, 260/280 and 260/230 absorption readings were measured. If the two measurements were above 1.8 the sample was deemed pure enough for RT-qPCR analysis.

2.2.10 cDNA Synthesis

cDNA was synthesized in 20µl aliquots from 1µg RNA using 4µl q-Script (Quanta Biosciences) according to the manufacturer's instructions.

The technique generates complimentary double stranded DNA for the RNA collected; using reverse transcriptase for complimentary single stranded DNA synthesis and DNA polymerase to convert this to double stranded DNA.

2.2.11 Designing primers for gene analysis

Primers were designed using the Primer Blast software. The transcript ID for the gene of interest was entered into Primer Blast. The parameters for the primers were set such that; had a length between 190-210bp, a Tm between $58^{O}C$-$62^{O}C$ with a max Tm difference of $2^{O}C$ and must span an exon-exon junction. The primers for genes used are given below:

Primers:

Glut1 for	GGAATCGTCGTTGGCATCCT
Glut 1 rev	CGAAGCTTCTTCAGCACACTC
LDHa for	CTGGGAGAACATGGCGACTC
LDHa rev	ATGGCCCAGGATGTGTAACC
Rplp0 for	CATGTCGCTCCGAGGGAAG
Rplp0 rev	CAGCAGCTGGCACCTTATTG
IL12 for	AGTCCCGAAACCTGCTGAAG
IL12 rev	GCTCCCTCTTGTTGTGGAAGA
IL10 for	CCTGCTCTTACTGACTGGCA
IL10 rev	CCAAGTAACCCTTAAAGTCCTGC
PHD3 for	CTGGTCCTGTACTGCGGGAG
PHD3 rev	GACCCCTCCGTGTAACTTGG
NOS2 for	GTCAACTGCAAGAGAACGGAGAAC
NOS2 rev	CTGCGGGGAGCCATTTTG
PFKL for	GCGGCGTGTGTTCATTGTAG
PFKL rev	CACTTCTCGTTCCGGAGCAC
PKM1 for	AAACAGCCAAGGGGGACTAC
PKM1 rev	TTATAAGAGGCCTCCACGCTG
PKM2 for	GCTATTCGAGGAACTCCGCC
PKM2 rev	AAGGTACAGGCACTACACGC

2.2.12 SYBR Green system for RT-qPCR analysis

SYBR Green system (Quanta Biosciences) was used with Perfect CTa SYBR Green FastMix medium ROX reagent. This system uses a method whereby SYBR green dye fluoresces when bound to double stranded DNA. Sequence amplification during PCR cycles results in a net increase in fluorescence that can be detected by the cycler. Each reaction consisted of 5µl FastMix reagent with 10pmol For and Rev primer with 100ng cDNA made up to 10µl with RNAase free water. Samples were ran on a 7900HT Fast Real Time PCR system (Applied Biosciences). Derived values were normalized to the expression of the control gene Rplp0, which is a ribosomal protein. Sample preparation and amplification cycles were performed as per the manufacturer's instructions.

2.2.13 Flow Cytometry Analysis

After stimulation, cells were flushed from wells by repeat pipetting and put into 5ml FACS tube. Cells were washed 1X with PBS buffer and spun down at 300g for 3 mins. For pS6 staining cells were resuspended in 1ml 0.5% paraformaldehyde (PFA) to fix cells for 15mins at 37^{O}C. Cells were then washed 2X with PBS buffer, resuspended in 90% methanol to permeabilise cells and placed at -20^{O}C for 15mins. Cells were then washed 1X in FACS buffer (PBS with 20% RPMI media) and resuspended in 100µl FACS buffer containing 1% FC block and left for 10 mins at 4^{O}C. Primary pS6 antibody was added to cells in 5ml FACS tube at 1/100 dilution plus required extracellular antibodies at concentrations indicated in table 2.2. Tubes with antibodies were left at 4^{O}C for 20 mins. Cells were then washed 1X with FACS buffer, resuspended in 100µl FACS buffer containing PE anti-rabbit secondary at 1/100 dilution and left at 4^{O}C for 20 mins. Cells were washed 1X with FACS buffer and samples were ran on a FACS Canto II(BD) using FACSDiva software and data was analysed using FlowJo^{TM} software (Treestar, Oregan).

2.2.14 Glucose uptake single cell assay

After stimulation of cells with required reagents cells were flushed from wells by pipetting, put in 5ml FACS tubes and spun down at 300g for 3 mins. They were washed 2X with glucose free media, resuspended in 500µl glucose free media containing 100µM 2-(*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NDBG) and left at 37^{O}C for 1 hour. Following this incubation cells were washed 2X in FACS buffer and protocol for normal FACS staining was followed as previously described, though these cells were never fixed.

2.2.15 Metabolic Flux analysis with Seahorse XF^{e}24 Extracellular Flux Analyser

Mature BMDCs were plated ($4x10^5$ cells/well) in Seahorse culture plate (pre-coated with Poly-L-Lysine to adhere cells) in 0.25ml culture media with GMCSF at 2ng/ml. After 1 hour a further 0.25ml culture media was added to wells to stimulate cells with media also containing reagents as required with 2ng/ml GM-CSF. After 20 hours stimulation media was removed and replaced with XF media (Seahorse Bioscience) supplemented with GMCSF (2ng/ml) and glucose (10mM); for appropriate wells DMOG and rapamycin were also added to continue inhibition. 2ng/ml GMCSF was used as results in the laboratory suggested unstimulated cells became less viable with 1ng/ml under conditions in the Seahorse assay. The cell plate was kept at 37°C for 30 minutes in a non-CO_2 maintaining incubator before insertion into the Seahorse XFe24.

2.2.16 Data Manipulation for Seahorse analysis

The Seahorse XFe24 takes measurements of the extracellular acidification rate (ECAR) and the oxygen consumption rate (OCR) every 7.5 minutes. Over the course of analysis four inhibitors are added to determine which processes in metabolism are responsible for the ECAR and OCR rates, four initial measurements are taken, then two at each inhibition state. The inhibitors, added in the listed order, are oligomycin (inhibits the F_0/F_1 ATPase), p-trifluoromethoxy carbonyl cyanide phenyl hydrazine (FCCP – an uncoupling agent), antimycin A & rotenone (inhibit

complex 3 and 1 respectively - blocking oxidation of NADH and succinate) and 2-deoxy-D-Glucose (2DG - which inhibits glycolysis).

The basal rate of cells ECAR and OCR was taken from the last of the four initial measurements, to allow cells and the plate (which absorbs CO_2 and O_2) to equilibrate. As seen in figure 2.1 the addition of Antimycin A and Rotenone gives the minimal OCR in the system. To calculate the use of oxygen by oxidative phosphorylation the average of the OCRs under antimycin A/rotenone inhibition was subtracted from the basal OCR. The addition of 2DG inhibits glycolysis such that the minimum rate of glycolysis can be observed (Fig 2.2). For the acidification due to glycolysis the average of ECAR under 2DG inhibition was subtracted from the basal PPR. The addition of oligomycin inhibits F_0/F_1 ATPase which allows for the maximal rate of glycolysis to be observed (Fig 2.2). The rate for Glycolytic capacity was determined by getting the average rate from samples after the addition of oligomycin and taking the rate following 2DG inhibition from this. The ratio of ECAR/OCR was determined by dividing the glycolytic rate by the oxygen consumption rate.

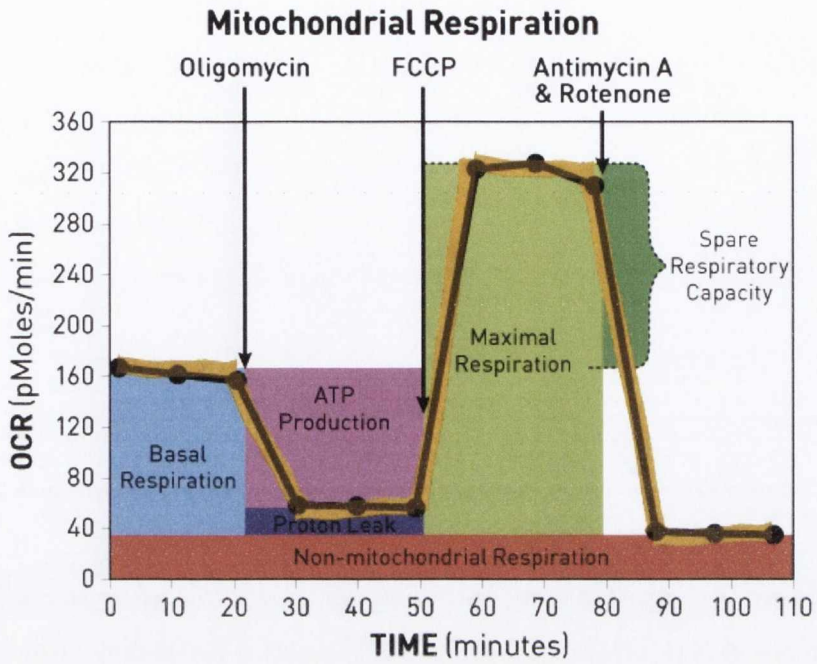


Fig 2.1 Example of direct OCR measurements taken by XFe24 metabolic Flux Analyser

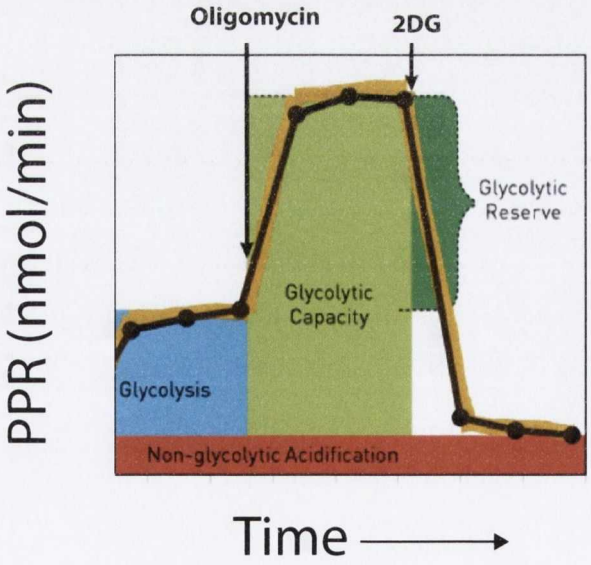


Fig 2.2 Example of direct PPR measurements taken by XFe24 metabolic Flux Analyser

2.2.17 Co-culture of OT-I CD8+ T-cells with BMDC's *in vitro*

Day 10 BMDC's were plated in a 24 well plate at $2x10^5$ cells/well and incubated for 6 hours with 1ug SIINFEKL OVA peptide and 2ng/ml GM-CSF. After the the incubation time DCs were washed and MHC class I-restricted, ovalbumin-specific, CD8+ T cells (OT-I cells) were isolated from spleen and lymph nodes of transgenic mice. Spleen and lymph nodes were mashed through a 70μm nylon mesh cell strainer. Red blood cells were lysed by the addition of 2ml sterile H_2O and immediately quenched by the addition of 10ml culture media. T cells were isolated using the Pan T cell Isolation Kit II (Miltenyi Biotec Inc.) and >90% CD8+ T cells were confirmed by FACS analysis. MACS buffer used was PBS containing 3.2mM EDTA. These OT-1 cells were then added to the wells containing BMDC's at required ratios. Following a 20-hour co culture cells were flushed from wells by pipetting and put in 5ml FACS tubes. The protocol for pS6 staining, which has previously been described, was then applied to these cells. If cells were to be analyzed for proliferation they were stained for flow cytometry as previously described. If IFNγ production was to be measured PMA (20ng/ml) and ionomycin (1ug/ml) and Golgi plug™ was added to T cell wells 4hours before removing and cells were stained for intracellular IFNγ.

2.2.18 CFSE staining

Cells were counted, put into a 15ml conical centrifuge tube and spun at 300g for 4 mins. Media was removed and cell pellet was washed once with 0% RPMI media, without disturbing the pellet. Cells were spun again and resuspended in 2mls 0% RPMI media containing 10uM CFSE (Invitrogen). Cells were left at $37^{O}C$ for 10 mins. CFSE staining was stopped by the addition of 10mls 10% media to cells. Cells were spun again at 300g 4 mins and counted.

2.2.19 *In vivo* model for T cell and BMDC interaction

Day 10 BMDCs were activated in a 50ml falcon tube with 100ng/ml GM-CSF and 1ug SIINFEKL OVA peptide for 1 hour. Cells were washed two times with RPMI

media and stained as described in 2.2.18 with cell tracker violet (Invitrogen). Following staining BMDCs were resuspended in PBS at a concentration of $50x10^6$. 2.5x105 DCs were injected into the lateral tarsal region using the Hock immunization method(Kamala, 2007). OT-1 specific T cells were isolated using MACS. These were then CFSE stained as described in 2.2.18 and resuspended in PBS. Mice were injected IV with different amounts of T cells ranging from $5x10^6$ to $2x10^5$. The following day popliteal and inguinal lymph nodes were isolated from injected mice and stained for pS6 as previously described.

2.2.20 Statistical Analysis

Data was analyzed using Graphpad Prism version 6.01. Data is presented in graphs as mean + SEM except when a duplicate dataset demands mean + ½ range. Multiple comparisons for large groups used ANOVA where variances were considered equal, with Turkey tests to compare all groups. Student T tests were used when comparing two datasets. Differences were considered significant at $p<0.05$

Chapter 3

A complex signaling circuit centered on mTORC1, HIF-1α and iNOS controls DC metabolism

3.1 Introduction

Otto Warburg discovered during the 1920s that cancer cells used anaerobic or "Warburg" metabolism to meet their energy demands, work for which he received a Nobel Prize (Vander Heiden et al., 2009). He had also shown that mixed lymphocytes used this form of metabolism, although the level of understanding of Immunology at the time prevented any more in depth experimentation. Recently the metabolism of immune cells has gained huge interest and the field of "Immunometabolism" has emerged. Many immune cells undergo a metabolic shift towards elevated glycolysis when activated including Th1, Th2, Th17 and cytotoxic T cells, NK cells and macrophages (Donnelly and Finlay, 2015). Dendritic cells also undergo a metabolic shift towards increased glycolysis and this metabolic shift has been shown to be required for DC activation. However the metabolic regulators involved in this process have yet to be fully established.

This chapter investigates the metabolic profile of activated DCs and characterizes the signaling molecules involved. In other immune cells the metabolic regulators involved have been identified. Studies in other immune cells have identified a number of key regulators. mTORC1 is a nutrient sensor that regulates various aspects of cellular metabolism, including glycolysis in both NK and T cells(Liu et al., 2015, Donnelly et al., 2014). The transcription factor c-Myc is important for early metabolic reprogramming in T cells while HIF-1α is important in IL-2 mediated reprogramming (Wang et al., 2011, Finlay et al., 2012). HIF-1α is also important in activated macrophage glycolytic metabolism (Tannahill et al., 2013). Both these transcription factors promote the mRNA expression of glucose transporters and glycolytic enzymes. This chapter investigates if these metabolic regulators play a role in DC metabolism.

3.2.1 The mTORC1 signaling pathway is activated in LPS stimulated BMDC's and Splenic DC's

The mTORC1-signaling pathway has previously been shown to be important in other immune cells for both control of metabolism and effector function (Xu et al., 2012). In DC's mTORC1 is known to be involved in cytokine production and co stimulatory molecule expression, though results are variable (Ohtani et al., 2008, Weichhart et al., 2008, Haidinger et al., 2010). The TLR4 agonist LPS is a potent signaling molecule which activates DC's. The question was asked if mTORC1 is active in LPS stimulated DCs. Splenic DC's and BMDC's were stimulated with LPS and mTORC1 signaling was measured either by western blot or a flow cytometry single cell assay. In splenic CD11c-hi/MHCII-hi DC's pS6 (phosphorylated ribosomal protein S6) was measured by intracellular staining. The level of mTORC1 activity can be assessed through the analysis of the levels of phosphorylated S6 ribosomal protein on Ser235/6 (pS6). S6 is phosphorylated by p70S6 kinase that is in turn directly activated by mTORC1 (Fig 3.1a). Inhibition of mTORC1 with rapamycin rapidly turns off downstream signaling resulting in the loss of pS6 within minutes and serves as a negative control for mTORC1 activity. Basal DC's stain predominantly negative for pS6 with a small proportion containing low levels of pS6 staining. The addition of LPS for 3 hours causes a large shift in pS6 staining in splenic DC's. This shift is completely blocked by the addition of the mTORC1 inhibitor rapamycin (Fig 3.1b) thus confirming pS6 staining reflects mTORC1 activity. In BMDC's stimulated for 10, 30, and 60 minutes with LPS there is a time dependent increase in phosphorylation of the mTORC1 substrate pS6K(T389) compared to BMDC's with no LPS stimulation (Fig 3.1c).

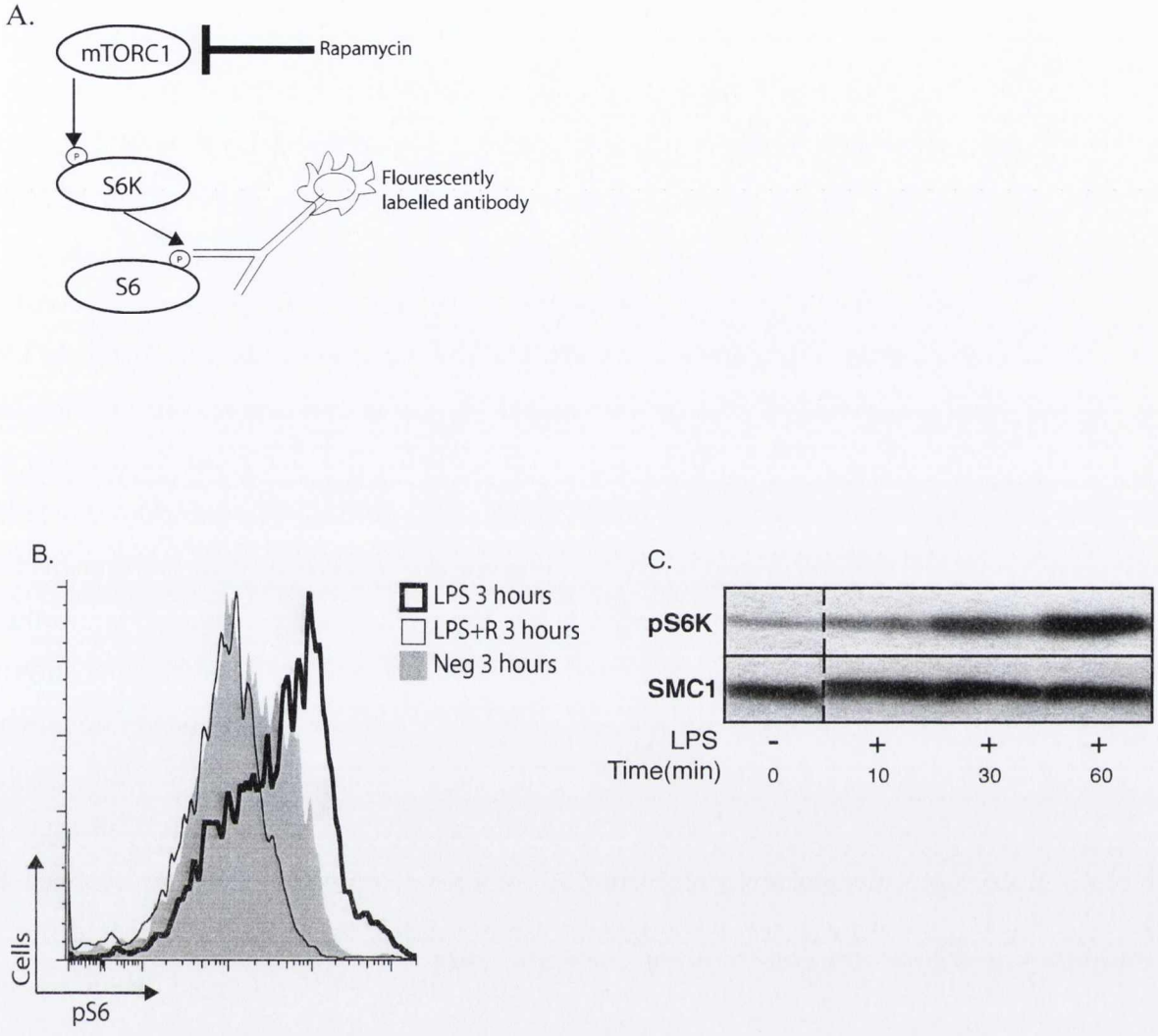


Fig 3.1. LPS stimulation of BMDC's and splenic DC's induces mTORC1 activity. (A) A schematic of the downstream signaling from mTORC1 to pS6 (B) Freshly isolated whole splenocytes were left unstimulated or stimulated for 3 hours with LPS (100ng/ml) +/- RAPA (20nM) and analysed by flow cytometry. (C) 10 day BMDC's were activated with LPS (100ng/ml) for the given time points and analysed for pS6K expression by western blot.

3.2.2 mTORC1 is required for the metabolic shift in activated BMDC's

Activated, effector T cells, B cells, macrophages and dendritic cells all undergo a metabolic shift towards increased glycolysis (Palsson-McDermott and O'Neill, 2013). This glycolysis takes place in an oxygen rich environment and is called aerobic glycolysis or Warburg metabolism, as seen in cancer cells. In effector T cells mTORC1 is necessary for this metabolic shift but the pathways involved in other immune cells are less well established (Waickman and Powell, 2012). In LPS stimulated dendritic cells an increase in glycolysis and decrease in OxPhos had previously been published (Krawczyk et al., 2010). This first paper showed that the metabolic shift was dependent on the PI3K/AKT pathway. We undertook a detailed study of the role for mTORC1 in the metabolic changes that accompany DC activation. LPS stimulated BMDC's were analyzed on the Seahorse Extracellular Flux Analyzer^{TM} in the presence or absence of the mTORC1 inhibitor rapamycin. The Seahorse machines measure simultaneously dissolved oxygen and free protons in a small volume above cells. This can then be used to calculate oxygen consumption rate (OCR) and extra cellular acidification rate (ECAR) a measure of OxPhos and glycolysis respectively. Additional information can be obtained by adding certain metabolic inhibitors, which allows the identification of different metabolic rates (see section 2.2.16). Unstimulated BMDCs have an ECAR reading of approximately 20mpH/min, demonstrating that these cells are quite glycolytic even under resting conditions. However with the addition of LPS these cells significantly increase their glycolytic rate showing that they are undergoing a metabolic switch towards elevated glycolysis. Adding rapamycin completely blocked this increase in glycolysis showing the requirement of mTORC1 in this process (Fig3.2a,c). The addition of the ATP synthase inhibitor oligomycin allows the calculation of the maximal rate of glycolysis for the cell or the glycolytic capacity. Oligomycin blocks ATP synthase and thus there was a decrease in OCR. FCCP uncouples the electron transport chain and allows the measurement of maximal oxygen consumption while the addition of antimycin A and rotenone blocks the electron transport chain (Fig 3.2b). By taking the rate following antimycin A/rotenone addition from the basal OCR rate you get a measure for oxidative phosphorylation (OxPhos). The addition of LPS blocked mitochondrial oxygen consumption and hence a block in OxPhos, demonstrated by the

characteristic flattening in the seahorse trace for LPS stimulation in Fig3.2b. Stimulating BMDCs with LPS in the presence of rapamycin blocked this decrease in OxPhos showing that mTORC1 was required in this process (Fig3.2d). Unstimulated and LPS treated BMDCs have a similar glycolytic capacity, however rapamycin treated BMDCs have a decreased glycolytic capacity showing that rapamycin was affecting the glycolytic machinery within the cell (Fig3.3a). Oligomycin blocks ATP synthase and thus there was a decrease in OCR. FCCP uncouples the electron transport chain and allows the measurement of maximal OCR or oxidative phosphorylation. Addition of rotenone blocks complex I and antimycin A blocks complex III allowing measurement of OCR when mitochondrial OxPhos is completely inhibited. Dividing the ECAR rate by the OCR rate gives you a ratio that indicates the relative amounts of glycolysis to OxPhos the cell is undergoing at any one time. Using this measurement it was clear that LPS stimulation of BMDCs induces a metabolic shift towards elevated glycolysis that is dependent on mTORC1 signaling (Fig3.3b).

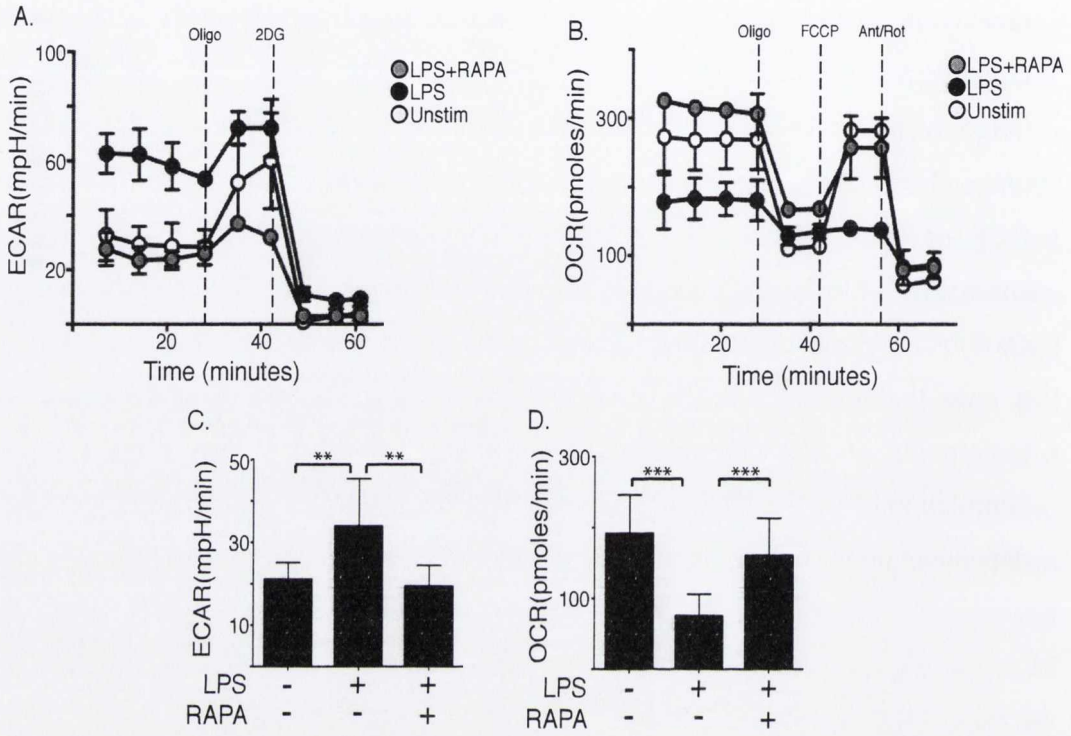


Fig 3.2 mTORC1 is required for the metabolic shift in LPS stimulated BMDCs, representative Seahorse traces. (A-D) BMDCs stimulated for 24 hours +/- LPS (100ng/ml), +/- RAPA (20nM) and analyzed using a Seahorse Extracellular Flux analyzer. (A-B) Representative traces for ECAR and OCR. (C-D) Pooled data N=5 ***p<0.001, **p<0.01, *p<0.05.

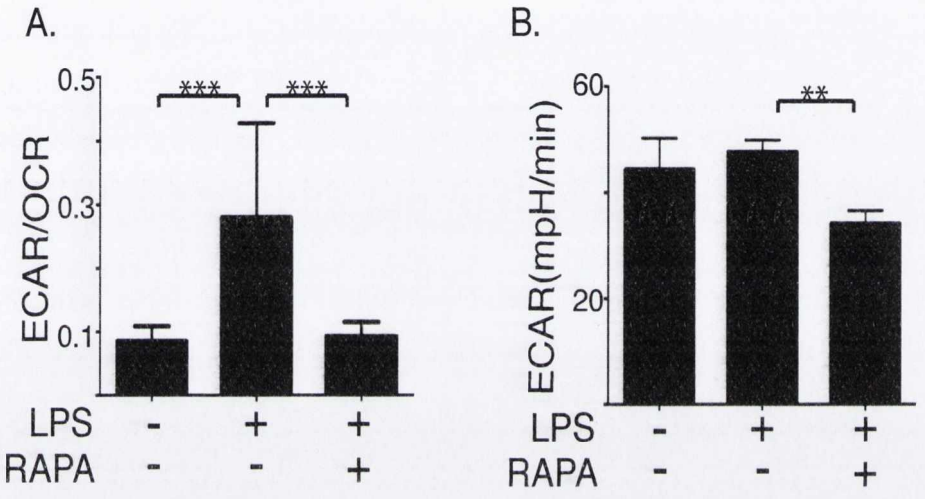


Fig 3.3 mTORC1 is required for the metabolic shift in LPS stimulated BMDCs, pooled data. BMDCs stimulated for 24 hours +/- LPS (100ng/ml), +/- RAPA (20nM) and analyzed using a Seahorse Extracellular Flux analyzer. Pooled data N=5. (A) ECAR/OCR. (B) Glycolytic capacity. ***$p<0.001$, **$p<0.01$, *$p<0.05$.

3.2.3 mTORC1 is required for the expression of key glycolytic genes in LPS stimulated DCs

In T cells there is a metabolic shift towards increased glycolysis that involves glycolytic reprogramming, as these cells significantly increase the glycolytic machinery within the cell (Cao et al., 2014). Glycolytic machinery refers to glycolytic enzymes and glucose transporters that control the rate of glycolysis. After establishing that the increase in glycolysis in LPS stimulated DCs is mTORC1 dependent, we next determined whether there is an mTORC1 dependent increase in the glycolytic machinery. The glucose transporter Glut 1 has been shown in other immune cells as the main inducible glucose transporter upon activation (Macintyre et al., 2014). There was a significant increase in the expression of GLUT 1 upon LPS stimulation that was completely blocked by the addition of rapamycin (Fig3.4a). There are several enzymes involved in the glycolytic pathway. The expression of some of the most important glycolytic enzymes were examined in LPS stimulated DCs. Lactate dehydrogenase (LDHA) converts pyruvate to lactate, an important step for anaerobic glycolysis (He et al., 2015). Phosphofructose kinase (PFK) catalyses the conversion of fructose-6-phosphate to fructose-1, 6-bisphosphate, a rate limiting step in glycolysis (Al Hasawi et al., 2014). The enzyme pyruvate kinase muscle isozyme 2 (PKM2) catalyses the conversion of phosphoenolpyruvate to pyruvate, the last step of glycolysis. The switch in expression from the alternative isoform PKM1 to PKM2 is important in controlling macrophage glycolysis (Palsson-McDermott et al., 2015). These three glycolytic genes were increased with LPS stimulation in DCs in an mTORC1 dependent manner (Fig3.4b-d).

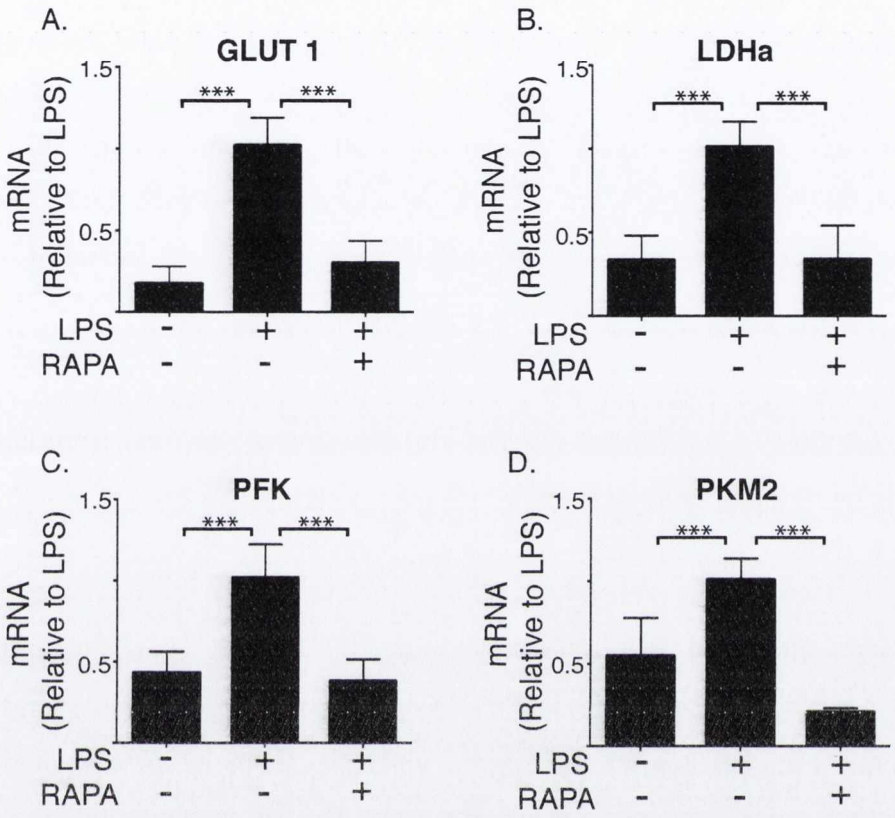


Fig 3.4 mTORC1 is required for the expression of key glycolytic genes in LPS stimulated DCs. (A) BMDCs stimulated for 24 hours +/- LPS (100ng/ml), +/- RAPA (20nM) and analysed by SYBR Green qPCR for indicated gene expression. Genes were normalized using the control gene RPLP0. Pooled data n=3. ***p<0.001, **p<0.01, *p<0.05.

3.2.4 There is a time dependent increase in glycolytic gene expression in LPS stimulated DCs.

mTORC1 was required for the increased expression of the glycolytic machinery in activated DCs. We next wanted to investigate at what point following LPS activation do DCs increase the expression of these glucose transporters and glycolytic enzymes. As seen in Fig3.5a-d all genes investigated had similar expression patterns. Increased expression above unstimulated levels was only observed up to 8 hours after 16 hours LPS stimulation. At both 16 and 24 hours the LPS induced expression of these genes was mTORC1 dependent.

3.2.5 mTORC1 is required for the stabilization of the transcription factor HIF1α in LPS stimulated BMDCs

DCs undergo mTORC1 dependent metabolic reprogramming by increaseing the expression of multiple glycolytic enzymes and glucose transporters. In other immune cells the two transcription factors that have been implicated in glycolytic metabolism are c-Myc and HIF1α. In CD8+ T cells the initial increase in glycolysis following TCR activation is c-Myc dependent while to maintain glycolysis CD8+ CTLs increase expression of HIF1α (Wang et al., 2011, Finlay et al., 2012). A study last year in DCs showed that expression of c-Myc decreased in DCs during development and is lost completely in mature DCs(Kc et al., 2014). With c-Myc not expressed in activated DCs the expression of HIF-1α was investigated in LPS stimulated DCs. HIF-1α is a transcription factor for many genes involved in the glycolytic pathway and glucose transport (Lum et al., 2007). Under normoxic conditions HIF-1α protein is usually unstable as prolyl hydroxylases tag HIF-1α for proteasomal degradation (Jaakkola et al., 2001). It has previously been reported that LPS activation of DCs induces HIF-1α stabilization but the exact mechanism remains unclear (Jantsch et al., 2008). Given that our data showed mTORC1 dependent increases in numerous HIF-1α target genes (GLUT1, LDHA, PFK) we hypothesized that HIF-1α might be important for LPS induced glycolysis in BMDC. HIF-1α protein expression was analysed at various timepoints following LPS activation of DC. There was no evidence of HIF-1α protein 4 hours after LPS

stimulation. HIF-1α protein was detected 8 hours and 24 hours after DC activation (Fig 3.6a). LPS induced HIF-1α protein expression at 8 hours and 24 hours was mTORC1 dependent (Fig 3.6b). PHD3 is a known target gene of HIF-1α(Pescador et al., 2005). In LPS activated DCs there was a significant increase in PHD3 expression. This was a good indicator of HIF-1α activity as this increase in expression was completely lost in HIF-1α knockout DCs (Fig3.7a). PHD3 expression was used to look at when HIF-1α activity was increased following LPS stimulation. There was no increase in PHD3 expression at 4 or 8 hours but from 16 hours on there was a significant increase in expression indicating that LPS stimulation was inducing HIF-1α activity. The addition of rapamycin blocked the increase in PHD3 expression at both 16 and 24 hours (Fig3.7b). HIF-1α is usually regulated at the posttranscriptional level but there have been previous reports in both macrophages and DCs that LPS stimulation increases HIF-1α mRNA levels(Spirig et al., 2010). In our hands there does not appear to be any significant increase in HIF-1α mRNA levels above basal at any time point with LPS stimulation. Rapamycin inhibition of mTORC1 had no affect on HIF-1α mRNA (Fig3.7c). The prolyl hydroxylase inhibitor DMOG can be used to induce HIF-1α stabilization (Milkiewicz et al., 2004). The addition of this compound was sufficient to stabilize HIF-1α at 4 hours even in the absence of mTORC1 signaling, before LPS induced HIF-1α stabilization (Fig 3.7d). Therefore pharmacological stabilization of HIF-1α is sufficient to induce HIF-1α in DC confirming that HIF-1α is solely regulated at the posttranscriptional level.

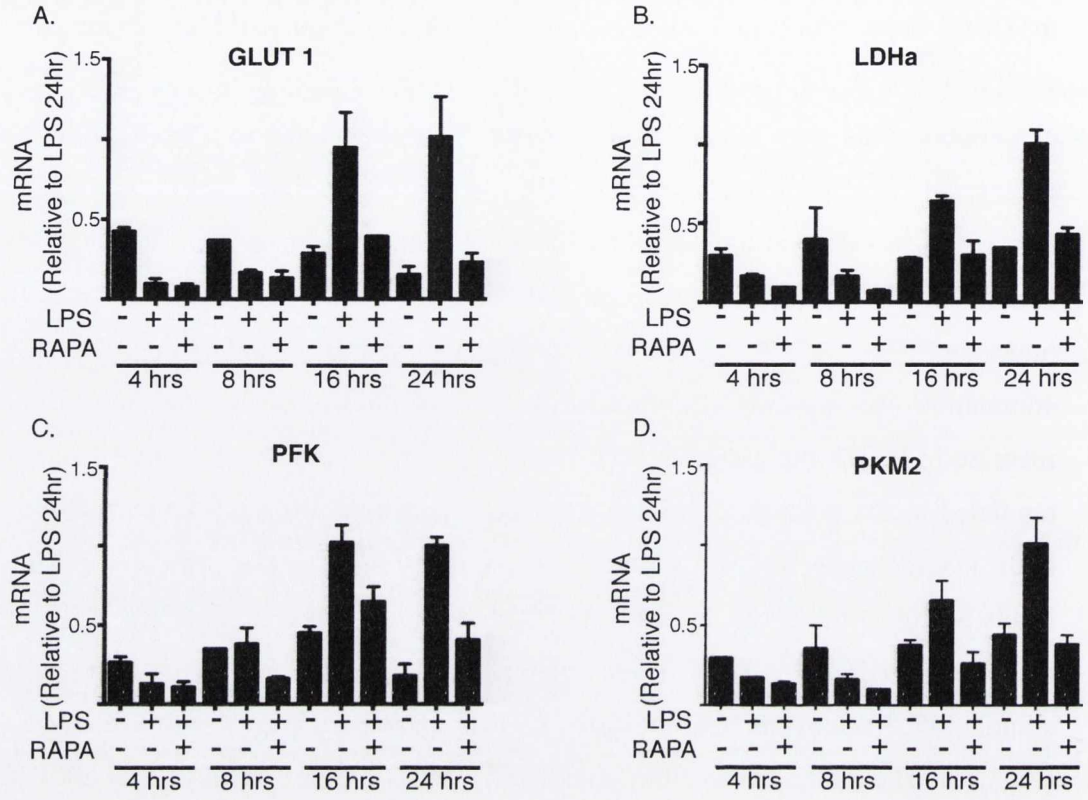


Fig 3.5 Glycolytic reprogramming occurs over the course of 16 hours. (A-D) BMDCs stimulated for the indicated time points with +/- LPS (100ng/ml), +/- RAPA (20nM) and analysed by SYBR Green qPCR for indicated gene expression. Genes were normalized using the control gene RPLP0. N=1.

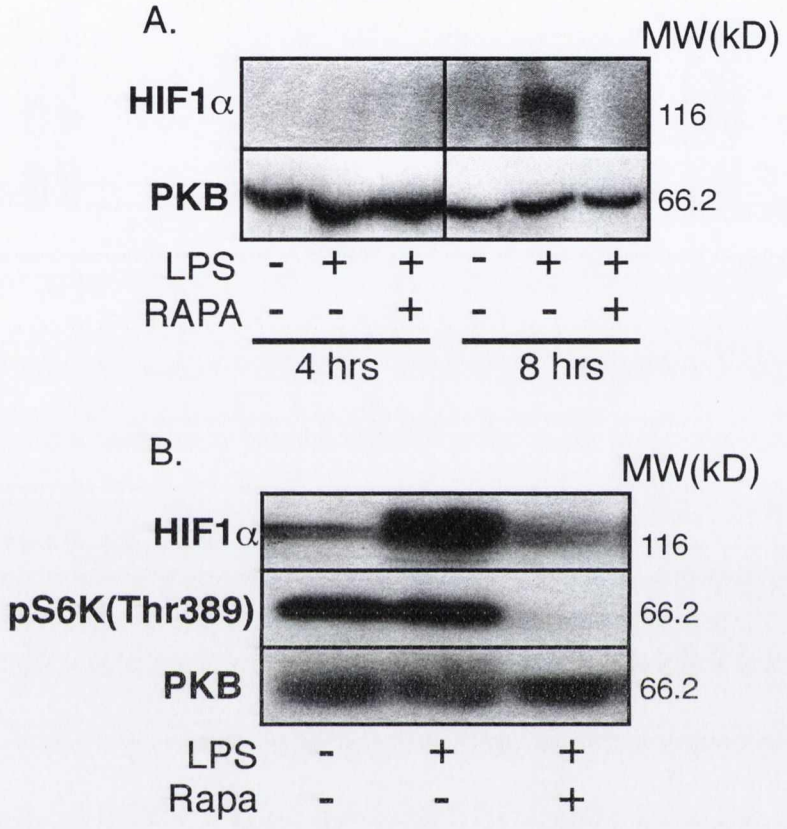


Fig 3.6 mTORC1 is required for HIF-1α protein stabilization in LPS stimulated DCs. (A-B) DCs were stimulated for 4, 8 or 24 hours +/- LPS +/- RAPA, lysed and analysed by western blot for indicated protein expression.

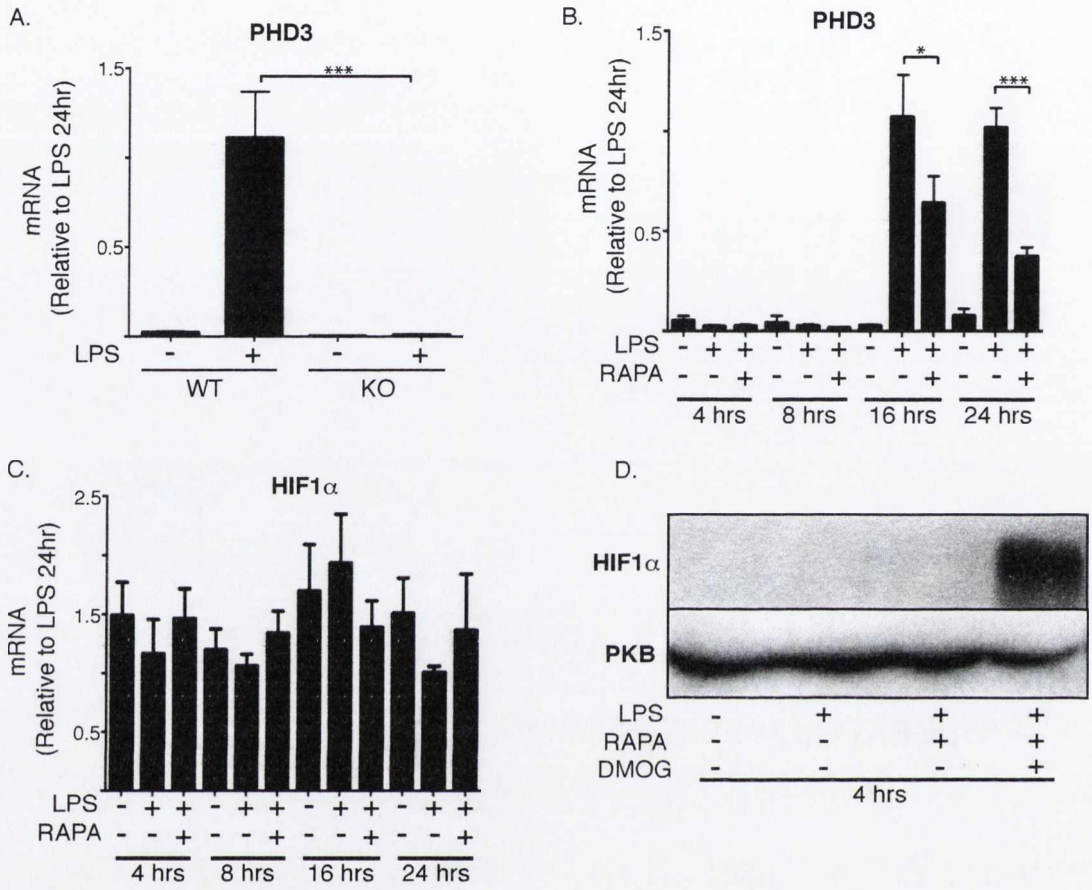


Fig 3.7 LPS stimulation induces HIF-1α activity that is mTORC1 dependent (A) HIF-1αflx/flx Cre- (WT) or HIF-1αflx/flx VAV/Cre+ (KO) BMDCs were stimulated for 24 hours +/- LPS (100ng/ml). PHD3 expression was measured by SYBR green qPCR. N=3 (B-C) BMDCs were stimulated for the indicated time points with +/- LPS (100ng/ml +/- RAPA (20nM). Indicated gene expression was measured using SYBR green qPCR. N=2. (D) DCs stimulated for 4 hours +/- LPS, +/- RAPA and +/- DMOG and analysed for the indicated proteins by western blot. ***p<0.001, **p<0.01, *p<0.05.

3.2.6 HIF-1α is required for the increased expression of key glycolytic enzymes and glucose transporters

LPS stimulated DCs undergo metabolic reprogramming that results in increased expression of glycolytic enzymes and glucose transporters. The increased expression of these genes was mTORC1 dependent. Given that we showed that the stabilization of HIF-1α was also mTORC1 dependent we hypothesized that HIF-1α was also required for the expression of these glycolytic genes. To conclusively prove that HIF-1α is required for the metabolic shift HIF-1α null BMDCs were used. These mice delete HIF-1α in all cells from the hematopoietic stem cell lineage(Georgiades et al., 2002). Although there is evidence that mTORC1 is required for normal DC development, HIF-1α is not required for DC development so this system produces fully functional DCs(Sathaliyawala et al., 2010). The expression of GLUT 1, PFK and PKM2 was examined following LPS stimulation in both wild type and HIF-1α null DCs. The expression of all these glycolytic genes was significantly decreased in LPS stimulated HIF-1α null DCs (Fig3.8a-c).

3.2.7 The transcription factor HIF-1α is required for the LPS induced increase in glycolysis

Cellular metabolism was next examined in LPS stimulated HIF-1α null BMDCs compared with WT DCs. HIF-1α wild type and HIF-1α null DCs were stimulated for 24 hours and metabolism was analysed using the Seahorse extracellular flux analyser. The increase in glycolysis that occurs in LPS stimulated DCs was not observed in the absence of HIF-1α (Fig3.9a). However LPS does inhibit the rate of OxPhos in HIF-1α null BMDCs (Fig3.9b). When looking at the overall ECAR/OCR ratio its clear that HIF-1α null DCs do not undergo a glycolytic shift to the same extent as seen in LPS stimulated wild type DCs (Fig3.9c).

3.2.8 mTORC1 is required for iNOS expression and Nitric oxide production in activated DCs

mTORC1 controlled HIF-1α activity was required for the glycolytic shift in activated DCs. mTORC1 was also required for the decrease in OxPhos observed in LPS stimulated DCs. In the literature iNOS production of NO has been shown to be required for LPS induced elevated glycolysis and decreased OxPhos (Everts et al., 2012). Experiments were designed to investigate the dynamics of iNOS expression and how this relates to the mTORC1 dependency for BMDC metabolic shift. NO is unstable in aqueous solutions so to measure the levels in culture the Griess reaction is used to measure nitrite, one of the stable and primary breakdown products of NO (Bryan and Grisham, 2007). In LPS stimulated DCs no increase in nitrite production was observed until 16 hours after LPS stimulation, with an even greater increase observed at 24 hours. At both time points the increase in nitrite production was partially blocked by rapamycin addition, similar to published data (Fig3.10a) (Amiel et al., 2012). The expression of iNOS mRNA was next investigated over time. There was observable increases in iNOS expression even at 4 hours after LPS stimulation, though there was significantly more iNOS mRNA at 16 and 24 hours. At the earlier time point's mTORC1 inhibition partially but not significantly blocks iNOS mRNA expression, while at 16 and 24 hours the LPS induced expression of iNOS was mTORC1 dependent (Fig3.10b). Next iNOS protein expression was analysed over time. There were low levels of iNOS protein observable in both LPS treated and rapamycin treated DCs 6 hours after stimulation. There was a substantial increase in iNOS protein expression at 24 hours that was significantly inhibited by rapamycin addition (Fig3.10c). Together these data argue that there is only a significant level of iNOS expression and activity in LPS stimulated BMDCs after 16 hours when there is an mTORC1 induced increase in iNOS mRNA and protein. Importantly when the rate of OxPhos at 8 hours was analysed there was no decrease observed. This shows that the decrease in OxPhos correlates with increased NO production that occurs after 16 hours LPS stimulation (Fig 3.10d).

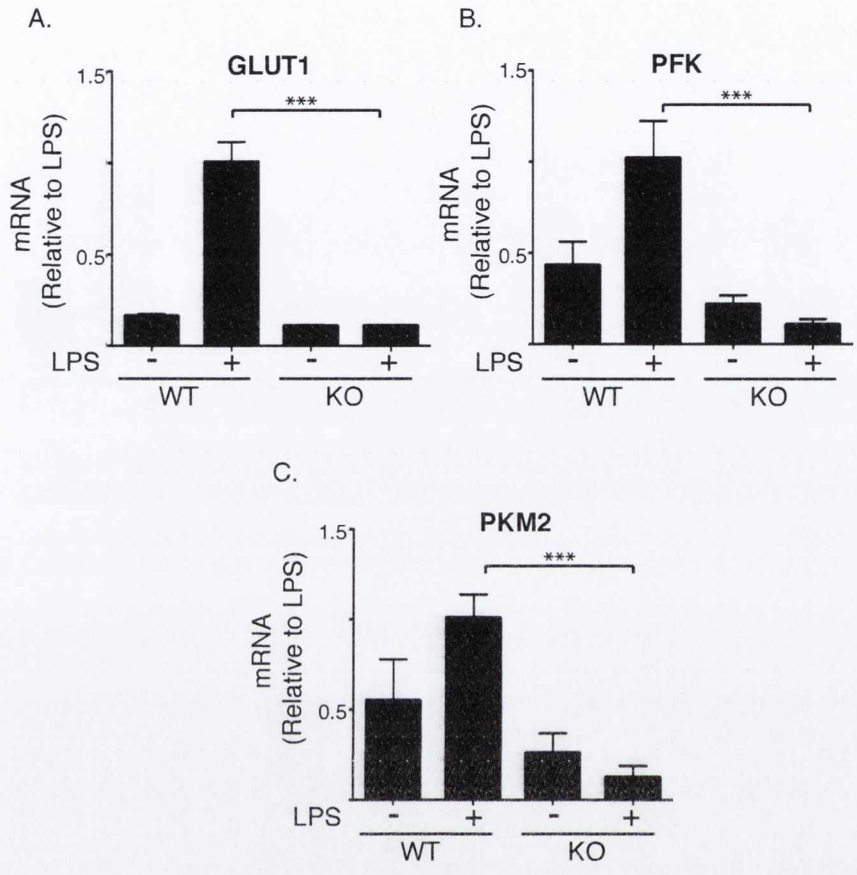


Fig 3.8 HIF-1α is required for the expression of key glycolytic genes in LPS stimulated DCs (A-C) HIF-1αflx/flx Cre wild type (WT) or HIF-1αflx/flx Vav-Cre null (KO) BMDCs stimulated for 24 hours +/- LPS (100ng/ml) and analysed by SYBR Green qPCR for indicated gene expression. Genes were normalized using the control gene RPLP0. Pooled data n=3. ***p<0.001, **p<0.01, *p<0.05.

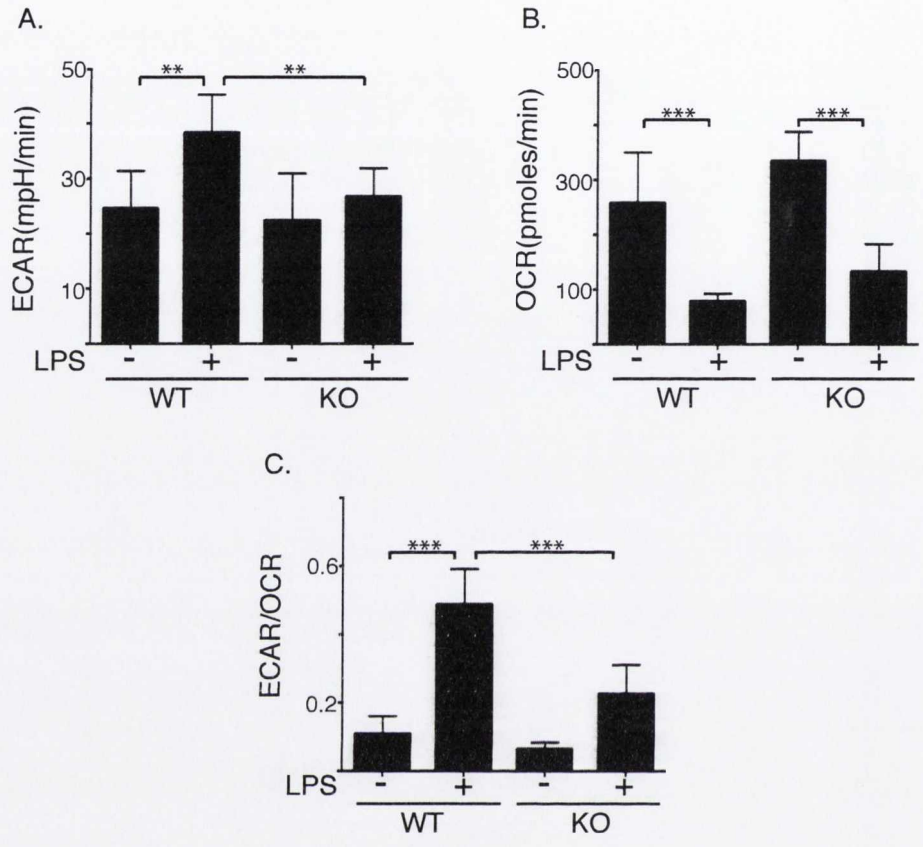


Fig 3.9 HIF-1α is required for increased glycolysis in LPS stimulated DCs. (A-C) HIF-1αflx/flx cre wild type (WT) or HIF-1αflx/flx Vav-Cre null (KO) BMDCs stimulated for 24 hours +/- LPS (100ng/ml), +/- RAPA (20nM) and analysed using a Seahorse Extracellular Flux analyser. Pooled seahorse data n=2 for ECAR, OCR and ECAR/OCR. ***$p<0.001$, **$p<0.01$, *$p<0.05$.

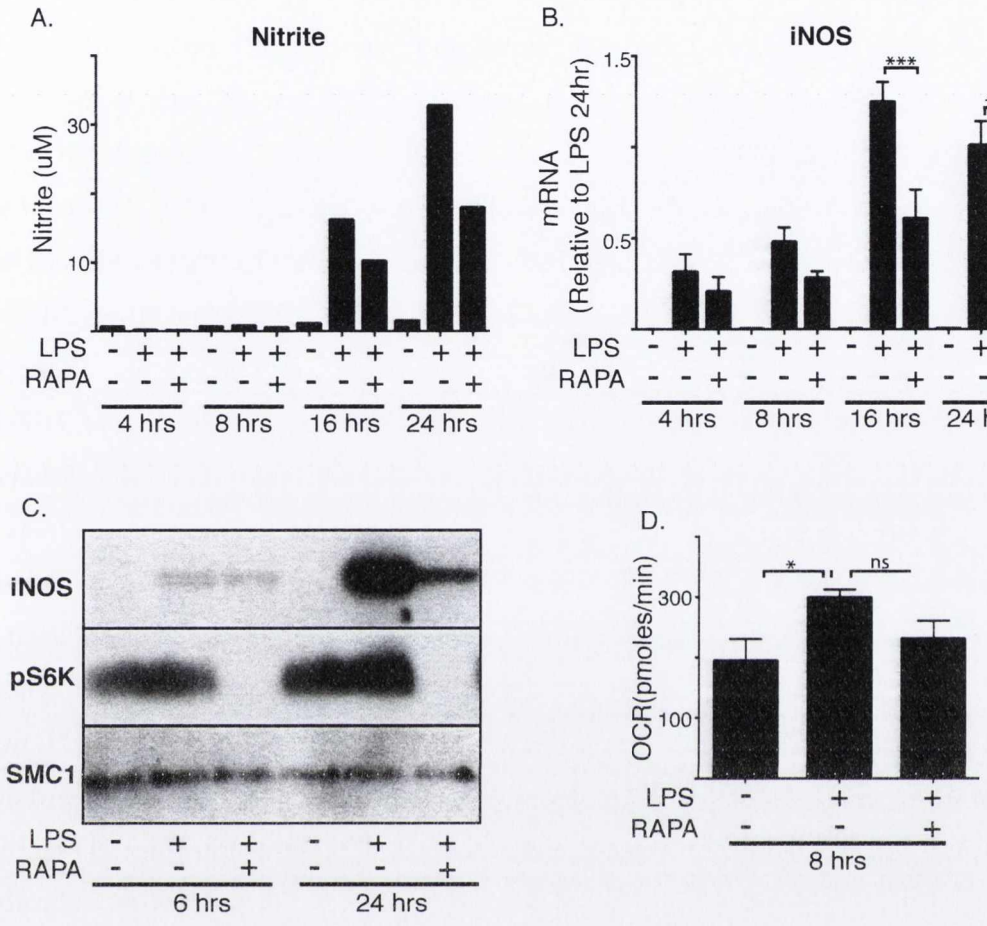


Fig 3.10 iNOS expression is mTORC1 dependent in LPS activated DCs. BMDCs were stimulated for the indicated time points +/- LPS (100ng/ml +/- RAPA (20nM). (A) Nitrite production was measured using the Griess reagent. N=1 (B) iNOS expression was measured using SYBR green qPCR. N=2 (C) iNOS expression analysed by western blot. (D) OCR measured using Seahorse machine. Pooled data N=3. ***p<0.001, **p<0.01, *p<0.05.

3.2.9 Nitric oxide is required for HIF-1α activity in LPS stimulated DCs

Nitric oxide is an important signaling molecule in the body as well as having a role in the protection against bacteria and parasites (Tennyson and Lippard, 2011). Cells can upregulate the expression of the enzyme iNOS that in turn leads to the production of high levels of NO (Lu et al., 2009). As mentioned already iNOS has been shown to be required for the metabolic switch in activated DCs. However we have shown that both mTORC1 and HIF-1α are required for the metabolic shift and the expression of key glycolytic genes. There is no direct mechanism for iNOS or NO to control the expression of glycolytic genes or glucose transporters. Therefore it was hypothesized that there may be a link between iNOS/NO and HIF-1α expression. In the literature there seems to be a complicated relationship between NO and HIF-1α. Low levels of NO inhibit HIF-1α protein stabilization, whereas at higher concentrations of NO it actually promotes the stabilization HIF-1α presumably by inhibiting the prolyl hydroxylases (Sogawa et al., 1998, Metzen et al., 2003). Indeed reactive oxygen species are known to inhibit prolyl hydroxylases and stabilize HIF-1α (Nishi et al., 2008). The iNOS specific inhibitor SEITU was used to investigate whether iNOS activity is required for HIF-1α activity. Adding SEITU to LPS activated DCs completely blocked NO production (Fig 3.11a). LPS induced PHD3 expression is blocked in the absence of NO production indicating that NO is required for HIF-1α activity (Fig 3.11a). LPS induced HIF-1α protein stabilization is also blocked with iNOS inhibition (Fig 3.11b). The effects of SEITU on HIF-1α activity do not involve the inhibition of mTORC1 signaling, as the levels of pS6K remain unaltered following the inhibition of iNOS. These data show that iNOS and NO are required for HIF-1α stabilization and activity. Next it was determined whether NO is sufficient to stabilize HIF-1α protein expression. Experiments were designed to determine if NO can stabilize HIF-1α expression in rapamycin treated BMDC that normally have low levels of HIF-1α and iNOS expression. SNAP is a nitric oxide donor when in aqueous solutions. Adding SNAP to LPS and rapamycin treated DCs results in HIF-1α stabilization. This result shows that endogenous NO is sufficient to stabilize HIF-1α even in the absence of mTORC1 signaling (Fig 3.12).

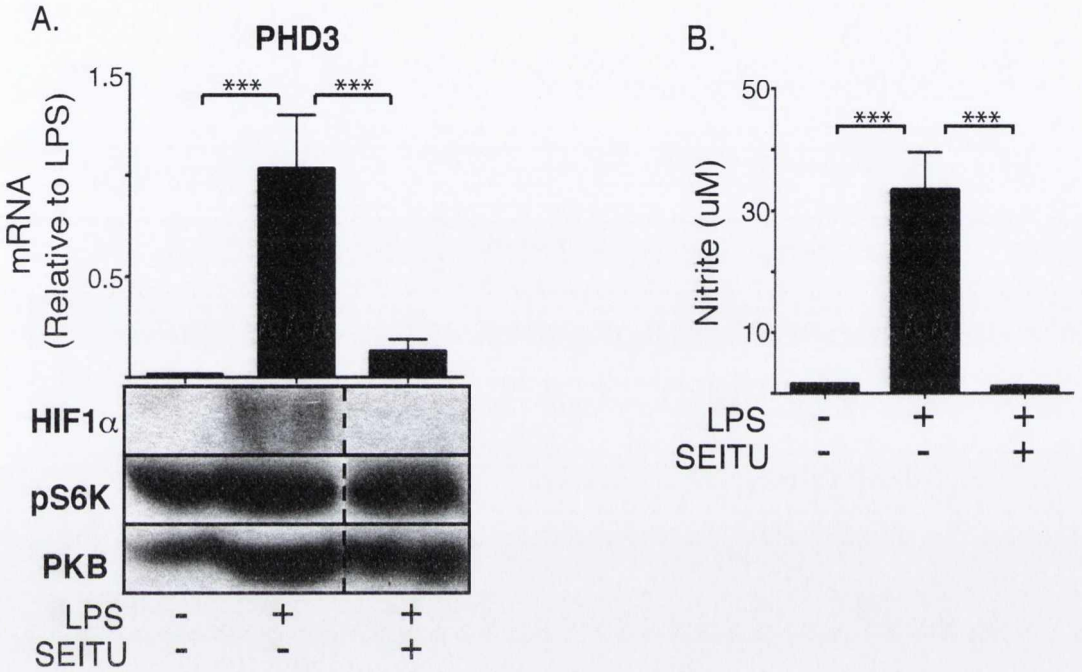


Fig 3.11 Inhibiting iNOS blocks HIF-1α activity in LPS activated DCs. (A) DCs were stimulated for 24 hours +/- LPS +/- SEITU, lysed and analysed by SYBR green qPCR for PHD3 expression or by western blot for indicated protein expression. Pooled data N=3. (B) DCs were stimulated for 24 hours +/- LPS +/- SEITU and NO measured in the supernatant using the Griess reagent. Pooled data N=3. ***$p<0.001$, **$p<0.01$, *$p<0.05$.

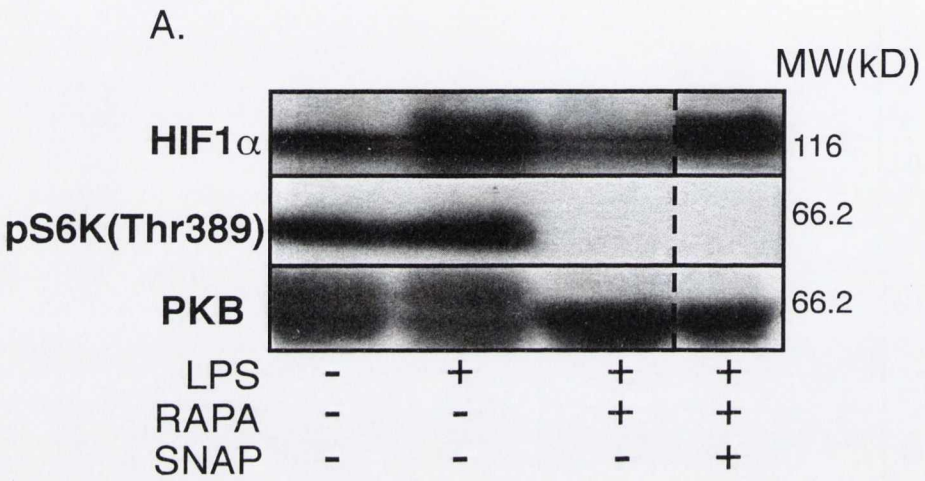


Fig 3.12 The addition of exogenous nitric oxide stabilizes HIF-1α in the absence of mTORC1 signaling. (A) DCs were stimulated for 24 hours +/- LPS, +/- RAPA, +/- SNAP, lysed and analysed by western blot for indicated protein expression.

3.2.10 HIF-1α is required for NO production in LPS stimulated DCs

The data shows that NO is required for HIF-1α stabilization in activated DCs. However iNOS is also a known target gene of HIF-1α in other cell types(Matrone et al., 2004). We wanted to investigate further the relationship between these two metabolic regulators and how their expression is linked. As described previously the prolyl hydroxylase inhibitor DMOG can be used to stabilize HIF-1α protein. Analysis of PHD3 expression shows that the addition of DMOG to activated DCs is sufficient to reestablish HIF-1α activity in the absence of mTORC1 signaling. Western blot data also shows that DMOG leads to an accumulation of HIF-1α protein even in rapamycin treated BMDCs. Rapamycin addition blocks iNOS expression, as has previously been shown. When DMOG was added to DC treated with LPS and rapamycin iNOS was expressed to similar levels as in DCs stimulated with LPS alone (Fig 3.13a). A similar trend is observed when nitrite production was analysed. Rapamycin blocked nitrite production as previously demonstrated. The addition of DMOG reversed the rapamycin effects and DCs produce nitrite to equivalent levels to DC stimulated with LPS alone showing that stabilizing HIF-1α is sufficent for NO production (Fig 3.13b). To conclusively prove that HIF-1α is required for NO production in activated DCs we analysed HIF-1α null BMDCs. In LPS stimulated HIF-1α null DCs there was a significant decrease in nitrite levels compared to wild type DCs (Fig 3.14a). HIF-1α protein levels are nearly completely gone in the HIF-1α null DCs, showing high knock out efficiency. The expression of iNOS is significantly down in the knockout when compared to the wild type, however iNOS expression is not completely blocked. The addition of rapamycin to the HIF-1α null DCs reduced iNOS expression even further (Fig 3.14b). To see if the HIF-1α requirement for iNOS expression was at the transcriptional level we looked at iNOS mRNA expression in both wild type and HIF-1α null DCs. There is a substantial decrease in iNOS mRNA expression in LPS activated HIF-1α null DCs compared wild type DCs (Fig 3.14c).

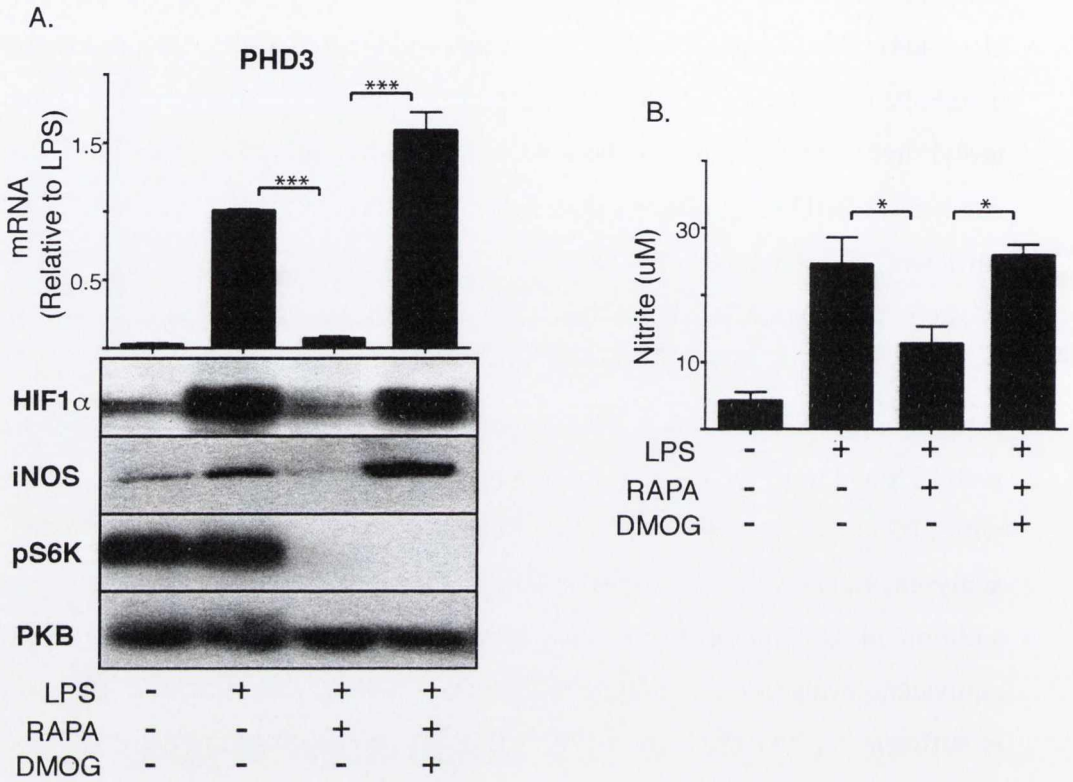


Fig 3.13 DMOG induces iNOS expression and nitric oxide production in the absence of mTORC1 signaling. (A) DCs were stimulated for 24 hours +/- LPS, +/- RAPA, +/- DMOG, lysed and analysed by SYBR green qPCR for PHD3 expression or by western blot for indicated protein expression. Pooled data N=3. (B) DCs were stimulated for 24 hours +/- LPS, +/- RAPA, +/- DMOG and NO measured in the supernatant using the Griess reagent. Pooled data N=3. ***$p<0.001$, **$p<0.01$, *$p<0.05$.

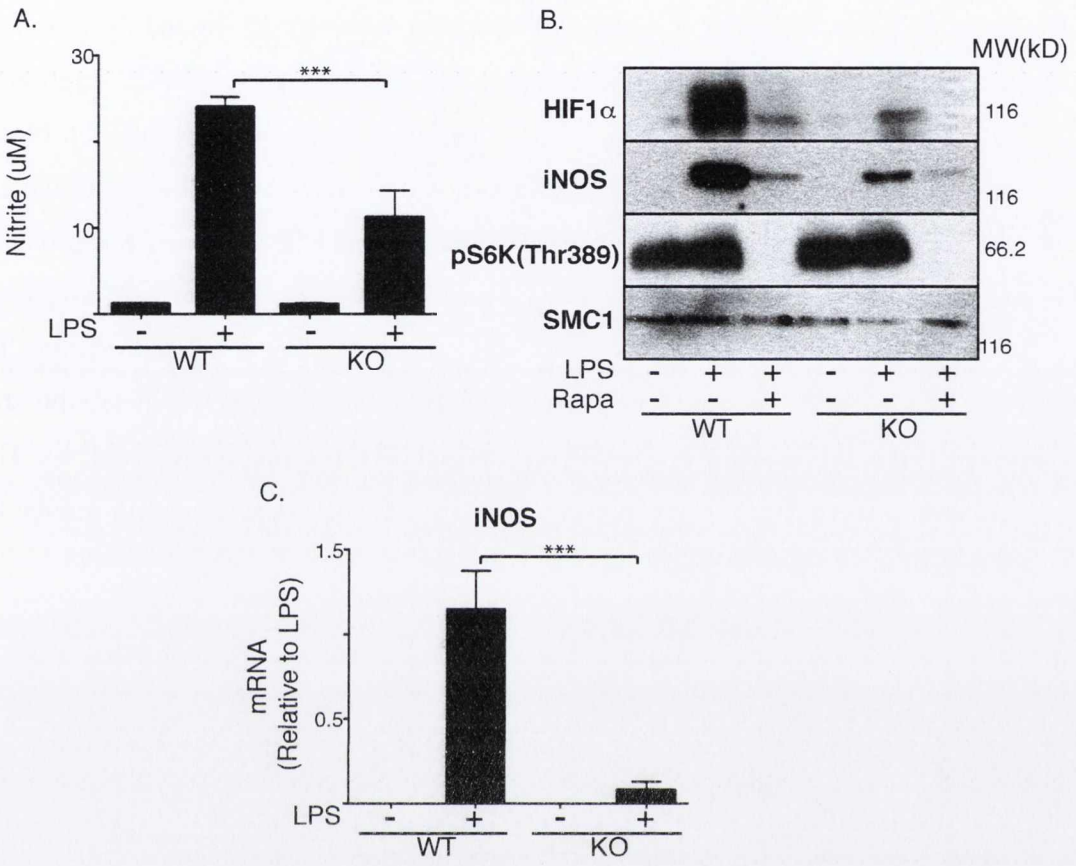


Fig 3.14 HIF-1α is required for iNOS expression and nitric oxide production in LPS stimulated DCs. (A) HIF-1α wild type (WT) or HIF-1α null (KO) BMDCs stimulated for 24 hours +/- LPS, +/- RAPA and NO measured in the supernatant using the Griess reagent. N=4 (B) HIF-1α wild type (WT) or HIF-1α null (KO) BMDCs stimulated for 24 hours +/- LPS, +/- RAPA lysed and analysed for the indicated protein expression using western blot. (C) HIF-1α wild type (WT) or HIF-1α null (KO) BMDCs stimulated for 24 hours +/- LPS, lysed and analysed by SYBR green qPCR for PHD3 expression. N=3 ***p<0.001, **p<0.01, *p<0.05.

3.2.11 Inducing nitric oxide production blocks OxPhos, even in the absence of mTORC1 signaling

This data shows that there is a complex interplay between HIF-1α and iNOS in LPS stimulated DCs. DMOG addition induces HIF-1α stabilization, iNOS expression and NO production even when mTORC1 signaling is blocked. Rapamycin blocks the decrease in OxPhos, as we had previously shown We wanted to investigate if DMOG induced iNOS expression is sufficient to inhibit OxPhos in the presence of rapamycin. The addition of DMOG in the presence of rapamycin leads to a decrease in OxPhos, to the same level as seen in LPS treated DCs alone. When looking at the ECAR/OCR ratio it's clear that DMOG addition reestablishes the metabolic shift towards glycolysis, even in the absence of mTORC1 signaling (Fig 3.15b). This data shows that HIF-1α stabilization and NO production is sufficient to induce the inhibition of OxPhos in BMDCs.

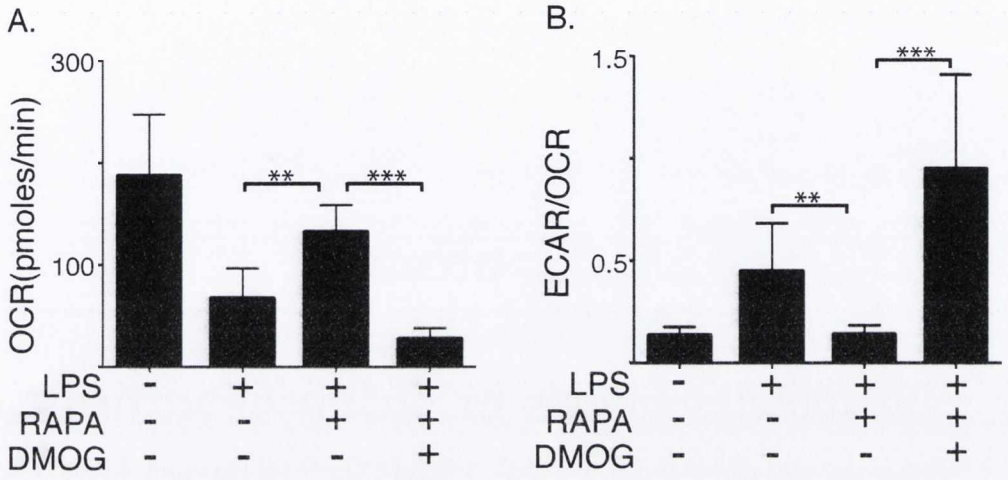


Fig 3.15 DMOG reverses the rapamycin inhibition of DC metabolism. (A-B) BMDCs stimulated for 24 hours +/- LPS (100ng/ml), +/- RAPA (20nM) +/- DMOG (200uM) and analysed using a Seahorse Extracellular Flux analyser. Pooled seahorse data n=2 for ECAR, OCR and ECAR/OCR. ***p<0.001, **p<0.01, *p<0.05.

Chapter 4

Nutrients in the microenvironment impact on mTORC1/HIF-1α and iNOS signaling in DCs

4.1 Introduction

Activated DC's undergo a metabolic shift towards elevated glycolysis, as shown in chapter 3. The metabolic regulators involved have been identified as mTORC1, iNOS and HIF-1α. mTORC1 is required for the activity of both iNOS and HIF-1α. mTORC1 also has an important role as a nutrient sensor in the cell responding to the availability of nutrients such as amino acids and glucose(Howell and Manning, 2011). DCs usually sense antigens in the periphery and migrate to lymph nodes were they interact with T cells. The DC must be able to respond to changes within the cellular microenvironment as it migrates. Nutrient availability can fluctuate within different microenvironments, particularly during inflammation and at tumor sites (Al-Zoughbi et al., 2014). How extracellular levels of nutrients impacts on mTORC1 signaling within activated DCs has not been studied.

This chapter will investigate the importance of amino acid availability on activated DCs. Amino acids are required for protein synthesis and other cellular processes. How amino acids signal to mTORC1 has previously been described in chapter 1. This chapter will also investigate how amino acid availability impacts on the metabolic regulators downstream of mTORC1 as this would link nutrient supply directly to DC metabolism. Understanding this link would help understand how DCs respond to changing amino acid availability during inflammation.

The second half of this chapter will investigate how activated DCs respond to glucose deprivation. Although the body has evolved specific mechanisms to maintain glucose homeostasis the demand for glucose increases at tumor sites and from activated immune cells (Ganeshan and Chawla, 2014). Other cells that enter into these microenvironments could become glucose deprived. We have shown that activated DCs increase their rate of glycolysis and expression of glucose transporters. Any decrease in glucose availability could impact on DC metabolism and the metabolic regulators involved.

4.2.1 mTORC1 signaling is sensitive to amino acid levels in the DC microenvironment

mTORC1 has well established functions as a nutrient sensor in many cell types. However the availability of amino acids overrides all other nutrient signals and switches off mTORC1 signaling (Beugnet et al., 2003). Recently it has emerged that in T cells amino acid availability is essential for T cell activation. Intriguingly it is only one amino acid transporter, Large amino acid transporter 1 (LAT1), which is required for the uptake of leucine and glutamine that is required for T cell activation (Sinclair et al., 2013). Experiments were designed to investigate if mTORC1 similarly acted as a nutrient sensor in DCs. Hanks buffered saline solution (HBSS) and dialysed FCS were used to ensure no amino acids were present in the media. Rapamycin and AZD, which is another mTOR specific inhibitor, were used as negative controls to show levels of pS6K when mTORC1 signaling was inhibited. Removing amino acids switches off mTORC1 to the same level as with the addition of rapamycin or AZD. As mentioned previously leucine and glutamine were found to be the important amino acids for maintaining mTORC1 signaling in T cells. Valine, arginine, and isoleucine have also been implicated as important for mTORC1 signaling in other cell types (Jewell et al., 2013). Adding these five amino acids to media was sufficient to maintain mTORC1 signaling in DCs. The addition of just leucine and glutamine to DCs was enough to maintain the same level of mTORC1 signaling as seen when the five aforementioned amino acids were added (Fig4.1). This shows that amino acid deprivation blocks mTORC1 signaling and in turn would affect DC metabolism, as mTORC1 is required for the metabolic shift.

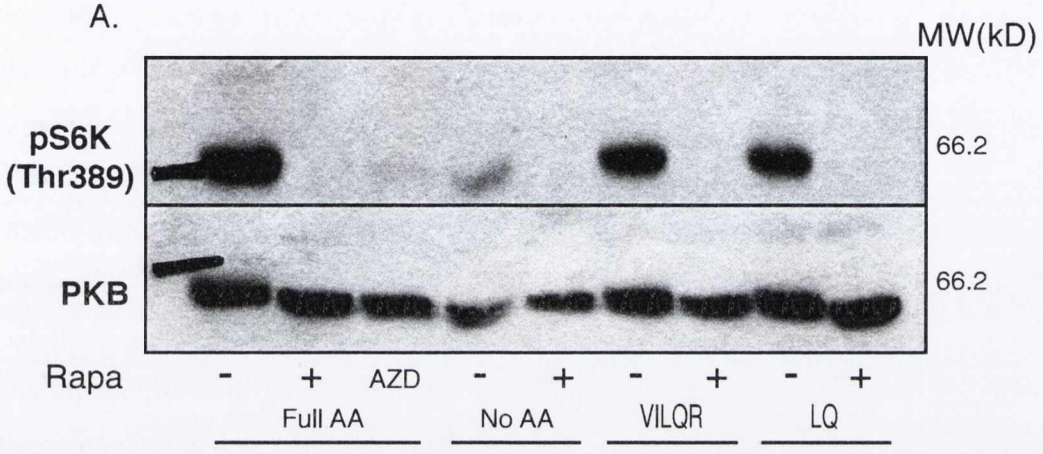


Fig 4.1 Amino acid availability impacts on mTORC1 signaling in DCs. (A) DCs were washed twice in HBSS and incubated for 30 minutes with the indicated amino acid combinations. Cells were then lysed and analysed by western blot for pS6K and PKB. AZD-8055 (AZD). Valine (V), isoleucine (I), leucine (L), glutamine (Q), arginine (R).

4.2.2 Arginine availability impacts on HIF-1α stabilisation, independently of mTORC1 signaling

Arginine is a semi essential amino acid in mammals as in adults the body usually produces sufficient amounts (Appleton, 2002). Arginine availability can decrease in the body as serum levels are depleted in cancer patients (Vissers et al., 2005). The anti-inflammatory M2 macrophage upregulates the enzyme arginase 1 that converts arginine to ornithine and urea that also decreases arginine levels in the cellular microenvironment (Roszer, 2015). The enzyme iNOS catalysis the conversion of L-arginine to citrulline with NO produced as a byproduct of the reaction. We have established that NO is required for HIF-1α stabilization in activated DCs. Given that arginine is required for NO production we wanted to investigate how arginine availability impacts on HIF-1α stabilization. To look at this we acquired media that had all amino acids besides arginine. Removing arginine from the media had no effect on mTORC1 signaling in DCs (Fig 4.2a). Arginine in the microenvironment was required for NO production in activated DCs as in the absence of arginine NO levels were significantly decreased (Fig 4.2b). PHD3 expression was completely blocked in the absence of arginine, demonstrating that arginine availability is required for HIF-1α activity (Fig 4.2c).

4.2.3 Arginine in the microenvironment is required for increased glycolysis in LPS activated DCs

Arginine is required for the stabilization of HIF-1α in activated DCs. HIF-1α is required for the increase in glycolysis observed in LPS stimulated DCs. We wanted to investigate if arginine availability impacted on the glycolytic shift. BMDCs were stimulated with LPS in media +/- arginine for 24 hours and DCs glycolytic rate under these conditions was investigated at 24 hours. Arginine withdrawal blocked the increase in glycolysis, demonstrating that availability of amino acids in the microenvironment can impact on DC metabolism (Fig 4.3).

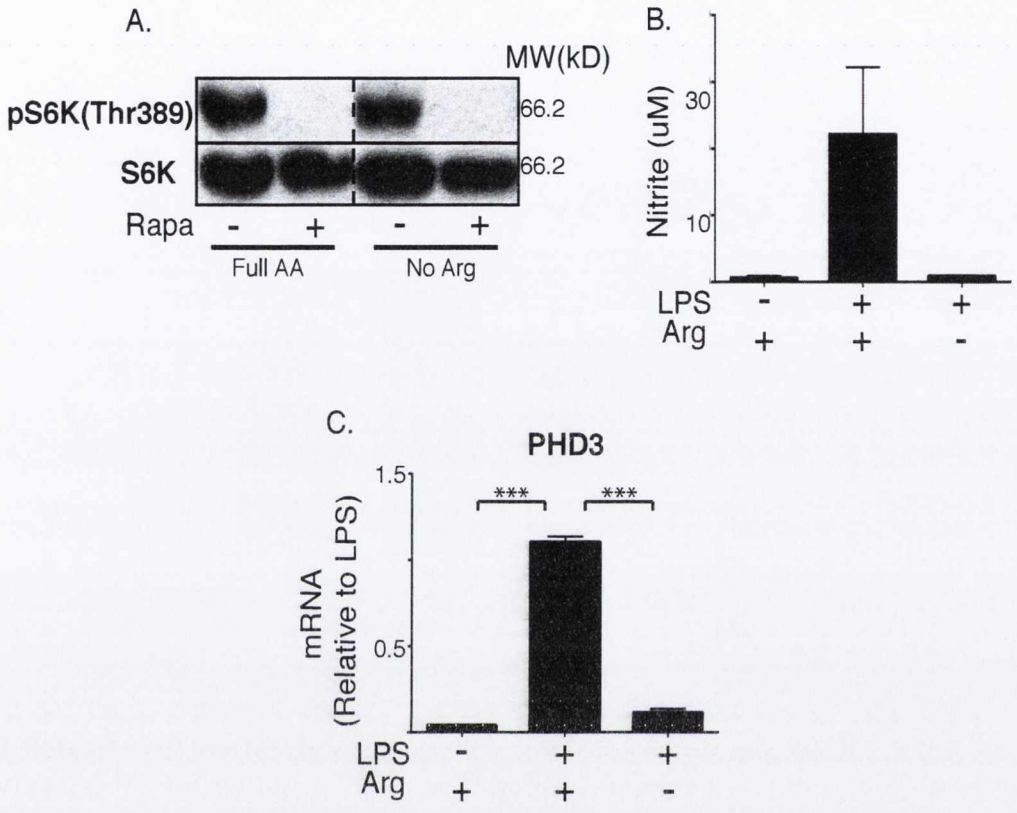


Fig 4.2 Arginine is required for HIF-1α activity in LPS stimulated DCs. (A) DCs were washed twice in HBSS and incubated for 30 minutes +/- arginine. Cells were then lysed and analysed by western blot for pS6K and total S6K. (B-C) DCs were stimulated for 8 hours in normal media. Cells then washed twice with SILAC media and then incubated for 16 hours in SILAC media +/- arginine. NO was measured using the Griess reaction. PHD3 expression measured using SYBR green qPCR. N=2. ***$p<0.001$, **$p<0.01$, *$p<0.05$.

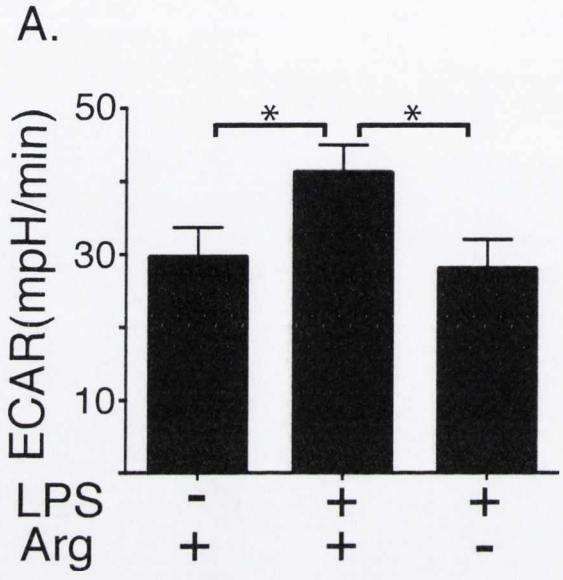


Fig 4.3 Removing arginine blocks the increase in glycolysis in activated DCs (A) BMDCs stimulated for 24 hours +/- LPS (100ng/ml), +/- arginine and analysed using a Seahorse Extracellular Flux analyser. Pooled seahorse data N=2 for ECAR. ***p<0.001, **p<0.01, *p<0.05.

4.2.4 mTORC1 signaling in DCs is sensitive to glucose levels in the microenvironment

Glucose is the main carbon source used as fuel by most cells. In blood the normal glucose concentration is around 5mM. The body has evolved an extremely efficient system of regulation that prevents glucose levels from fluctuating too much. When this system fails this can result in diabetes and the symptoms associated with this disease. Although the body is extremely adept at maintaining homeostasis, glucose levels can vary in localized microenvironments such as in inflamed areas or around tumors (Hirayama et al., 2009). Activated immune cells significantly increase their uptake of glucose and it is possible that these activated cells could reduce glucose levels in the microenvironment. mTORC1 has previously been described as sensor of glucose availability in multiple cell types including T cells (Rolf et al., 2013). Glucose withdrawal can inhibit mTORC1 following a decrease in ATP/ADP ratio and the activation of AMPK (Gleason et al., 2007). We wanted to investigate the sensitivity of mTORC1 signaling in DCs to glucose levels. It has been shown that inhibiting glycolysis immediately following LPS stimulation blocks DC activation (Krawczyk et al., 2010). To avoid this BMDCs were activated for 8 hours in the presence of 10mM glucose. After 8 hours cells were washed in glucose free media and then media was added containing 10mM, 2mM or 1mM glucose. Cells were then lysed 16 hours later. DCs in 2mM and 1mM glucose had no detectable levels of pS6. The levels of pS6k were significantly decreased in both 2mM and 1mM glucose. Taken together this shows that glucose deprivation blocked mTORC1 signaling in DCs (Fig4.4).

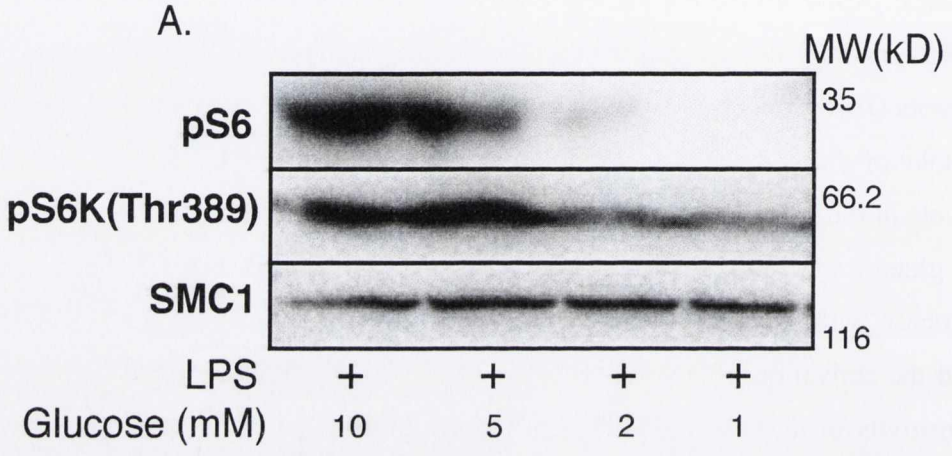


Fig 4.4 Glucose levels impact on mTORC1 signaling in DCs. (A) DCs were stimulated for 8 hours in normal RPMI. At 8 hours cells were washed with glucose free media and then left in media containing the indicated concentrations of glucose. Cells were then lysed at 16 hours later and analysed for pS6, pS6K and PKB expression.

4.2.5 Glucose deprivation blocks HIF-1α activity and NO production

mTORC1 signaling in BMDCs is sensitive to glucose levels in the microenvironment, with mTORC1 signaling switched off in a glucose concentration of 2mM. mTORC1 is also required for HIF-1α activity. We wanted to investigate if the effects glucose deprivation has on mTORC1 similarly impacted on HIF-1α activity. Cells were stimulated for 8 hours in normal media. After 8 hours cells were washed in glucose free media and then media was added containing 10mM, 2mM or 1mM glucose. PHD3 expression was significantly decreased in 2mM and 1mM glucose in activated DCs. This showed that glucose is required for HIF-1α activity (Fig 4.5a). HIF-1α is required for NO production in activated DCs. NO production was significantly reduced in 2mM and 1mM glucose in activated DCs, again highlighting the link between HIF-1α activity and NO production (Fig 4.5b).

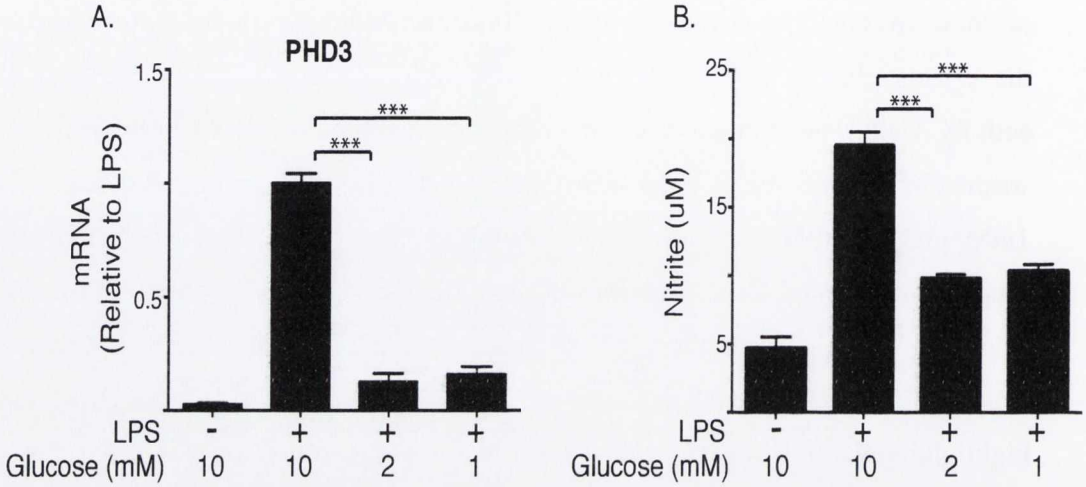


Fig 4.5 Glucose deprivation impacts on HIF-1α activity and NO production in LPS stimulated DCs. (A-B) DCs were stimulated for 8 hours in normal media. Cells then washed twice with glucose free media and then incubated for 16 hours in glucose free media with the indicated concentrations of glucose added. NO was measured using the Griess reaction. PHD3 expression measured using SYBR green qPCR. N=2. ***p<0.001, **p<0.01, *p<0.05.

4.2.6 Glucose deprivation inhibits HIF-1α activity in LPS stimulated DCs through mTORC1 independent mechanisms

A second strategy to investigate the requirement of glucose for HIF-1α activity was developed. Glucose was replaced with galactose as an alternative fuel source. Galactose can be metabolized through the Leloir pathway to UDP-glucose. This is then converted to glucose-6-phosphate and enters the glycolytic pathway(Sellick et al., 2008). However galactose is metabolized much slower than glucose and cells using galactose as a fuel source cannot maintain elevated levels of glycolysis. It has been shown that inhibiting glycolysis immediately following LPS stimulation blocks DC activation (Krawczyk et al., 2010). To avoid this we activated DCs for 8 hours in the presence of 10mM glucose. At 8 hours DCs were washed in glucose free media and then new media was added either containing 10mM glucose or 10mM galactose. Interestingly this approach did not inhibit mTORC1 activity. This is likely to reflect that galactose generates pyruvate that is metabolized in the mitochondria to generate ATP, thus avoiding a decrease in ATP and AMPK activation. However when PHD3 expression was analysed there was substantial decreased expression in DCs with glucose replaced with galactose. This shows glucose availability can regulate HIF-1α activity, independent of mTORC1 (Fig 4.6a). Having observed normal mTORC1 activity in galactose cultured BMDC, we next wanted to investigate NO production under these conditions. There was a significant decrease in NO levels from DCs in glucose when compared to those in galactose. This data suggests a direct link between HIF-1α activity and iNOS expression. This result also argues that mTORC1 controls NO production at least in part through promoting HIF-1α activity (Fig 4.6b). To show that these results were due to a lack of glucose, rather than a direct effect of galactose, both galactose (10mM) and glucose (2mM) were added to DCs. NO production in 2mM glucose plus galactose is returned to the same level seen in 10mM glucose, showing that these results are a result of glucose deprivation rather than a direct effect of galactose (Fig 4.7a).

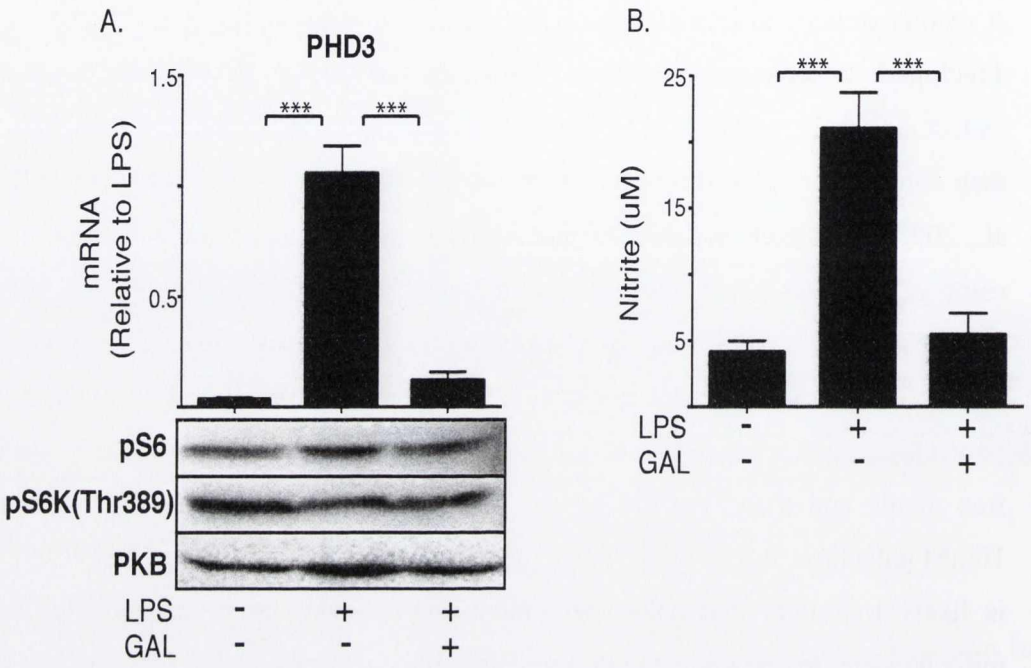


Fig 4.6 Replacing glucose with galactose blocks HIF-1α activity independently of mTORC1 (A-B) DCs were stimulated for 8 hours in normal media. Cells then washed twice with glucose free media and then incubated for 16 hours in glucose free media with either 10mM glucose or 10mM galactose. Indicated protein expression was measured by western blot. NO was measured using the Griess reaction. PHD3 expression measured using SYBR green qPCR. N=3. ***p<0.001, **p<0.01, *p<0.05.

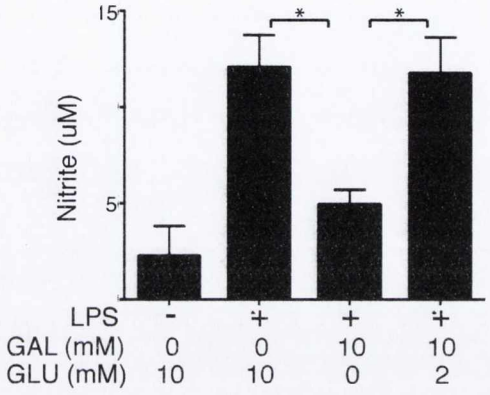


Fig 4.7 Adding both glucose and galactose is sufficient to reestablish HIF-1α activity (A-B) DCs were stimulated for 8 hours in normal media. Cells then washed twice with glucose free media and then incubated for 16 hours in glucose free media with the indicated concentrations of glucose and galactose. NO was measured using the Griess reaction. PHD3 expression measured using SYBR green qPCR. N=2. ***p<0.001, **p<0.01, *p<0.05.

4.2.7 Inhibiting O-linked glycosylation blocks HIF-1α activity

Removing glucose and replacing it with galactose blocks HIF-1α activity, independent of mTORC1 signaling. This effect is a direct result of glucose removal as adding back low doses of glucose was sufficient to reestablish HIF-1α activity. This suggested that glucose in the microenvironment was having an effect on signaling within the DC. About 5% of glucose taken up by cells is used for glycosylation (Bond and Hanover, 2013). There was a recent study that linked O-linked glycosylation to HIF-1α activity in cancer cells(Ferrer et al., 2014). Switching the fuel source from glucose to galactose would be predicated to block O-linked glycosylation (Goldberg et al., 2006). Experiments were designed to investigate this as a possible mechanism for inducing HIF-1α activity in DCs. The enzyme O-linked N-acetylglucosamine (O-GlcNAc) transferase (OGT) is required to catalyze the addition of O-GlcNAc to serine/threonine residues(Bullen et al., 2014). We used an established inhibitor of OGT ST045849 (OGTi) to investigate if O-linked glycosylation was playing a role in HIF-1α activity(Filhoulaud et al., 2009). Adding OGTi blocked HIF-1α protein stabilization when compared to levels in LPS stimulated BMDCs. HIF-1α activity was also blocked as PHD3 expression was significantly reduced. OGTi does not block mTORC1 signaling, as there are similar levels of pS6 in LPS treated with or without OGTi (Fig 4.8).

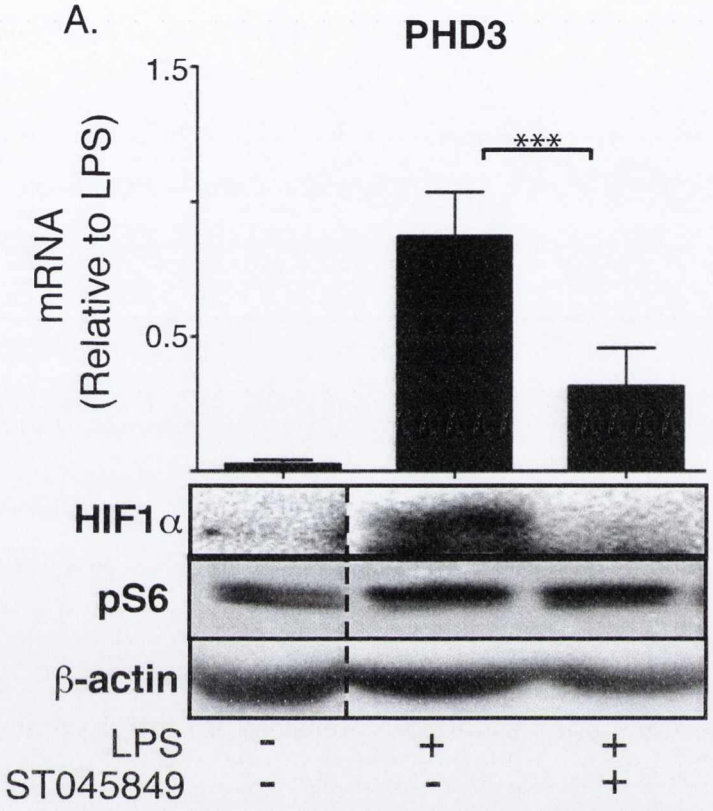


Fig 4.8 Inhibiting O-linked glycosylation blocks HIF-1α activity (A) DCs was stimulated for 8 hours after which ST045849 (1uM) or DMSO vehicle control was added. Cells were lysed at 24 hours. Indicated protein expression was analyzed by western blot. PHD3 expression measured using SYBR green qPCR. N=3. ***p<0.001, **p<0.01, *p<0.05.

4.2.8 Glucose is required for the metabolic shift in LPS stimulated DCs

Glucose is required for HIF-1α stabilization, independently of mTORC1. We hypothesized that replacing glucose with galactose would prevent the LPS induced metabolic shift. Therefore, detailed metabolic analysis of LPS activated DCs in the presence of galactose was performed. While LPS stimulated DCs increased rates of glycolysis in the presence of glucose, when cultured in galactose rates of glycolysis were dramatically decreased. This is consistent with previous observations that culturing cells in the presence of galactose substantially reduces lactate production (Fig 4.9a) (Aguer et al., 2011). Giving BMDCs both 10mM galactose and 2mM glucose, the 2mM glucose was sufficient to reestablish the levels of glycolysis seen in DCs stimulated in 10mM glucose only (Fig 4.10a). Interestingly galactose cultured DC did not reduce the rate of OxPhos in response to LPS (Fig 4.9b). Indeed DCs in the presence of 10mM galactose had increased levels of OxPhos, even relative to unstimulated DCs (Fig 4.10b). In DCs that had both 10mM galactose and 2mM glucose, 2mM glucose was sufficient to allow DCs decrease their rate of OCR to the same levels of LPS stimulated DCs in 10mM glucose (Fig 4.10b). This demonstrates that glucose is required for the metabolic shift seen in activated DCs.

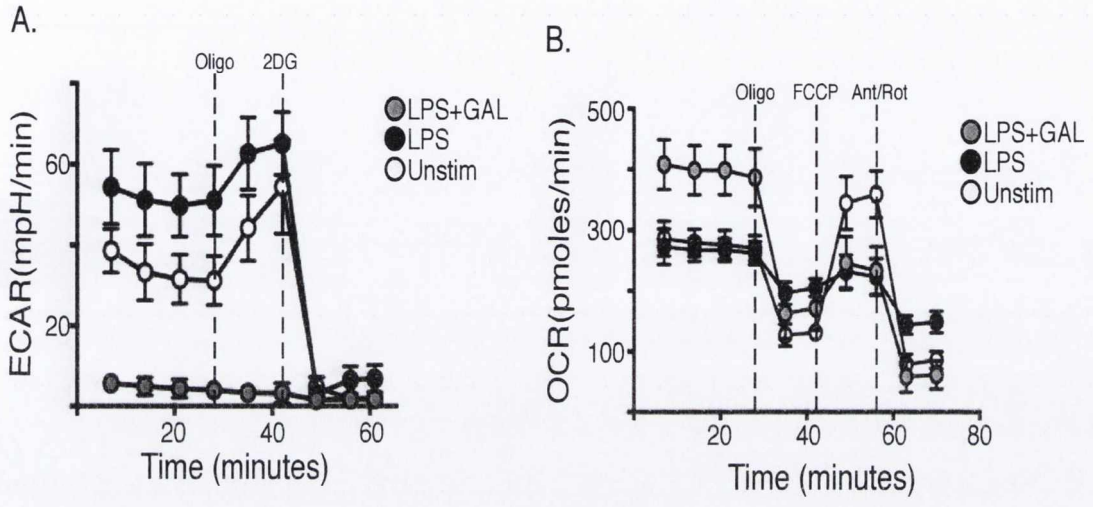


Fig 4.9 Replacing glucose with galactose blocks the metabolic shift in LPS stimulated DCs (A-B) BMDCs stimulated for 24 hours +/- LPS (100ng/ml), with 10mM glucose (unstim and LPS) or 10mM galactose (LPS+GAL) and analysed using a Seahorse Extracellular Flux analyser. Representative Seahorse traces for ECAR and OCR.

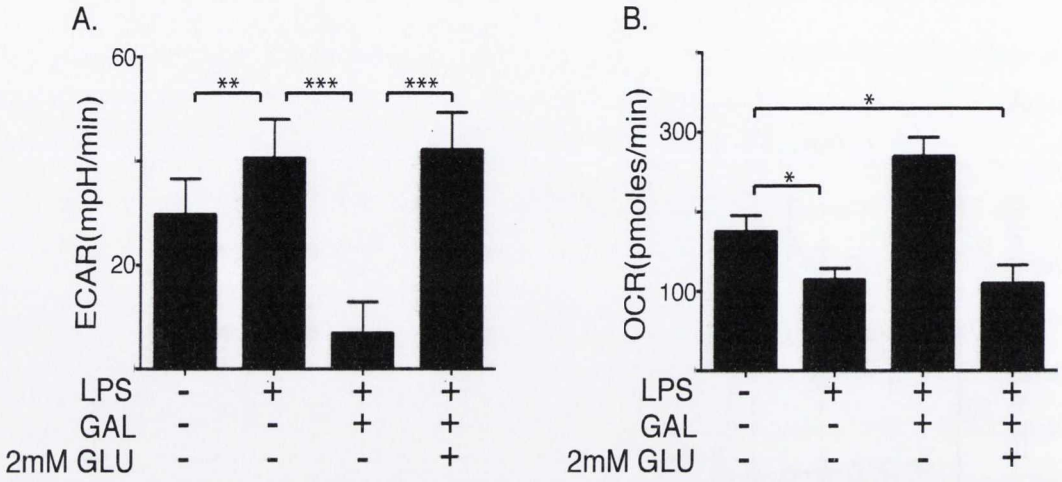


Fig 4.10 Glucose is required for the metabolic shift in LPS activated DCs (A-B) BMDCs stimulated for 24 hours +/- LPS (100ng/ml), with 10mM glucose (unstim and LPS), 10mM galactose (LPS+GAL) or 10mM galactose+ 2mM glucose (LPS+GAL+2mM GLU) and analysed using a Seahorse Extracellular Flux analyser. Pooled data N=2. ***$p<0.001$, **$p<0.01$, *$p<0.05$.

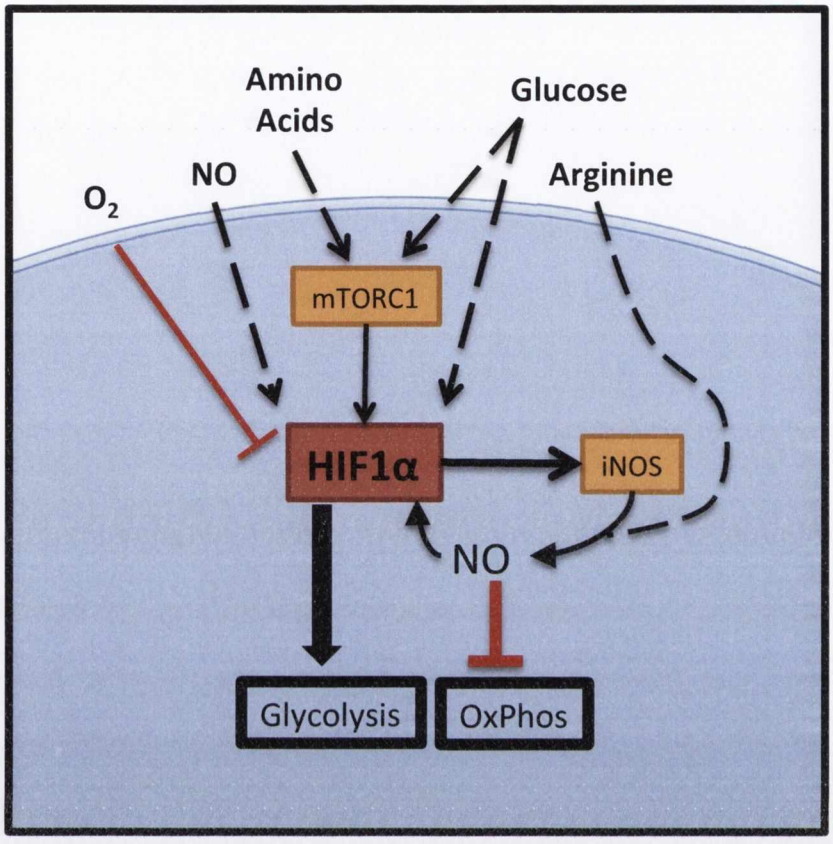


Fig 4.11 Schematic of how nutrient availability impacts on DC metabolism. White space indicates cellular microenvironment and nutrients available. Blue space indicates signaling events within activated DCs.

4.3 Discussion

This study presents the novel finding that 24 hour LPS activated DCs undergo glycolytic reprogramming characterized by increased expression in glycolytic genes and glucose transporters. These transcriptional changes are dependent on the metabolic regulators mTORC1 and HIF-1α. Previous work had demonstrated that elevated glycolysis in 24 hour activated DCs required NO production by the enzyme iNOS. This model proposed that following NO dependent inhibition of OxPhos increased pyruvate, the product of glycolysis, cannot be metabolized in the mitochondria and so is converted to lactate instead. This model suggests that the increased conversion of pyruvate to lactate accounts for the observed increase in glycolysis (Everts et al., 2012). However data presented in this PhD demonstrates that glycolytic reprogramming to increase the cellular glycolytic machinery is important for the observed changes in the rates of glycolysis. Our data shows that elevated glycolysis in 24 hour activated DC requires mTORC1 signaling. These data have been confirmed by another study that was published during the course of my PhD (Amiel et al., 2012). Our data makes the novel finding that mTORC1 is also required for DC glycolytic reprogramming. mTORC1 dependent glycolytic reprogramming has been shown in other activated effector immune cells such as T cells and NK cells (Finlay et al., 2012, Donnelly et al., 2014). In these cells there is an increase in glycolytic capacity, which is a measure of the glycolytic machinery within the cell that is blocked with the addition of rapamycin. However although mTORC1 is required for glycolytic capacity in LPS stimulated DCs, there was no difference in glycolytic capacity between unstimulated and LPS stimulated DCs even though there is a substantial increase in glycolytic genes. This has not been investigated fully but may indicate that genes other than those analyzed are those that determine the maximum glycolytic rate for DC. HIF-1α is an important transcription factor that promotes the expression of numerous glycolytic genes and glucose transporters (Liu et al., 2012). This metabolic regulator is required for elevated glycolysis in a number of immune cells including CD8+ T cells, Th17 cells and macrophages (Finlay et al., 2012, Dang et al., 2011, Cramer et al., 2003). HIF-1α has recently been shown to be required for DC glycolysis in response to Poly I:C (Pantel et al., 2014). Therefore combined our data argues for a key role for HIF-1α in promoting elevated rates of glycolysis in response to multiple TLR receptor

ligands. The data presented here represents a detailed metabolic study in LPS stimulated DCs. Recent research from the Pearce lab demonstrated that changes in metabolism occur immediately after DC activation. This study found that the rate of glycolysis increases minutes after DC activation and interestingly this process was found to be both mTORC1 and HIF-1α independent(Everts et al., 2014). These changes in metabolism occur too rapidly to involve any changes in mRNA or protein expression. Instead the translocation of the glycolytic enzyme Hexokinase II to the mitochondrial outer membrane results in increased glycolytic efficiency. Therefore while mTORC1 and HIF-1α are required to maintain increased glycolysis at 24 hours after activation they are dispensable for the increased rates of glycolysis that occur directly following activation. The data suggests that there are two distinct phases of glycolysis in activated DCs controlled by distinct mechanisms.

Our data has established a novel signaling circuit involving mTORC1/HIF-1α/iNOS that controls activated DC metabolism. There is a complex interplay between these metabolic regulators with each required for increased glycolysis and decreased OxPhos following LPS stimulation. Research published during the course of my PhD showed that nitric oxide is required for increased glycolysis and decreased OxPhos in DCs (Everts et al., 2012). Data in this PhD shows that mTORC1 and HIF-α is required for the metabolic shift in activated DCs and demonstrates an intimate relationship between these metabolic regulators. In astrocytes addition of rapamycin decreased iNOS mRNA expression (Lisi et al., 2011). This is consistent with a decrease in iNOS mRNA levels presented here following LPS stimulation in the presence of rapamycin. Indeed work published during the course of this PhD showed that the mTORC1 dependent decrease in OxPhos following LPS stimulation of DCs was due to decreased expression of iNOS (Amiel et al., 2014). Data presented in this PhD for the first time shows a requirement of NO for HIF-1α stabilization in activated DCs and links it directly to DC metabolism. LPS treated macrophages induce HIF-1α accumulation through a mechanism that requires reactive oxygen species (Nishi et al., 2008). Indeed nitric oxide itself has been shown in macrophages to stabilize HIF-1α (Sandau et al., 2001). A paper that was investigating cerebral ischemia showed that HIF-1α could bind to the HRE in the iNOS promoter, showing that during specific cellular events iNOS was a HIF-1α target gene (Matrone et al., 2004). Indeed macrophages in a hypoxic

microenvironment require HIF-1α for NO production (Takeda et al., 2010). However data presented here shows a novel involvement of HIF-1α dependent iNOS production in activated DC metabolism. Surprisingly in HIF-1α null DCs there is still a significant though reduced decrease in OxPhos, even though iNOS and NO production are substantially decreased, the remaining decrease in OxPhos was reversed by rapamycin addition. iNOS expression is significantly reduced in HIF-1α null DCs but iNOS expression is decreased even further with rapamycin addition. This suggests an mTORC1 dependent, HIF-1α independent mechanism for NO production. In macrophages LPS induced expression of iNOS requires octamer-binding factor 2 (Oct-2) (Lu et al., 2009). Oct-2 is expressed in B cells and the CNS but also in some cells of the myeloid lineage (Corcoran et al., 2004). A possible mechanism for the mTORC1 dependent but HIF-1α independent expression of iNOS involves mTORC1 dependent expression of the transcription factor octamer-binding factor 2 (Oct-2). In LPS treated macrophages rapamycin addition inhibits Oct-2 and hence decreases iNOS expression (Chou et al., 2011). Our data presented here did not find an increase in HIF-1α mRNA between unstimulated and LPS stimulated. This suggests that HIF-1α expression in activated BMDCs is controlled exclusively at the posttranscriptional level. In T cells HIF-1α expression requires both increased mTORC1 dependent transcription and translation (Bollinger et al., 2015, Nakamura et al., 2005). A previous study in LPS stimulated BMDCs showed a modest increase in HIF-1α mRNA (Jantsch et al., 2008). The direct link between mTORC1 and HIF-1α stabilization in DCs remains undetermined.

Data presented in this PhD shows that glucose in the cellular microenvironment is required for HIF-1α activity in activated DCs, through both mTORC1 dependent and independent mechanisms. This link between glucose availability and HIF-1α expression is entirely novel in the context of DC metabolism. Glucose deprivation of CD8+ CTLs leads to an inhibition of mTORC1 through a mechanism that involves a decrease in the ATP pool and the activation of the the energy sensor AMPK (Rolf et al., 2013). Glucose deprivation of DCs also leads to mTORC1 inhibition and decreased HIF-1α activity suggesting that glucose deprivation inhibits mTORC1 in activated DCs through a similar mechanism. Indeed in cancer cells the accumulation of HIF-1α protein requires glucose even in a hypoxic environment confirming HIF-1α as an important glucose and energy sensor

(Vordermark et al., 2005). Alternative strategies to investigate glucose deprivation and directly inhibit glycolysis have previously been described. This involves using the alternative carbon source galactose. Galactose is metabolized through the Leloir pathway with the end product G6P then entering the glycolytic pathway to produce pyruvate(Ross et al., 2004). The conversion of galactose to G6P is rate limiting and hence cells cultured in galactose cannot maintain elevated glycolysis (Aguer et al., 2011). A key difference in this strategy of glucose deprivation is that galactose provides pyruvate for mitochondrial OxPhos so these cells avoid ATP depeletion and sustain mTORC1 activity (Donnelly et al., 2014). Activated DCs in galactose have minimal rates of glycolysis but an increased OxPhos rate. Galactose treatment also did not effect mTORC1 signaling compared to DCs in the presence of glucose. However interestingly HIf-1α activity and NO production were completely blocked. This suggested a direct link between glucose availability and HIF-1α activity independent of mTORC1. Adding 2mM glucose to DCs in 10mM galactose reestablishes HIF-1α activity. However data presented here shows that DCs in 2mM glucose have inhibited mTORC1 signaling and that DCs in galactose have normal mTORC1 signaling. This suggests that glucose in this setting is contributing to some form of signaling rather than just as an energy source. Between 2-5% of intracellular glucose is used for glycosylation (Bond and Hanover, 2013). Evidence has emerged that links nutrient sensing to O-linked glycosylation (Zachara and Hart, 2004). Indeed in mesangial cells the availability and uptake of glucose is directly linked to the amount of glucose that enters the hexosamine biosynthetic pathway which produces O-GlcNAc the carbohydrate required for O-linked glycosylation (Goldberg et al., 2006). Recently a paper linked O-linked glycosylation to HIF-1α stability in cancer cells (Ferrer et al., 2014). In these cancer cells there is increased levels of O-GlcNAc. Increased O-GlcNAc is linked to reduced levels of α-ketoglutarate, the cofactor required for hydroxylation of HIF-1α and hence its degradation. The exact mechanism linking O-GlcNAc to α-ketoglutarate remains unknown. Our data shows that inhibiting OGT, the enzyme required for O-linked glycosylation, blocks HIF-1α activity independent of mTORC1. To our knowledge this is the first time that glucose availability and O-linked glycosylation has been linked to HIF-1α activity in any immune cell.

This study demonstrates that mTORC1 signaling in DCs is sensitive to amino acid availability in the cellular microenvironment. However data presented in this PhD also shows the novel finding that arginine in the microenvironment is required for HIF-1α activity and increased DC glycolysis, independently of mTORC1. As described previously mTORC1 is exquisitely sensitive to amino acid availability in the cellular microenvironment (Jewell et al., 2013). A paper from the Murphy group showed that leucine and glutamine are essential for mTORC1 signaling as in their absence mTORC1 failed to interact with RHEB and remained inactive (Nicklin et al., 2009). Recently in T cells uptake of these amino acids by the amino acid transporter SLC7A5 was shown to be required for mTORC1 signaling and T cell activation(Sinclair et al., 2013). Our data shows similar results as completely removing all amino acids blocks mTORC1 signaling in DCs, whereas adding just leucine and glutamine was sufficient to maintain mTORC1 signaling. A study in human monocyte derived DCs showed that valine was also required to maintain mTORC1 signaling perhaps indicating that different amino acids are required for mTORC1 signaling in different DC subsets (Kakazu et al., 2007). Work in hamster ovary cells had previously shown that removing arginine had an inhibitory affect on mTORC1 signaling (Hara et al., 1998). Our data in DCs show that removing arginine does not affect mTORC1 signaling. However data presented in this PhD shows the novel finding that removing arginine blocks NO production and hence HIF-1α activity. It has been known for many years that arginine is required for nitric oxide production (Hibbs et al., 1987). Evidence in this PhD directly links arginine, a nutrient in the cellular microenvironment, to elevated glycolysis in activated DCs.

Chapter 5

Nutrient availability in the microenvironment impacts on DCs ability to induce T cell responses

5.1 Introduction

A consensus view is emerging in the field of immunometabolism that the metabolism of immune cells is not just linked to energy generation within these cells but is directly linked to their effector function. Work in our lab has linked mTORC1 and HIF-1α directly to CTL effector function and migration (Finlay et al., 2012). We have also established that mTORC1 is essential for IFNγ and granzyme B production in NK cells (Donnelly et al., 2014). In macrophages HIF-1α is required for IL-1β production as well as elevated glycolysis (Tannahill et al., 2013). There are numerous other examples linking metabolism directly to immune cell effector function in the literature. Chapter 3 and 4 describe key roles for mTORC1/HIF-1α/iNOS in controlling BMDC metabolism. The data also show this signaling axis is acutely sensitive to conditions in the local microenvironment. This chapter will explore whether this signaling axis also has an important role in controlling DC function. Nutrient availability, particularly at tumor sites and at sites of inflammation, has emerged as an important factor in the outcomes of an immune response (Chang et al., 2015). We have established a nutrient sensing signaling circuit centered on HIF-1α that controls DC metabolism. In this chapter we will also investigate if nutrient availability has any effect on DC function. DCs induce T cell responses by providing essential signals to the T cell. These involve "signal 1" that is the antigen presented on the cell surface in a complex involving MHC molecules. Co-stimulation by co-stimulatory molecules provide "signal 2". The cytokines that the DC secretes contributes to "signal 3" that determines the type of T cell response induced for example Th1 vs. Th2.

5.2.1 Glucose removal does not affect DC viability

We have shown that glucose is required for HIF-1α stabilization and for elevated glycolysis in activated DCs. DCs in the absence of glucose also have significantly decreased NO production. In the absence of NO production DCs have increased viability days after activation (Amiel et al., 2014). We wanted to examine if removing glucose, and hence a decrease in NO production, had any effect on DC viability. As previously described BMDCs were activated in glucose for 8 hours. After 8 hours DCs were washed with glucose free media and then placed in media with either 10mM glucose or 10mM galactose and analysed for viability 24 hours, 48 hours and 72 hours after DC activation. Viability was determined by forward and side scatter analysis by flow cytometry. There was no difference in viability in all conditions 24 hours and 48 hours after DC activation. There was a decrease in viability in all conditions 72 hours after activation, with the lowest viability in LPS stimulated DCs in the presence of glucose. However the difference between the viability of DCs in glucose or galactose was not significant (Fig 5.1).

5.2.2 DCs in the absence of glucose express more MHC-II on the cell surface

DCs inducing T cell responses must present antigen to T cells at the cell surface. To induce CD4+ T cell responses DCs load antigen onto major histocompatibility complex class II (MHC-II) molecules inside the cell and then traffic this antigen/MHC complex to the surface of the cell. Immature DCs maintain high expression of MHC-II on their cell surface. Upon activation the surface expression of MHC-II increases significantly (Cho and Roche, 2013). Measuring the surface expression of MHC-II in our different conditions would give us an indication of the ability of the DC to present antigen. This is called "signal 1" and is essential for inducing T cell responses (Reis e Sousa, 2006). We set up our glucose/galactose experiments as previously described and measured MHC-II expression by flow cytometry. Similar to what had previously been published there was significant increases in MHCII expression with LPS stimulation. There was a clear trend of increasing MHC-II expression 24 hours, 48 hours and 72 hours after activation with a significant difference at 48 hours (Fig 5.2). The surface expression of MHC-I is highly expressed on immature DC and only increases marginally with activation.

For this reason MHC-I expression was not measured in this study (Gil-Torregrosa et al., 2004).

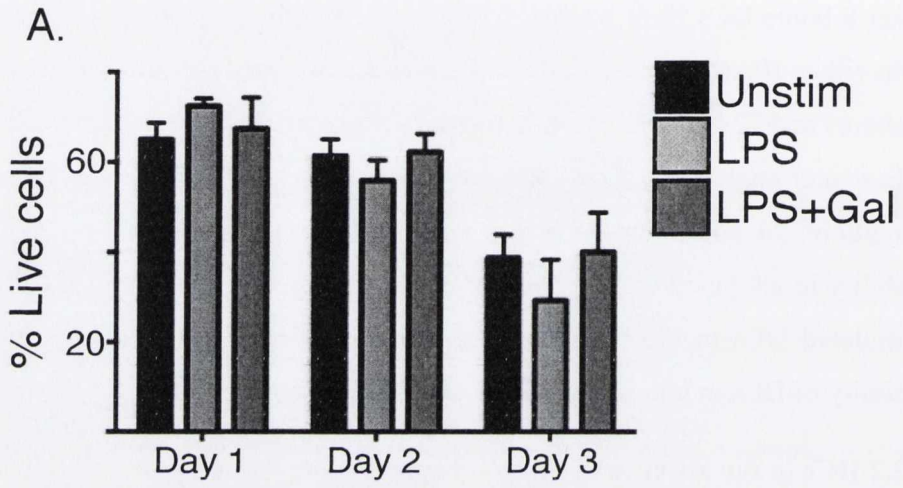


Fig 5.1Glucose removal does not affect DC viability. DCs were stimulated +/- LPS for 8 hours in normal media. Cells were then washed and placed in media containing either 10mM glucose or 10mM galactose and analysed by flow cytometry for viability using SSC-A and FSC-A. (A) Pooled data N=6.

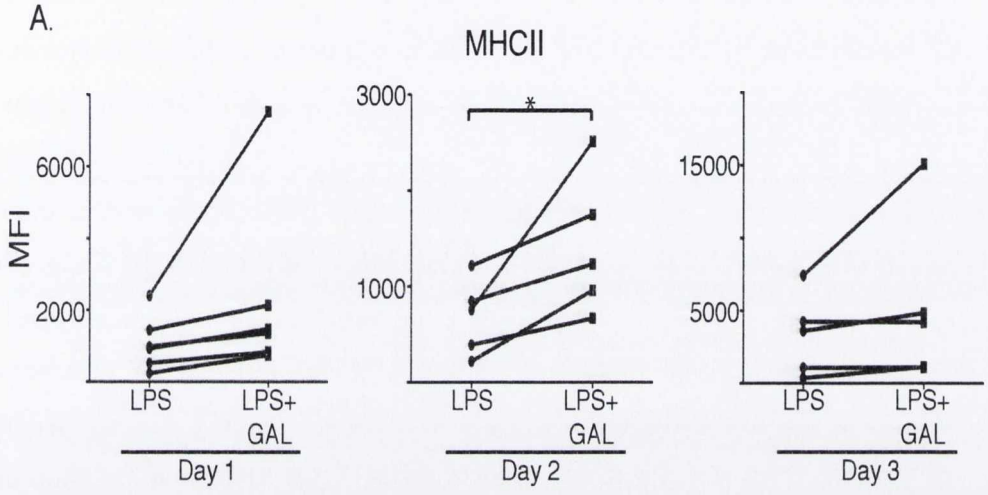


Fig 5.2 DCs express more MHC-II in the absence of glucose. DCs were stimulated +/- LPS for 8 hours in normal media. Cells were then washed and placed in media containing either 10mM glucose or 10mM galactose and analysed by flow cytometry for MHC-II expression (A) Pooled data N=5. ***$p<0.001$, **$p<0.01$, *$p<0.05$.

5.2.3 HIF-1α null DCs have increased expression of MHC-II

BMDCs in the absence of glucose have increased expression of MHC-II. In chapter 4 it was shown that glucose is required for HIF-1α stabilization and function through both mTORC1 dependent and independent mechanisms. Using HIF-1α null DCs experiments were designed to investigate if the increase in MHC-II expression in the absence of glucose was due to the absence of HIF-1α activity under these conditions. HIF-1α null DCs were activated in 10mM glucose and expression of MHC-II was compared to the expression in wild type DCs. Similar to in the glucose deprivation studies LPS stimulation induced a significant increase in MHCII expression in both the wild-type and HIF-1α null DCs. There was a trend of increased expression 24 hours and 72 hours after activation with a significant increase observed at 24 hours. There was large variability in expression levels 48 hours after activation with individual replicates having either a large increase in expression in the KO while other individual replicates having slightly decreased expression (Fig 5.3). Overall there is a trend of increased MHC-II expression in the HIF-1α null DCs, similar to that seen in DCs that had been activated in the absence of glucose.

5.2.4 Glucose removal increases co-stimulatory molecule expression in LPS stimulated DCs

The data shows that removing glucose blocks the metabolic shift in DCs. There also is a clear link between glucose availability and HIF-1α activity in activated DCs. Next experiments were designed to investigate if glucose deprivation and the resultant decreases in HIF-1α signaling had any impact on co-stimulatory molecules CD40, CD80 and CD86. These co-stimulatory molecules are required to provide "signal 2" to T cells when inducing T cell responses. T cells that receive "signal 1" in the absence of "signal 2" do not fully activate and enter a tolerogenic or anergic state (Bakdash et al., 2013). Previous studies have shown that inhibiting mTORC1 in DCs increases expression of co-stimulatory molecules on DCs following activation (Amiel et al., 2012). Although replacing glucose with galactose does not block mTORC1 signaling, it does block HIF-1α activity. We wanted to see if there

was any link between glucose availability and co-stimulatory molecule expression. BMDCs were switched into media containing 10mM glucose or galactose 8 hours after LPS stimulation and analyzed for co-stimulatory molecule expression 1, 2 and 3 days after activation. In the presence of glucose LPS stimulates a large increase in CD80 and CD86 expression on day 1 that decreases with time returning to close to unstimulated levels by day 3. However when BMDCs were placed in galactose there was a similar LPS induced increases in CD80 and CD86 that was maintained over the course of 3 days. Therefore on day 2 and day 3 there are significant differences in CD80 and CD86 expression in glucose cultured versus galactose cultured BMDCs (Fig 5.4, Fig 5.5). Analysis of DCs that are double positive for CD80 and CD86 expression yielded similar results (Fig 5.6). In contrast to the data for CD80 and CD86 glucose availability did not affect CD40 expression. LPS induced a large increase in CD40 expression on day 1 in both the presence of glucose and galactose. The expression of CD40 was maintained at the same levels on day 2 and day 3 in both conditions, indicating that glucose availability does not impact on CD40 expression to the same extent it does with CD80 and CD86 (Fig 5.7).

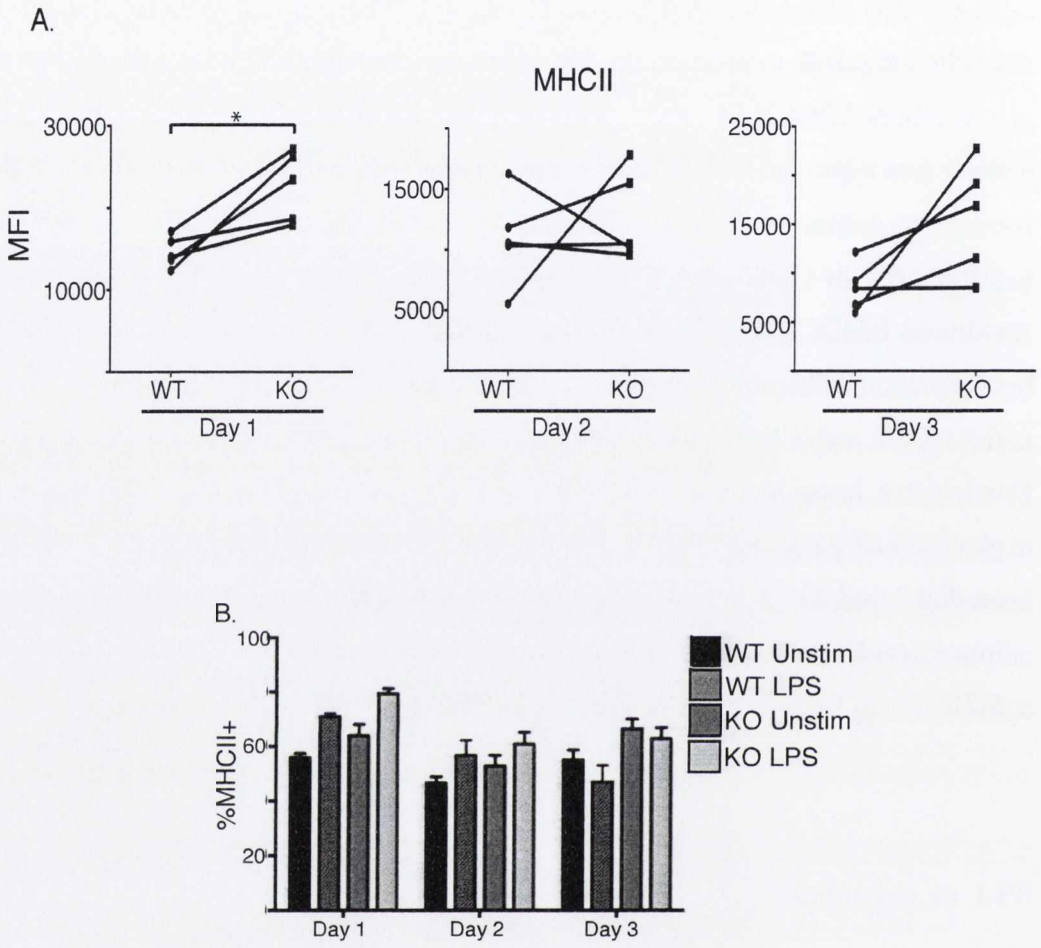


Fig 5.3 HIF-1α null DCs have increased MHC-II expression. HIF-1α null DCs were stimulated +/- LPS and analysed by flow cytometry for MHC-II expression day 1, day 2 and day 3 after DC activation. (A) Pooled data N=5. ***$p<0.001$, **$p<0.01$, *$p<0.05$.

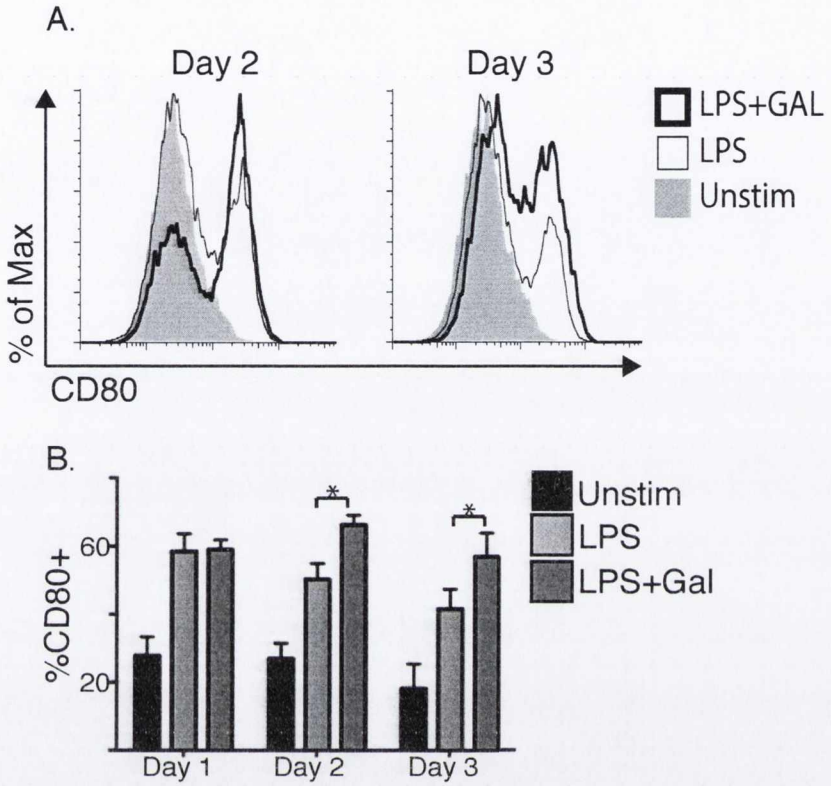


Fig 5.4 Glucose deprivation sustains CD80 expression. DCs were stimulated +/- LPS for 8 hours in normal media. Cells were then washed and placed in media containing either 10mM glucose or 10mM galactose and analysed by flow cytometry for CD80 expression (A) Representative histogram. (B) Pooled data N=6. ***p<0.001, **p<0.01, *p<0.05.

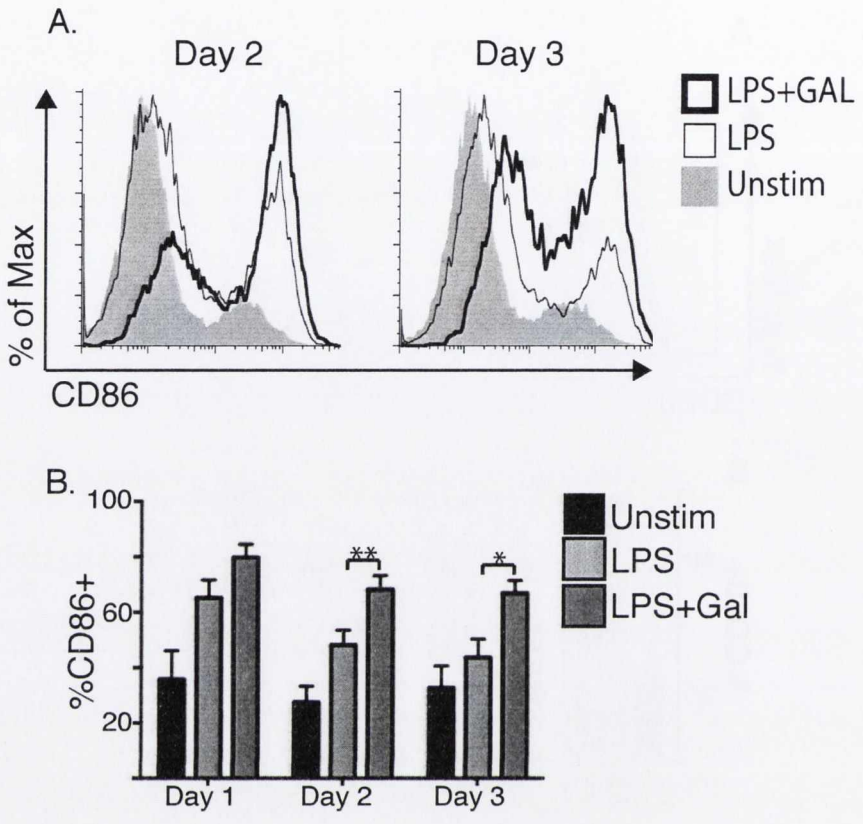


Fig 5.5 Glucose deprivation sustains CD86 expression. DCs were stimulated +/- LPS for 8 hours in normal media. Cells were then washed and placed in media containing either 10mM glucose or 10mM galactose and analysed by flow cytometry for CD86 expression (A) Representative histogram. (B) Pooled data N=6. ***p<0.001, **p<0.01, *p<0.05.

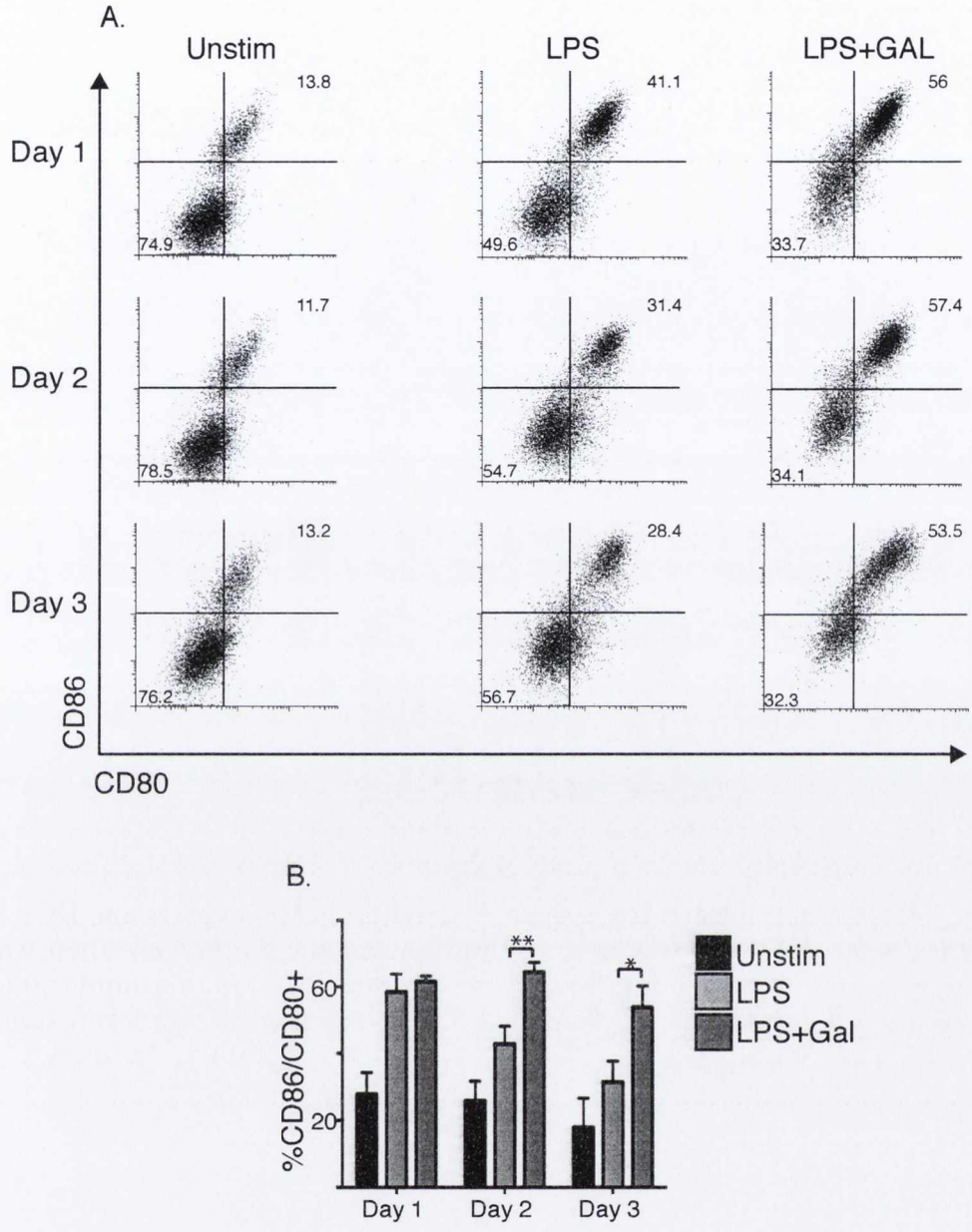


Fig 5.6 Glucose deprivation sustains CD80/CD86+ DCs after activation. DCs were stimulated +/- LPS for 8 hours in normal media. Cells were then washed and placed in media containing either 10mM glucose or 10mM galactose and analysed by flow cytometry for CD80/CD86+ cells (A) Representative dot plots. (B) Pooled data N=6. ***$p<0.001$, **$p<0.01$, *$p<0.05$.

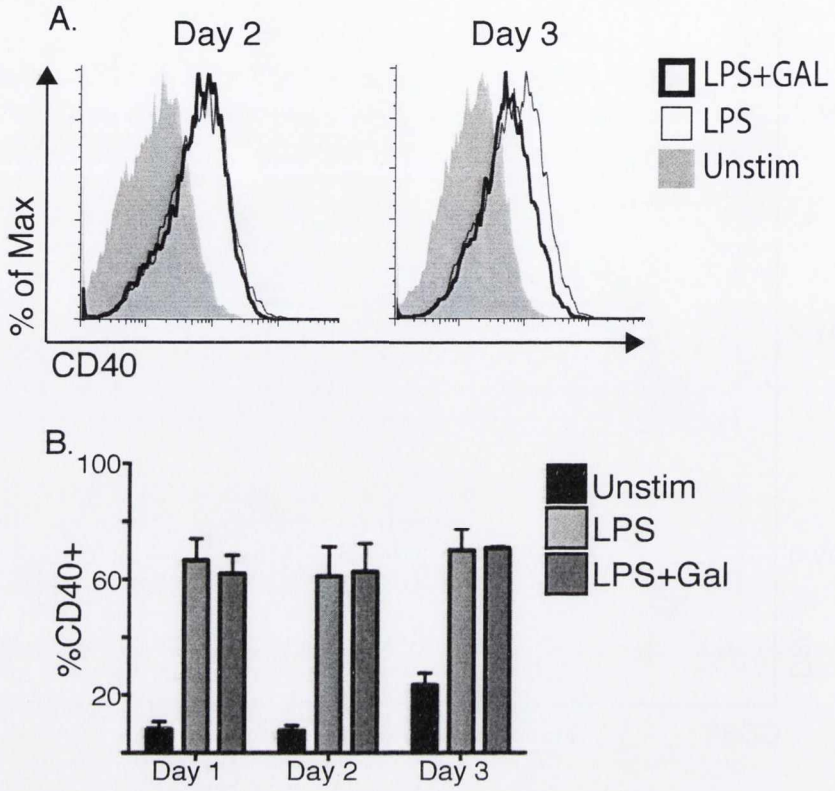


Fig 5.7 Glucose deprivation does not affect CD40 expression. DCs were stimulated +/- LPS for 8 hours in normal media. Cells were then washed and placed in media containing either 10mM glucose or 10mM galactose and analysed by flow cytometry for CD40 expression (A) Representative histogram. (B) Pooled data N=6. ***p<0.001, **p<0.01, *p<0.05.

5.2.5 HIF-1α null DCs have increased expression of CD80 and CD86

The results from these experiments show that glucose deprived LPS stimulated BMDCs have increased expression of CD80 and CD86. Under these conditions mTORC1 signaling is normal but HIF-1α activity is lost. We wanted to investigate whether the deletion of HIF-1α would have similar effects on co-stimulatory molecule expression to when the HIF-1α gene was present. The percentage of CD80/CD86+ DCs decreased over time after LPS activation. In contrast the percentage of CD80/CD86+ cells in HIF-1α null DCs was maintained 48 hours and 72 hours after activation, with a significant difference between HIF-1α KO and WT BMDCs observed at 72 hours (Fig 5.8). This was a similar trend to what we observed in DCs activated in the absence of glucose. CD40 expression did not change in DCs if there was glucose present or not. The same effect is seen in HIF-1α null and WT DCs that express equivalent levels of CD40 expression at all time points investigated (Fig 5.9).

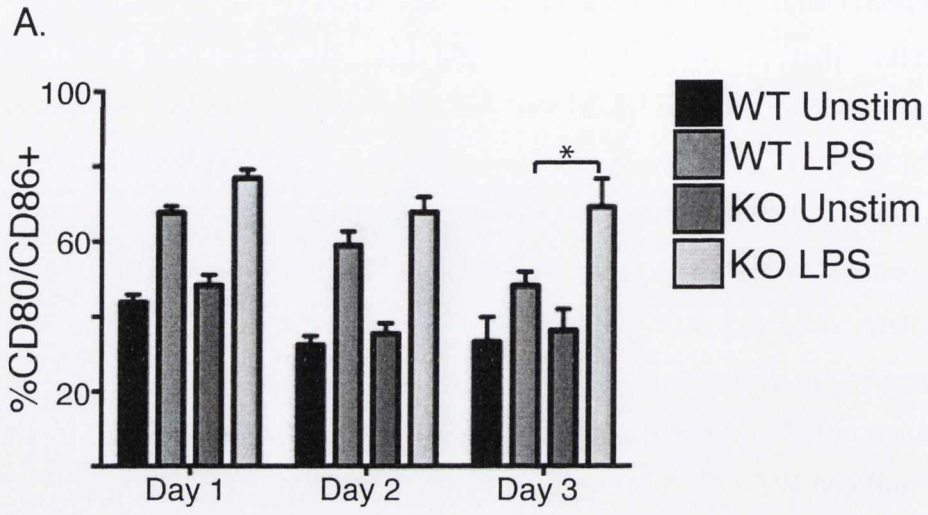


Fig 5.8 HIF-1α null DCs have increased CD80 and CD86 expression. HIF-1α null DCs were stimulated +/- LPS and analysed by flow cytometry for %CD80/CD86+ cells day 1, day 2 and day 3 after DC activation. (A) Pooled data N=5. ***p<0.001, **p<0.01, *p<0.05.

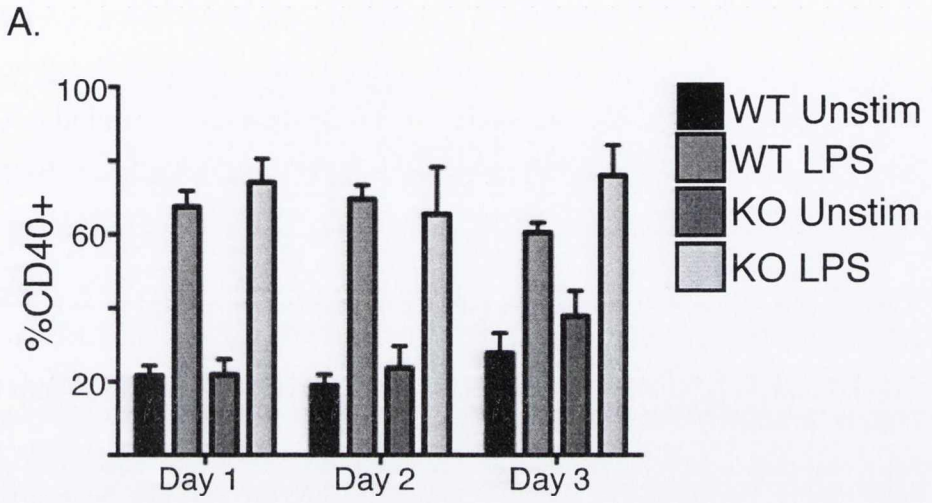


Fig 5.9 CD40 expression is not affected in HIF-1α null DCs. HIF-1α null DCs were stimulated +/- LPS and analysed by flow cytometry for CD40 expression day 1, day 2 and day 3 after DC activation. (A) Pooled data N=5.

5.2.6 mTORC1 regulates the expression of cytokines IL-10 and IL-12

When DCs are activated they secrete an array of cytokines such as the pro-inflammatory cytokines IL-6, TNF-α and IL-12 as well as anti-inflammatory cytokines such as TGF-β and IL-10. Il-12 has various roles including activating NK cells and providing "signal 3" to T cells during antigen specific T cell activation (Curtsinger et al., 2003). IL-10 dampens immune responses by inhibiting cytokine secretion and co-stimulatory molecule expression. mTORC1 has been implicated in the control of both IL-10 and IL-12 in both human and murine myeloid cells (Weichhart et al., 2011, Ohtani et al., 2008). Experiments were designed to investigate the link between the mTORC1/HIF-1α/iNOS signaling axis and BMDC cytokine production. The data show that mTORC1 inhibition by rapamycin blocked the LPS induced expression of IL-10 mRNA (Fig 5.10a). IL-12 has two subunits, the p40 subunit that is shared with IL-23 and the p35 subunit that is specific to IL-12. We used primers for the p35 subunit to look at IL-12 specific expression. There is an increase in expression of IL-12 with 24 hour LPS stimulation that is further increased when mTORC1 signaling is blocked (Fig 5.10b). The data argues that mTORC1 signaling inhibits the expression of IL-12p35 mRNA. However when the protein levels of the active form of IL-12, IL-12p70, were analysed after 24 hours, no difference was observed with or without rapamycin (Fig 5.10c). To investigate the discrepancy between mRNA analysis by qPCR and protein analysis by ELISA the levels of IL-12p35 mRNA was determined at various timepoints following LPS stimulation. IL-10 mRNA expression was induced 4 hours after LPS stimulation and reached a peak after 16 hours. Rapamycin addition blocks the expression of IL-10 at all time points (Fig 5.11a). In contrast, IL12p35 expression peaks between 4 and 8 hours and importantly this early burst of IL-12p35 expression was insensitive to rapamycin. IL-12p35 mRNA expression decreases substantially after 16 and 24 hours. mTORC1 signaling only negatively impacts upon IL-12p35 expression after 24 hours. The initial burst of IL12 produced by DC following activation can be considered innate IL12 that is important for activation of NK cells. In contrast at later timepoints DC will be interacting with T cells and the IL-12 produced will be delivered directly into the T cell microenvironment (5.11b).

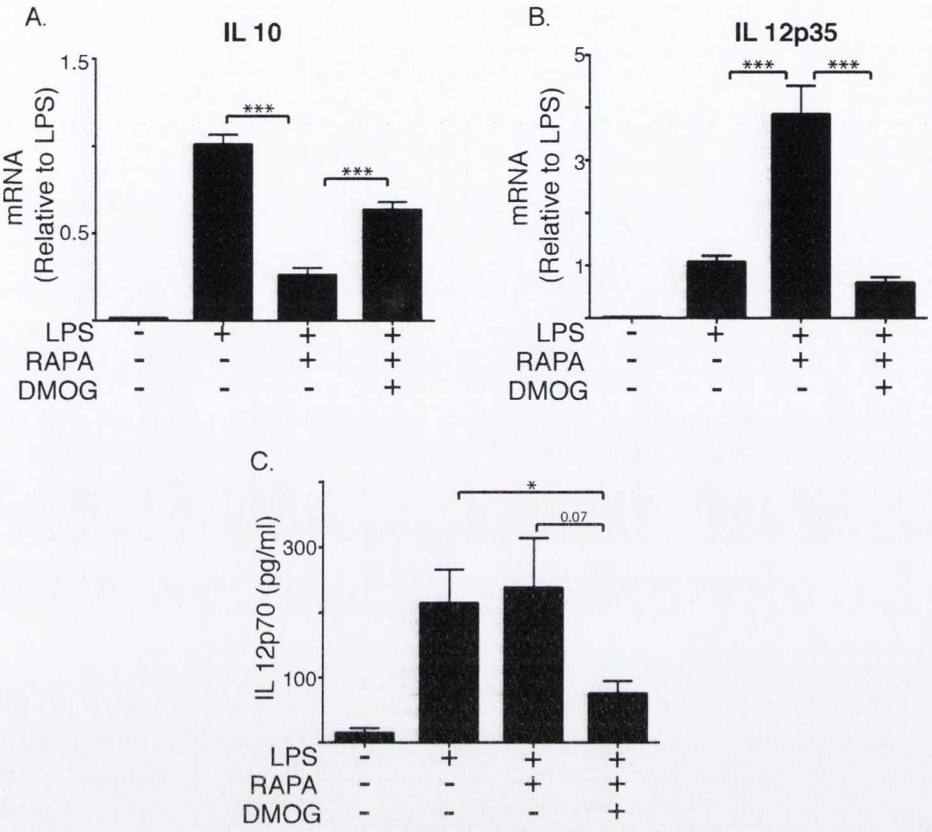


Fig 5.10 mTORC1 is involved in IL-10 and IL-12 expression in LPS stimulated DCs. DCs were stimulated +/- LPS, +/- RAPA for 24 hours (A-B) Cells were lysed and analysed for indicated mRNA expression using SYBR green qPCR. N=5 (C) Supernatants were analysed for IL-12p70 expression by ELISA. N=4 ***p<0.001, **p<0.01, *p<0.05. ns=not significant.

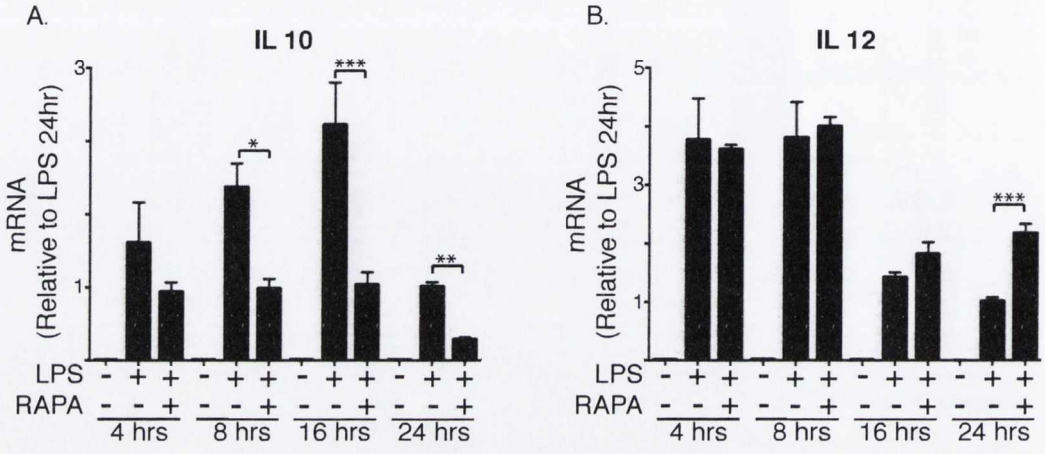


Fig 5.11 The dynamics of IL-10 and IL-12 expression is altered with mTORC1 inhibition. DCs were stimulated +/- LPS, +/- RAPA for the indicated time points (A-B) Cells were lysed and analysed for indicated mRNA expression using SYBR green qPCR. N=2. ***p<0.001, **p<0.01, *p<0.05. ns=not significant.

5.2.7 HIF-1α impacts on IL-10 and IL-12 expression in LPS activated DCs

mTORC1 is required for HIF-1α activity following LPS activation. There is evidence in the literature that links HIF-1α to cytokine production, for example IL-1β production in macrophages (Tannahill et al., 2013). We wanted to investigate if HIF-1α activity downstream of mTORC1 plays any role in the expression of IL-10 and IL-12. To assess whether HIF-1α was sufficient to alter the expression of IL-10 and IL-12 we used the HIF-1α activator DMOG, previously described in chapter 3, to induce HIF-1α expression in activated BMDCs that lack mTORC1 signaling due to the addition of rapamycin. IL-10 expression is blocked with rapamycin addition as previously shown. DMOG induced HIF-1α stabilization was sufficient to significantly increase the expression of IL-10, although not to the same levels as seen in LPS stimulated DCs alone (Fig 5.12a). There was a further increase in IL-12 mRNA expression with mTORC1 inhibition as previously described. This increase in IL-12 expression was completely blocked following DMOG induced HIF-1α activation with expression levels similar to levels seen in LPS only conditions (Fig 5.12b). These data argue that HIF-1α activity downstream of mTORC1 is sufficient to repress IL-12p35 expression. The data also suggests that mTORC1 induces IL-10 expression through both HIF-1α dependent and independent mechanisms. The levels of IL-12p70 were analysed and as before no significant differences were observed between LPS +/- rapamycin. However in contrast DMOG inhibited IL-12p70 expression (Fig 5.12c). We propose that mTORC1/HIF-1α inhibit IL-12 mRNA expression. During the normal LPS response HIF-1α activity is only observed after 16 hours, correlating with decreased IL-12p35 expression. We have shown that at earlier timepoints following LPS stimulation DMOG is sufficient to induce HIF-1α protein expression. Therefore in LPS+DMOG treated BMDCs HIF-1α is active for the duration of the 24 hour period. The decreased IL-12 protein measured in these cells by ELISA likely reflects HIF-1α mediated inhibition of IL-12p35 mRNA expression over the full 24 hour stimulation. This data argues that mTORC1/HIF-1α signaling inhibits IL-12 production. To further establish this link between HIF-1α and IL-10/IL-12 expression HIF-1α null DCs were analysed. There was a small but significant decrease in IL-10 expression when comparing LPS stimulated wild type DCs to HIF-1α null DCs. The level of IL-10 expression was further decreased in the HIF-1α null DCs with rapamycin addition (Fig 5.13a). The

expression of IL-12 mRNA was significantly increased in HIF-1α null DCs when compared to WT DCs. This level of expression was similar to levels observed with rapamycin addition in WT DCs. Indeed there was no further increase in IL-12 expression in HIF-1α null DCs with rapamycin addition (Fig 5.13b).Together these data argue an important role for mTORC1/HIF-1α signaling for repressing IL-12 production and suggests mTORC1 promotes IL-10 expression through multiple mechanisms.

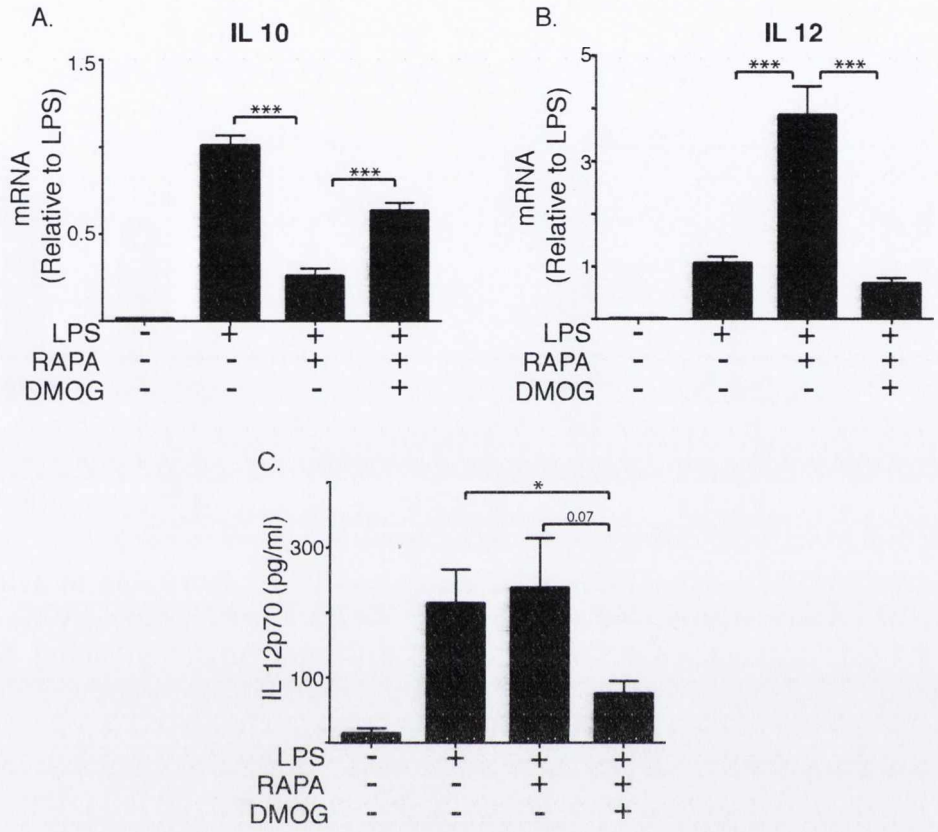


Fig 5.12 DMOG induced HIF-1α activity increases IL-10 and blocks IL-12 expression. DCs were stimulated +/- LPS, +/- RAPA +/- DMOG for 24 hours (A-B) Cells were lysed and analysed for indicated mRNA expression using SYBR green qPCR. N=3 (C) Supernatants were analysed for IL-12p70 expression by ELISA. N=4 ***p<0.001, **p<0.01, *p<0.05.

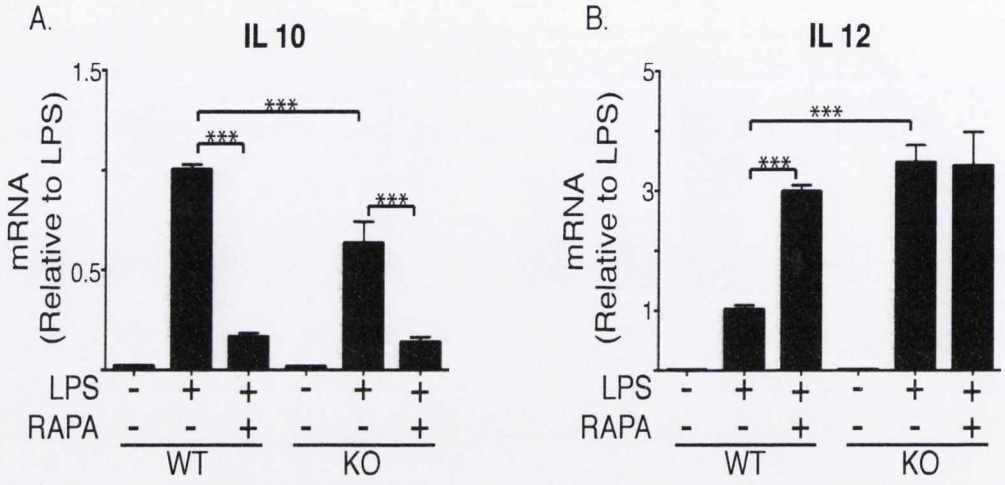


Fig 5.13 HIF-1α deletion impacts on IL-10 and IL-12 expression in activated DCs. DCs were stimulated +/- LPS, +/- RAPA for 24 hours (A-B) Cells were lysed and analysed for indicated mRNA expression using SYBR green qPCR. N=3. ***p<0.001, **p<0.01, *p<0.05.

5.2.8 iNOS activity is linked to IL-10 and IL-12 expression

The data shows that HIF-1α activity, downstream of mTORC1, is involved in controlling the balance of IL-10 and IL-12 expression. HIF-1α expression is controlled by numerous signals within DCs. We have shown that there is a complex interplay between HIF-1α and iNOS, with HIF-1α required for iNOS expression and NO required for HIF-1α stabilization. Experiments were designed to investigate if inhibiting iNOS, and thus blocking HIF-1α stabilization, had any affect on cytokine expression. The established iNOS inhibitor SEITU was used, that completely blocks NO production (Fig 3.9). Using this inhibitor there was a significant decrease in IL-10 expression when compared to levels in LPS stimulated DCs (FIG 5.5a). Blocking NO production also increased IL-12 mRNA expression (Fig 5.5b). This data further confirms a role for HIF-1α in controlling DC cytokine production.

5.2.9 Glucose deprived DCs sustain the ability to induce T cell proliferation

Glucose regulates the function of activated DCs in terms of signal 1-3 that are required to induce a full T cell response. The data shows that glucose regulates discrete signals but has no affect on others. Thus the impact that glucose would have on DC induced T cell responses was not clear. Experiments were designed to address this question. To investigate this we used OT-1 T cells that are CD8+ T cells that have a T cell receptor (TCR) specific for a peptide, SIINFEKL, derived from the protein ovalbumin. DCs were pulsed with SIINFEKL peptide and activated with LPS for 8 hours. At 8 hours DCs were washed with glucose free media and placed in media containing 10mM glucose or galactose. When adding OT-1 T cells on day 1, 2 and 3 after activation DC media was replaced with RPMI media in all conditions that contained normal concentrations of glucose. OT-1 T cells were stained with CFSE to allow analysis of T cell proliferation. T cells were analyzed 48 hours after co-culture with DCs for CFSE staining. This experimental design is illustrated in Fig 5.15. Effects on T cell activation were first investigated. CD69 is one of the earliest surface markers unregulated on T cells following activation(Ziegler et al., 1994). Glucose removal had no effect on CD69 expression

on T cells when added to DCs activated for 24, 48 or 72 hours (Fig 5.16). Similarly the ability of DCs to induce an increase in T cell size was not affected on any day showing that DCs in the presence or absence of glucose retained the ability to activate T cells (Fig 5.17). Experiments were designed to investigate the affect glucose removal had on T cell proliferation. Looking at the pooled data for T cells added to DCs on day 1 of DC activation there was no difference between either glucose and galactose conditions. Indeed a large % of T cells have undergone proliferation showing that DCs are efficient at inducing T cell proliferation 24 hours after activation. However the ability of DCs to induce T cell proliferation when cultured in the presence of glucose decreased dramatically 48 hours and 72 hours after activation. In stark contrast to this DCs cultured in the absence of glucose retained the ability to induce T cell proliferation with dramatic differences between the two conditions on day 2 and day 3 after DC activation (Fig 5.18). This effect on T cell proliferation is a glucose specific effect as culturing DCs in both 10mM galactose and 2mM glucose leads to T cell induced proliferation levels similar to that seen when DCs are cultured in 10mM glucose only (Fig 5.19).

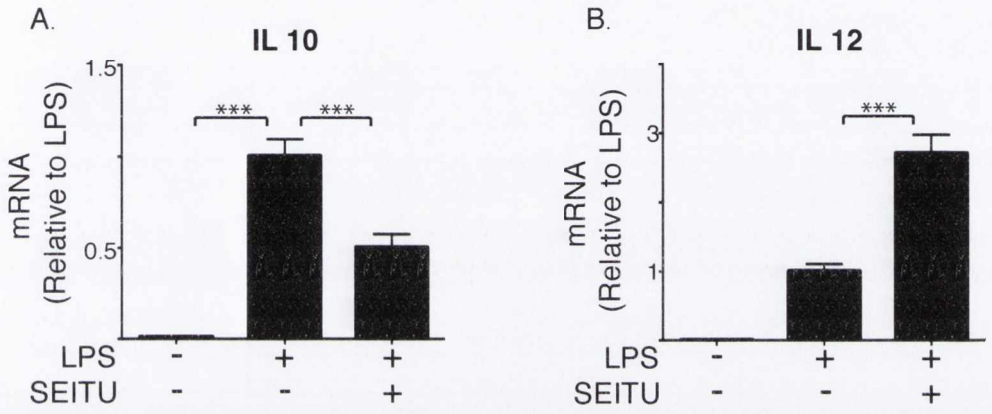


Fig 5.14 Inhibiting iNOS impacts on IL-10 and IL-12 expression in activated DCs. DCs were stimulated +/- LPS, +/- SEITU for 24 hours (A-B) Cells were lysed and analysed for indicated mRNA expression using SYBR green qPCR. N=3. ***p<0.001, **p<0.01, *p<0.05.

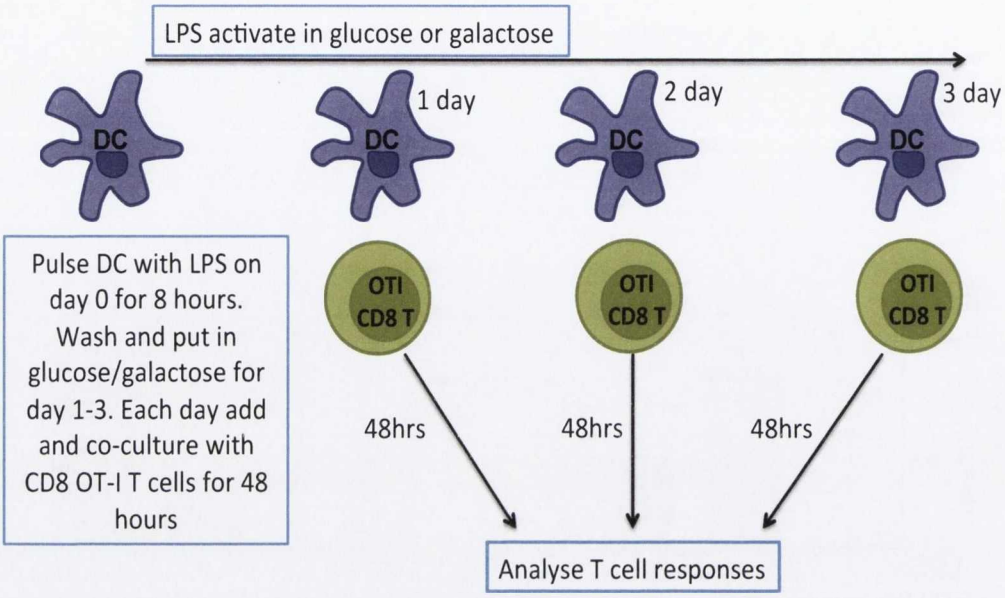


Fig 5.15 Experimental design for analyzing DC induced T cell responses. DCs were activated with LPS plus SIINFEKL peptide in the presence of glucose for 8 hours. At 8 hours DCs were washed in glucose free media and then placed in media containing 10mM glucose or galactose for day 1-3. On each day OT-I T cells were added to wells containing DCs in normal glucose media. T cell responses were then analysed 48 hours later.

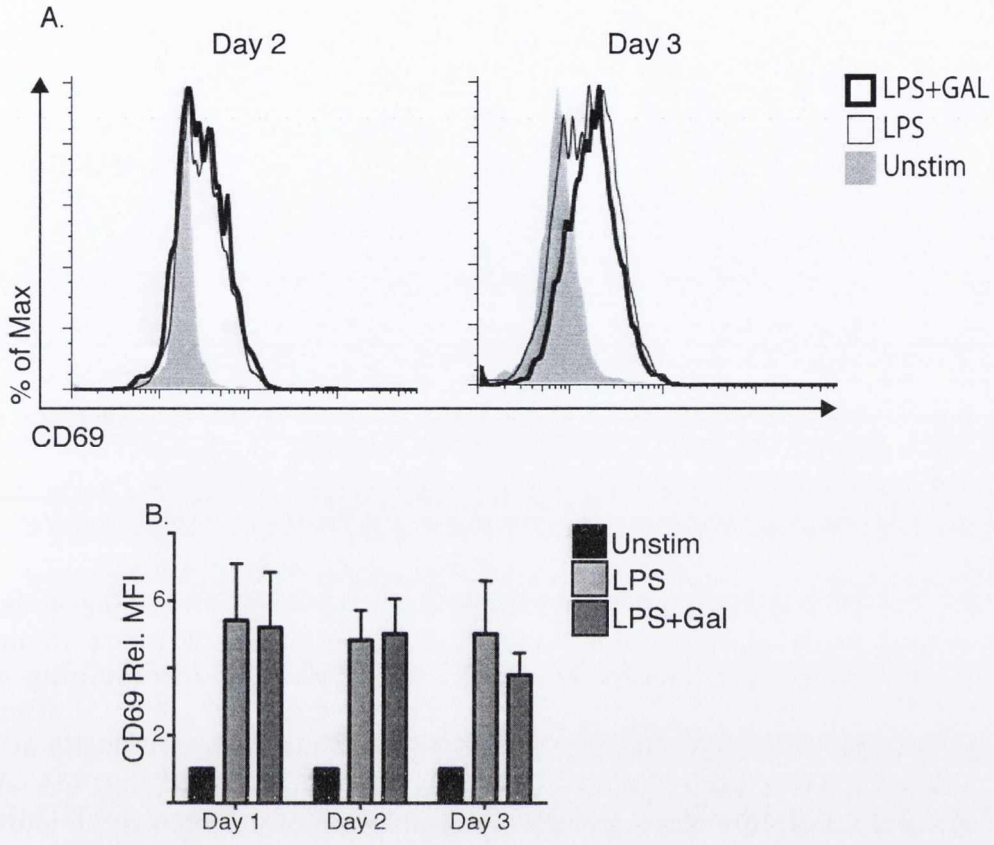


Fig 5.16 Induced T cell expression of CD69 is unchanged when cultured with DCs in the presence or absence of glucose. DCs were stimulated +/- LPS +/- SIINFEKL peptide for 8 hours in normal media. Cells were then washed and placed in media containing either 10mM glucose or 10mM galactose. On day 1, 2 and 3 after DC activation, OT-1 T cells were added in fresh media to DC wells at a 1:5 ratio. 48 hours later T cells were analysed for CD69 expression by extracellular staining. (A) Representative density plot. (B) Pooled data N=6.

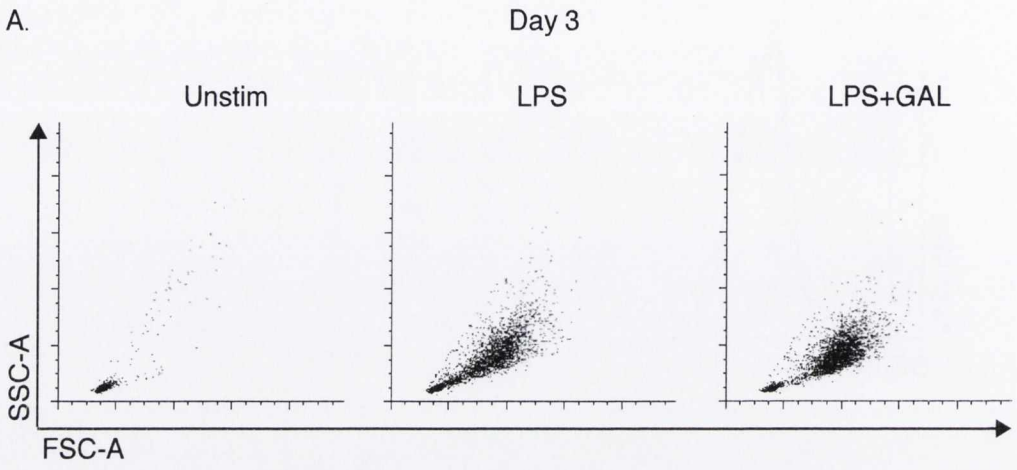


Fig 5.17 DCs cultured with or without glucose induce T cell blastogenesis. DCs were stimulated +/- LPS +/- SIIFEKL peptide for 8 hours in normal media. Cells were then washed and placed in media containing either 10mM glucose or 10mM galactose. On day 1, 2 and 3 after DC activation, OT-1 T cells were added in fresh media to DC wells at a 1:5 ratio. 48 hours later T cells size was analysed by plotting SSC-A and FSC-A. (A) Representative density plot for T cell size from T cells that had been added to DCs 3 days after DC activation.

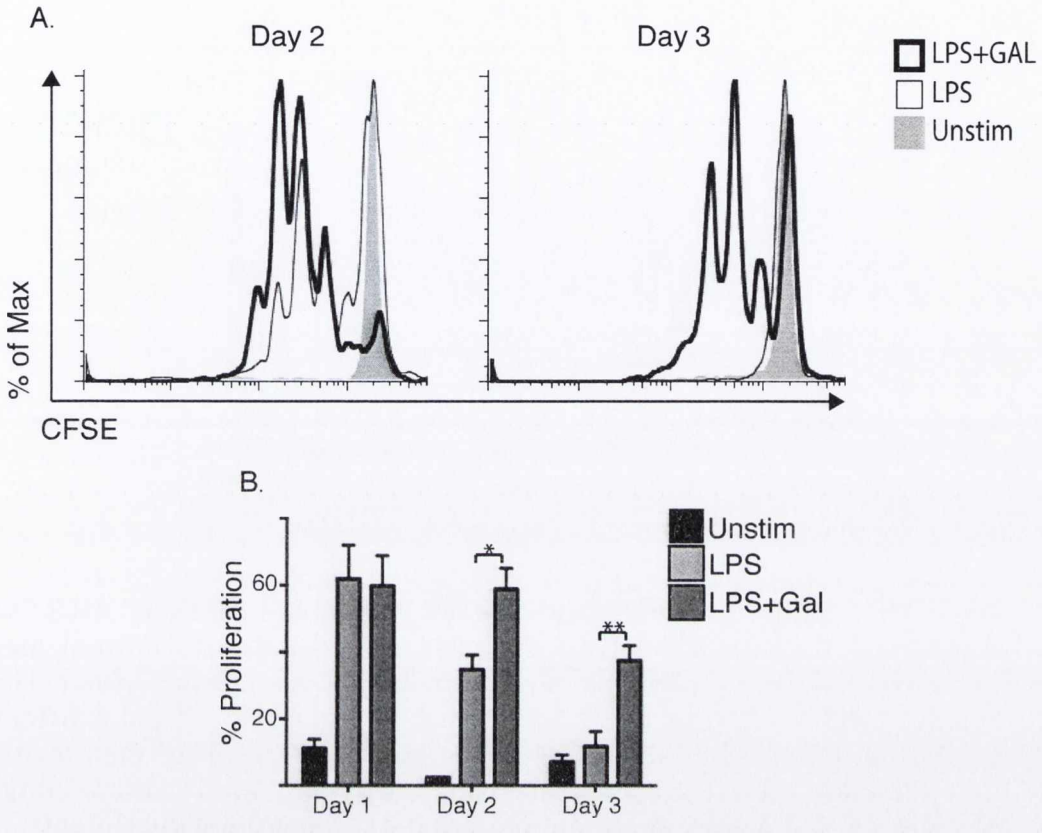


Fig 5.18 DCs in the absence of glucose sustain the ability to induce T cell proliferation. DCs were stimulated +/- LPS +/- SIIFEKL peptide for 8 hours in normal media. Cells were then washed and placed in media containing either 10mM glucose or 10mM galactose. On day 1, 2 and 3 after DC activation, OT-1 T cells stained with CFSE were added in fresh media to DC wells at a 1:5 ratio. T cells were analysed for CFSE staining 48 hours later. (A) Representative histogram. (B) Pooled data N=6. ***$p<0.001$, **$p<0.01$, *$p<0.05$.

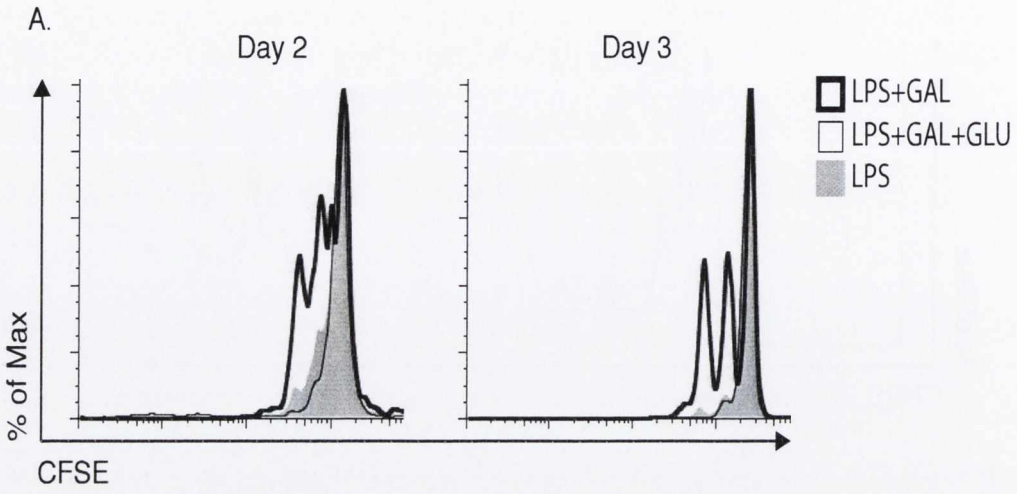


Fig 5.19 Adding back glucose blocks T cell proliferation. DCs were stimulated +/- LPS +/- SIIFEKL peptide for 8 hours in normal media. Cells were then washed and placed in media containing either 10mM glucose, 10mM galactose +/- 2mM glucose. On day 1, 2 and 3 after DC activation, OT-1 T cells stained with CFSE were added in fresh media to DC wells at a 1:5 ratio. T cells were analysed for CFSE staining 48 hours later. (A) Representative histogram of 2 separate experiments.

5.2.10 HIF-1α null DCs sustain the ability to induce T cell proliferation

DCs in the absence of glucose maintain their ability to induce T cell proliferation. Our data from HIF-1α null DCs regarding the effect on signals 1-3 suggested that HIF-1α null DCs may exhibit the same ability regarding inducing T cell responses. Experiments were designed to investigate this using HIF-1α null DCs. The same experimental design was used as shown in 5.15 except all experiments were done in the presence of glucose. There was no difference in T cell size at any day investigated showing that HIF-1α null DCs induced T cell blastogenesis equivalently to WT DCs (Fig 5.20). Similar effects were seen for CD69 expression on T cells during the same experiments (data not shown). Next the ability of HIF-1α null DCs to induce T cell proliferation was investigated. Both WT and KO DCs induced similar levels of T cell proliferation when T cells were added 24 hours after activation. There is a marked decrease in WT DCs ability to induce T cell proliferation 48 and 72 hours after DC activation, similar to what was shown previously. HIF-1α null DCs 48 hours and 72 hours after activation retained the ability to induce T cell proliferation to similar levels seen in 24 hour activated DCs (Fig 5.21). This shows that deleting HIF-1α has an impact on DC induction of T cell proliferation, similar to the effect of glucose removal.

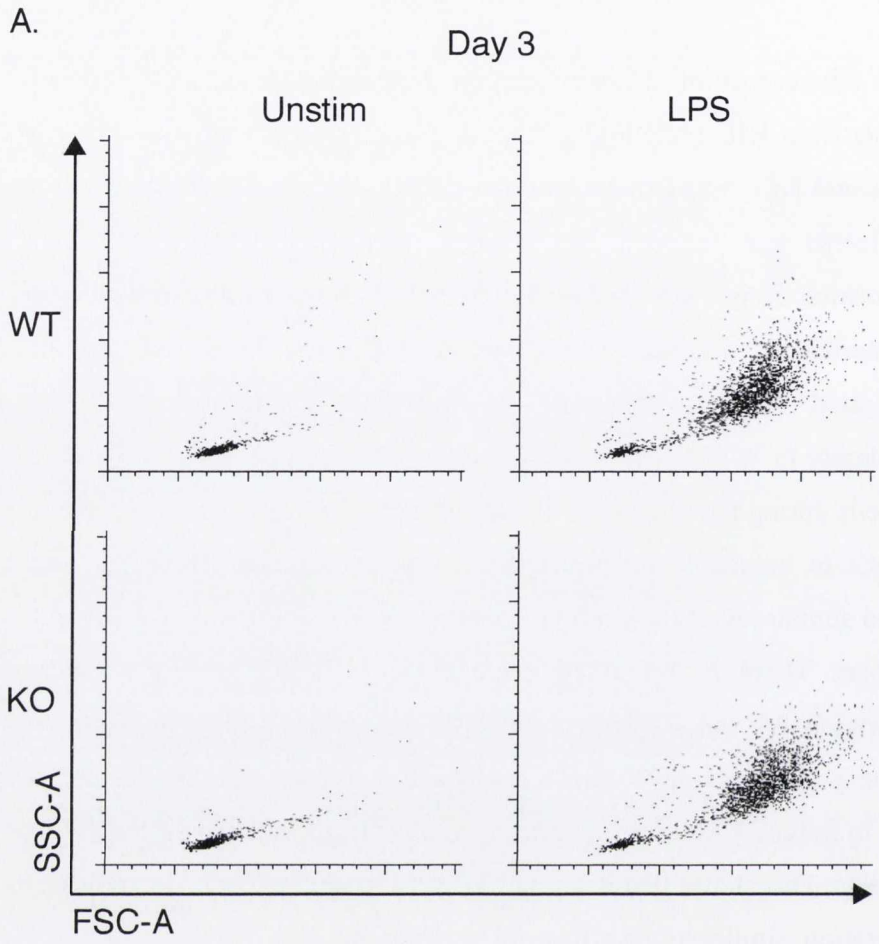


Fig 5.20 HIF-1α null DCs induce no difference in T cell size. HIF-1α null DCs were stimulated +/- LPS +/- SIINFEKL. On day 1, 2 and 3 after DC activation, OT-1 T cells were added in fresh media to DC wells at a 1:5 ratio. 48 hours later T cells size was analysed by plotting SSC-A and FSC-A. (A) Representative density plot for T cell size from T cells that had been added to DCs 3 days after DC activation.

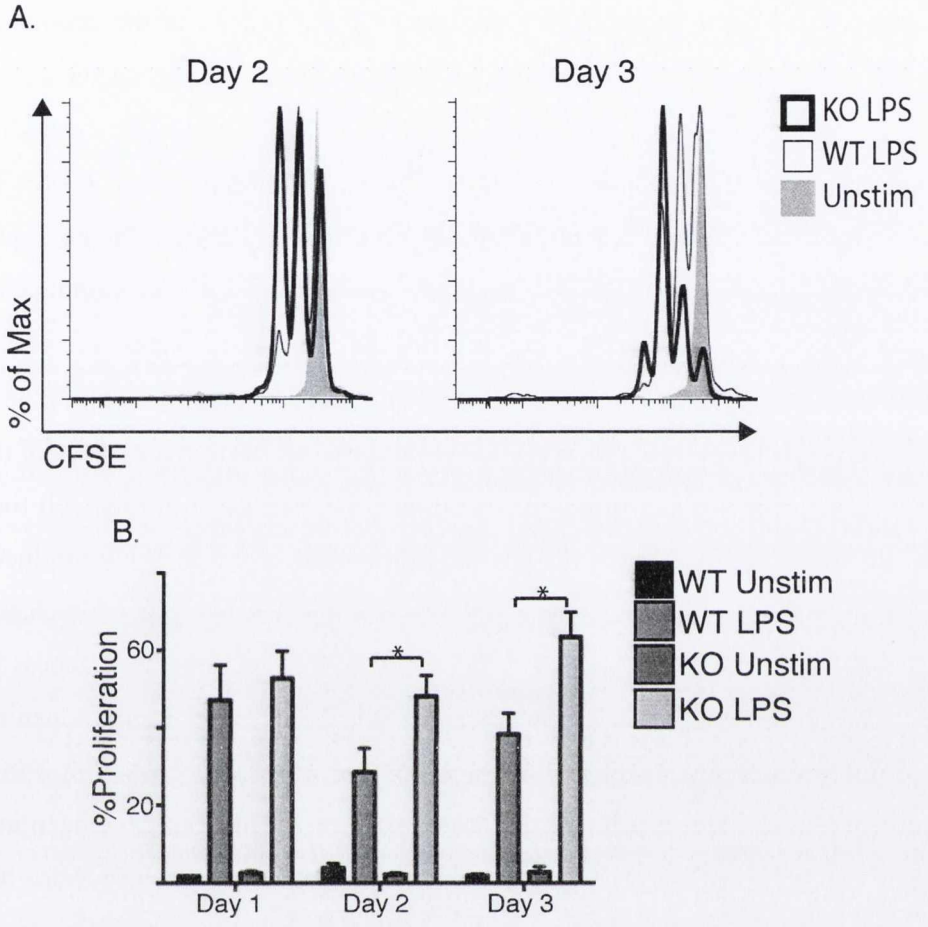


Fig 5.21 HIF-1α null DCs sustain the ability to induce T cell proliferation. HIF-1α null DCs were stimulated +/- LPS +/- SIINFEKL. On day 1, 2 and 3 after DC activation, OT-1 T cells stained with CFSE were added in fresh media to DC wells at a 1:5 ratio. T cells were analysed for CFSE staining 48 hours later. (A) Representative histogram. (B) Pooled data N=6. ***$p<0.001$, **$p<0.01$, *$p<0.05$.

5.2.11 In the absence of glucose DCs are able to sustain IFNγ production in T cells

One important effector function of activated CD8+ T cells is the production of IFNγ. IFNγ has many functions in the body ranging from increasing the activity of NK cells and macrophages, to inducing co-stimulatory molecule expression on several cell types (Schoenborn and Wilson, 2007). IFNγ secretion by CD8+ T cells is important for clearing an infection (Billiau and Matthys, 2009). The next question was to ask if glucose availability affects the capacity of DCs to stimulate T cell effector function such as IFNγ production. DCs were activated as previously described and OT-1 T cells were added on day 1, 2 and 3 after activation. 48 hours after T cell addition T cells were stimulated with phorbol myristate acetate (PMA) and ionomycin for 4 hours to induce maximal IFNγ production in the cell that was analysed by intracellular staining. The most important event in IFNγ production is the opening of the IFNγ gene locus. This is accomplished by epigenetic changes to the chromatin at the IFNγ promoter that allows RNA polymerases access to the gene and induce expression (Schoenborn et al., 2007). Importantly opening the locus requires continuous cell proliferation (Denton et al., 2011). Adding PMA and ionomycin allows the measurement of the number of T cells that have been induced to open the IFNγ locus. DCs activated in the presence of glucose were able to induce a large increase in IFNγ production within T cells but this ability decreased over time. DCs in the absence of glucose also induced high IFNγ production in T cells but in contrast this ability to induce IFNγ production was maintained over time (Fig 5.22).

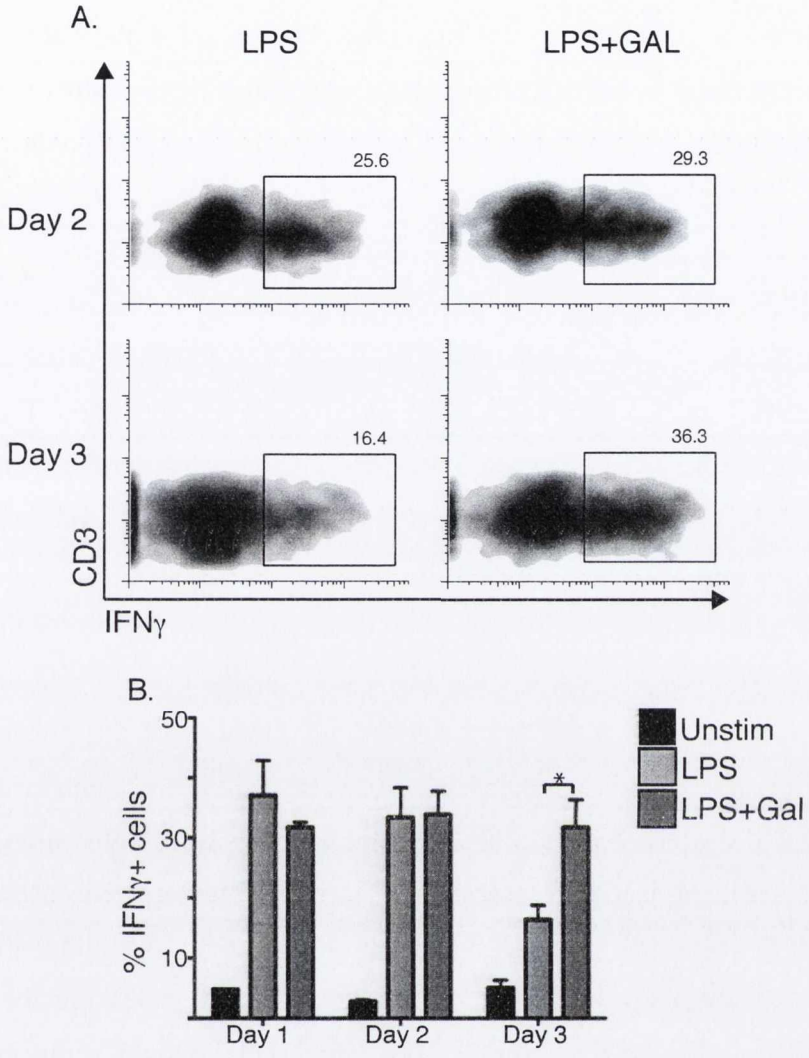


Fig 5.22 DCs induce increased IFNγ production in T cells in the absence of glucose. DCs were stimulated +/- LPS +/- SIIFEKL peptide for 8 hours in normal media. Cells were then washed and placed in media containing either 10mM glucose or 10mM galactose. On day 1, 2 and 3 after DC activation, OT-1 T cells were added in fresh media to DC wells at a 1:5 ratio. 48 hours later T cells were stimulated with PMA and ionomycin for 4 hours and analysed for IFNγ production by intracellular staining. (A) Representative density plot. (B) Pooled data N=6. ***$p<0.001$, **$p<0.01$, *$p<0.05$.

5.2.12 Increasing the T cell:DC ratio blocks DC mTORC1 signaling *in vitro*

DCs activated in the absence of glucose are able to maintain their ability to induce T cell proliferation and IFNγ production. During these experiments the T cell:DC ratio was kept at 5:1. From imaging studies done in intact lymph nodes it has been shown that DCs can interact with up to 12 T cells at one time when presenting antigen (Bousso and Robey, 2003). In some cases a cluster of T cells completely surround the DC. T cells when activated dramatically increase their uptake of nutrients such as glucose and amino acids (Jacobs et al., 2008, Sinclair et al., 2013). We hypothesized that DCs surrounded by a cluster of activating T cells would become nutrient deprived due to competitive uptake of nutrients by metabolically active T cells. In this scenario nutrient deprivation would turn off mTORC1 and HIF-1α signaling in the DC specifically. We and others have shown switching off mTORC1 and HIF-1α in DCs leads to a DC that is better able to stimulate T cells responses. We hypothesized that increasing the T cell:DC ratio could limit nutrient availability to the DC specifically and potentially impact on T cell effector function. Our hypothesized model is shown in Fig 5.23. Indeed there is some evidence in the literature that suggests the T cell:DC ratio influences T cell proliferation (Hopken et al., 2005). As mentioned previously mTORC1 is an important nutrient sensor within DCs with low glucose and amino acid deprivation both switching off mTORC1 signaling. Experiments were designed to investigate if increasing the T cell:DC ratio had any effect on DC mTORC1 signaling which would be an indication of nutrient deprivation specifically within the DC. To investigate this DCs were stimulated with SIINFEKL peptide and LPS for 6 hours. At 6 hours cells were washed and OT-1 T cells were added at different ratios. 18 hours later DCs were analyzed by intracellular staining for pS6 levels. There was a clear decrease in pS6 signaling from the 2:1 ratio with increasing T cells added. A significant decrease was seen at the 20:1 ratio indicting that mTORC1 signaling in DCs is affected by T cell number (Fig 5.24).

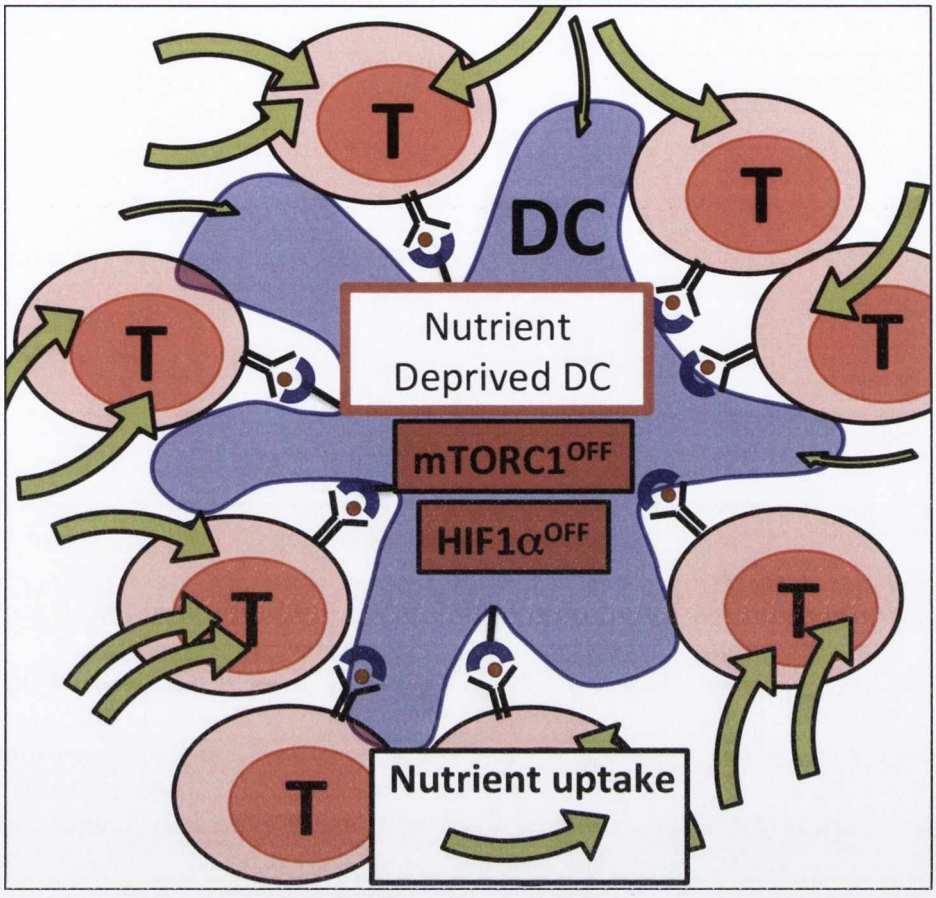


Fig 5.23 A schematic of how T cells interacting with DCs could control nutrient availability specifically within the DC. A DC presenting antigen can interact with up to 12 T cells at the same time. Activated T cells dramatically increase their uptake of nutrients. With the DC surrounded by T cells the DC may become nutrient deprived. This in turn would switch off mTORC1 signaling and HIF-1α signaling specifically in the DC. This would lead to a DC that is better able to induce T cell responses.

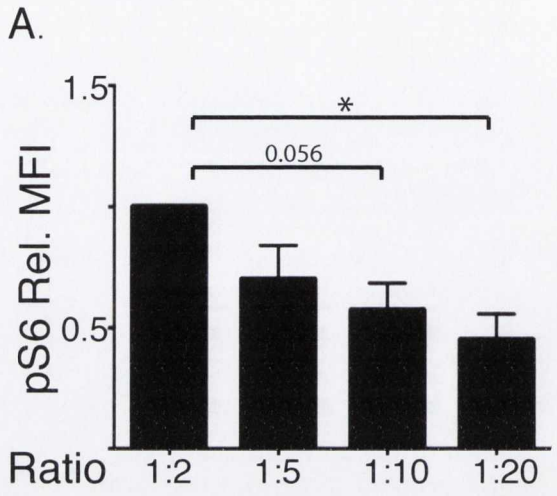


Fig 5.24 mTORC1 signaling in DCs is blocked with increasing T cell:DC ratio. DCs were stimulated +/- LPS +/- SIIFEKL peptide for 6 hours in normal media. Cells were then washed and OT-1 T cells added in fresh media to DC wells at the indicated ratios. 18 hours later T cells were analysed for pS6 production by intracellular staining. (A) Pooled data N=3. ***p<0.001, **p<0.01, *p<0.05.

5.2.13 NO production is decreased with increasing T cell numbers

Although there is some evidence that CD4+ T cells can express iNOS there is no evidence in the literature that CD8+ T cells can produce NO (Jianjun et al., 2013). NO production in DCs requires both mTORC1 and HIF-1α. By looking at NO production in our co-culture model we could get an indication of the level of mTORC1 and HIF-1α activity within DCs. To do this $1x10^5$ DCs were cultured at the indicated T cell:DC ratios as previously described. 24 hours after initial DC activation supernatants were removed from the wells and analysed for NO production by the Griess reaction. There was a clear stepwise decrease in NO production with increasing T cell:DC ratio with a threefold difference in NO production from the 1:1 ratio compared to the 20:1 ratio (Fig 5.25).

5.2.14 Glucose uptake in DCs is not affected by the T cell:DC ratio

mTORC1 signaling in DCs is blocked at high T cell:DC ratios. mTORC1 can be blocked by decreasing the availability of glucose. We wanted to investigate if glucose uptake within DCs was affected by increasing the T cell:DC ratio. To do this a fluorescent glucose analog called 2NBDG was used. Co-cultures with DCs and T cells were set up as previously described. At 24 hours from initial DC activation 2NBDG made up in RPMI media was added directly to the well, without disturbing the DCs or T cells, for one hour. Cells were then removed from the well and analyzed for 2NBDG fluorescence by flow cytometry. There was a trend of decreasing 2NBDG intensity in DCs but no significant decrease (Fig 5.26a). In T cells there was a trend of increasing 2NBDG intensity but no significant increase (Fig 5.26b).

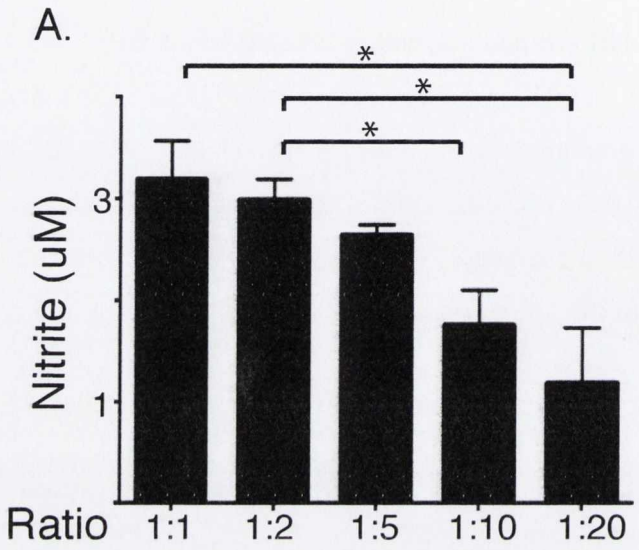


Fig 5.25 NO production in DCs is blocked with increasing T cell:DC ratio. DCs were stimulated +/- LPS +/- SIIFEKL peptide for 6 hours in normal media. Cells were then washed and OT-1 T cells added in fresh media to DC wells at the indicated ratios. 18 hours later NO production was measured by the Griess reaction. (A) Pooled data N=4. ***$p<0.001$, **$p<0.01$, *$p<0.05$.

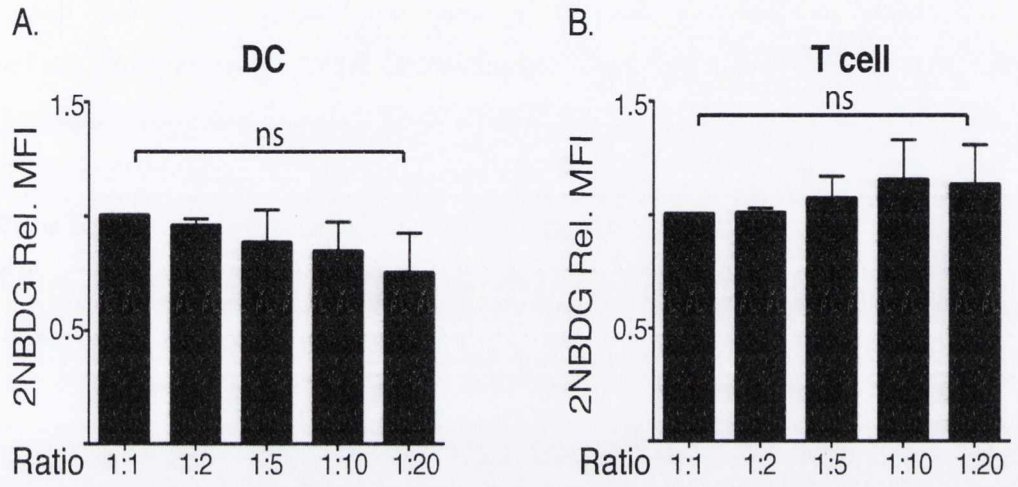


Fig 5.26 Glucose uptake is not significantly affected by the T cell:DC ratio. DCs were stimulated +/- LPS +/- SIIFEKL peptide for 6 hours in normal media. Cells were then washed and OT-1 T cells added in fresh media to DC wells at the indicated ratios. Fluorescent 2NBDG was added to the wells for 1 hour. Cells were then removed from wells and analysed for 2NBDG intensity by flow cytometry. (A) Pooled data N=3. ***$p<0.001$, **$p<0.01$, *$p<0.05$, ns=not significant.

5.2.15 The DC:T cell ratio affects T cell IFNγ production

DCs activated in the absence of glucose induce increased T cell proliferation and IFNγ production. The DC:T cell ratio impacts on mTORC1 and HIF-1α signaling specifically within the DC with high DC:T cell ratio inhibiting mTORC1 signaling and hence HIF-1α. It remained to be seen if the DC:T cell ratio had any effect on T cell effector function. We repeated the same experiment as previously described varying the DC:T cell ratio in different wells. After 18 hours from the initial addition of T cell to wells containing DCs, IFNγ production was analysed by intracellular staining. There was a dose dependent increase in the number of IFNγ+ T cells with increasing T cell:DC ratio with a significant increase observed at a 20:1 ratio (Fig 5.27b). There was also a dose dependent increase in the MFI of IFNγ+ cells showing that not only is there an increase in the number of IFNγ+ T cells but IFNγ+ cells are also producing more at higher T cell:DC ratios (Fig 5.27c).

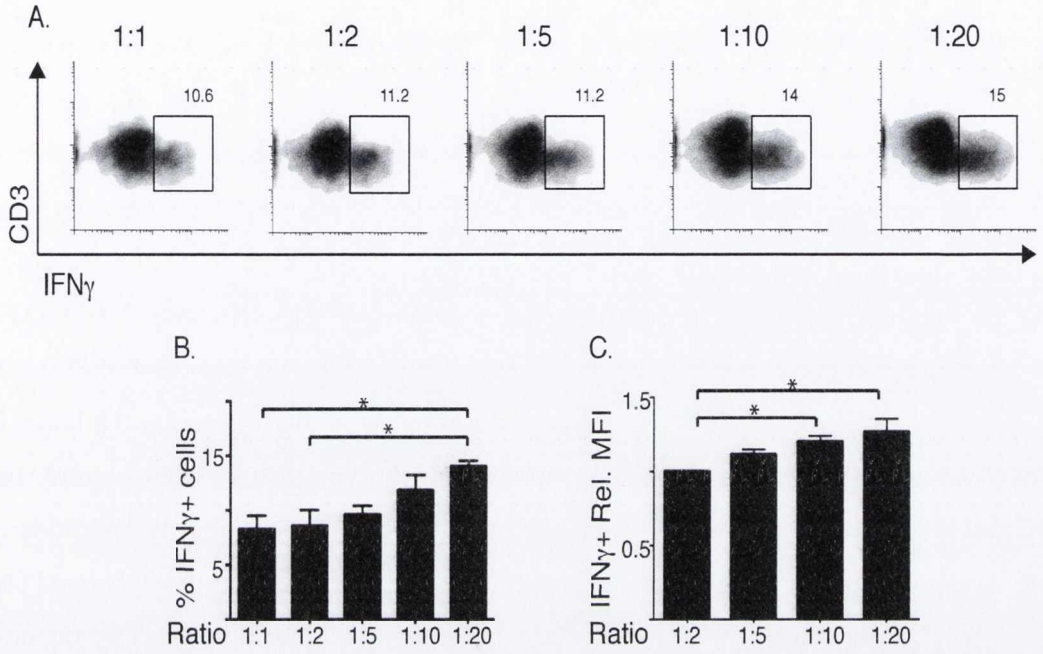


Fig 5.27 Increasing T cell:DC ratio leads to increased T cell IFNγ production. DCs were stimulated +/- LPS +/- SIINFEKL peptide for 6 hours in normal media. Cells were then washed and OT-1 T cells added in fresh media to DC wells at the indicated ratios. 18 hours later T cells were analysed for IFNγ production by intracellular staining. (A) Representative density plot. (B) Pooled data N=4. ***p<0.001, **p<0.01, *p<0.05.

5.2.16 mTORC1 signaling in DCs is blocked at high T cell:DC ratios *in vivo*

High T cell:DC ratios inhibit DC mTORC1 signaling *in vitro*. We next wanted to investigate whether T cells can inhibit mTORC1 in DC *in vivo*. To do this we developed a strategy to analyse T cell DC interactions within draining lymph nodes. DCs were stimulated for 1 hour with SIINFEKL and LPS. DCs were then washed extensively and stained with cell tracker violet. DCs were then injected into the lateral tarsal region using the Hock immunization strategy (Kamala, 2007). OT-1 T cells were then purified and CFSE stained. 4 hours after the injection of DCs, OT-1 cells were injected IV at different concentrations into different mice. 18 hours later the popliteal and inguinal lymph nodes were harvested and whole lymph nodes were stained to analyse pS6 staining in the injected DCs. Some lymph node cells were used for a rapamycin control to show the level of pS6 staining when mTORC1 signaling was blocked. The red dashed line in Fig 5.18 indicates this rapamycin control. In lymph nodes where there was a T cell:DC ratio of less than 10 there where high levels of pS6 staining when compared to the rapamycin control. However in lymph nodes that had a T cell:DC ratio of greater than 10 the pS6 levels in DCs were centered around rapamycin levels indicating that at high T cell:DC ratios mTORC1 signaling within DCs is turned off (Fig 5.28).

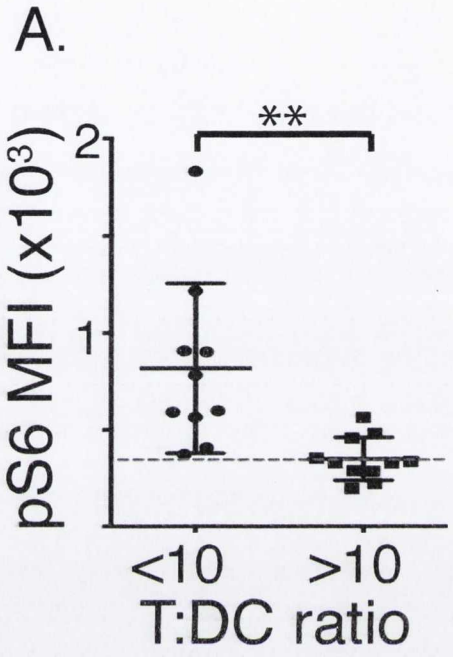


Fig 5.28 High T cell:DC ratios block mTORC1 signaling in DCs *in vivo*. DCs were stimulated for 1 hour with SIINFEKL and LPS. Cells were then stained with cell tracker violet and injected into the lateral tarsal region. OT-1 T cells were purified, stained with CFSE and injected IV 4 hours later. 18 hours after last injection popliteal and inguinal lymph nodes were harvested and pS6 was measured in DCs by flow cytometry. Red dashed line indicates rapamycin control. Lymph nodes were taken from 5 mice. ***$p<0.001$, **$p<0.01$, *$p<0.05$.

5.3 Discussion

Data presented in this PhD makes the novel finding that glucose and glycolysis is anti-inflammatory in DCs. Our data for the first time shows that glucose in the microenvironment inhibits DCs ability to induce T cell responses. However in the field of immunometabolism the overwhelming evidence suggests that glucose availability and glycolysis is required for immune cell effector function and promoting a pro-inflammatory phenotype. In CD4+, CD8+ and T_H17 T cells directly inhibiting glycolysis either by glucose deprivation, replacing glucose with galactose or adding the glycolysis inhibitor 2DG specifically blocks effector function (Chang et al., 2013, Finlay et al., 2012, Shi et al., 2011). In NK cells replacing glucose with galactose or adding 2DG blocks effector functions (Donnelly et al., 2014). Additionally in macrophages adding 2DG blocks IL-1β production (Tannahill et al., 2013). In DCs adding 2DG with LPS blocks co stimulatory molecule expression and cytokine production, showing that DCs require increased glycolysis to fully activate(Krawczyk et al., 2010). This was further investigated in a recent paper showing that increased glycolysis minutes after LPS stimulation in DCs meets the anabolic demands required for expansion of the Golgi and endoplasmic reticulum, needed for cytokine production (Everts et al., 2014). Evidence in the literature and also presented in this PhD argues that there are two stages of metabolic changes in activated DCs. The initial increase in glycolysis that happens minutes after TLR stimulation requires TBK1-IKKε-AKT and HKII leading to increased glycolytic efficiency and so increased flux through the glycolytic pathway. This initial stage is independent of mTORC1 and HIF-1α. The second stage presented in this PhD requires both mTORC1/HIF-1α/iNOS and represents complete glycolytic reprogramming with increased expression of glycolytic genes and glucose transporters. Blocking the initial increase in glycolysis will block DC activation (Krawczyk et al., 2010, Everts et al., 2014). However our data shows that blocking the second stage of metabolic changes in activated DCs maintains a pro-inflammatory DC phenotype and prolongs DC induced T cell responses. This is a completely novel finding and to our knowledge is the first time that glycolysis and glucose availability has been directly linked to anti-inflammatory functions. In the field of immunometabolism mTORC1 signaling is mainly linked with pro-inflammatory functions. This is certainly true in effector T

cells and NK cells (Xu et al., 2012, Donnelly et al., 2014). However there is growing evidence that mTORC1 plays an anti-inflammatory role in both DC's and macrophages. In macrophages inhibiting mTORC1 promotes the M1 pro-inflammatory phenotype (Mercalli et al., 2013). In both cell types inhibiting mTORC1 promotes elevated expression of pro-inflammatory cytokines and increased co-stimulatory molecule expression (Yeung et al., 2008, Weichhart et al., 2008, Baker et al., 2009). Rapamycin is used as an immunosuppressant in kidney transplant patients (Saunders et al., 2001). However in 66% of these patients they experience at least one of the conditions pneumonitis, glomerulonephritis or chronic inflammatory anemia that are inflammatory conditions (Saemann et al., 2009). Myeloid DC's analyzed ex vivo in patients given rapamycin display an increased pro-inflammatory phenotype (Weichhart et al., 2011). Data presented in this PhD shows that inhibiting mTORC1 blocks IL-10 expression that supports data previously published showing similar results (Katholnig et al., 2013a). A paper has shown that mTORC1 controlled IL-10 expression in DCs is STAT3 dependent (Haidinger et al., 2010, Saraiva and O'Garra, 2010). In macrophages IL-10 expression requires Twist 2 and C-Maf in an mTOR dependent process (van den Bosch et al., 2014). Data in this PhD establishes a link between HIF-1α and cytokine production in activated DCs. Hif-1α deletion partially blocks IL-10 expression in activated DCs while rapamycin addition in HIF-1α null DCs further blocks IL-10 expression. This suggests that IL-10 production is partially dependent on HIF-1α in activated DCs but that there is also a HIF-1α independent mTORC1 dependent mechanism for IL-10 expression. In an airway allergy model macrophage expression of IL-10 required HIF-1α (Toussaint et al., 2013). It has also been shown that the IL-10 gene promoter has a hypoxia response element (HRE) showing that HIF-1α has the potential to act as a transcription factor for IL-10 (Cai et al., 2013). Adding the mTORC1 specific inhibitor rapamycin leads to an increase in IL-12 mRNA expression at 24 hours. Increased IL-12 expression in DCs with mTORC1 inhibition has previously been shown both in human DCs and murine DCs (Haidinger et al., 2010, Katholnig et al., 2013a). However there is a discrepancy in the protein data, as mTORC1 inhibition does not increase protein expression of IL-12. Data presented here shows that although there is an increase in IL-12 mRNA expression at 24 hours, there is no increase in IL-12p70 protein expression at this time point with rapamycin treatment consistent with a previously

published study in BMDCs (Amiel et al., 2012). Our data investigating the dynamics of IL-12 expression in BMDCs may explain this result as mTORC1 inhibition does not effect IL-12 mRNA expression at earlier time points. This suggests that there are two stages of IL-12 expression in DCs, an initial burst immediately after activation when a large amount of IL-12 is secreted and then a second stage at later time points after activation when IL-12 expression levels are reduced and negatively controlled by mTORC1. This initial burst of IL-12 occurs when DCs are within the periphery and functions to stimulate innate cells such as neutrophils and NK cells (Moreno et al., 2006, Sarhan et al., 2015). At later time points DCs will have reached the draining lymph nodes and will be interacting with T cells. While the expression of IL-12 is reduced at this point, the cytokine will be delivered directly to interacting T cells. Indeed these close contacts between DCs and T cells in the lymph node can last for extended periods of time (Celli et al., 2007). Measuring IL-12 protein by ELISA gives values for IL-12 produced over the full time course and so mTORC1 dependent differences in IL-12 protein would be lost. Data presented here makes the novel finding that HIF-1α deletion enhances IL-12 expression. Indeed the data argues that HIF-1α mediates the effects of mTORC1 on IL-12 mRNA expression. IL-12 mRNA expression becomes mTORC1 dependent at the same time point after activation when HIF-1α protein is expressed. Interestingly when HIF-1α protein stabilization is pharmacologically induced for the full activation period by the addition of DMOG. There is a clear decrease in IL-12 protein by ELISA, confirming that HIF-1α can inhibit IL-12 protein production. There is some evidence in the literature that shows deleting HIF-1α in macrophages leads to increased IL-12 expression (Ramanathan et al., 2009). DMOG blocking IL-12 expression could also be independent of HIF-1α as there is a link in the literature between hydroxylation and inhibition of NK-κB signaling that could explain this result (Scholz and Taylor, 2013). Indeed the addition of DMOG protected mice in a model of colitis showing its powerful anti-inflammatory properties (Cummins et al., 2008). Future work would employ intracellular staining for IL-12 to analyze precisely the amount of IL-12 protein being produced at a given time after DC activation. Adding rapamycin to activated DCs effectively blocks mTORC1 signaling but has no effect on mTORC2 signaling (data not shown). Recently two papers appeared that linked mTORC2 inhibition to promoting an enhanced DC inflammatory phenotype. Importantly mTORC2 inhibition lead to increased IL-12

expression. It would be interesting to repeat the IL-12 results using a pan mTORC1 and mTORC2 inhibitor such as TORIN to see if that had an effect on IL-12 expression (Wei et al., 2015, Raich-Regue et al., 2015).

This study makes the unique insight that glucose availability and HIF-1α expression promote an anti-inflammatory phenotype in BMDCs. Removing glucose or deleting HIF-1α both lead to enhanced DC ability to induce T cell responses. Much like the discussion about glycolysis, HIF-1α expression in immune cells is overwhelmingly associated with pro-inflammatory functions. In CD8+ T cells it is required for effector function and migration (Finlay et al., 2012). HIF-1α is also required for IL-17 production and T_H17 cell differentiation (Shi et al., 2011). Interestingly a recent paper showed that hypoxia induced HIF-1α expression in T_H1 cells suppressed IFNγ production that was dependent on IL-10 production, showing that HIF-1α expression is not universally pro-inflammatory in T cells (Shehade et al., 2015). Deleting HIF-1α in macrophages blocked the shift to glycolysis and is required for pro-inflammatory cytokine production (Cramer et al., 2003). A more recent paper showed that succinate was required for HIF-1α stabilization and IL-1β production (Tannahill et al., 2013). However not all evidence in the literature links HIF-1α to an exclusively pro-inflammatory role in macrophages. Macrophage expression of HIF-1α in a breast cancer tumor model was associated with inhibited T cell responses. This paper showed that deleting HIF-1α decreased iNOS expression and NO production. Arginine uptake was also decreased, as it was no longer needed as a precursor to NO production. These two processes were directly linked to inhibited T cell proliferation at the tumor site (Doedens et al., 2010). Other papers have also shown that NO production and arginine depletion inhibits T cell proliferation, both of which could help explain our T cell proliferation data as removing glucose or deleting HIF-1α blocks NO production in DCs (Sato et al., 2007, Rodriguez et al., 2007). Glucose deprivation and deletion of HIF-1α maintains CD80 and CD86 expression for days after activation. This is a similar result to what was observed previously with mTORC1 inhibition in LPS stimulated DCs suggesting that HIF-1α downstream of mTORC1 is mediating these results on co-stimulatory molecule expression (Amiel et al., 2012). Some other papers have looked at the effect of HIF-1α activity upon co-stimulatory molecule expression in DCs. A paper using three different pharmacological inhibitors of HIF-1α found that inhibiting HIF-1α lead to

a block in co-stimulatory molecule expression at 24 hours. However they did not use any genetic modulation within DCs and did not rule out "off target" effects from the inhibitors used (Spirig et al., 2010). Indeed one of the inhibitors used in this study, YC-1, was originally identified as an activator of soluble guanylate cyclase and to increase cGMP levels (Chun et al., 2004). Another paper used a knockdown approach to target HIF-1α. They saw quite dramatic decreases in CD80 and CD86 expression with HIF-1α knockdown in hypoxia environments but the effect in normoxia was marginal (Jantsch et al., 2008). Some reports have also linked mTORC1 to the expression of negative regulators of T cell activation. The surface expression of programmed death ligand 1 (PD-L1) can be induced by LPS activation of DCs (Planes et al., 2014). PD-L1 binds to its receptor PD-1 on T cells and reduces T cell proliferation (Freeman et al., 2000). Several studies in DCs have shown that rapamycin inhibition of mTORC1 leads to a reduction in expression of PD-L1 and hence a DC with greater ability to induce T cell responses (Haidinger et al., 2010). Cancer cells upregulate PD-L1 expression that contributes to their ability to evade an immune response and antibodies blocking PD-L1 are showing great potential as therapies in the clinic (Iwai et al., 2002, Brahmer et al., 2012). Recently it was shown in myeloid derived suppressor cells (MDSC) that HIF-1α was required for PD-L1 expression. Indeed the same study showed that HIF-1α was also required for PD-L1 expression in DCs (Noman et al., 2014). Future work will investigate if HIF-1α dependent expression of PD-L1 in BMDCs plays a role in our data showing that in the absence of HIF-1α or glucose DCs are better at inducing T cell responses.

Data presented in this PhD presents a new model of T cell and DC interactions. This model explains how T cell deprivation of nutrients in the microenvironment impacts on mTORC1 signaling specifically in the DC which amplifies DCs ability to induce a robust T cell response. Some excellent papers have been published that have analysed DC T cell interactions in the lymph node. DCs presenting antigen establish stable interactions with T cells that last a long time (Celli et al., 2007). Indeed T cells that recognize their antigen increase the time they spend in a particular lymph node from 24 hours to 3-4 days (Bousso, 2008). It has also been shown that DCs can interact with up to 12 T cells at the one time and in some cases T cells form clusters around the DC, completely surrounding the cell (Bousso and Robey, 2003).

Activated T cells also dramatically increase their uptake of nutrients such as amino acids and glucose (Sinclair et al., 2013, Maciver et al., 2008). We developed a hypothesis that involved multiple T cells surrounding a DC that was presenting antigen. These T cells would become activated and start taking up a significant proportion of the available nutrients in the microenvironment. With the DC surrounded by T cells the DC may become nutrient deprived. This in turn would turn off mTORC1 and HIF-1α signaling specifically in the DC. This would allow the T cell and DC to align their metabolic states to optimize their pro-inflammatory functions. Therefore through a metabolically controlled feed forward mechanism activated T cells can shape the T cell response. Our data shows that the DC:T cell ratio largely influences the levels of mTORC1 signaling in the DC during DC T cell interactions. Evidence in the literature suggests that increasing the DC:T cell ratio is overwhelmingly pro-inflammatory. Increasing the DC:T cell ratio leads to increased T cell proliferation (Shimabukuro-Vornhagen et al., 2013, Hopken et al., 2005). It also has been linked to increasing the CTL response. A study investigated activated DCs ability to stimulate CD8+ T cells and induce CTL formation. They found that induction of the CTL response was completely inhibited at a DC:T cell ratio of 1:2 again linking the DC:T cell ratio to pro-inflammatory signals (Repnik et al., 2008). For the first time we have correlated nutrient availability in the microenvironment, the DC:T cell ratio, mTORC1 signaling within the DC and T cell effector function. However much work must be done to further prove our model of DC and T cell interactions. Analyzing T cell responses at different DC:T cell ratios in the presence of HIF-1α null DCs would be one way to investigate if altering HIF-1α stabilization downstream of mTORC1 could impact on T cell responses independent of DC:T cell ratio. Ideally analyzing T cell responses in DCs with constitutively active mTORC1 signaling, such as using TSC 1/2 null DCs, could show if maintaining mTORC1 signaling even at high DC:T cell ratios blocked the increase in T cell IFNγ production. However disrupting mTORC1 signaling during DC development leads to defective DC development. These DCs spontaneously activated but had impaired ability to induce T cell responses (Wang et al., 2013). Overall data presented here establishes a new model for DC T cell interactions in the lymph node that explains how these cells can synergize their metabolic needs to best induce T cell activation. This model predicts metabolic regulation of DC to be a mechanism

to distinguish *bona fide* responses (large DC-T clusters) from weak responses (small clusters) and to modulate DC function accordingly.

Chapter 6

Discussion and future prospective

6.3 Discussion

This PhD has examined in detail the metabolism of activated BMDCs and how this impacts on BMDC function. As mentioned already BMDCs are considered a model for Tip DCs, a specialized form of monocyte derived DCs (Xu et al., 2007, Dominguez and Ardavin, 2010). Infection with the bacterium *L monocytogenes* is known to induce differentiation of monocyte derived DCs (Serbina et al., 2003). The Pearce group investigated the metabolic profile of monocyte derived DCs induced after *L monocytogenes* infection in mice. They found that these DCs exhibited a similar metabolic profile of increased glycolysis and decreased OxPhos to that shown in LPS stimulated BMDCs. This showed for the first time that the metabolic changes in activated BMDCs observed *in vitro* could be replicated in monocyte derived DCs after *in vivo* infection. Interestingly this paper also examined the metabolic profile of conventional DCs isolated from the spleen after *L monocytogenes* infection and ex vivo LPS stimulation and found these cells exhibited no increase in glycolysis or decrease in OxPhos (Everts et al., 2012). However a more recent paper showed that splenic conventional DCs do increase their rate of glycolysis immediately after LPS stimulation indicating that these cells might only transiently increase their metabolism (Everts et al., 2014). A study investigating splenic CD11c+ MHCII+ splenic DCs 18 hours after polyI:C injection found that these DCs had increased glycolysis and decreased OxPhos. However this study did not distinguish between different subtypes of DCs in the spleen (Pantel et al., 2014). Although the data is variable for the ability of conventional DC to undergo a metabolic shift it does appear that inhibiting mTORC1 signaling in these cells does promote a pro-inflammatory phenotype. In myeloid DC rapamycin inhibition promotes increased expression of IL-12 and increased expression of CD80 and CD86 (Haidinger et al., 2010). This suggests that our model of T cell control of nutrients in the DC microenvironment will also have an effect on these cells to promote an enhanced ability to induce T cell responses. Although the mechanism may be different and not related to the metabolic state of these DCs mTORC1 signaling inhibition will still have the effect of driving their ability to promote a more pro-inflammatory DC response. Future work will address if this is true by isolating these cells and investigating their response in our T cell DC interaction model. There have been few studies done into DC metabolism in

humans but a few recent reports have appeared examining human monocyte derived DC metabolism in the context of DC tolerance. Tolerogenic DCs are resistant to activation stimuli, induce reduced effector T cell responses and instead promote T_{REG} differentiation (Maldonado and von Andrian, 2010). Dexamethasone and vitamin D induced tolerogenic human monocyte derived DCs displayed increased rates of OxPhos, spare respiratory capacity and glycolytic capacity. Indeed blocking fatty acid oxidation partially restored DCs ability to stimulate T cell responses. This study also showed that LPS stimulation of DCs ex vivo lead to increased glycolytic rate and lactate production but not a full decrease in OxPhos probably due to the fact that these cells do not produce much NO (Malinarich et al., 2015). A separate study found that vitamin D induced human monocyte derived DCs had both increased OxPhos and glycolysis. Interestingly this study found that these DCs required glucose and increased glycolysis to induce the tolerogenic phenotype that was PI3K/mTOR dependent (Ferrer et al., 2014). Much work needs to be done in establishing the metabolic profiles of the different DC subsets in both humans and mice. Indeed our work showing that nutrients in the microenvironment impacts on DC metabolism suggest that the location and inflammatory state of the tissue will have enormous bearing on the DC metabolic state. However these initial results suggest that metabolic changes in DCs will be cell type specific.

Work presented in this PhD has established that nutrient availability in the cellular microenvironment influences DC metabolism and their ability to induce T cell responses. Our data has shown that T cell dependent depletion of nutrients from the DC microenvironment influences nutrient sensing signaling within the DC using both *in vitro* and *in vivo* approaches. DCs are located throughout most tissues in the body (Shortman and Liu, 2002). Depending on where the DC is and the immune state of the microenvironment nutrient availability can vary in places where DCs are present. However there is now a wealth of data that suggests T cells also need sufficient availability of nutrients to activate and evidence is emerging that T cell activation is impaired at sites were nutrient availability has become limiting. DCs are known to infiltrate the tumor microenvironment and evidence suggests that there is a positive correlation between the number of DCs at a tumor site and inhibition of tumor progression (Lijun et al., 2012, Reichert et al., 2001). Indeed there is evidence that DCs can stimulate naïve T cells to activated T cells directly

within the tumor microenvironment without migration to secondary lymph nodes (Thompson et al., 2010). It has been known for a number of years that there is decreased nutrient availability at tumor sites (Vaupel et al., 1989). The inducible enzyme indoleamine 2,3 dioxygenase (IDO) is expressed in several cell types during different inflammatory events such as infection by microbial pathogens and at tumor sites (Li et al., 2012). IDO expression catalyzes the degradation of the essential amino acid tryptophan and is associated with potent anti-inflammatory properties (Mbongue et al., 2015). IDO expression at tumor sites leads to immune evasion by tumors and tumor progression showing how nutrient availability can greatly impact on immune responses (Huang and Mellor, 2014). Arginine levels are decreased in the plasma from cancer patients (Vissers et al., 2005). This is most likely due to increased expression of the enzyme arginase I at tumor sites by tumor cells themselves or from cells such as myeloid derived suppressor cells (MDSC) (Rodriguez and Ochoa, 2008). Increased expression of arginase I by these cells inhibits T cell responses in the tumor microenvironment (Rodriguez et al., 2004). This again highlights how important nutrient availability is for T cell activation. Much like activated immune cells, most cancer cells have elevated levels of glycolysis (Gatenby and Gillies, 2004). Glucose deprivation is a hallmark of the tumor microenvironment due to uptake by cancer cells and also altered blood flow at tumor sites (Vaupel and Hockel, 2000, Vaupel et al., 1989). Recent work has shown that competition for glucose at tumor sites between tumors and infiltrating T cells limits the T cell response and contributes to tumor immune evasion (Chang et al., 2015, Ho et al., 2015). This was the first time that evidence showed direct competition for nutrients in the inflammatory microenvironment influenced the immune response. In Warburg metabolism the end product of glycolysis is lactate (Palsson-McDermott and O'Neill, 2013). Lactate or lactic acid contributes to the low pH or acidosis observed at inflammatory and tumor sites (Lardner, 2001). Though initially viewed as just a waste product of glycolysis, recent investigations have found it can have significant effects on cellular processes and signaling, for example inhibiting the glycolytic enzyme phosphofructokinase (Leite et al., 2011). In T cells lactic acid has been shown to inhibit proliferation and cytokine production (Fischer et al., 2007). Recently lactic acid was also shown to inhibit CD4+ T cell motility in a model of rheumatoid arthritis and this was directly linked to an inhibition of glycolysis (Haas et al., 2015). In DCs endogenously produced

lactate in high-density cultures was shown to inhibit pro-inflammatory cytokine production with these DCs producing more IL-10 (Nasi et al., 2013). Studies have also shown that DC function at tumor sites was inhibited due to tumor-derived lactic acid (Gottfried et al., 2006). Interestingly recent evidence has shown that lactate can stabilize HIF-1α protein in macrophages and promote M2 polarization (Colegio et al., 2014). Given our data showing that HIF-1α is anti-inflammatory in BMDCs it would be interesting to investigate in the future if lactic acid during inflammatory events influenced HIF-1α signaling in DCs and their effector function. The tumor microenvironment and sites of inflammation are known to exhibit oxygen deficiency or hypoxia (Finger and Giaccia, 2010). Hypoxia is known to induce metastasis in cancer cells but can also affect immune cell function (Chang and Erler, 2014). Tumor hypoxia can modulate T cell trafficking and results in the recruitment of T_{REG} cells that contribute to tumor immune evasion (Facciabene et al., 2011). Low oxygen levels decrease IFNγ and IL-2 production in T cells but actually increases their lytic ability towards target cells (Caldwell et al., 2001). Hypoxia induced HIF-1α expression in macrophages lead to reduced T cell responses and increased tumor progression (Doedens et al., 2010). DCs in a hypoxic environment exhibited enhanced migration and decreased pro-inflammatory cytokine production (Kohler et al., 2012). In this PhD GM-CSF differentiated BMDCs were used in all experiments. DCs can be differentiated from progenitor cells using either GM-CSF or Flt3 ligand. A study comparing murine DCs differentiated with either GM-CSF or Flt3 found that Flt3 BMDCs closely represented steady state tissue resident DCs whereas GM-CSF BMDCs were closely related to TNFα/iNOS producing DCs (Tip DCs) that emerge after inflammation (Xu et al., 2007). Tip DCs are important for clearing bacterial infections due their production of NO but some reports suggest that they are also involved in inducing maximal T cell responses during viral infection (Serbina et al., 2003, Aldridge et al., 2009). Tip DCs are also considered a specialized type of monocyte derived DC (Dominguez and Ardavin, 2010). Monocyte derived DCs are known to differentiate at sites of inflammation and from there can traffic to draining lymph nodes to induce T cell responses (Leon et al., 2007). Interestingly monocyte derived DCs are also capable of activating memory T cells in the periphery without the need to traffic to draining lymph nodes during viral infection (Wakim et al., 2008). The evidence above shows that DCs exist in and migrate to many different

locations within the body. They are also present at sites of inflammation and at tumor sites. Our model for T cell depletion of nutrients in the DC microenvironment as a feed forward mechanism for enhanced T cell responses suggest this could happen anywhere where a DC has the potential to interact with multiple T cells. However the evidence presented above shows that T cells need a sufficient supply of nutrients to activate and hence for our model to take an effect. So at tumor sites or inflamed tissue, even though limited nutrients may enhance DC pro-inflammatory function it may not result in increased T cell responses as the T cells themselves will be inhibited by the lack of nutrients. Our model will only have an effect when the T cell has access to sufficient nutrients to meet its demand for activation for example when DCs are interacting with T cells in the lymph node as we have shown or potentially when activating memory T cells in the periphery. Work in the future will address the sites in the body where our model has an effect and the influence this has on the T cell response.

Our data shows that nutrient availability and DC metabolism are integrally linked to sustaining DCs ability to induce T cell responses. DCs ability to induce T cell responses makes them an attractive target for vaccine strategies in clinical interventions. Traditionally vaccines were based either on live attenuated organisms which were less pathogenic than the original pathogen or on killed organisms that could safely generate an immunological response. Today most vaccines are based on well-defined recombinant products that are usually proteins expressed by the pathogen (Plotkin, 2005, Pulendran and Ahmed, 2011). Recently investigation into the potential of DCs in cancer vaccines has started to increase (Palucka and Banchereau, 2012). Excitingly the first DC cancer vaccine was approved by the FDA for the treatment of metastatic prostate cancer in 2010. This treatment, called Sipuleucel-T, is an *ex vivo* based strategy where the patients own monocytes are isolated from their body and treated ex vivo with a prostate cancer specific peptide and GM-CSF to aid differentiation of DCs. The treated DCs are then added back to the patient with results indicting significant increased survival in patients receiving this therapy (Kantoff et al., 2010). Other *ex vivo* based DC strategies for the treatment of metastatic melanoma have also showed efficacy in the clinic (Fay et al., 2006). The potential to target mTORC1 signaling in DCs to improve vaccine

efficacy was recently shown in mice using an *ex vivo* based vaccination approach. DCs pulsed with OVA *ex vivo* and injected into mice containing melanoma cells expressing OVA lead to a reduction in tumor volume and mass (Amiel et al., 2012). Our data suggests that inhibiting HIF-1α, iNOS or glycolysis itself *ex vivo* would also be beneficial for a DC vaccination strategy and could be of interest to investigate in the future. A second DC vaccination strategy involves targeting DCs directly *in vivo*, usually using monoclonal antibodies against DC specific surface markers (Tacken and Figdor, 2011). This work was pioneered by the Steinman group who showed that conjugating a DC targeting antibody to an appropriate antigen could elicit strong immune responses from both CD4+ and CD8+ T cells (Bonifaz et al., 2004). This strategy was tested in the clinic using an antibody targeting the DC receptor DEC-205 and the tumor antigen NY-ESO-1 along with a TLR7/8 stimulant as an adjuvant. The results from this trial showed this strategy was safe in humans and caused tumor regression in a subset of patients (Dhodapkar et al., 2014). Our results suggest that targeting inhibitors of mTORC1/HIF-1α to DCs *in vivo* in conjugation with an appropriate antigen could be a strategy to boost DC vaccination efficacy. Data presented here showing increased IL-12 production when these two metabolic regulators are inhibited would suggest that this would drive an enhanced T_H1 and CTL response *in vivo*. Targeting specifically to the DC is essential, as inhibitors for mTORC1 or HIF-1α would block T cell function if given systemically. The induction of a cellular immune response rather than a humoral immune response is thought to be important for vaccines against HIV, malaria, TB and cancer (Pulendran and Ahmed, 2011). Although DC based vaccines have shown promise the efficacy has been poor in the clinic, particularly for cancer vaccines (Radford et al., 2014). This is most likely due to tumor immune evasion and inhibition of T cell responses. Indeed a phase I clinical trial found using an *ex vivo* DC vaccination strategy in combination with CTLA4 inhibition lead to complete clearance of melanoma in a subset of patients studied (Ribas et al., 2009). In the future combinatorial strategies could be tested that used either an *ex vivo* or DC targeted strategy employing an inhibitor of DC metabolism to boost the pro-inflammatory phenotype of the cell with an appropriate antigen.

Concluding remarks

Data presented in this PhD has established a new paradigm in the field of immunometabolism. For the first time we have shown that competition for nutrients during DC T cell interactions impacts on mTORC1/HIF-1α signaling within the DC. Inhibiting these signaling pathways in the DC promotes a pro-inflammatory phenotype and leads to a DC that sustains the ability to induce T cell responses over a longer time period. Our model merges these two novel findings and shows how T cells and DCs can synergize their metabolic states so that they can maximize their pro-inflammatory potential. This model predicts that metabolic regulation of DC pro-inflammatory outputs represents a mechanism to distinguish robust immune responses from weak/self reactive responses, which is critical to ensure an appropriate immune response Our research helps explain how DC T cell interactions in lymph nodes promote robust T cell responses. If validated, this model of T cells modulating the DC microenvironment will change our perception of the cellular interactions occurring during T cell activation and will represent a previously unappreciated level of reciprocal signaling between T cells and DC that impacts upon the T cell response. This data adds to the emerging view in the field that cellular metabolism has a role that extends beyond simply allowing the cell to meet energy and biosynthetic demands, and represents a fundamental physiological mechanism to control immune responses.

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