2018

Characterizing The Role And Regulation Of Glycogen Metabolism In Dendritic Cell Immune Responses

Phyu Myat Thwe
University of Vermont

CHARACTERIZING THE ROLE AND REGULATION OF GLYCOGEN METABOLISM IN DENDRITIC CELL IMMUNE RESPONSES

A Dissertation Presented

by

Phyu Myat Thwe

to

The Faculty of the Graduate College

of

The University of Vermont

In Partial Fulfilment of the Requirements
For the Degree of Doctor of Philosophy
Specializing in Cellular, Molecular, and Biomedical Sciences

October, 2018

Defense Date: May 2nd 2018
Dissertation Examination Committee:

Eyal Amiel, Ph.D., Advisor
Matthew Poynter, Ph.D., Chairperson
Jonathan Boyson, Ph.D.
Tom Jetton, Ph.D.
Jay Silveira, Ph.D.
Cynthia J. Forehand, Ph.D., Dean of the Graduate College

ABSTRACT

Dendritic cells (DCs) are the most potent professional antigen presenting cells (pAPCs) of the immune system and play a fundamental role in coordinating innate and adaptive immune responses. Through the expression of a wide array of pattern recognition receptors (PRRs), such as toll-like receptors (TLRs), DCs recognize a variety of microbial pathogens and infectious stimuli. Stimulation of DCs through TLR ligation results in a rapid series of activation-associated events, termed "maturation," which include the upregulation of surface co-stimulatory molecule expression, inflammatory cytokine secretion, and stimulation of naïve T cells via antigen presentation by MHC molecules.

Activation of DCs through TLRs is coupled with an increased metabolic demand fulfilled by a rapid change in DC glucose metabolism and characterized by increased aerobic glycolysis rates. TLR-driven glycolytic reprogramming plays an essential role in generating building blocks required for high level protein synthesis associated with maturation. Although glucose imported from extracellular environments has been broadly considered as the major driver of glycolytic metabolism in immune cells, the contributions of intracellular glucose stores to these processes are not well-defined. The role of intracellular stores of glucose, in the form of glycogen, is widely appreciated in non-immune systems. However, very little is known about the implication of glycogen metabolism in DC immune responses. This work unveils the role and potential regulatory mechanisms of glycogen metabolism in support of DC effector function.

The first part of this work primarily focuses on our characterization of the role of glycogen metabolism in early DC activation responses; while in the last chapter, we describe a potential regulatory mechanism of DC glycogen metabolism by activation-associated nitric oxide (NO) production. In this work, we tested the overarching hypothesis that DC-intrinsic glycogen metabolism supports the early glycolytic reprogramming required for effector responses and that nitric oxide can regulate this metabolism. We demonstrate that DCs possess the enzymes required for glycogen metabolic machinery and that glycogen metabolism supports DC immune effector response, particularly during early activation and in nutrient-limited environments. More importantly, we uncover a very intriguing metabolic phenomenon, in which DCs engage in the differential metabolic pathways driven by carbons derived distinctively from glycogen and free glucose. Our studies present the fundamental role and regulatory mechanisms of DC-intrinsic glycogen metabolism and underline the differential utilization of glycogen and glucose metabolism to support their effector responses. Overall, this work adds to a growing field of immuno-metabolism an improved understanding of an intricate layer of metabolic mechanisms that immune cells undertake in response to immune stimuli.

CITATIONS

Materials from this dissertation have been published in the following form:

Thwe, PM., Pelgrom, L., Cooper, R., Beauchamp, S., Haines, J.A., D'Alessandro, A., Everts, B., and Amiel, E.. (2017). Cell-intrinsic glycogen metabolism supports early glycolytic reprogramming required for dendritic cell immune responses. Cell Metabolism. 26(3): 558-567.e5.doi: 10.1016/j.cmet.2017.08.012. PMID: 28877459

Thwe, PM. and Amiel, E.. (2018). The role of nitric oxide in metabolic regulation of dendritic cell immune function. Cancer Letters. 2018 Jan 1; 412:236-242. doi:10.1016/j.canlet.2017.10.032. PMID: 29107106.

Thwe, PM. and Amiel, E.. (2018). Analysis of Glycogen Metabolic Pathway Utilization by Dendritic Cells and T Cells Using Custom Phenotype Metabolic Assays.

AND

Material from this dissertation has been submitted to Journal of Leukocyte Biology (JLB) for publication in the following form:

Thwe, PM., Fritz, D., Galasso, N., Ojemann, A., Sepaniac, L., Adamik, B., Snyder, JP., Hoyt, L., Poynter, M., and Amiel, E.. (2018). Syk-dependent Glycolytic Reprogramming in Dendritic Cells Regulates IL-1β production to Fungal-associated Ligands in a TLR-independent Manner.

AND

Material from this dissertation is in preparation for publication in the following form:

Thwe, PM., Rodriguez, P., Snyder, J., Davidson, C., Hasslet, N., Cummings, K., and Amiel, E.. (2018). Nitric oxide regulates glycogen metabolism utilization in LPS-activated dendritic cells.

ACKNOWLEDGEMENTS

First of all, I would like to thank my graduate mentor, Eyal Amiel, for all the support, time, and energy he has given me over the past few years. I cannot express how grateful I am to him for taking me in into his lab. He accepted me while knowing that I had no prior experience in mammalian cell cultures, let alone immunology and metabolism. I thank him for his support and patience in helping me to improve my scientific writing skills. I am also grateful to have been given the opportunity to participate in starting up his lab from the ground-up. Lastly, I thank him immensely for guiding me and setting an example for how to think critically and function as a scientist. Thank you, you've raised me well in the scientific realm!

I would like to thank to Jon Boyson, my first-year academic advisor and my committee member, for believing in me throughout my entire process and guiding me through the trials. I am immensely indebted to Stephen Everse. He remained an incredible advisor to me, even beyond his responsibility as our first-year course director. I would not have made it to this finish-line if it weren't for his wise advice and belief in my potential. Thank you both, Jon and Stephen, for your continued motivation since year one of my graduate career! I would like to thank Matt Poynter, my committee chair, for his support and suggestions about experimental strategies, personal and work life – I am forever thankful! I would also like to thank Jay Silveira and Tom Jetton, my committee members, for your insightful suggestions. Also, a big thanks to the Immuno-biology group for the vibrant and helpful suggestion in every lab meeting I've presented – I've learned so much from these meetings.

This journey wouldn't have been possible without the support of my graduate community. Diana (lab cousin) and Princess (lab sista), Myles (my other lab cousin), and Coop (lab baby), thank you all so much for creating this fun and upbeat environment in Rowell. I wish you all the best of luck in your graduate and professional careers. I would also like to thank my fellow graduate students, Vicki, Devin, Abbas, and Mike, in the Immuno-biology group, with a special thanks to Vicki for being my awesome first-year student mentor. She has always been there not just for my first year, but pretty much throughout this entire journey.

Finally, I'd like to thank my parents for their unconditional love and support for letting me pursue my dream education and career. I would like to extend my thanks to my Boston family for sharing their cheerful spirits and meditation practice with me. My greatest gratitude to my life-partner in crime, Aung, for his love and support (and for his patience in putting up with me all of these years). I cannot imagine how I could have made it through this journey without him by my side. Thank you for being there for me through thick and thin.

Lastly, this acknowledgement section will not be complete without expressing my gratitude to Marcus Thali, who was a standing member of the CMB admission committee during the year I came for the recruitment weekend. This entire Ph.D. process and my dream to go back into clinical diagnostics would not all have come true if he had not believed in my potential and convinced the entire committee that I am worth the investment. Thank you so much!!

TABLE OF CONTENTS

LIST OF TABLES

TABLE **PAGE**

LIST OF FIGURES

FIGURE **PAGE**

CHAPTER 1

Comprehensive Literature Review

1.1: Brief overview of the innate immune system

The innate immune system, recognized as the very initial phase of immune response, is the first line of defense against infection and elicits non-specific immunity against invading pathogens (Janeway, 2005). Early recognition of inflammatory stimuli and inflammation responses of the innate immune system is orchestrated by both cellular and non-cellular factors. First, epithelial cell surfaces provide a protective layer and the first line of defense against pathogen invasion. The cellular part of the innate immune system includes innate immune cells, such as neutrophils, macrophages, dendritic cells, eosinophils, and basophils (Abbas and Janeway, 2000; Nathan, 2002), and these cells are readily stationed at points of entry in mammalian organisms and work in a coordinated fashion to protect against infections. Once the epithelial barrier is breached and a focus of infection is established, innate immune cells act as first responders in initial recognition and capture of invading pathogens, secretion of anti-microbial and chemoattractant proteins, and elimination of microbial and cellular debris (Nowarski et al., 2013). Soluble components of the innate immune system include a cascade of opsonin proteins, which make up the "complement system," and proteolytic enzymes and antimicrobial proteins secreted by epithelial cells and leukocytes (Janeway, 2005).

1.1.1: Cells of the innate immune system

Leukocytes of the immune system are systematically derived from hematopoietic precursors originated from bone marrow, which then develop into two major progenitors, namely common myeloid progenitors (CMP) and common lymphoid progenitors (CLP). Committing to one or the other of the lineage progenitors results in the loss of developing

into alternative cell lineages in response to the initial differentiation signals. In general, cells of myeloid origin mainly constitute innate immunity and are canonically described as the first responders to infectious stimuli, whereas CLPs generally give rise to B and T lymphocytes, major players of the adaptive immunity (Kondo, 2010). There is also a well-appreciated role for some classes of non-myeloid innate immune cells of lymphoid origin, such as innate lymphoid cells (ILCs) (Kumar, 2014) and natural killer cells (Abbas and Janeway, 2000; Janeway and Medzhitov, 2002) in early inflammatory responses. Accumulation of phagocytes at the site of infection is one of the first and early characteristics of innate defense mechanisms against invading pathogens (Abbas and Janeway, 2000; Janeway, 2005; Janeway and Medzhitov, 2002). Phagocytes are largely classified into granulocytic and mononuclear phagocytes.

Granulocytic phagocytes are comprised of neutrophils, eosinophils, basophils, and mast cells. Neutrophils are the most efficient phagocytes among these granulocytes and represent the most abundant population in the circulation, constituting about 50-70% of total peripheral white blood cells (Mayadas et al., 2014). Also known as polymorphonuclear leukocytes (PMNs) due to their characteristic multi-lobed nuclei, these short-lived, highly motile cells are recruited to the sites of infection by chemo-attractants released from activated endothelial layers and tissue-resident innate immune cells. After phagocytosis, they destroy invading pathogens by several mechanisms, such as lysosomal acidification, respiratory burst, and a release of neutrophil extracellular traps (NETs), a meshwork of intracellular contents containing chromatin and DNA (Mayadas et al., 2014; Nowarski et al., 2013; Segal, 2005). Eosinophils, as motile as neutrophils, can migrate from the circulation to tissues. Their specialized role is defense

against multicellular parasites, such as worms, by releasing intracellular toxic granules that contain cytolytic enzymes, proteases, and cytokines, resulting in membrane damage and death of invading parasites (Weller and Spencer, 2017). Unlike neutrophils and eosinophils, basophils are rather immobile. Basophils contain intracellular granules potent against eradicating helminth and parasitic infections. Histamine is one of the most well-recognized of the basophilic granular contents, responsible for triggering allergy symptoms (Voehringer, 2013). Another type of granulocytes functionally similar to basophils and believed to share the ontogeny with basophils are called mast cells. Although the developmental process between mast cells and basophils are not fully understood, both cell types are known to cause IgE mediated allergic responses and potently induce TH2 mediated adaptive immunity (Siracusa et al., 2013; Voehringer, 2013).

The mononuclear phagocytic system consists of monocytes, macrophages, and dendritic cells, and originates from hematopoietic cells of bone marrow. One of the well-appreciated functional aspects of these cells is their specialized ability in antigen processing and presentation (Haniffa et al., 2015; Hume, 2006, 2008). Monocytes are the most abundant mononuclear phagocytes (5-10%) in the circulation whereas macrophages and DCs primarily reside in tissue niches. In most cases, monocytes serve as immediate precursors for both macrophages and DCs, which then migrate into tissue environments. Macrophages, a major phagocyte population in many tissues, are appreciated as the most efficient phagocytes in eliminating cellular debris as well as pathogenic particles. Although the phagocytic ability of DCs is not as potent as macrophages, their specialized ability to traffic to secondary lymphoid organs and their role as key antigen presenting

cells is fundamental in coordinating both innate and adaptive immune responses (Haniffa et al., 2015; Janeway, 2005; Janeway and Medzhitov, 2002). The antigen processing and presenting function of macrophages and DCs is discussed at length in the following subsections.

1.1.2: MHC molecules and Antigen Presenting Cells (APCs)

Major histocompatibility complex (MHC) molecules, encoded by a cluster of genes called the MHC, also known as human leukocyte antigens (HLA) and located on chromosome 6 short arm, are critically involved in presenting antigenic peptides on the surface of antigen presenting cells to T cells, which will screen presented peptides for self- versus non-self-antigenic signatures (Cunliffe and Trowsdale, 1987; Matzaraki et al., 2017). Subsequent to T cell receptor engagement with cognate peptide/MHC complexes, DCs assist in the induction of T cell-mediated adaptive immune responses (Abbas and Janeway, 2000; Janeway, 2005; Matzaraki et al., 2017; Roche and Furuta, 2015; Rock et al., 2016). The mechanisms of antigen processing and presentation via MHC differ greatly between the two major classes of MHC molecules, classified as MHC class I and II. For MHC class I presentation, intracellular proteins of endogenous origins or derived from intracellular pathogens are degraded in the cytosol or nucleus and threaded into the endoplasmic reticulum (ER) lumen via a transporter associated with antigen presentation (TAP). Class I molecules are also assembled in the ER lumen by partial folding with the aid of TAP, which helps bind MHC with the two chaperone proteins, calreticulin and ERp57. Calreticulin, ERp57, and an additional chaperone called PDI help stabilize MHC I. Peptides translocated via TAP are sometimes trimmed by ER

aminopeptidase associated with antigen processing (ERAAP). The modified peptides then bind to MHC-I peptide binding region, forming a peptide-MHC complex ready to be exported out of the ER and expressed on the cell surface for antigen presentation to CD8+ T cells (Neefjes et al., 2011; Porgador et al., 1997; Rock et al., 2016).

Similar to the MHC-I assembly, MHC-II molecules are also assembled in the ER, however, in a different fashion than the former. Exogenous proteins such as extracellular microbial pathogens are processed and presented by the MHC class II pathway. Extracellular microbes endocytosed by immune cells are degraded into peptides in phago-lysosomes and late endosomes. In the ER lumen, MHC-II molecules are assembled in alpha and beta chains, in association with the invariant chain (Ii), which is then cleaved into a Class II associated Ii peptide, called CLIP. CLIP is bound in the peptide-binding region of MHC molecules and keeps the MHC from pre-mature binding to the antigenic peptides originated in the ER. The MHC molecules are then transported into endosomes where the processed peptides are located. With the help of HLA-DM, which facilitates the exchange of CLIP, the exogenous peptide is degraded in the phago-endosomal pathway. The fully assembled MHC-peptide complex is then transported onto the cell surface to present the peptide to CD4+ T cells (Neefjes et al., 2011; Roche and Furuta, 2015).

Any nucleated cells in our body express MHC class I on their surfaces. However, a class of cells that possess an ability to present antigenic peptides to CD8+ T cells and initiate adaptive immune responses by stimulating T cells are termed antigen presenting cells (APCs). APCs include fibroblasts, epithelial cells, endothelial cells, mesenchymal

cells, and immune cells such as dendritic cells, macrophages, and B cells (Rock et al., 2016; Steinman, 2001).

While all nucleated cells express MHC-I, those expressing high levels of MHC-II are further classified as "professional" antigen presenting cells (pAPC) due to their specialized ability to present antigens from both endogenous and exogenous sources (Sprent). Efficient stimulation of T cells is proposed to require two major signals, both of which are provided by pAPCs. The first signal is presentation of antigenic peptides to T cells via MHC molecules. APCs with an ability to provide co-stimulatory signals, also known as second signals, are considered to initiate a much stronger T cell response, and for this process, pAPCs are well-equipped to express co-stimulatory ligands, such as CD40, CD80, and CD86 (Lipscomb and Masten, 2002). It has also been proposed that a third signal, cytokines released from both APCs and T cells, enhance the process, although certain schools of thought refute this idea (Abbas and Janeway, 2000). In general, the major elements of pAPCs are characterized by the ability to uptake invading pathogens; process pathogen-associated proteins into peptides ready to be presented via MHC-I/ II; the surface expression of co-stimulatory molecules; and the ability to migrate to the T cell-rich lymphoid areas and present processed peptides to T cells; thereby initiating adaptive immune responses.

Although recent studies showed that other hematopoietic innate immune cells such as basophils, mast cells, and eosinophils express MHC II molecules and are proposed to be categorized as APCs, it is still unclear whether these cells could be defined as pAPCs (Abbas and Janeway, 2000; Kambayashi and Laufer, 2014). Nevertheless, dendritic cells (DCs), macrophages, and B cells are long-considered as the

major canonical pAPCs. The degree of antigen presentation capacity vastly differs among the three types of pAPCs, which is likely due to the inherent functional differences and their distinct cellular shapes and structures.

1.1.2.1: B cells

B cells primarily function as antibody producing cells of the immune system. In contrast to macrophages and DCs, B cells have a relatively limited capacity to phagocytose/engulf large particulate antigens, which is considered one of the important attributes of an APC. B cell interaction with antigen is manifested by the binding of surface immunoglobulins to the antigenic particles released from macrophages, which release antigenic protein molecules during phagocytic/macropinocytic processes (Ronchese and Hausmann, 1993; Unanue, 1984). Therefore, the antigen presenting ability of B cells is reported to be facilitated by external help from macrophages or DCs. Despite this, the potency of B cells to effectively stimulate naïve T cells is still very poor (Lassila et al., 1988; Ronchese and Hausmann, 1993). Several studies have later supported the idea that antigen specific B cells, not naïve B cells, can activate T cells that have been stimulated by the same antigen (Lassila et al., 1988; Ronchese and Hausmann, 1993).

B lymphocytes are primarily known as antibody-producing cells. During development, B cells undergo VDJ recombination, a process that involves rearrangements of Variable (V), Diversity (D), and Joining (J) gene segments to generate diverse B cell receptors (BCR). Upon successful VDJ recombination, matured B cells express immunoglobulin D and M (IgD and IgM) (LeBien and Tedder, 2008). Unlike the term “maturation” in DCs, B cell maturation refers to a developmental process, in which

developing B cells undergo VDJ rearrangements of immunoglobulin (Ig) chains of a BCR. B cells have several different functions including, but not limited to, antibody-secretion, cytokine production, and antigen presentation (Hoffman et al., 2016). In regard to antigen presentation functionality, BCRs capture antigens and become clustered on the B cell surface at which stage, B cells become activated (Hoffman et al., 2016; LeBien and Tedder, 2008). Clustered BCRs are internalized, and activated B cells bearing antigen-containing BCRs in the intracellular compartments then travel to T- and B-cell borders of lymph nodes. Because B cells also express co-stimulatory molecules required for antigen presentation, they can stimulate antigen-specific T cells, not naïve T cells (Hoffman et al., 2016). Some of the activated B cells are then transformed into plasma cells, forming primary foci in the lymph node, whereas other B cells form germinal centers in primary follicles, where somatic hypermutation and the generation of high-affinity antibody production occurs (Abbas and Janeway, 2000; Janeway, 2005; LeBien and Tedder, 2008).

1.1.2.2: Macrophages

Macrophages are of myeloid origin and perform a diverse array of functions in the immune system. Like DCs, macrophages are also tissue resident innate immune cells highly specialized in phagocytosis and clearance of microbial organisms (Condeelis and Pollard, 2006; Hashimoto et al.; Janeway, 2005). Due to their best ability to clear engulfed particles and antigens, macrophages are implicated in tissue homeostatic functions, such as tissue remodeling and resolution of inflammation after infection or

tissue injury. Activated macrophages are rich in lysosomes that contain hydrolytic enzymes to break down engulfed materials.

Lysosomal digestion is a major pathway for processing antigens in macrophages (Banchereau and Steinman, 1998a). Although macrophages are unquestionably better pAPCs than B cells, there are still ongoing debates about whether or not macrophages are less efficient in antigen presenting capacity than DCs. Since the functional roles of macrophages in the innate immune system are diverse, they possess the flexibility to respond to a broad range of stimuli. The exposure to particular types of stimuli is thought to initiate the generation of two primary macrophage populations (Pearce and Pearce, 2013). Such a phenomenon is called macrophage polarization, in which the exposure to IFNγ and TLR-agonists can produce "M1" or classically activated macrophages, whereas "M2" or alternatively activated macrophages are induced by stimulation with canonical Th-2 cytokines IL-4 and IL-13 (Elhelu, 1983; Galván-Peña and O'Neill, 2014; Hashimoto et al., 2011). A more detailed description of M1 and M2 macrophages in regard to their metabolic and functional differences will be later discussed in the comprehensive metabolism review of this chapter –Section 1.3.1.1 Metabolic reprogramming in macrophages.

1.1.2.3: Dendritic cells (DCs)

DCs, first characterized by Paul Langerhans in the 1800's as Langerhans cells (LCs) in the tissue epidermal layer (Silvano, 2014), were later illustrated in the 1970's by Ralph Steinman as cells possessing a strange dendritic branch-like membrane protrusion with the ability to present exogenous antigens to T cells in an MHC-dependent manner

(Silvano, 2014). DCs are primarily considered as tissue resident cells and largely exist in various lymphoid organs, epithelial layers, and connective tissues, whilst a small percentage is found in peripheral blood (Banchereau and Steinman, 1998a; Steinman, 2001, 2012; Steinman and Hemmi, 2006). Because of the heterogeneous nature of their localization in tissues/organs and a diverse expression of surface markers, DCs have been categorized into a number of different subsets (Lipscomb and Masten, 2002), which are discussed in the following section.

As sentinels of the innate defense line, DCs constantly sample particulates in tissue micro-environments and possess a high capacity for phagocytosis of both exogenous and endogenous sources of antigens (Banchereau and Steinman, 1998a; Steinman, 2001). They can also capture antigens via receptor-mediated mechanisms due to a high expression of a variety of pattern recognition receptors, expressed both intracellularly and on the surface, such as toll-like receptors (TLRs), C-type lectin receptors (CLR), and NOD-like receptors (NLRs) (Hammer and Ma, 2013). When resting DCs encounter pathogens, they become activated and subsequently downregulate the expression of PRRs. Capture of a pathogen prompts DCs to engage in a process called maturation, characterized by upregulation of surface MHC-I and –II molecules, co-stimulatory molecules (CD40, CD80, and CD86), and the secretion of chemokines and pro-inflammatory cytokines. DCs at this stage are termed “mature” DCs and are capable of delivering Signal 1 and Signal 2 for naïve T lymphocyte activation. One of the critical functions of mature DCs is the migration to draining lymphoid tissues where they present antigenic peptides to naïve T cells. DC migration to secondary lymphoid organs is driven by upregulation of chemokine receptor CCR7 upon PRR signaling and maturation

(Banchereau and Steinman, 1998b; Steinman, 2001). Two striking qualities of mature DCs is that a single DC can interact with multiple T cells at the same time and that DCs innately possess copious amount of MHC-class II containing lysosomal compartments (MIIC) (Nijman et al., 1995; Pierre et al., 1997), which are considered "specialized" components for antigen presentation, compared to other pAPCs. On these accounts, DCs are considered the most efficient pAPCs in the immune system (Banchereau and Steinman, 1998a; Nijman et al., 1995). This dissertation will thoroughly explore the metabolic regulations of TLR- and fungal-ligand-activated DCs in their effector responses.

1.1.3: DC subsets and heterogeneity

DCs exist as a heterogeneous population of cells originating from the hematopoietic lineage (Hashimoto et al., 2011). Regardless of the shared characteristics in processing and presenting antigens and the ability to travel to secondary lymphoid organs to activate T cells, the class of DCs still represent functionally and phenotypically diverse subsets in different tissues sources (Steinman, 2012). After developing from macrophage-dendritic cell precursors (MDP), developing DCs lose granulocyte components and become committed to the DC lineage. Cells at this stage are termed as common DC precursors (CDP), which then give rise to fully developed DCs of different subsets under the influence of distinct growth factors (Hashimoto et al.). Under certain conditions, DCs can also develop directly from monocyte precursors and are termed as monocyte-derived DCs (mDCs). A similar phenomenon is also seen in a subset of macrophages that are originated from monocytes (monocyte-derived macrophages). An

illustration of DC development and a general characterization of DC subsets are depicted in

Figure 1 -1. *Major subsets of DCs and developmental pathways*

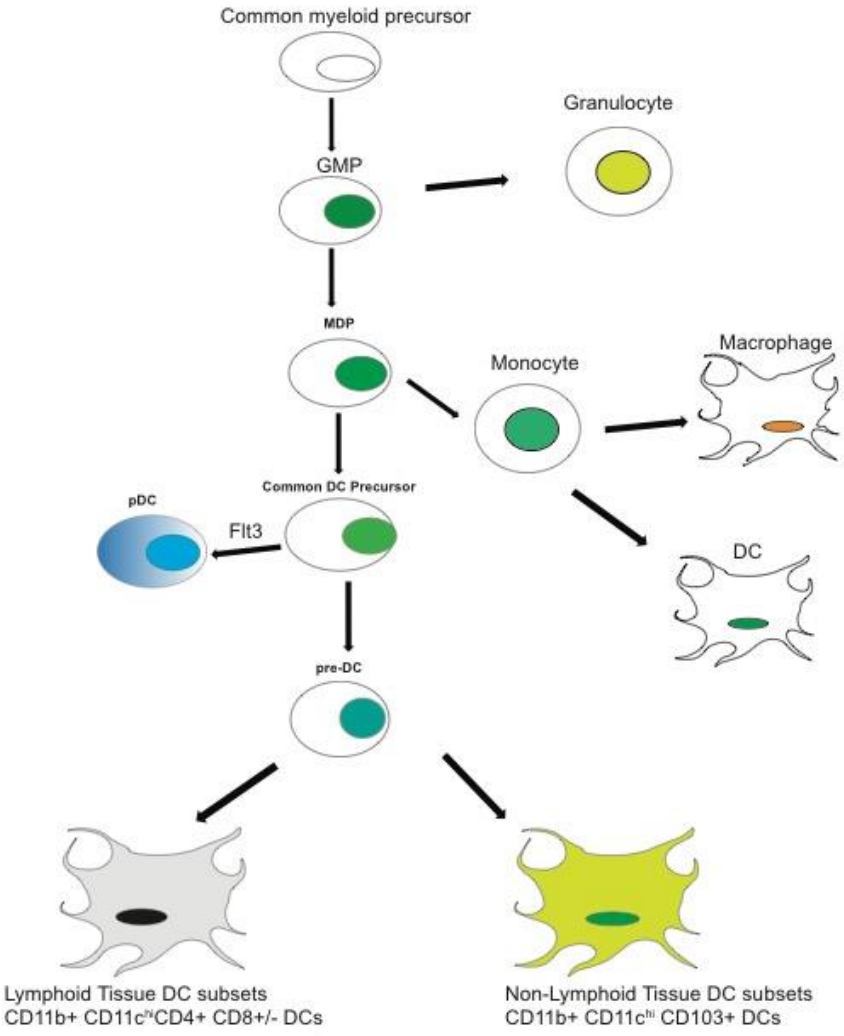


Figure 1 -1. Major subsets of DCs and developmental pathways

(illustration adapted from Hashimoto *et al.* (Hashimoto et al., 2011))

To date, DCs are broadly categorized into conventional or classical DC (cDCs) and plasmacytoid DC (pDCs) subsets (Steinman, 2012). cDCs can be of lymphoid or non-lymphoid origins (Sichien et al., 2017; Silvano, 2014). Two major subsets of cDCs are: $CD11b^+$ cells that induce $CD4^+$ T cell activation and differentiation, and $CD11b^+$ $CD8\alpha^+$ DCs that are specialized in cross-presentation of antigens and activation of $CD8^+$ T cells (Crowley et al., 1989; den Haan et al., 2000; Hashimoto et al.; Silvano, 2014; Vremec et al., 1992). Initially thought to arise from lymphoid tissues, pDCs, one of the non-classical DC types, were later discovered to be originated from bone marrow precursors as well. These pDCs then circulate in peripheral blood and migrate to lymphoid tissues upon encountering antigens (Banchereau and Steinman, 1998a). pDCs are a primary source of type-1 IFN upon viral infections (Chistiakov et al., 2015; Diebold et al., 2004; Nakano et al., 2001; Saas et al., 2017; Siegal et al., 1999) and highly express TLR7 and TLR9 (Steinman, 2012). A common trait of both cDCs and pDCs is that both cell types are dependent on growth factor fms-like tyrosine-3 ligand (Flt3L) for their development (Guo et al., 2016; Kingston et al., 2009), a model used for both *in vitro* and *in vivo* generation of cDCs and pDCs from mouse. Another major type of non-classical DCs is monocyte-derived DCs (mDCs). mDCs are also known as inflammatory DCs (iDCs) and found in the peripheral blood. As the name implies, mDCs are transformed from circulating $CD14^+$ monocytes in the event of infection and represent prototypical inflammatory DCs, that are believed to migrate to peripheral tissues upon activation (Chow et al., 2017; Collin et al., 2013; Hashimoto et al.; Lee and Iwasaki, 2007; Mildner and Jung, 2014). In addition to these major subtypes, there are still several subsets of tissue DCs throughout our body localizing in skin (Langerhans cells), intestine

(CD103^{+}DCs), and lungs (Hashimoto et al.) by bearing different markers on their surface. The lack of specific lineage markers on DCs has posed a difficult problem in characterizing the underlying molecular mechanisms for differentiating specific DC subsets.

Two primary models used in the field of mouse DCs are *in vitro* generation of DCs from bone marrow hematopoietic stem cells by using the cytokines granulocyte-macrophage-colony-stimulating-factor (GM-CSF) and Flt3 Ligand. The former is generally termed bone-marrow derived DCs (BMDCs) and the latter produces Flt3L DCs (Merad et al., 2013; Mildner and Jung, 2014). Regardless of diversity, a number of DC subsets share common immune characteristics with BMDCs; thus, the use of BMDCs as an experimental model provides us with insightful information about DC biology and functionality. Tissue resident DCs such as splenic or lymphoid DCs can also be isolated directly from organs and tissues by isolation of CD-marker specific DCs. For instance, CD11c^{+} splenic DCs and CD11b^{+} CD8α^{+}/CD8α^{-} DCs are isolated from spleen connective tissues and lymph nodes, respectively. On the other hand, *in vitro* generation of human DCs greatly differ from murine DC isolation. A commonly-used method for isolating human DCs is *in vitro* culture differentiation of CD14+ circulating monocytes with cytokines GM-CSF and IL-4 (Collin et al., 2013; Sallusto and Lanzavecchia, 1994, 2018). The immunophenotypic outcome of the two widely-accepted models of mouse and human DCs, BMDCs and GM-CSF/IL-4 derived DCs respectively, is thought to closely reflect that of mDCs (or iDCs) *in vivo* (Mildner and Jung, 2014; Thwe and Amiel, 2018). More recent discoveries of DC subsets localized in different tissue niches, with distinct cell surface markers and ontogeny, have been made with the aid of technological

advances (Chow et al., 2017; See et al., 2017; Silvano, 2014; Steinman, 2012; Villani et al., 2017; Young, 2012). Along with these discoveries comes the necessity to explore the functional details of these subsets and compare them with previously defined DC models.

1.2: Pattern recognition receptors (PRRs)

Pattern recognition receptors (PRR), originally proposed by Janeway to possess the ability to distinguish self from non-self-molecules, are well-appreciated as major drivers in recognizing evolutionarily conserved microbial pathogens and triggering effector responses of innate immunity (Takeuchi and Akira). PRRs are a family of germ-line encoded receptors located both intracellularly and extracellularly in or on innate immune cells. While PRRs were initially thought to be expressed on macrophages and DCs only, PRRs such as TLRs have been found on other cell types such as epithelial cells and fibroblasts, which also play an important role in barrier defense (Mogensen, 2009). PRRs serve as initial sensors of microbial organisms by recognizing conserved molecular structural components called pathogen associated molecular patterns (PAMPs). They can also recognize host cell-derived damage associated molecular patterns (DAMPs) resulting from host cellular and tissue damage regardless of the presence or absence of infection (Takeuchi and Akira). The initial recognition of microbial stimuli by PRRs is important in controlling infections (Mogensen, 2009) and maintaining cellular homeostasis to prevent catastrophic outcomes in the host. Strategic localizations of PRRs both inside and outside of a cell provide the host an advantage to clear any invading inflammatory stimuli in a timely manner. PRRs are generally classified into five major family members, which include toll-like receptors (TLR), C-type lectin receptors (CLRs),

retinoic acid inducible gene (RIG)-1 like receptor (RLRs), NOD-like receptors (NLRs), and the AIM-2 receptors (ALRs) (Brubaker et al., 2015; Takeuchi and Akira). A detailed outline of PRR families, with their cellular localization and ligand recognition is mentioned in Table *1*.

Activation of PRRs via binding of a cognate ligand leads to a dynamic array of transcriptional and translational changes resulting in innate inflammatory responses, such as a release of pro-inflammatory cytokines, chemoattractants, (Brubaker et al., 2015) and cell adhesion molecules accompanied by increased expression of immunoreceptors (Mogensen, 2009). Downstream effects of PRR activation are orchestrated by a number of intracellular signaling pathways, which, in fact, are primarily initiated by the association of receptors with intracellular adaptor(s), that relay signals from the receptors to downstream effector proteins/enzymes. Some adaptors share a common domain with more than one receptor, thus the signals between different categories of receptors are more efficiently relayed to downstream targets via conserved and convergent signaling processes. Innate immune responses cannot be achieved by receptors alone but rather through the protein-protein interactions between receptors and adaptor molecules. Albeit not all receptors require adaptor molecules, they play a crucial role in ligand recognition (Brubaker et al., 2015); detailed functions of some major adaptor molecules will be discussed later in this chapter. Among the above-mentioned PRR family members, our studies primarily investigate the metabolic regulation of DCs activated via toll-like receptor 4 (TLR4) and a C-type lectin receptor family member, Dectin-1.

Table 1: Summary of major PRRs, cognate ligands, and cellular localizations

Type of PRRs	**Localization**	**Ligand**	**Origin of ligand**
TLRs			
TLR1 (works in concert with TLR2)	Plasma membrane	Triacyl Lipoprotein	Bacteria
TLR2	Plasma membrane	Lipoprotein	Bacteria, Virus, parasite, self-antigen
TLR3	Endolysosome	dsRNA	Virus
TLR4	Plasma membrane	LPS	Bacteria, virus, self-antigen
TLR5	Plasma membrane	Flagellin	Bacteria
TLR6 (works in concert with TLR2)	Plasma membrane	Diacyl lipoprotein	Bacteria, virus
TLR7	Endolysosome	ssRNA	Virus, Bacteria, self-antigen
TLR8 (human)	Endolysosome	ssRNA	Virus, Bacteria, self-antigen
TLR9	Endolysosome	CpG-DNA	Bacteria, virus, protozoa, self

TLR10	Endolysosome	Unknown	Unknown
TLR11	Plasma membrane	Profilin-like molecule	Protozoa
TLR12	Unknown	Unknown	Protozoa
TLR13	Unknown	Unknown	Unknown
RLR			
RIG-I	Cytoplasm	Short dsRNA	RNA and DNA viruses
MDA-5	Cytoplasm	Long dsRNA	RNA viruses
AIM2	Cytoplasm	dsDNA	Bacteria, viruses
NLR			
NOD1	Cytoplasm	LPS	Bacteria, self
NOD2	Cytoplasm	LPS	Bacteria, self
CLR			
Dectin-1	Plasma membrane	β-glucan	Yeast and hyphae of fungi
Dectin-2	Plasma membrane	Mannose, α-linked mannans	Fungal yeasts and Hyphae
DC-SIGN	Plasma membrane	Glycan	Self, Viruses, Fungi (Garcia-Vallejo and van Kooyk, 2013)
MINNCLE	Plasma membrane	SAP130	Fungi, Self-antigen

1.2.1: Toll-like receptors (TLRs)

TLRs are the most studied PRRs in the field of innate immunity. The discovery of mammalian's toll protein is linked to the finding of Drosophila Protein *Toll*, which was originally observed to play a role only in fruit fly development. Right before the discovery of the function of *Toll,* IL-1R type 1 was identified to be implicated in T cell activation as well as in acute phase inflammatory reactions. IL-1R was soon revealed to be homologous to the Toll of Drosophila, which was then reassessed and shown to be also involved in fruit fly immunity against fungal pathogens. The cytoplasmic tails of TLRs and IL-1R have been shown to possess a conserved homology domain, known as the TIR domain, which is responsible for initiation of signaling cascades (Akira, 2003; Akira and Takeda, 2004; O'Neill et al., 2013). At the extracellular region, leucine rich repeats are conserved across all TLRs (O'Neill et al., 2013). While more than 20 TLRs were identified in invertebrate species, such as channel catfish (Quiniou et al., 2013), 11 TLRs have been discovered to date in humans and mice, with some controversial reports on the expression of TLR11, TLR12, and TLR13 in humans.

Regardless of their structural similarities in leucine rich repeat motifs in the extracellular domain, TLRs can recognize diverse arrays of ligands, which are differentially localized across the cell. TLR1, TLR2, TLR4, TLR5, TLR6 are located in the plasma membrane and generally recognize extracellular pathogens (Brown et al., 2011; Brubaker et al., 2015; Medzhitov, 2007; Takeda and Akira, 2005). TLR3, TLR7, TLR8, and TLR9 are found in the cytoplasm, localized across endosomal or lysosomal membranes, and recognize intracellular PAMPs, such as nucleic acid motifs expressed in

viral and bacterial genomes (Akira, 2003; Akira and Takeda, 2004; Janeway and Medzhitov, 2002; Takeda and Akira, 2004, 2005; Uematsu and Akira, 2007).

TLR2 recognizes a broad range of ligands originated from Gram positive bacteria, mycobacteria, fungi, and viruses. It heterodimerizes with TLR1 for recognition of triacylated lipopeptides or with TLR6 for recognition of diacylated lipopeptides (Lee and Iwasaki, 2007; Santos-Sierra et al., 2009; Shen et al., 2010; Takeda and Akira, 2005; Takeuchi and Akira, 2004). Among the TLRs, TLR2 recognizes the widest variety of ligands, even including glycosylphosphatidylinositol lipid from the parasite *Trypanosoma cruzi.* Of note, TLR1 and TLR6 are thought to be constitutively expressed on the surface of any cell types, whereas TLR2 is identified mostly on macrophages, dendritic cells, and endothelial cell, and TLR2 expression on these cells is highly regulated (Beutler, 2004; Brubaker et al., 2015; O'Neill et al., 2013; Takeda and Akira, 2005).

TLR3 is predominantly expressed in DCs (Muzio et al., 2000) and recognizes double-stranded RNA, a major component of many viruses and a molecular signature of an active viral replication cycle (Alexopoulou et al., 2001; O'Neill et al., 2013). Ligation of TLR3 receptor results in NF-κB activation and Type I IFN signaling, and abolishing the expression of TLR3 causes a defective defense against dsRNA viral infections and impaired immune responses to Poly IC, a synthetic ligand for TLR3 (Alexopoulou et al., 2001).

The first characterized TLR in mammals was TLR4. The importance of TLR4 was discovered in C3H/HeJ mice, harboring a mutation in a region of the TLR4 gene that was later identified to contain a LPS responsive area, and this mutation was manifested by LPS-hyporesponsiveness and increased susceptibility of these animals to endotoxic

shock elicited by infection with Gram-negative bacteria (Hoshino et al., 2016; Poltorak et al., 1998). This finding was also confirmed in TLR4 deficient mice (Hoshino et al., 2016; Poltorak et al., 1998; Rosenstreich et al., 1977). The structure and assembly of TLR4 is distinct from other TLR members because TLR4 itself does not directly bind to the LPS ligand. The binding of LPS to TLR4, however, is facilitated by two other accessory proteins, CD14, which is an essential receptor for LPS, and MD-2, complexed to TLR4 (da Silva Correia et al., 2001). This dissertation work uses TLR4 ligand stimulation as a model to investigate the influence of metabolic pathways in the regulation of DC immune responses during inflammatory conditions.

TLR5 recognizes the conserved flagellin protein that makes up flagella (Hayashi et al., 2001; Medzhitov, 2007; O'Neill et al., 2013; Uematsu et al., 2006; Yoon et al., 2012) in both Gram positive and negative bacteria (Hayashi et al., 2001). TLR5 is highly expressed in lamina propria cells in the intestine; however, intestinal epithelial cells, originally thought to have higher expression of TLR5, have been later shown to express low levels of TLR5 (Uematsu et al., 2006). TLR5 plays a crucial role in mucosal areas by not only initiating the innate immune responses against bacterial infection but also inducing adaptive T helper cell responses, particularly in TH-17 differentiation (Hawn et al., 2003; Uematsu et al., 2008; Uematsu et al., 2006), suggesting the important roles of TLRs beyond innate immunity.

Single stranded nucleic acids (ssRNAs), particularly guanosine and uracil rich nucleotides derived from viruses and bacteria are recognized by TLR7 and TLR8 (Diebold et al., 2004; Gorden et al., 2006; Heil et al., 2004; Jurk et al., 2002; O'Neill et al., 2013). The recognition of ssRNAs via TLR7 and/or TLR8 was originally thought to

be species specific, in which murine TLR8 was believed to be nonfunctional while, in human, both TLR7 and TLR8 can recognize single stranded viral RNA ligands (Heil et al., 2004; Jurk et al., 2002). However, Gorden *et al* demonstrated in a later study that murine TLR8 can be successfully activated by a synergistic action of a synthetic immune response modifier (IRM) and polyT oligodeoxynucleotides, TLR7/8 agonist (Gorden et al., 2006), suggesting the structural differences between TLR7 and TLR8 may be a culprit in the adjuvant requirement for TLR8 in recognizing its cognate ligands. ssRNAs exist in large quantities in human cells; however, TLR7 and/or TLR8 hardly detect host-derived ssRNAs most likely due to the localization of these receptors in endosomes (Takeda and Akira, 2005).

TLR9 recognizes unmethylated CpG nucleotide motifs derived not only from bacterial DNAs but also from viruses, such as Herpes simplex virus-1 and -2 and MCMV (Medzhitov, 2007; O'Neill et al., 2013). TLR9 distinguishes mammalian CpG due to the low frequency methylation of nucleotides (Hemmi et al., 2000). The discovery of TLR9 recognition of viral DNAs was elegantly demonstrated by a collaborative effort of Akira, Medzhitov, Takeda, and Colonna. Proper antiviral responses such as secretions of IFN-α, IFN-β, and other inflammatory cytokines involved in functional activation of NK cells require the ability of TLR9 to recognize viral DNAs (Krug et al., 2004a; Krug et al., 2004b; Lund et al., 2003). TLR9 is recruited to endosomal compartments, where it is complexed with DNA ligands with the aid of endosomal acidification during the process of endocytosis (Hemmi et al., 2000).

The role of TLR10 has not been extensively studied. TLR10 is detected in human macrophages, neutrophils, activated B cells, and plasmacytoid dendritic cells (Bourke et

al., 2003; Hasan et al., 2005; Lee et al., 2014). It shares the same locus with TLR1 and TLR6; therefore, it is postulated to heterodimerize with TLR1 (Akira and Takeda, 2004; Hasan et al., 2005; O'Neill et al., 2013). While the ligand for TLR10 is not well-defined, influenza viral infection in macrophages and B cells mediated via TLR10 is reported to induce potent innate immune responses (Fernandes et al., 2014). On the other hand, other studies have indicated the anti-inflammatory roles mediated by TLR10 (Oosting et al., 2014). These findings altogether suggest that TLR10 possesses both pro- and anti-inflammatory responses in modulating immune responses.

TLR11 and TLR12 have recently been shown to dimerize upon *Toxoplasma gondii* infection in mice (Andrade et al.). TLR11, however, exists in human as a pseudogene, and TLR12 is not entirely expressed in human (Andrade et al.; Quiniou et al., 2013). Although the expression of these two TLRs seem unimportant in humans, the fact that they play a critical role in rodent toxoplasma susceptibility suggest potential relevance to human immunology (Andrade et al.).

1.2.1.1: TLR signaling pathways

The cytoplasmic tail of TLRs is made up of a highly conserved Toll/IL1-R (TIR) domain, which forms a TIR-TIR structure upon dimerization of TLRs in encountering a cognate ligand. Such dimerization, such as homo-dimerization in TLR3 or hetero-dimerization in TLR2 with TLR1 or TLR6 (Ozinsky et al., 2000), initiates signal transduction in order to produce specific immune responses elicited by TLR-ligand interactions (Weber et al., 2005). Binding of adaptors at the TIR domains triggers a TLR signaling cascade. Five adaptor molecules involved in TLR signaling have been

discovered to date: myeloid derived differentiation factor 88 (MyD88); TIR-domain containing adaptor inducing IFN-β (TRIF); TIRAP/MyD88-adaptor like (Mal); TRIF-related adaptor molecule (TRAM); and Sterile-alpha and Armadillo motif-containing protein (SARM). Out of these five, MyD88 is recognized as a major signaling adaptor implicated in almost all TLRs, with the exception of TLR3. In general, two major signaling pathways are engaged upon TLR-ligand interactions: MyD88-dependent and MyD88-independent pathways.

MyD88 was the first TIR adaptor to have been discovered (Lord et al., 1990) and later recognized to play an essential role in TLR and IL-1 signaling (Bernard and O'Neill, 2013; Bonnert et al., 1997; Deguine and Barton, 2014; Medzhitov et al., 1998; Muzio et al., 1998; Muzio et al., 1997). MyD88 is comprised of two domains: a death domain (DD) in the N-terminal and a TIR domain in the C-terminal. TIR-TIR association between MyD88 and TLR domains phosphorylates IRAK, IL-1 receptor associated kinase-4, which then associates with TRAF6 and IRAK-1, subsequently diverging into two distinct pathways – activation of NF-κB and AP-1 (Medzhitov et al., 1998; Wang et al., 2001). Activation of NF-κB is achieved by a series of phosphorylation of molecules, such as a complex of TGF-β activated kinase (TAK) proteins and inhibitors of NFκβ (IKK) (Wang et al., 2001; Zandi et al., 1997). Activated NF-κB is then translocated to nucleus, where it induces the transcription of a number of genes for pro-inflammatory cytokines (Ghosh et al., 1998; Zandi et al., 1997). On the other hand, TAK1 complex activated by phosphorylated TRAF6/IRAK-1 triggers a series of phosphorylation events in MAPK Kinases, such as MAPKK, MKK3, and MKK7, eventually leading to activation of p38 and JNK (Wang et al., 2001), with a final outcome of the transcriptional

factor activator protein-1 (AP-1) activation. AP-1 is accountable for induction of several genes encoding inflammatory cytokines and acute phase reactant proteins (Lee and Kim, 2007).

In TLR7 and TLR9 activation, MyD88 signaling also induces the transcription factor Interferon regulatory factors (IRFs) for type I interferon production in addition to NF-κB dependent cytokines (Hacker et al., 2006; Oganesyan et al., 2006). The MyD88-dependent pathway in TLR2 activation requires the Mal/TIRAP adaptor, one of the five TLR adaptors with a TIR domain as mentioned above, for proper signal transduction (Santos-Sierra et al., 2009). Originally discovered as a bridging adaptor in the assembly of MyD88 complex to the TIR domain of TLRs (Gay et al., 2011), Mal has been later identified to function independent of MyD88 in TLR2 and TLR4 (Equils et al., 2004; Fitzgerald et al., 2001; Santos-Sierra et al., 2009).

The MyD88-independent pathway, originally discovered in MyD88 knock-out mice that display intact NF-κB and MAPK activities in response to LPS (Kawai et al., 1999), was later found to be facilitated by the TRIF adaptor molecule, one of the TIR domain containing adaptors homologous to MyD88 (Hoebe et al., 2003; Oshiumi et al., 2003; Yamamoto et al., 2003a). These findings were confirmed in TRIF3 knock-out mice, displaying impaired IFN production upon challenges with Poly I:C, a synthetic ligand for TLR3, indicating that TLR3 signaling is exclusively dependent on TRIF3 (Brown et al., 2011; Hoebe et al., 2003; Takeda and Akira, 2005; Yamamoto et al., 2003a). This pathway is interchangeably referred to as TRIF-dependent or MyD88-independent pathway. Both TLR3 and TLR4 engage in a MyD88-independent pathway, although TLR4 is unique among the TLR family in that also signals through the MyD88-

dependent pathway (Takeda and Akira, 2004; Yamamoto et al., 2003a). Association of TRIF in TIR-TIR domains initiates a series of phosphorylation cascades leading to the activation of IRF3, which induces IFN-β. IFN-β, in turn, activates STAT1 for induction of more IFN-inducible genes (Sato et al., 2003). Similar to Mal, which serves as a bridge in MyD88-dependent pathways, a second adaptor called TRAM was later found to be required for TRIF signaling in TLR4 activation (Yamamoto et al., 2003b).

1.2.2: C-type lectin receptors

C-type lectin transmembrane receptors (CLRs) are one of the family members of PRRs and mainly recognize carbohydrate cell wall components of microbial origin (Brubaker et al., 2015; Dennehy and Brown, 2007; Takeuchi and Akira). It has been demonstrated later that CLRs can also recognize non-carbohydrate structures, such as lipids, proteins, and nucleic acids (Brown et al., 2003b; Dambuza and Brown, 2015; Plato et al., 2013). The CLR family is comprised of relatively large members, including Dectin-1, Dectin-2, mannose, MCL (macrophage C-type lectin), DC-SIGN (dendritic cell specific ICAM3-grabbing non-integrin), and CLEC9A. CLRs are widely expressed in myeloid cells, predominantly in dendritic cells and macrophages, and to some extent in B cells and neutrophils (Dambuza and Brown, 2015). CLR activation is achieved through the recognition of polysaccharide antigens in C-type lectin domains of the receptors and generally gives rise to induction of NFκB or NFAT-mediated signaling pathways. Cytoplasmic tails of CLRs are composed of intracellular signaling motifs, such as ITAMs (immunoreceptor tyrosine-based activation motifs), ITIMs (immunoreceptor tyrosine-based inhibitory motifs), and non-immunoreceptor tyrosine-based motifs (Plato et al.,

2013). CLR signal transduction occurs either through a direct activation of these intracellular domains or indirectly via binding of adaptors to immuno-modulatory motifs (Plato et al., 2013; Takeuchi and Akira). The induction of CLRs via ITAMs generally leads to phagocytosis, cellular proliferation and differentiation, and migration (Plato et al., 2013). For instance, in myeloid immune cells, such as DCs and macrophages, CLR activation initiates inflammatory responses such as increased phagocytosis, respiratory burst, and pro-inflammatory cytokine release (Brown et al., 2003b; Dambuza and Brown, 2015; Plato et al., 2013). However, the activation of inhibitory motifs usually results in negative regulation of CLR-mediated signaling pathways (Kerrigan and Brown, 2010).

1.2.2.1: Dectin-1 receptor

Dectin-1 is the most well-characterized CLR family member. The expression of Dectin-1 was originally believed to be restricted to dendritic cells (Ariizumi et al., 2000); however, several studies have later indicated that it is also expressed on monocytes, macrophages, neutrophils, and occasionally on B cells, eosinophils, and a small population of T cells (Brown et al., 2003b; Drummond and Brown, 2011; Taylor et al., 2002). The extracellular portion of Dectin-1 receptor consists of carbohydrate binding domains, just like any other types of CLRs. However, the intracellular component of Dectin-1 consists of ITAM-like motifs at its cytoplasmic tail, which is unique compared to other CLRs operating via a true ITAM domain (Brown, 2006; Kerrigan and Brown, 2010; Underhill and Goodridge, 2007). Two major signaling pathways, known as spleen tyrosine kinase (Syk) and CARD, downstream of ITAMs are reported to be implicated in Dectin-1 activation, which will be explained in detail later.

Dectin-1 recognizes both 1,3- and 1,6- β glucans, which are carbohydrate polymers and major components of plant and fungal cell walls (Batbayar et al., 2012). These β glucan molecules possess an immuno-modulatory capacity through the dectin-1 receptor and elicit pro-inflammatory responses, such as release of cytokines, chemokines, and reactive oxygen species (ROS) in dendritic cells (Underhill et al., 2005).

Fungal cell walls are composed of approximately 60% of β-glucans, 10-20% of chitin, and about 40% of mannan proteins (Batbayar et al., 2012; Netea et al., 2008; Shepherd, 1987). The exposure of β-glucan on fungal cell wall surfaces allows Dectin-1 to serve as the primary receptor in recognizing yeast pathogens. Since some fungi exist only as yeasts while others, such as *Candida albicans,* can switch between yeast and hyphal forms, such morphological transformation changes the exposure of cell wall structural components on the surface. For instance, while the yeast form of *C.albicans* is primarily recognized by Dectin-1, its hyphae are recognized by Dectin-2, which preferentially binds mannose and alpha-mannans present in fungal hypha (Alexopoulou et al., 2001; Kapteyn et al., 2000; Shepherd, 1987). In that case, a morphologic change from yeast to hyphae can potentially alter the ultimate immune outcomes due to potentially different signaling mechanisms downstream of each receptor-ligand pair. In addition, some TLRs, such as TLR2, TLR4, TLR6, and TLR9, can also recognize phospholipo-mannan, O-linked mannans, zymosan, and fungal DNA, respectively (Netea et al., 2008). The redundant and/or collaborative recognition of highly complex fungal structural components by both CLRs and TLRs (Ferwerda et al., 2008; Netea et al., 2006) pose challenges in understanding immune responses elicited by a specific ligand-receptor pair. Of interest, although Dectin-1 is an innate immune receptor, it's implication in

generating trained innate immunity or innate immune memory was illustrated in human monocytes that are initially primed with β-glucan followed by subsequent re-activation with *C. albicans* yeast. Trained innate immunity is the memory responses of innate immune cells, whereby innate immune signaling can induce epigenetic changes that cause the innate immune cells to exhibit long-term effector responses upon challenge with microbial pathogens (Cheng et al., 2014). In this dissertation, we have undertaken to identify the downstream metabolic and immune responses induced by Dectin-1 specific ligands and have examined the similarities and differences between metabolic reprogramming in DCs driven by this canonical CLR as compared to TLRs.

1.2.2.5: Dectin-1 signaling pathway

Activation of CLRs is achieved through signal transduction via immuno-receptor tyrosine-based activation motifs (ITAMs) at the cytoplasmic tail of the receptor. Unlike signal transductions by the ITAMs of other CLRs, which is largely carried out by two domains of tyrosine phosphorylation sites of ITAM motifs, Dectin-1 signaling is uniquely transduced through a single tyrosine phosphorylation at its ITAM - hence namely ITAM-like motifs (Fuller et al., 2007; Rogers et al., 2005). Phosphorylation at ITAM-like motif recruits an adaptor molecule called spleen tyrosine kinase (Syk) (Brown, 2006; Fuller et al., 2007; Kerrigan and Brown, 2010; Plato et al., 2013; Rogers et al., 2005; Underhill et al., 2005), originally thought to be restricted only in the lymphocyte development and function (Mocsai et al., 2010), and later discovered to play a crucial role in signal transduction in non-lymphoid cells. The importance of Syk in Dectin-1 activation was elegantly demonstrated in Syk-/- mice challenged with Zymosan, a fungal cell wall

protein-carbohydrate complex extracted from *Saccharomyces* or *Candida albicans* with the potent immuno-stimulatory capacity, resulting in failure to produce inflammatory cytokines (Rogers et al., 2005). Phosphorylated Syk then induces NF-κB transcription via activation of CARD9, caspase-recruitment domain 9, which then forms a complex with Bcl-10 (B-cell lymphoma protein 10) (Gross et al., 2006; LeibundGut-Landmann et al., 2007). Syk-CARD9-Bcl10 axis in Dectin-1 activated DCs is crucial in initiating Th1 and Th17 differentiation in a MyD88-independent manner (Kingeter and Lin, 2012; LeibundGut-Landmann et al., 2007). Several studies have also reported that Syk-dependent Dectin-1 activation not only gives rise to inflammatory cytokine production but also generates ROS (Underhill et al., 2005) and activates the inflammasome complex. These findings indicate an interplay between the metabolic regulation of Syk-kinase and inflammasome signaling pathway. Our work here will set out to identify this association in the context of fungal infection.

A few years after the role of Syk in Dectin-1 signaling was identified, Gringhuis *et al* showed that Dectin-1 is also activated by Syk-independent pathway for NK-κB induction. Activation of Syk-independent pathway is achieved through Raf1 kinase signaling and is known as the non-canonical NF-κB activation pathway (Gringhuis et al., 2009). Raf1 signaling in Dectin-1 activation seems to crosstalk with TLR2 and TLR4. This is not surprising given the fact that Dectin-1 ligands are cooperatively recognized by TLRs, such as TLR2 and TLR4, due to the complex nature of fungal cell wall composition.

1.2.3: NLRP3 inflammasomes and IL-1β

Nucleotide binding oligomerization domain leucine rich repeats receptors (NLRs) are another family members of PRRs that can sense insults derived from both microbial and non-microbial origins of PAMPs and DAMPs. NLRs then initiate immune responses in the intracellular compartments; thus, these receptors are exclusively localized in the cytosol (Prochnicki et al., 2016; Sutterwala et al., 2014). NLRs consist of a number of subfamily members, categorized into four main types based on their N-terminal effector domains (Sutterwala et al., 2014; Ting et al., 2008). These members include NLRP that contains pyrin domain, CARD that contains caspase activation and recruitment domain, BIRC that has Baculoviral inhibitory repeats, and the NLR containing an acidic transactivation domain (Ting et al., 2008). Each NLR family member further consists of sub-family members, and approximately 22 total members of NLRs have been identified to date (Sutterwala et al., 2014). Upon receiving signals, which are either cellular stress and damage or microbial pathogens, the NLR members associate with other inflammatory proteins and form multiprotein complexes, known as inflammasomes. An inflammasome is typically composed of NLR or pyrin domain containing proteins, an adaptor protein called apoptosis associated speck-like protein containing domain (ASC), and cysteine protease Pro-caspase-1. Inflammasome formation results in the autocatalysis of caspase-1, which then catalyzes pro IL-1β and pro IL-18 and releases the active forms of these pro-inflammatory cytokines. Activation of inflammasomes is crucial in triggering acute inflammatory responses. To date, NLRP1, NLRP3, NLRC4, and AIM2 (absent-in-melanoma 2) have been characterized to form inflammasomes (He et al.; Lamkanfi and Dixit, 2014; Próchnicki and Latz, 2017; Prochnicki et al., 2016; Sutterwala

et al., 2014), although some studies have later reported the potential activation of inflammasomes mediated by NLRP6, NLRP7, and NLRP12 (He et al.; Sutterwala et al., 2014).

NLRP3 (NLR protein3) is the most studied member among the NLR family. Activation of NLRP3 inflammasome leads to caspase-1 activation, which results in the production of active IL-1β and IL-18. Active IL-1β is a highly potent pro-inflammatory cytokine and serves as one of the earliest inflammatory responses initiated by innate immune cells, thus playing a crucial role in early anti-microbial recognition and defense. IL-1β also mediates the release of other cytokines, such as TNF-α and IFN-γ from other cells. Not only does it recruit innate immune cells, such as macrophages, monocytes, and neutrophils, but also induces Th1 and Th17 responses of adaptive immunity (Lamkanfi and Dixit, 2014). Despite the well-known association of IL-1 activation and NLRP3 inflammsomes, IL-1β activation can also be achieved by an NLRP3 inflammasome-independent manner (Bossaller et al., 2012; Gringhuis et al., 2012).

Because of the potency and strength in amplification of inflammatory responses, IL-1β is not constitutively expressed but is synthesized as an inactive form. The inactive form protects this interleukin molecule from inadvertent inflammation initiation, until it is ready to be released into the extracellular milieu. Lacking a signal sequence (Auron et al., 1984; Lopez-Castejon and Brough, 2011), IL-1β secretion is distinct from the canonical protein ER-Golgi secretory pathway. Several mechanisms of IL-1β secretion have been proposed: exocytosis of secretory lysosomes, exosomes, or autophagosomes, plasma membrane shedding, and cellular exit through a specialized transporter across the plasma membrane (Lopez-Castejon and Brough, 2011). Although it is still not exactly

clear why several different mechanisms exist only to secrete a single cytokine of active IL-1β, it is possible that the types and strengths of stimuli could potentially influence specific immune mechanisms for this purpose (Lopez-Castejon and Brough, 2011; Martinon et al., 2002; Netea et al., 2010).

Due to highly potent inflammatory effects of inflammasome components, NLRP3 formation is tightly controlled (Eder, 2009; Lamkanfi and Dixit, 2014; Prochnicki et al., 2016; Sutterwala et al., 2014). While NLRP3 was thought to be triggered by potassium efflux, mitochondrial DNA, or reactive oxygen species, several lines of evidence have indicated the requirement of two signals for NLRP3 inflammasome formation (He et al.; Lamkanfi and Dixit, 2014; Próchnicki and Latz, 2017; Prochnicki et al., 2016; Sutterwala et al., 2014). Signal one is known as "priming," which is essentially a transcriptional regulatory checkpoint in generating pro IL-1 and pro-caspase-1. This step is mediated by PAMPs that induce the NF-κB dependent pathway via activation of TLRs and NLRs as well as cytokine receptors, such as IL-1R1 and TNF-R (Lamkanfi and Dixit, 2014; Sutterwala et al., 2014). Signal two represents "activation," a process characterized by the assembly of inflammasome components and caspase-1 activation, resulting in a release of active IL-1β . Activators for signal two can be both endogenous and exogenous molecules such as intracellular microbial infections, monosodium urate crystals and alike (alum and asbestos), toxins such as nigericin, and potassium depleting molecules such as adenosine triphosphate (ATP) (Sutterwala et al., 2014). Since NLRP3, in its inactive state, is known to be highly ubiquitinated under normal basal conditions, these activators then deubiquitinate NLRP3 and subsequently promote the full activation of NLRP3 inflammasomes (Juliana et al., 2012; Lin et al., 2014).

While the classical outcome of NLRP3 inflammsome formation is caspase-1 activation, along with processing of IL-1β and IL-18 from their inactive forms, active caspase-1 can also cause pyroptosis, a form of cell death depicted by membrane rupture and cell swelling. Although caspase-1-mediated pyroptosis is an alternative outcome of a canonical NLRP3 inflammasome pathway (Qiu et al., 2017; Sutterwala et al., 2014; Wellington et al., 2014), a non-canonical pathway of NLRP3 inflammasome formation is mainly carried out via caspase-11, whose activation is achieved by sensing intracellular LPS or a LPS component (acylated lipid A) and results in pyroptosis as well (Kayagaki et al., 2013; Yang et al., 2015).

In addition to the aforementioned two-signal tiered regulation of NLRP3 formation, metabolic regulations contributed by both the host and microbes also play a major role in proper NLRP3 inflammasome-mediated immune responses. With an emerging interest in cellular metabolic regulation of immune responses, NLRP3 inflammasome activation has been shown to be coupled with an increase in cellular glycolytic flux (Moon et al., 2015). NLRP3 inflammasome formation can be triggered in the events of perturbations in metabolic pathways and enzymatic activities. For instance, inhibition of hexokinase (HK), a major rate-limiting enzyme of glycolysis, by the bacterial-derived citrate metabolite (Wolf et al., 2016) or N-acetylglucosamine (NAG)-containing bacterial peptidoglycan (Spiro, 1958) can induce NLRP3 inflammasome processing. Studies have reported the induction of inflammasomes by an increased level of mitochondrial reactive oxygen species (ROS) (Zhou et al., 2010), which has also been demonstrated to be a product of caspase-1 activation in a NLRP3-independent fashion (Yu et al., 2014).

Although TLR-mediated priming signals for NLRP3 inflammasomes were extensively studied, only in recent years has significant attention been given to elucidating the mechanisms of inflammsome activation via other PRRs, particularly Dectin-1 activation (Cheng et al., 2011; Gross et al., 2009; Hise et al.; Kankkunen et al., 2010) in response to fungal pathogens. There is also an emerging interest in how cellular metabolism is implicated in this process. This dissertation will discuss the metabolic regulation of NLRP3 inflamasome activation mediated by Dectin-1 recognition of fungal ligand.

1.3. Metabolism overview

Metabolism in eukaryotic cells is categorized into catabolism and anabolism, in which biomolecules, such as carbohydrates, fatty acids, amino acids, and nucleic acids, are broken down and synthesized, respectively. While the catabolism-driven generation of energy, mostly in the form of ATP, is essential, the need to generate metabolic intermediates for molecular building blocks renders cellular metabolism a core regulator of cellular function and homeostasis. Metabolic pathways are orchestrated by a series of enzymatic reactions, with one or more regulatory (rate-limiting) enzymes serving as a check-point for many of those pathways. Metabolic reactions are highly regulated at both cellular and systemic levels, in response to the cues from cell-cell interactions and external stimuli, thus adding complexity to various metabolic networks. Therefore, the intricately interconnected and redundant metabolic networks allow for rapid cellular adaptation to environmental insults, such as metabolic pathway inhibition or nutrient

deprivation. This dissertation discusses the metabolic changes in immune cells, particularly myeloid cells, in response to infectious stimuli.

Major metabolic pathways include, glycolysis, the citric acid (TCA) cycle and oxidative phosphorylation, pentose phosphate pathway (PPP) or hexose monophosphate shunt, glycogen metabolism, fatty acid synthesis and degradation, and nucleotide synthesis. Glucose is a fundamental carbon source for rapid energy production and is primarily broken down via glycolysis, which yields 2 net ATP molecules. The end product of glycolysis, pyruvate, enters the TCA cycle and mitochondria for a complete oxidation of carbons, with a higher yield of 32 net ATPs. Pyruvate enters the TCA cycle after being converted to acetyl co-A in the cytoplasm or oxaloacetate (OAA) in mitochondria. Under certain conditions, such as limited oxygen supply, pyruvate is reduced to lactate, a condition well-known as anaerobic glycolysis in bacterial and yeast carbohydrate metabolism (Voet et al., 2013). Since ATP is not the only form of cellular energy, cells can also obtain NADPH via the PPP, an alternative pathway to glycolysis. Glucose-6-phosphate (G6P), liberated from glucose by the rate-limiting enzyme hexokinase, enters the PPP, one of the crucial anabolic pathways entirely responsible to generate not only NADPH, used for a number of anabolic processes such as fatty acid synthesis, but also ribose-5-phosphate for nucleic acid building blocks (Voet et al., 2013). Another potential fate of glucose is glycogen metabolism, in which excess cellular glucose is converted to glycogen for storage, which is then later utilized based on the physiological needs of the cell (Dashty, 2013; Schwartz, 1976). Details of glycogen metabolism will be extensively discussed later in this chapter. Additionally, G6P and pyruvate are considered key intermediates of glucose metabolism; the metabolic flux of

these molecules is one of the main focuses of this dissertation. While glycolysis, glycogen metabolism, and PPP occur in the cytosol, the TCA cycle operates in conjunction with electron transport chain/oxidative respiration in mitochondria.

From the perspective of cellular energy generation, fatty acid oxidation provides the highest energy yield to support ATP-dependent anabolic reactions and physiological processes. Each round of fatty acid β-oxidation results in acetyl-CoA, which is further oxidized in the TCA cycle electron transport chain, resulting in an extremely large amount of energy output. For instance, an approximate net total of ~106 ATP is generated from oxidation of one molecule of palmitate. Unlike β-oxidation, fatty acid synthesis occurs in the cytosol. The starting material acetyl-CoA, generated from mitochondria, is transported across the mitochondrial membrane in the form of citrate, which is then converted back to acetyl-CoA. While the fatty acid synthesis process is generally considered to be energy consuming, this pathway plays a crucial role in immune cell activation responses to infectious stimuli, which is discussed in great detail in the following subsection.

Amino acids are building blocks of a number of compounds such as proteins, polypeptides, and other biomolecules, such as cytokines, chemokines, and several inflammatory mediators. Besides the essential amino acids that are obtained from the diet, a number of glycolytic and the TCA cycle intermediates generate amino acids required for basal cellular functions. For instance, alanine can be generated from pyruvate, α-KG (keto-glutarate) from the TCA cycle can be converted to glutamate, which can also be converted to glutamine. It has been widely known that glutamine, in addition to glucose, is a primary fuel source among other amino acids for rapidly

proliferating and metabolically active cells, such as tumor cells and lymphocytes (Everts and Pearce, 2014; Galván-Peña and O'Neill, 2014; Matarese et al., 2014; Newsholme and Newsholme, 1989; Norata et al., 2015; Pavlova and Thompson, 2016; Pearce and Pearce, 2013; Saeed et al., 2014; Warburg et al., 1927).

1.3.1 Metabolic reprogramming in cancer and immune cells

The idea of cellular metabolic reprogramming was introduced in tumor cells by a German physiologist Otto Warburg, who demonstrated that cancer cells "ferment" glucose and heavily depend on glycolysis even in the presence of abundant oxygen in the circulation. Cancer cells are rapidly proliferating cells with an increasing demand of energy and metabolic substrates. From an energy standpoint, glycolysis is not energetically efficient compared to mitochondrial respiration. Yet, cancer cells are more dependent on glycolysis than non-transformed cells, which Warburg proposed was due to the impairment of the mitochondrial respiratory chain (Warburg, 1956; Warburg et al., 1927). However, this fact has been proven otherwise in most recent studies (Palsson-McDermott and O'Neill, 2013; Warburg, 1956). While the breakdown of glucose is generally considered as a catabolic process, an increase rate of glycolysis in these cancer cells serves to support anaplerosis, whereby metabolic intermediates are generated for biosynthetic materials required during cell proliferation (Palsson-McDermott and O'Neill, 2013).

The study of metabolism in immune cells dates back to the early 1910's, when Meyer and Lepine demonstrated the formation of two three-carbon sugars from glucose molecules in dog leukocytes (Levene and Meyer, 1912). It was in the 1920's when Otto

Warburg introduced the idea of a metabolic switch in cancer cells, that a link between leukocyte and cancer metabolism was established by two seminal findings of the Dutch and German scientists, who demonstrated a reduced rate of aerobic respiration and an increase in glycolysis in white blood cells isolated from exudates (Nagy and Haschemi, 2015). Due to technical disadvantages in isolating pure leukocyte populations and challenges in metabolism assays, metabolic studies in immune cells were not prioritized until a recent reemergence in the past few decades.

The first study on peripheral blood monocytes and polymorphonuclear phagocytes defined the cellular needs for active glycolysis in support of the immediate energy demand during an initial phase of phagocytosis, followed by a later phagocytic stage in which the cells uptake more oxygen via aerobic respiration (Sbarra and Karnovsky, 1959). Unlike lymphocytes and other myeloid cells, neutrophils are dependent almost exclusively on glycolysis due to a relatively small number of mitochondria (Fossati et al., 2003; Maianski et al., 2004; Rodriguez-Espinosa et al., 2015). Recent studies also showed that neutrophils undergo two different metabolic phases for neutrophil extracellular trap (NET) formation, in which the early stage does not require exogenous glucose whereas extracellular glucose serves as a major fuel source to drive the later phase (Rodriguez-Espinosa et al., 2015).

1.3.1.1 Metabolic reprogramming in macrophages

A link between the metabolism and immune responses in macrophages was demonstrated by the pioneering work of Hard in the late 1960's, which showed that activated macrophages consume low oxygen and depend on glycolysis (Hard, 1970).

After two decades, macrophage utilization of major nutrient sources and intermediary metabolites, such as glucose, glutamine, ketone bodies, and fatty acids, were thoroughly investigated by Newsholme (Newsholme et al., 1986). Consistent with the earlier findings of Hard and Cline, Newsholme's studies showed an increased glycolytic activity in macrophages upon activation. However, his work also showed that the Kreb's cycle in these macrophages is fueled mostly by glutamine, but very little by glucose, suggesting that OXPHOS is still functional in order to generate energy. His work also showed an increased phospholipid turnover, indicating the membrane recycling and biogenesis occurs during the activation process (Newsholme et al., 1986; Newsholme and Newsholme, 1989). It was not until a few years later that the differential metabolism between two populations of macrophages, namely M1 and M2, was discovered. Although originally recognized by their different responses to Th1 and Th2 cytokines, IFN-γ and IL-4/IL-13 respectively, these two types of macrophages can be metabolically discriminated based on their differential arginine utilization.

Classically activated (IFN-γ activation) or LPS-activated M1 macrophages upregulate iNOS, which breaks down arginine to citrulline and nitric oxide (NO), whereas alternatively activated (IL-4/IL-13 activation) M2 macrophages upregulate arginase 1 to produce urea, polyamines, and ornithine from arginine (Corraliza et al., 1995). Subsequent studies have reported that pro-inflammatory M1 macrophages depend on aerobic glycolysis and produce reactive oxygen species and NO, with very little mitochondrial activity. On the other hand, M2 macrophages, primarily considered to be involved in the resolution stage of inflammation, possess highly active mitochondrial OXPHOS function (Galván-Peña and O'Neill, 2014).

Identification of these two macrophage subsets has driven the field to meticulously analyze metabolic phenotypes between the two populations. Indeed, the glycolytic activity of M1 macrophages activated with LPS is different than that of M1 macrophages stimulated with IFN-γ (Galván-Peña and O'Neill, 2014; Tavakoli et al., 2013), due to the fact that LPS-activated macrophages have a higher expression of Glut1 and hexokinase 2 (HK2) as opposed to IFN-γ stimulated counterparts (Kelly and O'Neill, 2015; Rodriguez et al., 2017). These findings suggest differences in metabolic profiles in response to distinct stimuli. Regardless of activation of LPS and/or IFN-γ, pro-inflammatory M1 macrophages actively engage in the pentose phosphate pathway (PPP), a phenomenon not observed in M2 macrophages. This is essential for generating NADPH for the production of ROS and NO by M1 macrophages (Nagy and Haschemi, 2015). Instead, M2 macrophages down-regulate glycolysis and fully engage in OXPHOS. A common characteristic of M1 and M2 macrophages is that glucose still fuels both cell types although the Krebs cycle is disrupted in M1 macrophages, termed as TCA cycle break (Van den Bossche et al., 2017). This break occurs after citrate and succinate generation. The first break causes an accumulation of citrate (Jha et al., 2015), which is funneled towards fatty acid synthesis, essential in membrane biogenesis and prostaglandin formation. Citrate also supports the production of NO (Infantino et al., 2011; O'Neill, 2011) and itaconic acid, a metabolite newly discovered to be generated by immune response gene-1 (Irg-1) (Michelucci et al., 2013; Strelko et al., 2011) believed to possess anti-microbial activities (Michelucci et al., 2013; Naujoks et al., 2016). The second break is at succinate dehydrogenase enzyme leading to an accumulation of succinate (Lampropoulou et al., 2016), which acts as a metabolite sensor in inducing

hypoxia inducible factor-1α (HIF-1α) and formation of IL-1 in acute inflammatory responses to infection (Tannahill et al., 2013).

However, in M2 macrophages, the Krebs' cycle is completely intact, most likely due to the fact that these cells are heavily dependent on oxidative metabolism, which is shown to be supported by a combination of glucose, fatty acid oxidation, and glutamine. In contrast to M1 that favors fatty acid synthesis for inflammatory responses, M2 macrophages rely on fatty acid oxidation (FAO), which in turn is driven by fatty acid synthesis (FAS), a paradoxical nature of fatty acid metabolism in M2 macrophages most likely to support their longevity and tissue repair after the inflammatory phase (Everts et al., 2014; Galván-Peña and O'Neill, 2014; Huang et al., 2014; Odegaard and Chawla, 2011; Vats et al., 2006). FAO, however, plays a role in driving M2 polarization via activation of mammalian (mechanistic) target of rapamycin complex 2 (mTORC2) (Arts et al., 2016). Additionally, the TCA cycle in M2 macrophages is indirectly fueled by glutamine (Jha et al., 2015), a finding consistent with the pioneering study of Newsholme, who had defined the metabolite utilization in macrophages even before the characterization of M1 and M2 population was established (Newsholme et al., 1986; Newsholme and Newsholme, 1989). An illustrated summary of metabolic pathway utilization in M1 versus M2 macrophages is shown below (Figure 1 -*2*).

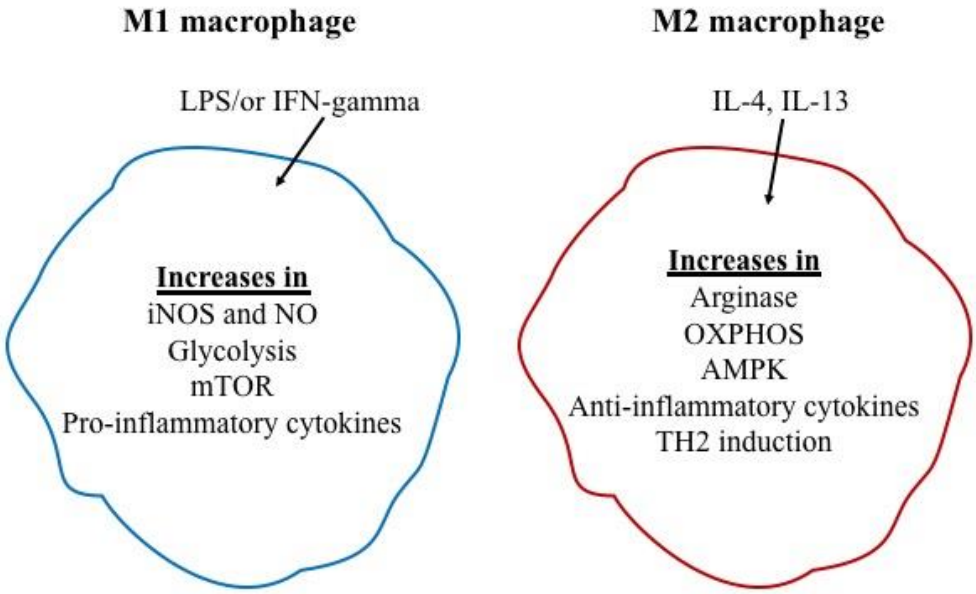


Figure 1 -2. Metabolic pathways utilized by M1 and M2 macrophages.
(Adapted from *Kelly and O'Neill, 2015 (Kelly and O'Neill, 2015)*)

1.3.1.2: Metabolic reprogramming in dendritic cells

Like macrophages, the metabolic status of DCs is highly regulated by immune activation conditions. Naïve or resting DCs have relatively little metabolic demands and generally undergo catabolic pathways, such as breaking down proteins and triacylglycerol to generate ATP via OXPHOS from mitochondria. In naïve DCs, glucose enters OXPHOS for complete oxidation. They also engage in FAO to support OXPHOS (Deberardinis et al., 2006). On the other hand, citrate generated by these cells is exported via citrate carriers across mitochondria into cytoplasm to convert to acetyl-CoA for use in FAS, which is then rapidly utilized in FAO, a phenomenon observed in macrophages (Everts and Pearce, 2014; Maroof et al., 2005; Pearce and Everts, 2015).

Activation of DCs by TLR agonists induces the cells to rapidly undergo a process called maturation characterized by upregulation of the expression of molecules essential for maturation responses, increased secretion of cytokines and chemokines, and migration to secondary lymphoid organs and ultimately presenting antigens to T cells (Banchereau and Steinman, 1998b; Steinman, 2001, 2012; Steinman and Banchereau, 2007; Steinman and Hemmi, 2006). In order to fulfill this anabolically demanding maturation process, activated DCs experience rapid metabolic changes, known as metabolic reprogramming (Everts et al., 2014; Krawczyk et al., 2010).

Similar to the Warburg's effects in cancer cell metabolism, activated DCs undergo a metabolic switch towards a rapid increase in glycolytic rate, a phenomenon termed "glycolytic burst" (Everts et al., 2014; Krawczyk et al., 2010). Instead of pyruvate then entering OXPHOS as is seen in resting DCs, a great portion of the pyruvate generated in activated DCs is converted to lactate, allowing the regeneration of NAD+ from this reaction to allow the cells to turn over NADH for ATP production via glycolytic burst. This glycolytic reprogramming occurs in two waves (Thwe and Amiel, 2018): an increased glycolytic flux at an early phase of activation (Everts et al., 2014) followed by a sustained glycolysis characterized by fundamental metabolic changes in a late activation stage (Krawczyk et al., 2010). The underlying cause of the two distinct metabolic changes is discovered to be due to an upregulation of inducible nitric oxide synthase (iNOS) that generates nitric oxide (NO), a cytotoxic molecule implicated in a number of physiological functions. An extensive detail on iNOS and NO is discussed in a later section.

Acute glycolytic burst during early activation is coupled with a transient increase in mitochondrial activity as evidenced by increased mitochondrial potentials and spare respiratory capacity (Everts et al., 2014; Pearce and Everts, 2015). Despite the fact that most pyruvate is shunted towards an increased formation of lactate, the increased glycolytic flux at this early stage seems to fuel the TCA cycle for a purpose other than generating ATP from that reaction, particularly to support the generation of TCA cycle intermediates for downstream activation processes. Evert *et al* elegantly demonstrated that citrate generated from the TCA cycle, driven by glycolytic flux, is transported across mitochondrial membranes into the cytosol for *de novo* fatty acid synthesis. Fatty acid generated from this step is believed to fulfill the need of biomass expansion in endoplasmic reticulum and Golgi organelles for increased synthesis of proteins and cytokines during the maturation process. Interrupting this process at any stage tremendously impairs the immune effector function of activated DCs (Everts et al., 2014). Because both the inflammatory BMDCs as well as monocyte-derived DCs share similar phenotypes with M1 macrophages, it is plausible to consider that the above-mentioned citrate production in DCs could represent a TCA cycle break seen in M1 macrophages. This remains an open question in the field of DC immuno-metabolism. Of note, DCs shift their fatty acid metabolism from the resting condition by suppressing FAO once the cells become activated by TLR agonists (Krawczyk et al., 2010). While a lot of studies have been reported on carbohydrate metabolism and fatty acid utilization in activated DCs, little is known about their amino acid metabolism. Although the role of glutamine is well-highlighted to be essential for rapidly proliferating cancer cells and lymphocytes as well as for supporting macrophage activation, its implication in DC

effector function needs to be investigated. Whether or not glutamine serves as a major amino acid source to generate NO in activated DCs is yet to be fully examined. A complete illustration of metabolic pathway utilization in activated DCs is portrayed below in Figure 1 -*3*.

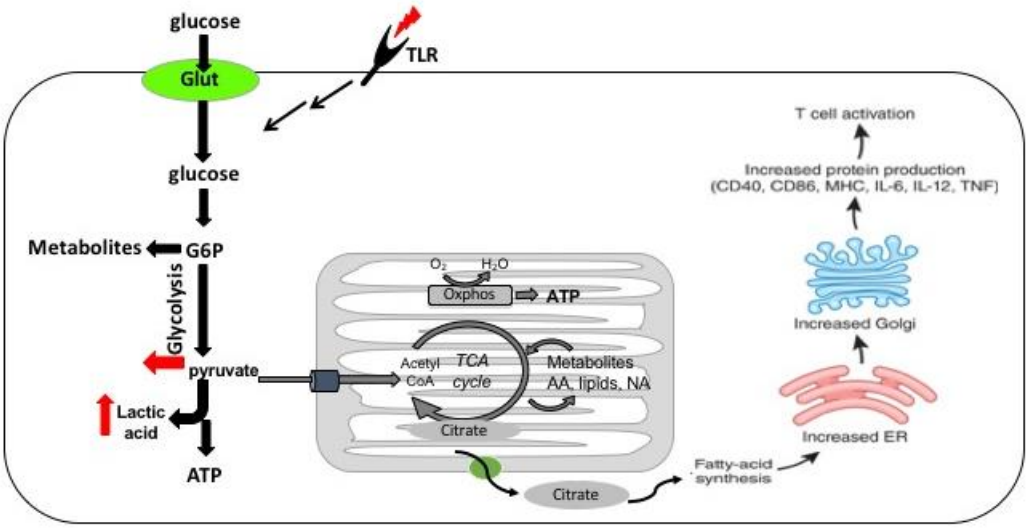


Figure 1 -3. Glycolytic reprogramming in dendritic cells activated via a TLR.
(Adapted from *O'Neil 2014 (O'Neill, 2014)*)

1.3.2: Epigenetic modification during metabolic reprogramming

Epigenetic modification is defined as changes in the gene expression and activities, without any changes in DNA sequences. Transcriptional accessibility to the genome by histone modification is mediated by acetylation/deacetylation, methylation, and SUMO-ylation of histones, and all these alterations are considered post-translational changes. In general, histone acetylation allows increased gene exposures to DNA binding proteins and factors, leading to transcriptional upregulation and changes in downstream cellular functions, whereas histone deacetylation leads to gene repression. On the other

hand, histone methylation can cause activation or repression of gene expression depending on the sites of modification and the number of methyl groups added to histones (Alvarez-Errico et al., 2015; Kimura, 2013).

Post-translational modification of proteins and metabolic enzymes, such as pyruvate kinase M2 (PKM2) isoform and peroxisome proliferator-activated-receptor γ (PPAR-γ) co-activator 1α (PGC-1α), by acetylation or methylation have been reported to alter metabolic pathways and cellular functions. For example, acetylation of PKM2 induces the accumulation of glycolytic intermediates, leading to uncontrolled tumor growth and poor cancer prognosis (Lv et al., 2011; Palsson-McDermott and O'Neill, 2013). In immune cells, these alterations can be triggered by a number of factors, including metabolites and metabolic changes occurring during immune reactions, which can cause consequent impacts on immune effector functions. Citrate generated from activated DCs and macrophages provides a great source of acetyl-CoA, which is believed to be further implicated in direct acetylation of histone molecules or of metabolic enzymes. A recent study on bone marrow-derived macrophages (BMDM) demonstrated a correlation between histone acetylation and an increased IL-6 expression in BMDMs treated with histone deacetylase (HDAC) inhibitors (Hu et al., 2016). In another example, increased expression of interferon gamma (IFN-γ) upon histone acetylation in activated CD4 T cells is driven by acetyl-coA turnover from glycolytic reprogramming in these cells (Peng et al., 2016). Metabolites such as lactate, although generally considered as a by-product, have also been reported to inhibit HDAC, thus promoting HDAC related gene expression in cancer cells (Latham et al., 2012). On the other hand, a few studies have also revealed the effect of deacetylation by a protein called Sirtuin on modulation of

DC effector function, such as cytokine release and the regulation of TH17 differentiation (Alvarez et al., 2012; Dominy et al., 2010; Yang et al., 2013). Besides acetylation and deacetylation reactions, increased di-methylation of histones in DCs and macrophages has been shown to induce the gene transcription as well as production of type I interferons in viral infections, eventually resulting in viral resistance (Fang et al., 2012a; Pearce and Everts, 2015; Saeed et al., 2014). These findings have highlighted that the immuno-metabolic activities of immune cells are implicated by another complex level of regulation by epigenetic changes.

The importance of epigenetic regulation in triggering trained innate immunity is highlighted by the work of Neetea, who elegantly demonstrated a link between metabolic reprogramming and epigenetic regulation to elicit "innate memory" in human monocytes (Cheng et al., 2014). Specifically, immune effector genes downstream of a canonical metabolic sensor, mTOR are highly methylated and acetylated in monocytes that are "trained," whereby these cells are initially primed with fungal pathogens, and subsequently and repeatedly challenged with the similar pathogen, to induce innate immune memory. These epigenetic changes are coupled with metabolic reprogramming, characterized by a metabolic switch that supports the survival of trained monocytes and their defense against infections (Cheng et al., 2014). Notably, recent studies have demonstrated that the TCA cycle intermediate fumarate, which is derived from an integrated effort of glycolysis, Kreb's cycle, and glutaminolysis, induces tri-methylation or di-methylation (Fang et al., 2012a) and acetylation of histones in monocytes, and that inhibition of this process impairs the metabolic and immune phenotypes in these cells (Arts et al., 2016; Fang et al., 2012b).

The fact that epigenetic modifications of innate immune cells induce a powerful impact on the immune responses along with metabolic regulation suggests a complex interaction of immuno-metabolic changes under epigenetic controls. A great body of work has demonstrated that post-translational modification of histones in immune-response related genes can regulate immune cell function. The link between metabolic regulation and epigenetic changes in immune cells has only recently gained interest in the field of immuno-metabolism and is a major area for future work.

1.4: Glycogen Metabolism

1.4.1: Overview of glycogen metabolism and regulation

Glycogen, a primary storage form of glucose in our body, is a highly complex polymer of glucose formed by α-1,6 glycosidic linkages at branch points and α-1,4 glycosidic long chains. As glucose is a fundamental energy source of living organisms, glycogen serves as an energy reservoir to provide a constant glucose supply (Adeva-Andany et al., 2016; Dashty, 2013; Roach, 2002). Cells such as neurons, muscle, and red blood cells are almost exclusively dependent on glucose for their primary fuel source; hence, glycogen metabolism is very active in these tissues (Voet et al., 2013). Disorders in glycogen metabolism were first described by McArdle as glycogen storage diseases, in which inherited defects in glycogen metabolic enzymes result in impaired glycogen breakdown that tremendously impact the energy metabolism and cellular functions in metabolically active tissues (Mc, 1951).

Glycogen metabolism is comprised of glycogen synthesis and breakdown pathways. Both pathways are synchronously orchestrated by a number of enzymes

localized in the cytoplasm. The synthesis of glycogen is initiated by a linkage of 4 UDP-(uridine 5'-diphosphate) glucose, formed by the help of phosphoglucomuase-1 from glucose-1 phosphate (G1P), which is converted from glucose-6-phosphate (G6P) derived from glucose that enters the cells via glucose transporters (Adeva-Andany et al., 2016). The initial glycosidic linkages are initiated by glycogenin, followed by elongation and branching of the polymer with the help of glycogen synthase (GYS), which is the rate limiting enzyme of the synthesis pathway, and branching enzyme. On the other hand, catabolism of glycogen happens in both cytoplasm and lysosomes. In the cytoplasm, glycogen phosphorylase (PYG), the rate limiting enzyme of the degradation pathway, releases G1P from α1,4-glycosidic linkage, and debranching enzyme catalyzes the branch point α1,6-glycosidic chains. G1P released from the glycogen polymer is then converted back to G6P, which in the liver is dephosphorylated in the endoplasmic reticulum by glucose-6-phosphatase; therefore, free glucose can be exported out of the cells by glucose transporters. Other tissues, however, do not express glucose-6-phosphatase and recycle the released G6P molecules for their cell-intrinsic utilization (Adeva-Andany et al., 2016; Roach, 2002; Roach et al., 2012). Glycogen breakdown also occurs in lysosomes where lysosomal α-glucosidase (acid maltase) catalyzes α-1,4 glycosidic linkages of glycogen (Adeva-Andany et al., 2016; Roach et al., 2012; Rosenfeld, 1975). The rate limiting enzymes of these two pathways exist in different isoforms based on the tissue distribution. Specifically, PYG exists in 3 isoforms: liver (PYGL), muscle (PYGM), and brain (PYGB or GPBB). Synthase, however, is expressed as GYS1, which is expressed in muscles and all other tissues except the liver, and GYS2, whose expression is restricted to the liver.

As both glycogenesis and glycogenolysis are high-energy metabolic processes and simultaneous reactions could be considered energetically wasteful based on unnecessary hydrolysis of UTP, several mechanisms are at play to tightly regulate these two pathways, particularly by reciprocal regulations of glycogen breakdown and synthesis. Early studies on glycogen regulatory mechanisms showed that covalent phosphorylation reactions of rate-limiting enzymes allowed them to be either in an active or inactive state based on their phosphorylation status (Roach, 2002; Roach et al., 2012; Wilson et al., 2010). Specifically, phosphorylated PYG, termed "phosphorylase *a*", is an active form of the enzyme, whereas phosphorylated glycogen synthase is an inactive form. Activities of these two key enzymes are again modulated by a number of proteins by which phosphorylation of one enzyme leads to a cascade of phosphorylation events. The downstream activation/inactivation of these key enzymes via post-translational phosphorylation is believed to be under the control of hormones such as insulin and adrenaline (Voet et al., 2013).

The correlation established between the hormonal regulation and the structural conformation/functional activities of the key glycogen enzymes in the early studies led to many seminal discoveries about phosphoprotein phosphatases and kinases (Carlson et al., 2018) that are directly implicated in the glycogen metabolic pathways via receptor- and messenger-mediated regulatory mechanisms (Carlson et al., 2018; Dashty, 2013). PYG activity is directly modulated by phosphorylase kinase, which in turn is activated by protein kinase A, a master regulatory protein of many signaling pathways. Dephosphorylation of PYG by protein phosphatase-1 (PP1) leads to inactivation of PYG, especially due to insulin-induced inactivation of phosphorylase kinase when the

circulating glucose levels are high. Phosphorylase activity is usually increased in fight/flight conditions by the influence of hormones, such as glucagon and adrenaline. A release of these hormones increases the levels of cyclic AMP, which in turn induces the activity of PKA, ultimately leads to a cascade of phosphorylation events (Voet et al., 2013). In contrast to the regulatory mechanisms of PYG, those of GYS are more complex and involve more than phosphorylation-dephosphorylation of enzymes. PP1 also plays an active role in synthesis by modulating the activity of glycogen targeting regulatory subunit called RGL, which in turn regulates PP1 activity. Interestingly, one of the mechanisms proposed in the field is a proportional increase in glucose transporter (GLUT) expression with a rise in blood glucose levels, leading to intracellular glycogen accumulation (Hansen et al., 1995; Kim et al., 2005; Ren et al., 1993; Rossetti et al., 1997). Additionally, two major players of cellular nutrient and metabolic homeostasis, AMPK, a key energy sensor protein kinase, and glycogen synthase kinase 3, GSK3, a master regulator of many signaling events, are also involved in the regulation of glycogen synthesis. Although insulin-induced inactivation of GSK3 by dephosphorylation in the liver is proposed to modulate glycogen synthesis (Roach, 2002; Voet et al., 2013), further elucidation is necessary to better understand GSK3 mediated mechanisms in a cell-specific manner. On the other hand, AMPK has long been demonstrated to be involved in energy metabolism, including glycogen metabolism (Roach, 2002; Roach et al., 2012).

Another form of regulatory mechanism is allosteric control of enzymes by metabolites, such as AMP, ATP, glucose, and G6P. Depending on the energy status of the cells, these allosteric molecules regulate the activities of the rate limiting enzymes via positive or negative feedback mechanisms. For instance, when there is a need for ATP

during low energy statuses (i.e., high AMP and low ATP), glycogen synthesis is inhibited but degradation is promoted. In this case, PYG is allosterically activated by AMP but inhibited by ATP (Roach, 2002; Roach et al., 2012). The complex interrelation between the allosteric modulation and protein kinase phosphorylation can be under hormonal or non-hormonal controls that may also involve regulation at the transcriptional level.

When discovered in the early 1900's, glycogen synthesis and degradation was initially thought to occur only in the cytoplasm. Interestingly, an accumulating body of work has strongly suggested a distinctive spatial occurrence of glycogen metabolism in a sub-cellular level (Baker et al., 2005; Fernandez-Novell et al., 1994; Fernandez-Novell et al., 1992; Fernandez-Novell et al., 1996; Garcia-Rocha et al., 2001; Wilson et al., 2010; Wilson et al., 2002). The findings in both liver and muscular systems unanimously agree that glycogen synthase is dispersed throughout the cells under various metabolic conditions. For instance, in hepatocytes, the insulin-induced blood glucose rise initiates the translocation of GYS to the cellular periphery, a location proximal to the membrane, and this is identified by a cluster of newly synthesized glycogen pools along with translocated GYS (Fernandez-Novell et al., 1994; Fernandez-Novell et al., 1992; Fernandez-Novell et al., 1996; Garcia-Rocha et al., 2001). A similar phenomenon has also been observed in muscle glycogen metabolism and has become broadly recognized as "compartmentalization" (Prats et al., 2005; Prats et al., 2011; Prats et al., 2009). While these studies were performed almost exclusively on GYS, the subcellular localization of phosphorylase enzyme has so far been investigated in yeasts (Wilson et al., 2010). As the differential localization of glycogen particles is found in a structure resembling vacuoles, the term "glycosome" (Parsons, 2004) has been coined in parasite metabolism (Rybicka,

1996). While the studies on Tryponosoma parasites mentioned that the composition of these glycosomes is not restricted to glycogen enzymes (Michels et al., 2006; Parsons, 2004), other schools of thought highlighted the exclusive role of glycogen in those specialized organelles (Rybicka, 1996). Another interesting fact is that vacuoles in yeast cells provide a specialized environment for glycogen storage (Roach et al., 1998; Wilson et al., 2010; Wilson et al., 2002). In animals, cellular autophagy plays a crucial role under nutrient-limited circumstances, in which autophagosomes fuse with intracellular vacuoles. Whether or not the above-mentioned glycosomal vacuoles are directly related to cellular autophagy in mammals raises an interesting question of the roles of these vacuoles and the purpose of compartmentalization of glycogen metabolism in cellular function. Nonetheless, these findings suggest a role for glycogen as more than a storage molecule and potentially indicate the parallel existence of glycogen degradation pathway compartmentalized away from synthesis, an interesting metabolic behavior known to be observed in neuronal and astrocyte biology that is later touched upon in this dissertation.

The studies of glycogen metabolism and its regulation in several species and a number of organ/systems can be dated back to as early as the 1900's (Young, 1957a, b). However, due to the existence of different isoforms of enzymes involved in the glycogen metabolic pathways, recent studies have newly identified distinct functional and regulatory mechanisms at a subcellular level of several different tissues and systems. These observations, indeed, alert the scientific community for the needs for more work to better understand the exact role(s) of glycogen, its metabolism, and regulation in the systems that have been previously overlooked in the past.

1.4.1 Glycogen metabolism in Skeletal Muscles, Astrocytes, and Neurons

In addition to the liver glycogen metabolism, skeletal muscles and the brain are two other major sites of active glycogen storage and metabolism. Skeletal muscle is well-known to possess a large pool of glycogen stores in support of continuous muscular contractile activities. Muscle cells actively engage in anaerobic glycolysis and glycogenolysis for a rapid turnover of glucose and ATP. Similar to the skeletal muscle system, the energy demand of the mammalian brain is immensely high and is fulfilled primarily by glucose as the main fuel source. Glucose is also required for generation of substrates, such as glutamate for neurotransmitter synthesis. Although the brain can use ketones and lactate under particular circumstances, glucose functions as an indispensable nutrient source. Hence, glycogen serves as the largest energy reserve in the brain. Both tissues express GYS1 and tissue specific isozymes of glycogen phosphorylase, namely PYGM in muscles and PYGB in the brain. Unlike the liver, the muscle and brain cells do not express glucose-6-phosphatase; therefore, individual glucose molecules released from these tissues are contributed towards their own metabolic needs and physiological functions (Dashty, 2013). Regardless of differential isoform expressions of glycogenolysis pathway, this subsection highlights a few interesting glycogen metabolic characteristics shared by both systems.

The utilization of glycogen metabolism in the skeletal muscles depends on muscular activities. Under resting or non-exercising conditions and the fed state, the intracellular glycogen level increases and glycogen breakdown is inactive. However, glycogen breakdown is initiated and muscular glycogen level is depleted rapidly after medium to strenuous muscular activities. Interestingly, prolonged exercise reverts the

glycogen level to a much higher content, coupling with a phenomenon termed supercompensation, in which intracellular glycogen depletion is compensated with an increased glycolytic activity (Nielsen et al., 2011; Prats et al., 2009). It is not clearly understood why and how these cells accumulate more glycogen than even before the energetic crisis in these cells. A potential mechanism is intracellular compartmentalization in muscle cells (Prats et al., 2005; Prats et al., 2011; Prats et al., 2009) that allows them to engage in parallel metabolic pathways of spatially defined domains. Skeletal muscle undergoes actin cytoskeletal filament rearrangements after exercise-induced glycogen depletion, resulting in the formation of distinct intracellular compartments harboring glycogen resynthesis processes (Prats et al., 2005; Prats et al., 2011; Prats et al., 2009). The idea of compartmentalization is closely related to a phenomenon termed "glycogen shunt", which refers to a continuous sequence of synthesizing and simultaneous breakdown of glycogen regardless of abundant glucose in the cellular microenvironment (Shulman et al., 2001; Shulman and Rothman, 2001). While the glycogen shunt could be beneficial for millisecond muscular contractions by sustaining required energy, the drawback is an excessive generation of lactate that eventually results in muscle fatigue (Shulman and Rothman, 2001). Nevertheless, the exact purpose and regulatory mechanisms of interrelation between compartmentation and glycogen shunt are not clearly understood.

Astrocytes contain a comparatively larger quantity of glycogen than neurons, although both cell types actively utilize glycogen metabolism for their functions (Hertz et al., 2007). The fact that glycogenolysis still occurs in the brain even in the presence of sufficient glucose strongly indicates the role of glycogen as more than a reservoir of

energy storage (Brown et al., 2005; Brown et al., 2003a; Schousboe et al., 2010; Swanson, 1992). Similar to skeletal muscle glycogen metabolism, glycogen shunt activity is also observed in neurons and astrocytes (Shulman et al., 2001; Shulman and Rothman, 2001). While there are controversial reports on whether or not the glycogen shunt is beneficial in light of short-term energy production (Obel et al., 2012), neuronal and astrocytic cells could still take advantage of the glycogen turnover for glutamate synthesis. Glutamate released from neurons to neuron-astrocytic synapses is taken up by astrocytes, which then convert glutamate to glutamine via the glutamate-glutamine cycle. Clearance of glutamate from these synapses is crucial for proper action potential propagations (Hertz et al., 2007; Schousboe et al., 2010). Because the transport of glutamate across the blood brain barrier and into the neurons is not as efficient as glucose, the cells normally depend on glycolysis and glycogenolysis to generate glutamate (Brown et al., 2005; Hertz, 2004; Hertz and Dienel, 2002; Rogers et al., 2005; Sickmann et al., 2012). On this account, the glycogen shunt is proposed to serve this purpose (Kreft et al., 2012), subsequently enhancing the rapid action potential firings upon neuronal stimulation. The net ATP generated from a long-term cyclical turnover of glycogen is also proposed to drive glutamate transporters (Walls et al., 2009). Although functionally different, pioneering studies on the glycogen shunt and intracellular compartmentation both in the brain and skeletal muscle systems underscore the complex role of glycogen in fundamental homeostasis of cellular metabolic activities.

1.4.2 Glycogen metabolism in cancer cells

Because cancer cells shift their metabolism to glycolysis, it is not surprising that glycogen is also involved in cancer cell biology. The implication of glycogen metabolism in certain cancer cells was examined as early as in the 1960's. Earlier studies discovered an accumulation of glycogen and increased glycogen synthesis in tumor and malignant cells, particularly during the active phase of cell cycle (Huang et al., 1978; Rousset et al., 1979; Rousset et al., 1981). Increased glycogen storage in actively proliferating cancer cells is believed to be due to a survival mechanism in low glucose conditions (Pelletier et al., 2012; Pescador et al., 2010), and such accumulation is found to be induced in hypoxic environments, via hypoxia-induicble-factor-1 (HIF1-α) (Favaro et al., 2012; Iida et al., 2012; Pelletier et al., 2012; Pescador et al., 2010; Zois et al., 2014). One of the most interesting findings from a recent study is that cancer cells display a temporal change in glycogen metabolism under hypoxic conditions. Upon acute exposure to hypoxia, cancer cells upregulate the transcription and translation of both GYS1 and PYGL, a phenomenon similar to the "glycogen shunt" described in the brain and skeletal muscles, with a subsequent accumulation of glycogen levels. A prolonged exposure of low oxygen levels causes a decrease in GYS1 activity, along with a gradual increase in PYG, resulting in a net breakdown of glycogen in cancer cells. Disruption of glycogen breakdown by blocking PYGL with pharmacologic or genetic means increases the cellular glycogen content and induces cell senescence (Favaro et al., 2012). This bi-phasic phenotype of temporal changes in glycogen metabolism sheds some light into why cancer cells store glycogen. Intriguingly, the glycogen shunt observed in cancer cells potentially serves as a homeostatic mechanism involving glycolysis, pentose phosphate

pathway, and gluconeogenesis by maintaining G6P and other glycolytic intermediates (Shulman and Rothman, 2017).

1.4.3 Glycogen metabolism in immune cells

Since the discovery of glycogen in the liver by Claude Bernard in the 1850's (Young, 1957b) preceeded by the establishment of glycogen metabolism in other tissues such as muscles and the brain, the presence of glycogen in immune cells was not examined until 1925 (Gibb and Stowell, 1949). The earliest studies identified glycogen as uniformed "clusters of granules" present in the blood cells of the peripheral circulation (Scott and Still, 1968). Due to technical challenges in isolating a pure population of leukocytes, glycogen content was examined mostly in mixed polymorphonuclear leukocytes or neutrophils (Wagner, 1946, 1947) although some studies later showed that lymphocytes also contain glycogen, with a much higher content in younger cells (Gibb and Stowell, 1949). More glycogen has been detected in lymphoproliferative diseases, such as chronic lymphocytic leukemia or Hodgkin's lymphoma (Quaglino and Hayhoe, 1959; Valentine et al., 1953). On the other hand, glycogen concentration in immune cells of myeloid lineage has been shown to start at a relatively low level but increase upon cellular maturity. Observations from earlier studies indicated a normal glycogen level in leukocytes of myeloid leukemia (Wagner, 1946, 1947), whereas a higher content was observed in polycythemia (Gibb and Stowell, 1949; Valentine et al., 1953; Wagner, 1947) or acute lymphoid leukemic patients (Cusso et al., 1975). Surprisingly, glycogen synthase activity is reported to be consistently low in acute leukemic WBCs (Cusso et al., 1975; Seitz, 1965). In all of these conditions, glycogen was measured in the total WBC

population, rather than in specific cell types. Therefore, it is impossible to determine which exact cell type has a faulty glycogen metabolic machinery directly attributable to a particular disease condition. As opposed to the variations of leukocyte glycogen levels in proliferative disorders, one consistent finding among endotoxin-induced inflammatory leukocytes was an initial spike of glycogen in the leukocytes located within inflammatory exudates (Gudewicz and Filkins, 1974; Scott and Cooper, 1974; Wulff, 1962), a phenomenon proposed to be due to "pre-phagocytic" response, (Scott and Cooper, 1974) most likely in preparation for phagocytosis and subsequent functional activities.

Unlike the structure of liver glycogen, the catalysis and synthesis of intracellular glycogen in leukocytes takes place uniformly across all glycogen particles in the "first-in last-out" behavior, characterized by the last glucose molecules being added to the polymer to be released first (Scott, 1968; Scott and Still, 1968). Although the appearance of glycogen particles in leukocytes is similar to and proposed as glycosome-like organelles as observed in parasites, the definitive structures of such in immune cells are yet to be proven.

As the fluctuation of leukocyte glycogen levels observed in various disease conditions can be explained by enzymatic expression, glycogen phosphorylase (PYG) was the first and only characterized enzyme in WBCs in earlier days. In the liver, the inactive form of PYG, *phosphorylase b,* is not affected by AMP, whereas in the muscles, the activity of *phosphorylase b* is strongly influenced by high AMP levels (Gella et al., 1978; Yunis and Arimura, 1964). In this respect, some reports have indicated that leukocyte PYG behaves similarly to the liver PYG, although other studies claimed the

behavior of leukocyte PYG conforming to that of muscle PYG (Yunis and Arimura, 1968).

The functional aspects of glycogen metabolism were widely studied almost exclusively in neutrophils possibly due to the fact that they are the most abundant cell type in peripheral blood circulation. The energy expenditure during neutrophil phagocytosis is shown to be fulfilled almost entirely by the breakdown of intracellular glycogen rather than from free glucose (Borregaard and Herlin, 1982; Sbarra and Karnovsky, 1959; Stossel et al., 1970). These findings were followed by the discovery of an interesting metabolic phenomenon wherein granulocytes utilize free glucose exported via glucose transporters to support chemotaxis and glycogen-derived glucose for phagocytosis (Weisdorf et al., 1982). A recent study on the differential nutrient utilization by neutrophils for neutrophil extracellular trap (NET) formation showed that exogenous glucose is required during the later stage, but not in the earlier phase, of NET formation (Rodriguez-Espinosa et al., 2015), implying the potential role that intracellular glycogen plays during the initial stage. Macrophages, one of the most abundant phagocytes, were also shown to use intracellular glycogen reserves for phagocytosis (Gudewicz and Filkins, 1976). Of interest, macrophage ingestion of extracellular glycogen that is originated from cellular debris, particularly from dead PMNs, in active inflammatory tissue environments is suggested to serve as a fuel source during host defense or tissue repair mechanisms (Gudewicz and Filkins, 1974). Very limited information regarding lymphocyte utilization of glycogen was available until a recent study reported the differential glycogen metabolism in T cells cultured in IL-2 versus IL-15 (Ma et al., 2018). In that case, IL-15 cultured memory CD8+ T cells maintain cellular homeostasis

and survival via a circuit of generating G6P from imported glucose, which in turn is rapidly utilized for synthesizing glycogen. The resultant glycogen is immediately broken down to regenerate G6P, which is then channeled into the PPP for NADPH and reduced glutathione (Ma et al., 2018). Similar to the glycogen metabolic circuit described in T cells, a majority of this dissertation focuses on continuous cycles of glycogen turnover in LPS-activated DCs.

1.5.1 Major Metabolic regulatory mechanisms in DCs

1.5.1.1: PI3k/Akt/mTOR/HIF1-α

With a growing interest in the immune cell utilization of metabolic pathways, significant advances have been made within the past decades towards our understanding of how these cells regulate their metabolic statuses. One major signaling pathway known as phosphatidyl inositol 3 kinase (PI3k), Akt, and mammalian (mechanistic) target of rapamycin (mTOR) - PI3k/Akt/mTOR- plays a crucial role in mediating a number of cellular processes such as nutrient uptake, cellular proliferation, cell death, anaplerosis, and cataplerosis. Conjugation of cognate ligands with PRRs activates PI3k, a conserved family member of intracellular lipid kinases ubiquitously expressed in many cells, and catalyzes the formation of PIP3, phosphatidyl inositol-3,4,5-triphosphate, from PIP2, phosphatidyl inositol bisphosphate. PIP3, which acts as a second messenger, phosphorylates PI3k-dependent-kinase-1 (PDK1) (Cao et al., 2016; Vanhaesebroeck et al., 2010; Weichhart and Saemann, 2008). At the same time, PIP2 and PIP3 activation recruits the serine threonine kinase Akt1 to the plasma membrane, where Akt is phosphorylated on the amino acid T308 residue by activated PDK1 and on Ser473 by

mTORC2, a kinase that is believed to be stimulated by PI3k via an unknown mechanism (Weichhart and Saemann, 2008; Yu and Cui, 2016). This leads to the full activation of Akt1 and subsequent Akt phosphorylation in many downstream proteins, such as glycogen synthase kinase 3 (GSK3), tuberous sclerosis 2 (TSC2), and forkhead box protein O1 (FoxO1). TSC2, a negative regulator of mTORC1, is inhibited upon its phosphorylation by Akt1, leading to mTORC1 activation. Thus, the PI3k/Akt signaling cascade ultimately leads to an activation of mTORC1 (Duvel et al., 2010; Weichhart and Saemann, 2008; Xu et al., 2012; Yu and Cui, 2016).

This signaling activity is important in many respects because mTORC1 is a conserved serine threonine kinase that plays a crucial role in nutrient sensing and regulating cellular metabolism. Stimulation of mTORC1 induces the expression of a number of genes and the synthesis of proteins involved in cellular proliferation, cytokine secretion, and anabolic processes. Two major targets of mTORC1 are S6K and 4E-BP1, and phosphorylation of these molecules leads to increased protein synthesis (Duvel et al., 2010; Xu et al., 2012).

The PI3k-Akt-mTORC1 pathway is essential for the *in vivo* development of mouse Langerhans cells and human pDCs (van de Laar et al., 2012) as well as in *in vitro* differentiation of human monocyte derived DCs cultured with GM-CSF and IL-4 (Haidinger et al., 2010; Sukhbaatar et al., 2016). Inhibition of this pathway with the mTOR inhibitor, rapamycin, or PI3k inhibitors causes deleterious effects on the development, differentiation, and survival of these cells. While mTOR was demonstrated to have negative effects on DC immune responses by downregulating pro-inflammatory cytokine secretion, surface co-stimulatory molecule expression, and ultimately reduce

DC ability in stimulation and differentiation of T cells (Amiel et al., 2012; Amiel et al., 2014), recent studies have shown the opposing effects of mTOR on both immune and metabolic phenotypes of immune cells (Amiel et al., 2012; Amiel et al., 2014). While the PI3k-Akt-mTOR pathway is the core of development, differentiation, and immune effector responses of dendritic cell, each of these components is vastly fundamental in regulation of metabolic outcomes in response to a variety of stimuli. In regard to cellular metabolism, PI3k-Akt-mTORC1 signaling pathway drives the LPS-activated DCs to fully commit to aerobic glycolysis (Amiel et al., 2012; Krawczyk et al., 2010), a phenomenon observed in mouse but not in human myeloid DCs (Amiel et al., 2012). Inhibition of mTORC1 by rapamycin reverts this metabolic phenotype back to mitochondrial respiration, by preserving the mitochondrial function and longevity, along with enhanced survival and effector function (Amiel et al., 2014; Krawczyk et al., 2010). mTORC1 activation in TLR-activated macrophages has been illustrated to induce hexokinase-1 dependent glycolytic reprogramming in support of NLRP3 inflammasome assembly and IL-1β production (Moon et al., 2015), an interesting metabolic phenotype presumed to be shared by activated DCs, in which, however, has yet to be examined.

A more in-depth analysis of PI3k-Akt driven metabolic changes in DCs reveals a spatio-temporal regulation, in which Akt is activated by TBK1-IKKε, two kinases directly downstream of TLR, leading to the Akt-driven translocation of hexokinase II to the outer mitochondrial membrane and a resultant increase in glycolytic flux (Everts et al., 2014). The TBK1-IKKε-Akt-driven early glycolytic flux occurs independently of mTOR or PI3K activation as evidenced by the fact that TBK1 and IKKε are rapidly phosphorylated in an PI3k/mTOR independent manner (Everts et al., 2014). Inhibiting

either Akt or TBK1 phosphorylation disrupts the acute metabolic changes required for *de novo* fatty acid synthesis, a process believed to be supported by the intermediates, citrate and NADPH, produced from the TCA cycle and the PPP respectively. Newly synthesized fatty acids are incorporated into the expansion of ER and Golgi organelles in support of maximizing the protein and cytokine outputs required for acute DC activation. This regulatory mechanism assures DCs to be able to replenish the TLR-driven rapid demand of metabolic intermediates by generating carbon substrates via both glycolysis and the TCA cycle (Everts et al., 2014). These findings present a new paradigm as to how we should reconsider the metabolic reprogramming in DCs insofar as the metabolic alterations are separated by temporally controlled signaling mechanisms. Regulations involving PI3k-mTOR come into play after the first wave of rapid glycolytic reprogramming (Amiel et al., 2012; Amiel et al., 2014; Everts et al., 2014; Krawczyk et al., 2010). While PI3k-mTOR-mediated signaling plays a minimal role in early metabolic reprogramming, this axis possesses a critical function in controlling the metabolic fate of GM-CSF DCs via its regulation of activation-induced nitric oxide (NO) production (Amiel et al., 2014; Krawczyk et al., 2010). The metabolic implication of NO-mediated regulation in NO-producing DCs is discussed in the following subsection.

mTOR activation in DCs also promotes anabolic pathways and glycolysis via induction of hypoxia inducible factor-1 (HIF-1α) and sterol regulatory element binding (SREB) to promote the synthesis of a cohort of nucleic acids, proteins, and amino acids required for activation processes. Here, HIF-1α activation by mTOR is of particular interest because its stimulation occurs in LPS-activated inflammatory DCs even under normoxic conditions (Corcoran and O'Neill, 2016; Jantsch et al., 2008). Similar to the

implications of mTOR in DC effector responses, activated HIF-1α enhances the survival, ATP production, expression of key maturation-associated molecules, and antigen presenting capability to T cells of LPS-activated DCs. Inhibition of HIF-1α causes detrimental effects on DCs (Jantsch et al., 2008). The immune responses supported by the activation of HIF-1α are directly coupled with the HIF-1α-driven surface expression of glut1 or generation of metabolic intermediates such as phosphoglycerate kinase1 (PGK1), lactate, and succinate. For instance, although not yet proven in DCs, the generation of succinate in support of IL-1β production in TLR-activated macrophages is dependent on HIF-1α activation (Corcoran and O'Neill, 2016; Tannahill et al., 2013). The mTORC1-HIF-1α axis also plays a crucial role in the trained immunity of Dectin-1-ligand (β-glucan) primed-monocytes by supporting aerobic glycolysis (Cheng et al., 2014), and this regulatory mechanism is shown to be fundamental in epigenetic reprogramming in these cells (Arts et al., 2016; Cheng et al., 2014).

1.5.1.2: Nitric oxide (NO)- mediated metabolic regulation

One of the downstream targets of mTORC1 upon TLR ligation in GM-CSF cultured murine DCs is upregulation of inducible nitric oxide synthase (iNOS), an enzyme responsible for catalyzing the generation of nitric oxide (NO) and citrulline from arginine. iNOS upregulation is under the direct regulation of HIF-1α, which in turn is activated by mTORC1. In other words, iNOS expression is shown to be most entirely dependent on PI3k-mTOR-HIF-1α signaling directly downstream of TLR activation (Everts et al., 2012). Interestingly, the signaling network of iNOS, HIF-1α, and mTOR

seems to be more complex than what we have learned from previous studies. A recent study elegantly demonstrated a feedback loop of iNOS expression by mTOR-mediated induction of HIF-1α, which in turn is stabilized by iNOS upregulation and NO production. Therefore, the TLR-driven glycolytic switch is coordinated by this mTOR-HIF-1α-iNOS circuit, the operation of which is again dictated by nutrient availability in tissue microenvironments (Lawless et al., 2017). While the murine GM-CSF cultured BMDCs upregulate iNOS a few hours after TLR activation, conventional DCs or human myeloid DCs do not express iNOS at all (Bogdan et al., 2000; Bonham et al., 1997; Bonham et al., 1996; Thwe and Amiel, 2018). A distinct subset of DCs, claimed to be present in both mouse and human and capable of generating both TNF-α and NO, is called TNF-α/iNOS producing DCs (Tip-DCs). Although human peripheral blood monocyte-derived DCs do not produce NO nor express iNOS in *in vitro* culture systems, several studies have identified the presence of diverse subsets of tissue resident DCs capable of generating NO in certain disease conditions, such as psoriatic inflammation (Haider et al., 2008; Lowes et al., 2005; Wilsmann-Theis et al., 2013).

NO displays multifaceted roles in several physiological functions, such as muscle relaxation/contractility, neuronal activities, and immune responses. NO is also known to impose anti-microbial effects on invading pathogens (Qualls et al., 2012). As diverse as its role can be in normal and/or abnormal physiological responses in the body, NO is largely implicated in DC metabolism and effector function. Among its pleotropic functions, NO is notorious as a mito-toxic molecule that has an ability to reversibly S-nitrosylate cytochrome C oxidase of the electron transport chain (ETC) (Clementi et al., 1998). Therefore, in TLR-activated BMDCs, glycolytic metabolism is sustained through

the course of activation as the mitochondrial OXPHOS respiratory chain is crippled from NO intoxication. On an important note, the TLR-driven initial glycolytic burst, along with a brief surge in mitochondrial activity during early activation, is believed to be under the NO-independent phenotype, which, however, is converted to a NO-dependent switch to aerobic glycolysis, characterized by a permanent damage to the mitochondria (Everts et al., 2014; Everts et al., 2012; Pearce and Everts, 2015). Although NO-producing DCs that are fully committed to Warburg metabolism are thought to be dependent on glycolytic ATP for their survival and activation, conflicting findings have indicated that ATP can be dispensable (Everts et al., 2012). Additionally, as a result of the NO-mediated mitochondrial inhibition, functional impairments of DCs manifest as reduced co-stimulatory molecule expression and diminished capability to prime T cells (Everts et al., 2012). Because NO is fairly permeable to phospholipid membrane bilayers and can easily travel to target neighboring cells, it can cause detrimental effects on the oxidative metabolism and function of bystander cells that do not produce NO. An example is a decrease in the proliferation of T cells exposed to NO-producing DCs potentially due to the fact that NO derived from DCs induces mitochondrial dysfunction and a shortened lifespan of T cells (Xu et al., 2001). This dissertation work discusses the impact of NO on DC utilization of glycogen metabolism during activation.

1.5.1.3: AMP-kinase (AMPK)

Another key metabolic sensor that acts in opposition to the mTOR regulatory mechanism is AMP kinase (AMPK). AMPK is generally activated when the ratio of cellular ATP to ADP drops (i.e., low energy status) although other mechanisms of activation can be brought about by the phosphorylation action of its upstream kinase

LKB1 or increased intracellular calcium (Hardie et al., 2012). With a primary purpose of maintaining cellular energy status, AMPK is well known to antagonize the anabolic events mediated by mTOR (O'Neill and Hardie, 2013). Thus, AMPK is recognized to play a central role in catabolic metabolism (Hardie et al., 2012; O'Neill and Hardie, 2013). Since AMPK upregulates OXPHOS, its activity in DCs is most prominent in resting/naïve conditions prior to encounter of pathogens. In line with this, TLR ligation of DCs has been shown to downregulate AMPK phosphorylation. As activated DCs undergo a rapid metabolic switch from OXPHOS to glycolysis, which is facilitated by the PI3k-Akt-mTOR axis, AMPK is considered as an inhibitor of DC activation responses (Krawczyk et al., 2010). In parallel with its role as an energy sensor, AMPK is also recognized as a glucose sensor, even under normal energy statuses, and the activation of AMPK via glucose sensing mechanism again antagonizes mTORC1 activities (Lin and Hardie, 2018). Inhibition of AMPK not only promotes glycolytic metabolism but also augments the production of inflammatory cytokines such as TNF-α, IL-12p40, and IL-6, along with decreased IL-10 secretion (Kelly and O'Neill, 2015; Krawczyk et al., 2010). Because AMPK can also be pharmacologically activated by drugs, such as metformin used as a first-line diabetes therapy, and multiple downstream targets of AMPK demonstrate its signaling complexity, this dissertation work rather focuses on the potential implications of AMPK on DC glycogen metabolism.

1.6: Perspective and scope of this thesis

Following the characterization that the Warburg phenomenon first described in cancer cells also occurs in activated immune cells, carbohydrate metabolism became the

most widely-studied topic in immune cells (Levene and Meyer, 1912; Newsholme et al., 1986; Palsson-McDermott and O'Neill, 2013; Schwartz, 1976; Stossel et al., 1970; Warburg, 1956; Warburg et al., 1927; Yunis and Arimura, 1964). As glucose is the primary carbon fuel substrate for many cell types, including immune cells, there has been a growing appreciation for the role of glucose metabolism in supporting the immune responses and survival of DCs within recent years. While most of the metabolic studies have focused on the TLR activation of DCs in the field, there has been a recent increasing attention towards understanding the metabolic responses to stimulation with ligands for other PRRs, such as C-type lectin receptors in fungal infections. While glucose metabolism has been well-characterized to play a central role in metabolic regulations of immune cell function, very few studies to date have investigated the role of glycogen metabolism in regulating glucose homeostasis in immune cells. A large body of work on liver, brain, muscle, and cancer cell glycogen metabolism has broadly demonstrated the cell- or tissue-specific roles and regulatory mechanisms of glycogen metabolism. Understanding cell-specific role for glycogen metabolism in cells of the immune system can make a substantial contribution to our evolving understanding of the importance of metabolic regulation of immunological processes. Therefore, we initially set out to identify metabolic reprogramming broadly in non TLR-ligand stimulation of DCs, and then advanced our research into how DC glycogen metabolism, and glycogen metabolism regulation, is implicated in their immune effector responses.

Chapter 2 of this dissertation determines whether DC stimulation with molecules extracted from fungal pathogens can alter glycolytic metabolism in a manner similar to that observed in TLR-induced DCs. Using an array of ligands for Dectin-1, which is the

most widely-used model in investigating C-type lectin receptor activation, we attempted to compare the nuances of metabolic regulation in TLR- and Dectin-1 mediated DC activation. On this account, Chapter 2 also establishes the glycolytic contribution to one of the important effector signatures of early innate inflammatory responses – inflammasome activation.

In the past decade, attention has been given towards comprehending glycolytic reprogramming of immune cells, which was thought to be driven primarily by glucose from the extracellular environment. One problem with this paradigm is that the inducible glucose transporter-1 (Glut1), which has been widely invoked as the central regulator of increased glycolytic flux in activated immune cells (Freemerman et al., 2014; Fu et al., 2004), is not readily expressed in the early phase of immune activation and thus cannot satisfy the acute glycolytic need of those cells undergoing the rapid metabolic demands associated with activation. In trying to resolve this problem with the prevailing paradigm, we were first interested in examining how DCs utilize intracellular fuel sources to drive glycolysis, particularly focusing on the early activation period before the Glut1-mediated glucose flux occurs. Chapter 3 of this dissertation describes our studies testing and establishing the overarching hypothesis that DCs possess cell-intrinsic glycogen metabolic machinery responsible for driving the early glycolytic reprogramming associated with initial immune responses by these cells. To our knowledge, we are the first to demonstrate that cell-intrinsic glycogen metabolism supports early glycolysis in TLR-activated DCs and that inhibiting glycogen metabolism can significantly impair a wide variety of DC immune effector functions.

One interesting feature of glycogen metabolism observed in cancer cells, neurons, astrocytes, and muscle is continuous cycles of glycogen synthesis and degradation, a phenomenon termed the "glycogen shunt," which was demonstrated in these systems to serve cell- or tissue-specific functional purposes, such as supporting the action potential firings in neurons and rapid muscular contractility in muscle. While, prior to this work, there was no formal understanding of this unusual metabolic phenomenon in immune cells, we formally demonstrate that the glycogen shunt phenomenon in TLR-activated DCs exists using a metabolomics approach, and that this pathway critically supports citrate generation in these cells, previously shown to be a signature metabolite of DC activation. The findings from our work has highlighted the need for reassessment of the role of cellular glycogen metabolism as more than a storage depot for their immune responses, such as the potential role of maintaining metabolic homeostasis via the glycogen shunt during immune activation.

Chapter 5 of this dissertation presents preliminary findings on the investigation of some of the regulatory mechanisms on DC metabolism mediated by nitric oxide production in these cells. A significant body of work has delineated the deleterious effects of NO on immune cell metabolism, such as inhibition of mitochondrial oxidative phosphorylation, enforcing the cells to permanently switch to glycolysis, and shortening cellular lifespan (Corinti et al., 2003; Everts et al., 2012; Thwe and Amiel, 2018). The divergent role of NO between murine and human models in regard to glycogen metabolism has not been previously investigated. This is fundamentally important because of the versatile roles that NO participates in, particularly pertaining to its ability to both enhance and suppress immune outcomes during infection. Therefore, in Chapter

5, we attempt to provide an overview of what is known about the pleiotropic effects of NO on DC metabolism and then go on to elucidate a potential role for NO-mediated regulation of glycogen metabolism in activated DCs.

In this body of work, we have embarked to investigate a more complex level of understanding carbohydrate metabolism and regulation in DC activation and immune effector function. Collectively, these studies have contributed to a novel understanding of the complexity by which glucose homeostasis is regulated through glycogen metabolism in DCs. Our findings have identified new pathways and metabolic regulatory checkpoints that impact the functional aspects of DCs in response to infectious stimuli.

CHAPTER 2

Syk-dependent Glycolytic Reprogramming in Dendritic Cells Regulates IL-1β production to Fungal-associated Ligands in a TLR-independent Manner.

Abstract

Dendritic cells (DCs) activated via TLR ligation experience metabolic reprogramming, in which the cells are heavily dependent on glucose and glycolysis for the synthesis of molecular building blocks essential for maturation, cytokine production, and the ability to stimulate T cells. Although the TLR-driven metabolic reprogramming events are well documented, fungal-mediated metabolic regulation via C-type Lectin Receptors such as Dectin-1 is not clearly understood. Here, we show that activation of DCs with fungal-associated ligands induces acute glycolytic reprogramming that supports the production of IL-1β via inflammasome formation. This acute glycolytic induction to fungal-associated ligands is depending on Syk signaling in a TLR-independent manner, suggesting now that different classes of innate immune receptors functionally induce conserved metabolic responses to support immune cell activation. These studies provide new insight into the complexities of metabolic regulation of DCs immune effector function regarding cellular activation associated with protection against fungal microbes.

Introduction

Dendritic cells (DCs) are professional antigen presenting cells (APCs) of the innate immune system. They play a critical role in initiating both innate and adaptive immune responses by serving as a bridge between these two systems (*1*, *2*). They express a vast array of pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), C-type Lectin receptors (CLRs), and mannose receptors (*3*, *4*). Recognition of microbial stimuli through these receptors induces DCs to upregulate transcription and translation of genes and proteins associated with their immune effector function, such as surface

expression of co-stimulatory molecules, MHC class II, and production of pro-inflammatory cytokines and chemokines. Subsequent to activation, DCs migrate to secondary lymphoid organs and present antigens to T cells, thereby initiating the adaptive immune responses (*1*).

Among the PRRs, CLRs can recognize various lengths of carbohydrates through binding of polysaccharide moieties in C-type lectin domains (CTLD) of the receptors. Dectin-1 is a C-type lectin receptor expressed primarily on myeloid cells, such as macrophages and DCs, and on a small subset of lymphocytes (*5, 6*). Among the complex multilayers of fungal cell walls, O-linked mannosylated proteins and mannans in the thin outermost layer are recognized by TLRs, whereas β-glucan, the most abundant layer of the fungal cell wall, is primarily recognized by Dectin-1 receptor (*7*). Innate immune responses to fungal infection are orchestrated by the cooperative recognition of PAMPs on the fungal cell wall via TLRs and CLRs. Previous studies have reported that fungal activation of immune cells induces their activation via stimulation of TLR2 and/or TLR4, in addition to Dectin-1 (*8*).

β-glucans capable of stimulating Dectin-1 exist in β1,3- and β1,6- linkages. These Dectin-1 agonists are commercially available from the preparation of yeast *Saccromyces cervisiae* or *Candida albicans*; *w*hile the β-glucan ligands such as zymosan (Z), a cell wall component derived from *Saccromyces cerevisiae,* has been shown to elicit pro-inflammatory immune responses in macrophages and DCs (*5, 9, 10*), these responses are mediated by both Dectin-1 and TLR2 activation (*11, 12*). However, studies have shown that whole glucan particles or depleted zymosan (ZD), which is deprived of TLR stimulatory capacity by treatment of Z with hot alkali, produce Dectin-1 specific

inflammatory responses, such as ROS and cytokine production (*13*, *14*). Furthermore, Dectin-1 specific responses are non-redundant with TLR-mediated innate immune responses, as Dectin-1-deficient mice display impaired immune responses against *C. albicans*, resulting in compromised resistance to fungal infection (*10*).

Stimulation of PRRs by pathogen associated molecular patterns (PAMPs), such as microbial pathogens, and/or danger associated molecular patterns (DAMPs), such as ATP and uric acid crystals, results in the release of pro-inflammatory cytokines by DCs and macrophages. Among these cytokines, IL-1β plays a crucial role in both local and systemic inflammation as one of the earliest inflammatory mediators released by activated innate immune cells. The secretion of biologically active IL-1β is mediated by the cleavage of pro-IL-1β by active caspase-1, and is tightly controlled by two distinct cellular signals. PRR activation for IL-1β expression serves as a first signal, which is followed by the second signal that causes the cleavage of pro-IL-1β by active caspase-1. Different forms of a heterogeneous group of multiprotein complexes, termed inflammasomes, are responsible for activing caspase-1. These inflammasome complexes consist of sensor proteins, such as NOD-like receptor family proteins (NLRs) that can detect PAMPs and/or DAMPs, an adaptor protein apoptosis-related speck like protein (ASC), a pyrin or caspase recruitment domain, and effector caspase-1. Inflammasome activation gives rise to autocatalysis and activation of caspase-1, which then exerts a catalytic activity on pro IL-1β (*15*-*17*). Among different types of NLR inflammasomes, activation of the NLRP3 inflammasome is most abundantly studied in the field of myeloid immune cells in response to various microbial pathogens, including fungi, and plays a documented downstream role in Dectin-1-initiated innate immune responses (*18*-

20). Recent studies have reported that NLRP3 inflammasomes are required for immune responses against *C. albicans* mediated by both Dectin-1 and TLR2; however, IL-1β secretion was completely ablated in Dectin-1 deficient mice compared to that in TLR2 knock out mice (*19, 21, 22*). Although the immune effector responses in these studies have been reported to require both TLR2 and Dectin-1 to fight fungal infections, Dectin-1–mediated effects on downstream NLRP3 inflammasome activation are much more pronounced (*21*).

Activation through TLRs causes DCs to exhibit rapid changes in cellular metabolism characterized most prominently by increased rates of aerobic glycolysis. TLR-driven glycolytic reprogramming critically supports the survival and immune function of DCs by satisfying both the energetic and nutrient substrate requirements associated with the rapid anabolic synthesis of proteins and effector molecules during DC activation (*23-26*). Pharmacologic or genetic approaches to inhibit the induction of aerobic glycolysis during DC activation results in attenuation of DC maturation and leads to subsequent adverse effects on DC effector functions, including inflammatory cytokine secretion, antigen presentation, and T cell stimulation (*25-27*). A prominent recent study has demonstrated that monocytes challenged with a β-glucan cell wall component of *Candida albicans* can also upregulate aerobic glycolysis in these cells, albeit the underlying mechanism for this process is epigenetically regulated after a long-term fungal challenge (*28*). Although activation of the NLRP3 inflammasome in macrophages is supported by aerobic glycolysis (*18*), and inhibition of glycolysis attenuates IL-1β release and inflammasome activation (*18, 29*), little is known about the dectin-1 specific acute glycolytic induction for inflammasome formation in DCs, nor the role of Dectin-1

in support of acute glycolytic reprogramming. Here, we report that DC stimulation with Dectin-1 specific ligands drives aerobic glycolysis for inflammasome formation, in a TLR-independent manner.

Upon activation of Dectin-1, the downstream adaptor motif of its cytoplasmic tail, which is similar to immunoreceptor tyrosine-based activation motifs (ITAMs), serves as a docking site for spleen tyrosine kinase (Syk) (*30*). Syk signaling plays an important role in lymphocyte proliferation and survival. In macrophages and DCs, phosphorylation of Syk induces inflammatory responses such as cytokine secretion (*31*), ROS production, and NLRP3 inflammasome activation (*30, 32*). On the other hand, the PI3k/Akt axis regulates the proliferation, survival, and metabolic activities in many cells (*33-35*). We have shown the activation of PI3k and Akt signal transduction during the TLR-driven glycolytic burst in DCs (*24*). However, whether the PI3k/Akt axis is associated with Syk activity in Dectin-1 stimulated DCs is not quite understood. Additionally, TLR signaling has been shown to depend on MyD88 (*36, 37*), which is an essential signaling adaptor molecule in most TLR signaling pathways. While some reports indicate the involvement of MyD88 in the immune responses to fungal pathogens (*31, 38, 39*), the role of MyD88 in Dectin-1 activation is still controversial (*38, 40*). In this report, we demonstrate that inflammasome activation by a Dectin-1 specific ligand is mediated by a Syk-dependent and PI3k/Akt-dependent glycolytic reprogramming that is independent of TLR-MyD88.

While the glycolytic reprogramming of DC effector function has been extensively studied in TLR-driven activation, DC metabolic regulation in response to fungal pathogens is understudied. In this study, we show that Dectin-1 driven glycolytic burst is regulated by Syk-kinase activation in a TLR-independent manner. Syk inhibition

abolished the Dectin-1 driven glycolytic burst and downstream IL-1β activation, while sparing MyD88-dependent TLR stimulation. Collectively, our study suggests that DCs engage in a Syk-dependent signaling mechanism responsible for driving the Dectin-1 specific glycolytic burst in support of IL-1β release. These findings show that CLRs, in addition to TLRs, are sufficient for driving early glycolytic reprogramming in DCs, and that this activity is important for sustaining the early inflammatory responses to fungal-associated ligands.

Materials and methods

Mice and reagents

C57BL/6J, TLR2$^{-/-}$, and MyD88$^{-/-}$ mice were purchased from Jackson Laboratory and bred in-house and maintained at the University of Vermont animals care facility under protocols approved by Institutional Animal Care and Use Committee. Endotoxin free LPS (*Escherichia coli* serotype O), Pam2Csk4, Zymosan (Z), Zymosan-depleted (ZD), Curdlan, and Whole Glucan Particles (WGP) were purchased from Invivogen. was from Selleckchem, 2-deoxy-glucose (2DG) was purchased from Sigma. Antibodies for flow cytometry: 7-Aminoactinomycin D (7-AAD), anti-CD11c, anti-CD86, anti-CD40, and anti MHC-II antibodies- were purchased from BD Biosciences and Biolegend. For Western blot analysis, all antibodies were from Cell Signaling except for Il-1 and Caspase-1antibodies which were from Santa Cruz. PI3K, Akt, and TBK1 pharmacological inhibtors (LY294002, Akti-1/2, and BX-795 respectively) were purchased from Selleckchem.

Mouse DC culture and Activation

Bone marrow derived DCs were generated as described in Lutz *et al (Lutz et al., 1999)*. Briefly, BM hematopoietic cells were differentiated in GM-CSF (20ng/uL; Peprotech) in complete DC medium (CDCM), comprised of RPMI1640, 10% FCS, 2mM L-glutamine, 1IU/mL Pen-Strep, 1mM beta-mercaptoethanol, for 7 days. On day 7, DCs were washed in CDCM and cultured at 2x10^5 cells per 200μL of media For intracellular cytokine staining, cells were activated for a total of 4 hours with an addition of Golgi plug (Biolegend) after the first hour of stimulation.

Western Blot Analysis

DCs lysed using lysis buffer with Pierce Protease and Phosphatase inhibitors. Protein levels were quantified using the Pierce BCA Assay kit prior to transfer to running on 12% SDS-PAGE gels and transfer to PVDF membranes.

Metabolism assays

Extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were measured with Metabolic Flux Analyzer (Seahorse Bioscience, North Billerica, MA 24XP and/or 96XP).

Flow Cytometry and Cytokine measurements

Above mentioned antibodies were used for flow cytometry. For intracellular staining of TNF- α and IL-12 (Biolegend), cells were fixed in 4% Paraformaldehyde, permeabilized in 0.2% saponin, and stained with the antibodies in FACS buffer (1%BSA in PBS). Samples were analyzed on a BD LSRII flow cytometer. For cytokine levels, supernatants

were collected as indicated time points and measured by Duo-set ELISA kits (R&D Systems).

Quantitative Real-time PCR of Il-1β expression

RNA was isolated with an RNAeasy Kit (Qiagen) and cDNA was synthesized with an iScript cDNA Synthesis Kit (Biorad). *Il-1β* Taqman primer probes (Applied Bioscience system) and AB7500 sequence detection system or QuantStudio 3.0 were used for relative mRNA expression. mRNA relative quantitative values were calculated based on 2(-ΔΔCT) and were normalized to untreated DCs.

Statistical Analysis

Data were analyzed with GraphPad Prism software (version 6.0). Samples were analyzed using Paired t-test, One-way and Two-way ANOVA. ANOVA tests were post-calculated by Tukey's multiple comparison test. Results are means +SD or +SEM, and statistical values are represented significant when *p* values were below 0.05.

Results

Dectin-1 mediated activation drives glycolytic reprogramming

TLR activation in DCs leads to acute glycolytic reprogramming, which is an essential process to generate metabolic intermediates for the synthesis of biomolecules required for DC effector responses. We have previously characterized that this glycolytic burst occurs in response to agonists across the TLR family of receptors (*24*). In contrast, relatively little is known about metabolic changes in DCs response to fungal pathogens

and fungal-associated ligands. Although studies with human monocytes activated with β-glucan from *Candida albicans* demonstrate a long-term switch from oxidative phosphorylation to aerobic glycolysis in these cells, this study delineates a long-term metabolic reprogramming associated with epigenetic regulation of myeloid cell metabolism and does not explicitly address acute metabolic regulation in these cells (*28*). While previous reports have described that cooperative activation of TLRs and Dectin-1 play an important role in innate immune responses to fungal-associated ligands, rapid metabolic changes in response to Dectin-1 -specific ligands have yet to be elucidated (*8, 12, 13*). In order to identify Dectin-1 -specific acute metabolic reprogramming in DCs and to determine the contribution of these metabolic changes to early immune responses by these cells, we utilized an array of ligands specific to TLRs alone (LPS), Dectin-1 alone (ZD, Curdlan, WGP), or ligands that interact with both simultaneously (Zymosan).

We first characterized the ability of these different agonists to induce acute glycolytic reprogramming, termed 'glycolytic burst', in GM-CSF-differentiated BMDCs by metabolic extracellular flux analysis (Agilent, Seahorse Biosciences). By stimulating the cells with LPS (TLR agonist), Zymosan (Z, TLR and Dectin-1 agonist), or Zymosan depleted (ZD, Dectin-1 –specific agonist), we were able to determine the ability of these receptors in mediating acute glycolysis induction. Consistent with our previous work in which TLR4 stimulation with LPS induced a rapid glycolytic burst, we observed a similar metabolic response to both Z and ZD stimulation (**Figure 2 -*1*A**). 2-deoxy-glucose (2DG), a synthetic molecule structurally similar to glucose that functions as an inhibitor of glycolysis, dramatically attenuated glycolytic burst induced by all three ligands, suggesting that Dectin-1 activation drives the aerobic glycolytic burst (**Figure 2 -*1*B**). To

verify that each member of our agonist panel was able to induce DC maturation comparably, we next characterized the induction of surface co-stimulatory molecule expression in BMDCs stimulated for 24 hours with each ligand (**Figure 2 -*1*C**). Futhermore, we observed a requirement for glycolytic metabolism to support activation-associated TNF-α and IL-12 production in response to each ligand (**Figure 2 -*1*D**). Consistent with our metabolic data (**Figure 2 -*1*A** and **Figure 2 -*1*B**), proper DC maturation by all ligands, including Dectin-1 specific ligand, ZD, requires glycolytic reprogramming (**Figure 2 -*1*C** and **Figure 2 -*1*D**).

Dectin-1 –dependent glycolytic burst and maturation requires Syk signaling

Dectin-1 -driven immune responses are documented to be mediated by both Syk-dependent and -independent mechanisms (*5, 41*). Based on this, we were interested in testing the requirement for Syk signaling in supporting Dectin-1-mediated glycolytic burst and effector function in DCs. We examined acute glycolysis induction in BMDCs stimulated with TLR and/or Dectin-1 ligands in the presence or absence of the Syk inhibitor, PRT. Interestingly, PRT completely ablated glycolysis induction in the cells stimulated by the Dectin-1 specific ligand, ZD, while only a partial inhibition was observed for the TLR-specific agonist LPS and dual agonist Z (**Figure 2 -*2*A**). Upregulated surface expression of the costimulatory molecules CD40 and CD86 in response to Dectin-1–specific ligands (ZD, Curdlan, WGP) was strongly attenuated by Syk inhibition, while TLR-driven maturation (LPS) was unimpaired, and dual-agonist maturation (Z) was only partially impaired, by Syk inhibition (**Figure 2 -*2*B**). These data

indicate Syk-dependent signals are sufficient to regulate Dectin-1-mediated glycolytic reprogramming and maturation in DCs.

Dectin-1 –mediated metabolic reprogramming is independent of TLR/MyD88 signals

In order to confirm that Dectin-1 specific metabolic reprogramming and activation was independent of TLR-signaling, we stimulated DCs from TLR2$^{-/-}$ and MyD88$^{-/-}$ mice and assessed their ability to induce acute glycolytic burst in response to Dectin-1–specific activation (**Figure 2 -*3*A**). We next activated WT, MyD88$^{-/-}$ and TLR2$^{-/-}$ BMDCs with ZD in the presence or absence of PRT and found that DCs exhibited comparable levels of glycolysis induction to ZD even in the absence of TLR2 or MyD88 expression (**Figure 2 -3B**). Syk inhibition completely ablated Dectin-1–mediated glycolysis induction in all genotypes (**Figure 2 -*3*B**). Furthermore, TLR2$^{-/-}$ BMDCs were unresponsive to canonical TLR2 agonist Pam2Csk4, and MyD88$^{-/-}$ BMDCs did not mature to both TLR2 and TLR9 agonists as expected, while all genotypes matured properly to LPS (presumably via the MyD88-independent TRIF/TRAM signaling pathway) and Dectin-1-specific ligands (**Figure 2 -*3*C**). These data confirm that Dectin-1/Syk mediated signals are necessary to drive DC metabolic reprogramming even in the absence of the TLR2/MyD88 signaling axis that is also well-documented to regulate innate immune responses to fungal-associated ligands.

Dectin-1 mediated glycolytic reprogramming requires a PI3K/TBK-1/Akt signaling axis

Syk signaling is known to play a crucial role in Dectin-1-mediated innate immune responses (*22, 30, 31, 42*). In previously published work, we have identified that

phosphorylation of Akt T308 is required for Hexokinase association with the mitochondria and glycolytic reprogramming in TLR-stimulated DCs (*24*). We thus hypothesized that similar signaling pathways may regulate acute metabolic responses to Dectin-1-specific activation of DCs. To test this, we first examined expression of total and T308-phosphorylated Akt in BMDCs stimulated with Z or ZD (**Figure 2 -*4*A**). We observed that Dectin-1 activation by both ligands induces the phosphorylation of Akt T308, suggesting that similar signaling mechanisms are likely involved in Dectin-1 mediated glycolytic reprogramming (**Figure 2 -*4*A**). PI3K inhibition resulted in reduced phosphorylation of Akt T308, suggesting that PI3K is involved in regulating Dectin-1–dependent signaling that leads to Akt T308 phosphorylation (**Figure 2 -*4*B**). This is in contrast to previously published data showing that PI3K is not required for acute glycolytic reprogramming to TLR stimulation in DCs (*24*). However, inhibition of TBK1 completely ablated the phosphorylation of Akt T308 in ZD activation (**Figure 2 -*4*C**), which is consistent with previously published data for TLR-mediated acute activation. Consistent with these data, Akt inhibition attenuated acute glycolytic reprogramming in both Z- and ZD–stimulated DCs, whereas PI3K inhibition significantly impacted glycolysis induction only in ZD-stimulated cells (**Figure 2 -*4*D**). In TLR-stimulated DCs, TBK1 phosphorylation of Akt has been demonstrated to regulate acute glycolytic burst independent of PI3k signaling, whereas the PI3k/Akt axis plays a major role in maintaining long-term glycolytic activity in these cells (*24*, *26*). In contrast to these findings for acute glycolytic induction to TLR-mediated stimulation, our data suggest that Dectin-1–dependent Akt T308 activation is mediated by both PI3k and TBK-1.

Dectin-1 –mediated acute glycolytic reprogramming is required for NLRP3 inflammasome activation

In macrophages, glycolytic reprogramming has been demonstrated to drive NLRP3 inflammasome activation initiated by two signal components, LPS as a first signal and ATP or Nigericin as a second (*18*). While Cheng *et al.* have shown that human monocytes challenged with β-glucan components of *C. albicans* upregulate aerobic glycolysis, these studies emphasized the long-term stimulatory effect of β-glucan-treated monocytes and the metabolic changes described in these cells have been documented to be regulated by epigenetic changes (*28, 43*). However, the requirement for Dectin-1 driven acute metabolic regulation for NLRP3 inflammasome activation has not been explicitly characterized in DCs. We hypothesized that the aerobic glycolysis observed in Dectin-1 activated BMDCs (**Figure 2 -*1*** and **Figure 2 -*2***) is required to support NLRP3 formation and IL-1β production in a Syk-dependent manner. To test this, we activated BMDCs with LPS, Z, and ZD in the presence or absence of 2DG for 6 hrs and examined the transcriptional expression of IL-1β (**Figure 2 -*5*A**). Interestingly, mRNA expression of IL-1β did not change regardless of glycolysis inhibition, indicating that IL-1β production is not regulated by glycolysis at the transcriptional level, a finding consistent with the lack of a glycolytic requirement for transcription of inflammatory genes in response to TLR stimulation of DCs (*24*). However, the levels of IL-1β in both culture supernatants (**Figure 2 -*5*B**) and cellular lysates (**Figure 2 -*5*C**) of Dectin-1-activated BMDCs stimulated with nigericin were significantly attenuated by 2DG-mediated glycolysis inhibition. Furthermore, Syk inhibition completely ablated IL-1β release from

Dectin-1–stimulated cells, further supporting the requirement of Syk-dependent signals for both glycolytic induction and NLRP3 inflammasome activation in these cells (**Figure 2 -*1*B**). These data suggest that Dectin-1 -mediated IL-1β production is regulated at the translational level in a Syk and glucose -dependent manner (**Figure 2 -*5*C**). In addition, NLRP3 induction was notably inhibited by 2DG during Dectin-1–mediated activation but not TLR activation (**Figure 2 -*5*D**).

Discussion

Stimulation of myeloid immune cells with fungal ligands induces metabolic reprogramming (*28, 44*). Several studies have also shown that fungal pathogen activation causes inflammasome formation (*17, 32, 42, 45*) and induces the Syk-kinase signaling pathway (*22, 30, 31, 42*). While these studies add significant value to the understanding of host immune responses against fungal pathogens, an integrated understanding of fungal-mediated metabolic changes in the regulation of specific immune outcomes in DCs, such as inflammasome activation, has not been previously described. In this study, we demonstrate that the Dectin-1-specific activation of DCs induces glycolysis-dependent IL-1β production via the Syk-mediated signal transduction pathway, which drives activation of the PI3K/TBK1/Akt axis. By activating BMDCs with Dectin-1-specific ligands, we have been able to discriminate between Dectin-1-specific glycolytic burst and effector responses from TLR-mediated effects. We show that the Dectin-1 specific ligands induce rapid glycolytic reprogramming in DCs, and DC maturation by Dectin-1 specific ligands is dependent on glucose in a TLR2 and MyD88–independent manner.

In this study, we have taken a reductionist approach using purified ligands to interrogate the contribution of specific receptors to anti-fungal metabolic responses. However, the composition of fungal cell walls is complex, and understanding the contribution of a single receptor to innate immune cell metabolism is a challenge. Recent work by Netea's group has elegantly shown that activation of monocytes with hyphae or yeast cells results in a differential utilization of metabolic pathways, in which yeast-activated immune cells are dependent on both glycolysis and TCA metabolism, while hyphae activation induces the cells to rely solely on glycolysis (*44*). Earlier studies have reported that *C.albicans* hyphae are primarily recognized via Dectin-2 receptors (*46*), suggesting that metabolic responses to nuanced components of fungal cell walls by different PRRs could initiate utilization of distinct metabolic pathways.

The TLR-driven early glycolytic reprogramming is mediated by Akt-dependent TBK1/IKKε signaling (*24*), and a long-term commitment to aerobic glycolysis is facilitated by PI3k/Akt/mTOR signaling (*25*). While MyD88, an upstream adaptor molecule of TLR-mediated signaling pathways, is central to TLR-driven effector responses, conflicting reports exist regarding the role of MyD88 in non-TLR signaling pathways such as Dectin-1-driven responses (*22, 38*). However, whether MyD88 is involved in Dectin-1-specific ligand recognition or a crosstalk between cooperative ligand recognition by TLRs and Dectin-1 is not clear. To address this, we examined if MyD88 is involved in Dectin-1-specific metabolic regulation of DCs. We found that the Dectin-1-specific ligand, ZD, specifically triggers rapid glycolytic burst independent of TLR2 or MyD88. These data indicate that Dectin-1-specific signals, via a Syk/PI3K/Akt signaling axis, are sufficient to initiate both DC glycolytic reprogramming and maturation

in response to activation by fungal-associated ligands (modeled in **Figure 2 -*6***). These findings are consistent with the recent work of Ding *et al.* in which they showed that PI3k/Akt signaling regulates the proliferation of cytotoxic T cells activated by human monocytes induced with Dectin-1 specific β-glucan molecules (*34*).

The pioneering work of Sousa *et al.* highlighted that Syk plays a central role for cytokine production by Dectin-1 activation (*31*). Syk phosphorylation is also crucial for Dectin-1-mediated inflammasome formation and IL-1β production (*22, 32*). Although the TLR-MyD88 axis is not essential for Dectin-1-mediated inflammasome formation in BMDCs, clear distinctions between Dectin-1- and TLR-mediated signaling components have not been fully established (*22*). This could be potentially due to the complex nature of fungal cell wall composition and the challenge of experimentally isolated single receptor-specific responses. Here, we show that Dectin-1 activation of BMDCs requires Syk signaling for glycolysis induction. Interestingly, we also show that Dectin-1-mediated Syk-dependent IL-1β production in BMDCs is heavily reliant on glucose and glycolytic reprogramming.

Glucose metabolism in TLR-mediated activation has been widely studied in DCs and innate myeloid immune cells alike (*47, 48*), while few studies delineate the glycolytic requirement during cell activation via CLRs such as Dectin-1. This work helps illustrate that glucose metabolism plays a central role in DC activation via distinct families of PRRs and that the upregulation of glycolysis is a classic metabolic hallmark in both TLR and CLR-mediated activation. Interestingly, recent studies suggest that there may be nuanced differences in cellular metabolic responses to distinct types of microbial-associated ligands. For instance, activation of human monocytes with Pam3Csk4 and

LPS induces differential metabolic responses, in which Pam3Csk4 upregulates both glycolysis and OXPHOS, whereas LPS induces aerobic glycolysis but not OXPHOS (*49*). Differential functional outcomes could also arise from cellular participation in divergent glucose metabolism. In an example of monocytes stimulated with TLR2 ligands, phagocytic activity of these cells is preferentially supported by OXPHOS whereas cytokine production is facilitated by glycolysis and OXPHOS (*49*). In parallel to TLR-mediated metabolic changes, recent work has highlighted the differential metabolic outcomes from dimorphic stages of *C. albicans*, leading to diverse functional responses (*44*). Of note, since we have recently shown that TLR-mediated DC activation relies on alternative metabolic pathways such as glycogen metabolism (*50*), it is tempting to hypothesize that Dectin-1 mediated responses may participate in similar aspects of activation-assoicated glucose metabolism.

In summary, our findings identify Dectin-1-specific acute glycolytic reprogramming events that support DC effector responses to fungal-associated ligands. However, the complex and redundant nature of the metabolic networks demands additional work at an organism level to better understand the regulatory mechanisms implicated in particular anti-fungal immune responses. With a rising interest in metabolic regulation and consequent immune outcomes from various pathogenic stimuli, our work adds to an increasingly nuanced understanding of metabolic regulation of DCs in response to different microbial stimuli. These studies, as well as those by others in the field, underscore the need for additional research to elucidate the complexity of metabolic regulation of innate immune cell responses to different pathogens.

Figures

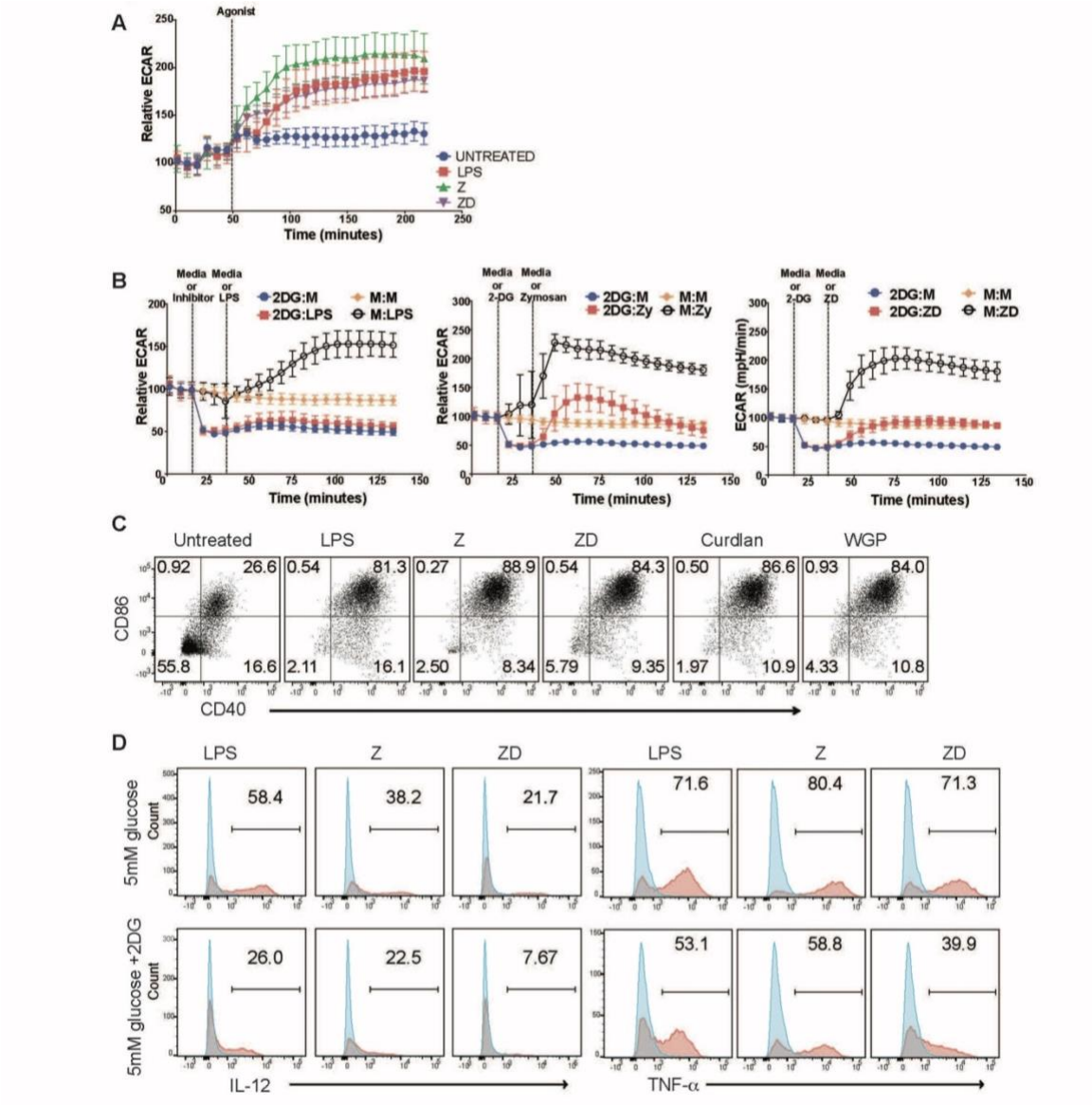


Figure 2 -1. Dectin-1 mediated activation drives glycolytic reprogramming.

(A) BMDCs were stimulated with LPS, Z, ZD for 24hours and extracellular acidification rate (ECAR) is measured in real time using a Seahorse Extracellular Flux Analyzer (Agilent, Seahorse Biosciences). (B) Relative ECAR of BMDCs stimulated with 2DG followed by media or corresponding ligands (LPS, Z, or ZD). (C) Surface expression of CD40 and CD86 on BMDCs stimulated for 24 hours with LPS, Z, ZD, Curdlan, or WGP. (D) Intracellular cytokine staining of TNF and IL-12 of BMDCs stimulated for 6 hours with LPS, Z, ZD in 5mM glucose in the presence or absence of 2DG. Blue peaks represent unstimulated. Representative of n=3.

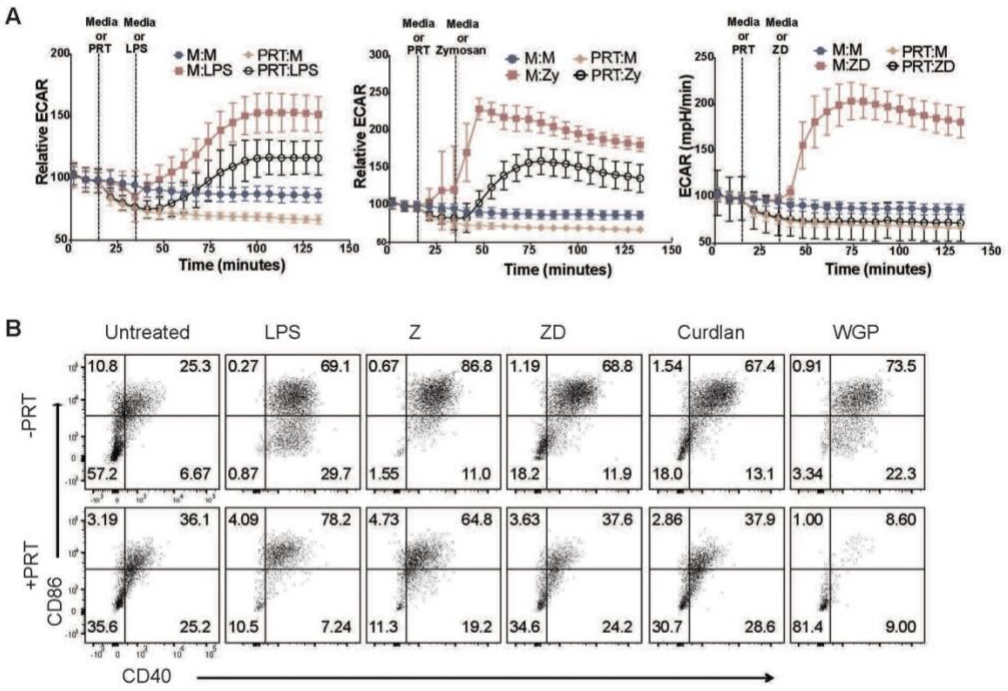


Figure 2 -2. Dectin-1 dependent glycolytic burst and maturation requires Syk signaling.

(A) Real-time Relative ECAR of BMDCs stimulated in the present or absence of Syk inhibitor (PRT) followed by LPS, Z (Zymosan) or ZD. (B) CD40 and CD86 surface expression of BMDCs stimulated for 24hours with LPS, Z, ZD, Curdlan, or WGP in the presence or absence of Syk inhibitor.

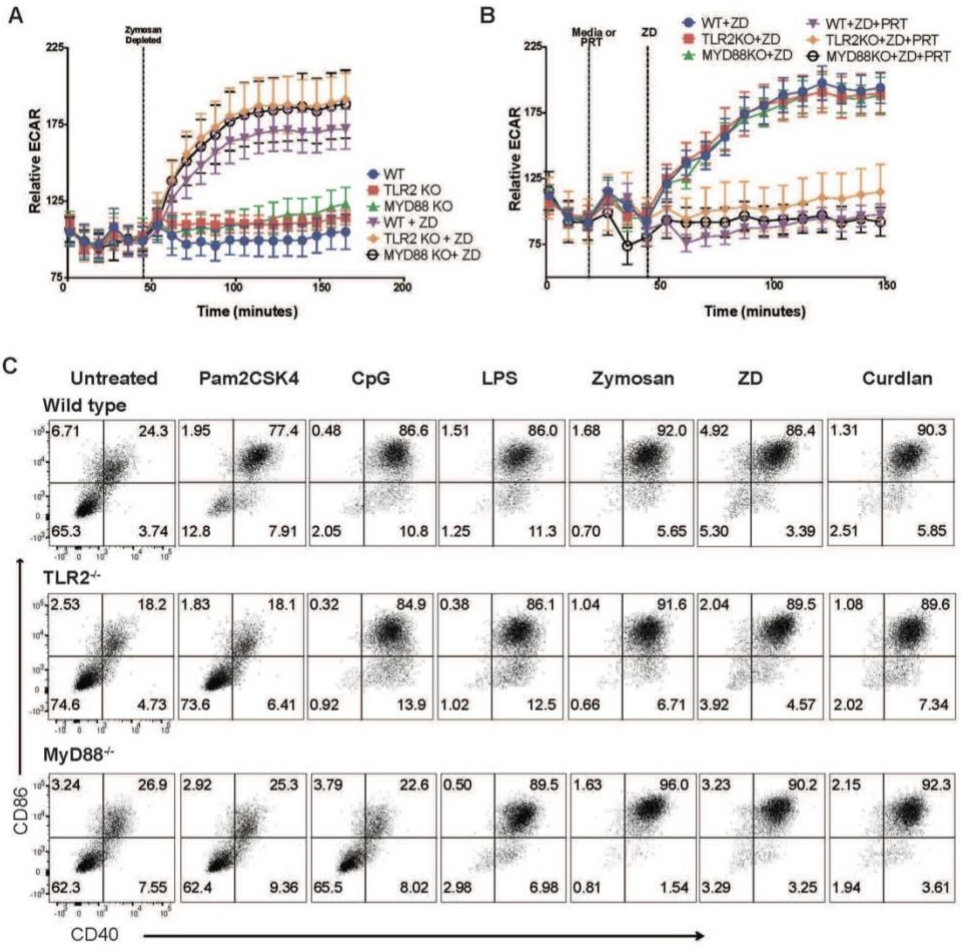


Figure 2 -3. Dectin-1 mediated glycolytic reprogramming is independent of TLR2/MyD88 signals.

(A) Relative ECAR of BMDCs from WT, TLR2$^{-/-}$, and MyD88$^{-/-}$ stimulated in real time response to ZD. (B) Relative ECAR of BMDCs from WT, TLR2$^{-/-}$, and MyD88$^{-/-}$ stimulated in real time response to PRT followed by ZD. (C) CD40 and CD86 surface expression of BMDCs WT, TLR2$^{-/-}$, and MyD88$^{-/-}$ stimulated for 24 hours with Pam2CSK4 (TLR2 ligand), CpG (TLR9 ligand), LPS (TLR4 ligand), Z (TLR2 and Dectin-1 ligand), ZD, or Curdlan (Dectin-1 ligands).

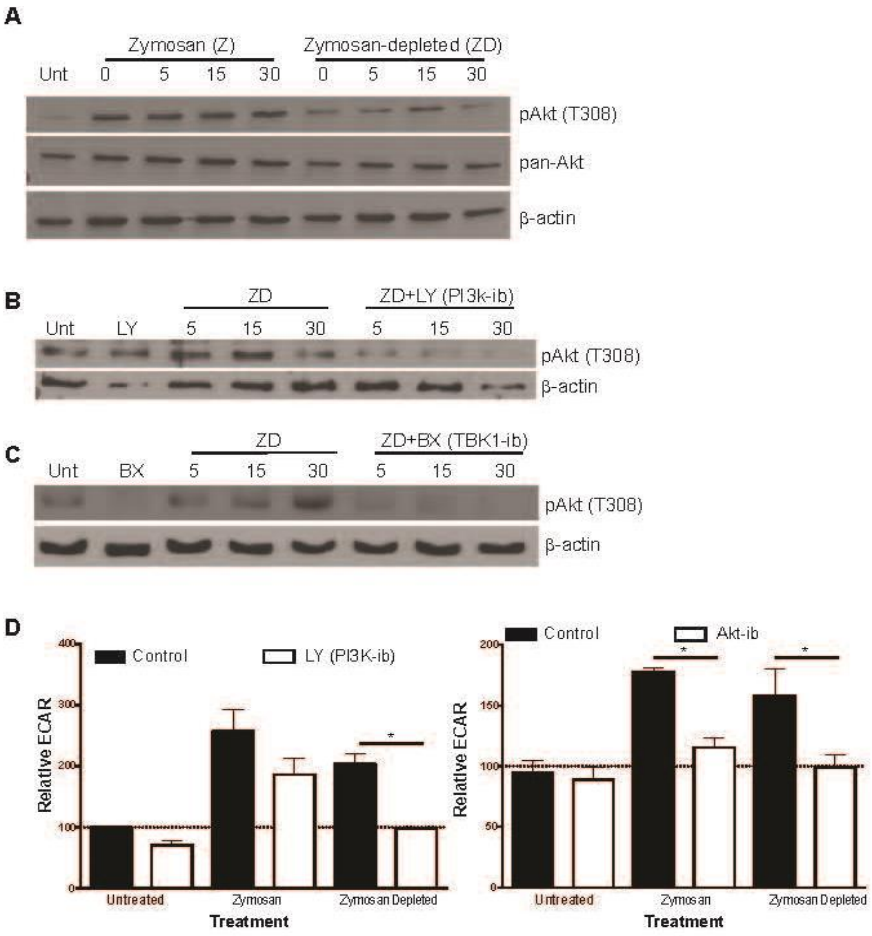


Figure 2 -4. Dectin-1 mediated glycolytic reprogramming requires a PI3k/TBK1/Akt signaling axis.

(A) Protein expression of pAkt (T308), pan Akt, and β-actin in BMDCs stimulated with Z and ZD for 5, 15, and 30 minutes. (B) Protein expression of pAkt (T308) in BMDCs stimulated for 5, 15, and 30 minutes with ZD in the presence or absence of PI3k inhibitor (LY). (C) Protein expression of pAkt (T308) in BMDCs stimulated with ZD in the presence or absence of TBK1 inhibitor (BX) for 5, 15, and 30 minutes. (D) Relative ECAR of BMDCs stimulated in real time response to Z and ZD in the presence or absence of PI3k or Akt inhibitors. Data represents n=4; * $p<0.05$ student t test.

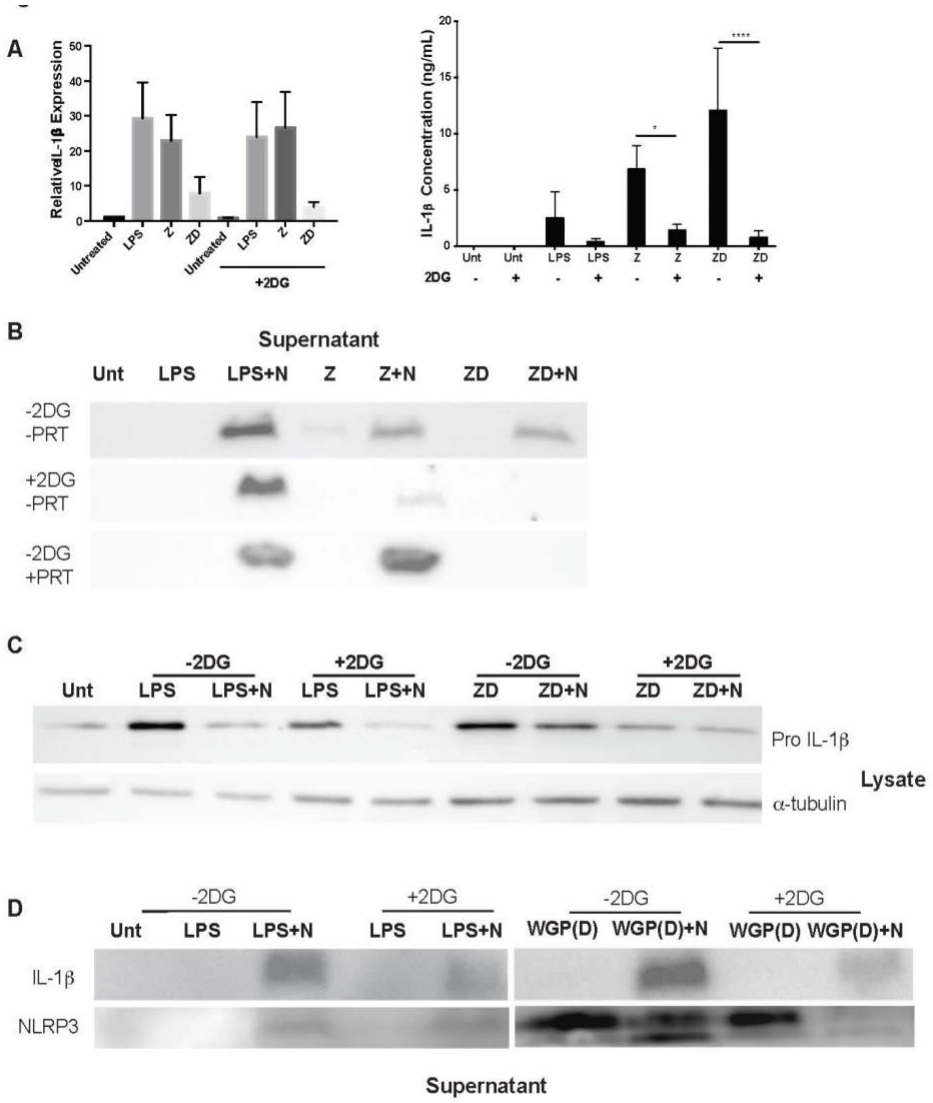


Figure 2 -5: Dectin-1 mediated acute glycolytic reprogramming is required for NLRP3 inflammasome activation.

(A) Relative mRNA expression of IL-1β in BMDCs stimulated for 6 hours with LPS, Z, ZD in the presence or absence of 2DG. (B and C) Protein expression of active IL-1β from culture supernatant (B) and pro-IL-1β from cell lysates (C) of BMDCs stimulated with LPS, Z, and ZD in the presence or absence of 2DG or PRT for 4 hours with an addition of nigericin in the last hour. (D) Protein expression of IL-1β and NLRP3 from culture supernatant of BMDCs stimulated with LPS and WGP particles in the presence or absence of 2DG for 4 hours with an addition of nigericin in the last hour.

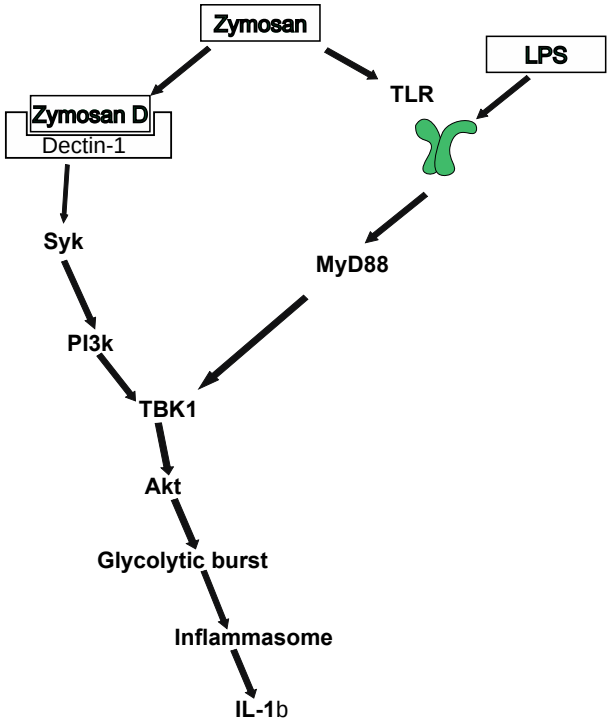


Figure 2 -6: Model of Dectin-1 -mediated glycolytic reprogramming and IL-1β production

References

1. J. Banchereau, R. M. Steinman, Dendritic cells and the control of immunity. *Nature* **392**, 245-252 (1998).
2. H. K. Lee, A. Iwasaki, Innate control of adaptive immunity: dendritic cells and beyond. *Seminars in immunology* **19**, 48-55 (2007).
3. A. Iwasaki, R. Medzhitov, Control of adaptive immunity by the innate immune system. *Nature immunology* **16**, 343-353 (2015).
4. S. W. Brubaker, K. S. Bonham, I. Zanoni, J. C. Kagan, Innate Immune Pattern Recognition: A Cell Biological Perspective. *Annual review of immunology* **33**, 257-290 (2015).
5. R. A. Drummond, G. D. Brown, The role of Dectin-1 in the host defence against fungal infections. *Current opinion in microbiology* **14**, 392-399 (2011).
6. D. M. Reid, N. A. R. Gow, G. D. Brown, Pattern recognition: recent insights from Dectin-1. *Current Opinion in Immunology* **21**, 30-37 (2009).
7. D. M. Arana *et al.*, The role of the cell wall in fungal pathogenesis. *Microb Biotechnol* **2**, 308-320 (2009).
8. G. Ferwerda, F. Meyer-Wentrup, B.-J. Kullberg, M. G. Netea, G. J. Adema, Dectin-1 synergizes with TLR2 and TLR4 for cytokine production in human primary monocytes and macrophages. *Cellular Microbiology* **10**, 2058-2066 (2008).
9. I. M. Dambuza, G. D. Brown, C-type lectins in immunity: recent developments. *Current Opinion in Immunology* **32**, 21-27 (2015).
10. P. R. Taylor *et al.*, Dectin-1 is required for [beta]-glucan recognition and control of fungal infection. *Nature immunology* **8**, 31-38 (2007).
11. B. N. Gantner, R. M. Simmons, S. J. Canavera, S. Akira, D. M. Underhill, Collaborative Induction of Inflammatory Responses by Dectin-1 and Toll-like Receptor 2. *The Journal of experimental medicine* **197**, 1107-1117 (2003).
12. M. G. Netea *et al.*, Immune sensing of Candida albicans requires cooperative recognition of mannans and glucans by lectin and Toll-like receptors. *The Journal of clinical investigation* **116**, 1642-1650 (2006).
13. J. C. Gensel *et al.*, Toll-Like Receptors and Dectin-1, a C-Type Lectin Receptor, Trigger Divergent Functions in CNS Macrophages. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **35**, 9966-9976 (2015).
14. D. K. Vijaya Kumar, W. A. Eimer, S. Ramakrishnan, Specificity of Toll-Like Receptor 2 and Dectin-1 Signaling in CNS Macrophages. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **35**, 16015-16017 (2015).
15. F. S. Sutterwala, S. Haasken, S. L. Cassel, Mechanism of NLRP3 inflammasome activation. *Annals of the New York Academy of Sciences* **1319**, 82-95 (2014).
16. T. Próchnicki, E. Latz, Inflammasomes on the Crossroads of Innate Immune Recognition and Metabolic Control. *Cell metabolism* **26**, 71-93 (2017).
17. T. Prochnicki, M. S. Mangan, E. Latz, Recent insights into the molecular mechanisms of the NLRP3 inflammasome activation. *F1000Research* **5**, (2016).

18. J. S. Moon *et al.*, mTORC1-Induced HK1-Dependent Glycolysis Regulates NLRP3 Inflammasome Activation. *Cell reports* **12**, 102-115 (2015).
19. M. Lamkanfi, R. K. S. Malireddi, T.-D. Kanneganti, Fungal Zymosan and Mannan Activate the Cryopyrin Inflammasome. *Journal of Biological Chemistry* **284**, 20574-20581 (2009).
20. S. Mariathasan *et al.*, Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature* **440**, 228-232 (2006).
21. A. G. Hise *et al.*, An Essential Role for the NLRP3 Inflammasome in Host Defense against the Human Fungal Pathogen Candida albicans. *Cell Host & Microbe* **5**, 487-497.
22. O. Gross *et al.*, Syk kinase signalling couples to the Nlrp3 inflammasome for anti-fungal host defence. *Nature* **459**, 433-436 (2009).
23. E. Amiel *et al.*, Inhibition of mechanistic target of rapamycin promotes dendritic cell activation and enhances therapeutic autologous vaccination in mice. *Journal of immunology (Baltimore, Md. : 1950)* **189**, 2151-2158 (2012).
24. B. Everts *et al.*, TLR-driven early glycolytic reprogramming via the kinases TBK1-IKKvarepsilon supports the anabolic demands of dendritic cell activation. *Nature immunology* **15**, 323-332 (2014).
25. B. Everts *et al.*, Commitment to glycolysis sustains survival of NO-producing inflammatory dendritic cells. *Blood* **120**, 1422-1431 (2012).
26. C. M. Krawczyk *et al.*, Toll-like receptor-induced changes in glycolytic metabolism regulate dendritic cell activation. *Blood* **115**, 4742-4749 (2010).
27. E. Amiel *et al.*, Mechanistic target of rapamycin inhibition extends cellular lifespan in dendritic cells by preserving mitochondrial function. *Journal of immunology (Baltimore, Md. : 1950)* **193**, 2821-2830 (2014).
28. S. C. Cheng *et al.*, mTOR- and HIF-1alpha-mediated aerobic glycolysis as metabolic basis for trained immunity. *Science* **345**, 1250684 (2014).
29. M. Xie *et al.*, PKM2-dependent glycolysis promotes NLRP3 and AIM2 inflammasome activation. *Nat Commun* **7**, 13280 (2016).
30. D. M. Underhill, E. Rossnagle, C. A. Lowell, R. M. Simmons, Dectin-1 activates Syk tyrosine kinase in a dynamic subset of macrophages for reactive oxygen production. *Blood* **106**, 2543-2550 (2005).
31. N. C. Rogers *et al.*, Syk-dependent cytokine induction by Dectin-1 reveals a novel pattern recognition pathway for C type lectins. *Immunity* **22**, 507-517 (2005).
32. P. Kankkunen *et al.*, (1,3)-beta-glucans activate both dectin-1 and NLRP3 inflammasome in human macrophages. *Journal of immunology (Baltimore, Md. : 1950)* **184**, 6335-6342 (2010).
33. S. Bhattacharyya *et al.*, Immunoregulation of dendritic cells by IL-10 is mediated through suppression of the PI3K/Akt pathway and of IkappaB kinase activity. *Blood* **104**, 1100-1109 (2004).
34. J. Ding *et al.*, β-Glucan enhances cytotoxic T lymphocyte responses by activation of human monocyte-derived dendritic cells via the PI3K/AKT pathway. *Human Immunology* **76**, 146-154 (2015).

35. D. L. Moyes *et al.*, Protection Against Epithelial Damage During Candida albicans Infection Is Mediated by PI3K/Akt and Mammalian Target of Rapamycin Signaling. *The Journal of infectious diseases* **209**, 1816-1826 (2014).
36. K. M. Dennehy, G. D. Brown, The role of the β-glucan receptor Dectin-1 in control of fungal infection. *Journal of leukocyte biology* **82**, 253-258 (2007).
37. G. M. Gersuk, D. M. Underhill, L. Zhu, K. A. Marr, Dectin-1 and TLRs Permit Macrophages to Distinguish between Different Aspergillus fumigatus Cellular States. *The Journal of Immunology* **176**, 3717-3724 (2006).
38. B. Li *et al.*, Yeast glucan particles activate murine resident macrophages to secrete proinflammatory cytokines via MyD88- and Syk kinase-dependent pathways. *Clin Immunol* **124**, 170-181 (2007).
39. A. Plato, J. A. Willment, G. D. Brown, C-Type Lectin-Like Receptors of the Dectin-1 Cluster: Ligands and Signaling Pathways. *International Reviews of Immunology* **32**, 134-156 (2013).
40. G. D. Brown *et al.*, Dectin-1 mediates the biological effects of beta-glucans. *The Journal of experimental medicine* **197**, 1119-1124 (2003).
41. N. C. Rogers *et al.*, Syk-Dependent Cytokine Induction by Dectin-1 Reveals a Novel Pattern Recognition Pathway for C Type Lectins. *Immunity* **22**, 507-517.
42. S. C. Cheng *et al.*, The dectin-1/inflammasome pathway is responsible for the induction of protective T-helper 17 responses that discriminate between yeasts and hyphae of Candida albicans. *Journal of leukocyte biology* **90**, 357-366 (2011).
43. S. Bekkering *et al.*, Metabolic Induction of Trained Immunity through the Mevalonate Pathway. *Cell* **172**, 135-146.e139 (2018).
44. J. Domínguez-Andrés *et al.*, Rewiring monocyte glucose metabolism via C-type lectin signaling protects against disseminated candidiasis. *PLoS pathogens* **13**, e1006632 (2017).
45. Y. He, H. Hara, G. Núñez, Mechanism and Regulation of NLRP3 Inflammasome Activation. *Trends in Biochemical Sciences* **41**, 1012-1021.
46. K. Sato *et al.*, Dectin-2 Is a Pattern Recognition Receptor for Fungi That Couples with the Fc Receptor γ Chain to Induce Innate Immune Responses. *Journal of Biological Chemistry* **281**, 38854-38866 (2006).
47. A. J. Freemerman *et al.*, Metabolic reprogramming of macrophages: glucose transporter 1 (GLUT1)-mediated glucose metabolism drives a proinflammatory phenotype. *The Journal of biological chemistry* **289**, 7884-7896 (2014).
48. B. Everts, E. J. Pearce, Metabolic control of dendritic cell activation and function: recent advances and clinical implications. *Frontiers in immunology* **5**, 203 (2014).
49. E. Lachmandas *et al.*, Microbial stimulation of different Toll-like receptor signalling pathways induces diverse metabolic programmes in human monocytes. *Nature microbiology* **2**, 16246 (2016).
50. P. M. Thwe *et al.*, Cell-Intrinsic Glycogen Metabolism Supports Early Glycolytic Reprogramming Required for Dendritic Cell Immune Responses. *Cell metabolism* **26**, 558-567.e555.

CHAPTER 3

Cell-intrinsic glycogen metabolism supports early glycolytic reprogramming required for dendritic cell immune responses

Summary

Dendritic cell (DC) activation by toll-like receptor (TLR) agonists causes rapid glycolytic reprogramming that is required to meet the metabolic demands of their immune activation. Recent efforts in the field have identified an important role for extracellular glucose sourcing to support DC activation. However, the contributions of intracellular glucose stores to these processes have not been well characterized. We demonstrate that DCs possess intracellular glycogen stores and that cell-intrinsic glycogen metabolism supports the early effector functions of TLR-activated DCs. Inhibition of glycogenolysis significantly attenuates TLR-mediated DC maturation and impairs their ability to initiate lymphocyte activation. We further report that DCs exhibit functional compartmentalization of glucose and glycogen –derived carbons, where these substrates preferentially contribute to distinct metabolic pathways. This work provides novel insights into nutrient homeostasis in DCs, demonstrating that differential utilization of glycogen and glucose metabolism regulates their optimal immune function.

Introduction

Dendritic cells (DCs) are canonical "professional antigen presenting cells" of the immune system and play a central role in coordinating both innate and adaptive immune responses (Banchereau and Steinman, 1998; Lee and Iwasaki, 2007; Lipscomb and Masten, 2002). DCs recognize microbial pathogens and other inflammatory stimuli through the expression of innate immune receptors including the Toll-like receptor (TLR) family (Akira and Takeda, 2004; Amati et al., 2006; Barton and Medzhitov, 2002). DC

activation by TLR signaling initiates a complex set of transcriptional and translational events that are characterized by the up-regulation of surface co-stimulatory molecule expression, inflammatory cytokine secretion, and the ability to stimulate T lymphocytes via antigen presentation by major histocompatibility (MHC) molecules.

TLR stimulation initiates a shift in DC metabolism characterized by upregulation of aerobic glycolysis, which plays a vital role in supporting the immune effector function and survival of both human and mouse DCs (Amiel et al., 2012; Amiel et al., 2014; Everts et al., 2012; Krawczyk et al., 2010). Rapid glycolysis induction supports the metabolic requirements associated with the high levels of protein synthesis that contribute to DC immune activity. The TLR-mediated "glycolytic burst" drives *de novo* fatty acid synthesis via glucose-dependent citrate metabolism, which supports the synthesis and secretion of inflammatory cytokines (Amiel et al., 2014; Rehman et al., 2013). Interrupting the glucose-to-citrate pathway significantly impairs DC maturation, cytokine secretion, and T cell stimulatory capacity (Amiel et al., 2014; Everts et al., 2012; Krawczyk et al., 2010).

Immune cells are thought to primarily support activation-associated glycolysis via increased expression of glucose transporters (Everts and Pearce, 2014; Fox et al., 2005; Pearce and Everts, 2015; Pearce and Pearce, 2013). Consistent with this, the role of the inducible glucose transporter, GLUT1, in regulating activation-associated glucose flux in both myeloid and lymphoid immune cells has been a major focus in the field (Freemerman et al., 2014; Macintyre et al., 2014). In DCs however, GLUT1 upregulation occurs several hours after TLR stimulation, while TLR-mediated glycolytic reprogramming happens within minutes of activation. Thus, the source of glucose

supporting the earliest events in DC activation, namely whether glucose is sourced from the extracellular environment or from intracellular pools, has not been fully defined. We propose that the DCs utilize intracellular glycogen reserves to fuel their metabolic needs during early immune activation and that glycogen metabolism is required by these cells to initiate proper immune effector responses.

Glycogen, a large branch-chained glucose polymer, has been extensively characterized in hepatocytes, muscle cells, and neuronal tissue where it serves as an intracellular carbon reservoir (Adeva-Andany et al., 2016; Roach et al., 2012; Voet et al., 2013). By expressing tissue-specific enzymes for glycogen synthase (GYS) and glycogen phosphorylase (PYG), rate-limiting enzymes of glycogen synthesis and break down respectively, cells in the liver, muscle, and brain store glucose in the form of glycogen to be utilized according to their specific metabolic demands (Adeva-Andany et al., 2016; Roach et al., 2012; Voet et al., 2013). During glycogenolysis, PYG isozymes break down glycogen into glucose-1-phosphate (G1P), which is subsequently converted into glucose-6-phosphate (G6P) and can serve as a direct substrate for further catabolism via glycolysis. In this manner, glycogen-storing cells, such as those in muscle and brain tissue, can maintain intracellular glycogen reserves for cell-intrinsic metabolic requirements (Adeva-Andany et al., 2016; Voet et al., 2013). The significance of cell-intrinsic glycogen metabolism in immune cells has not been well-characterized.

We demonstrate that DCs express specific isoforms of enzymes essential for glycogen synthesis and breakdown and that these cells require glycogen metabolism to support their immune function. Although the presence of glycogen in DCs has been previously implicated (Maroof et al., 2005), the direct role for glycogen in DC

metabolism and immune function has not been described. We propose that DCs use intracellular glycogen reserves to support early glycolytic metabolism that accompanies their activation. We show that disruption of glycogen metabolism significantly impairs DC maturation and immune effector function, particularly at early stages of activation and in glucose-restricted conditions. We further show that glycogen-derived carbons preferentially contribute to the TCA-dependent citrate pool compared to glucose catabolized directly by the cell. These findings elucidate a novel metabolic regulatory pathway in DCs, and provide new insights into energy and nutrient homeostasis in these cells in support of their immune activation.

Results and discussion

DCs express glycogen metabolic machinery and utilize cell-intrinsic glycogen metabolism upon activation

TLR stimulation drives DCs to undergo glycolytic reprogramming in order to meet cellular anabolic demands associated with activation (Amiel et al., 2014; Krawczyk et al., 2010). We performed a nutrient screening assay using single-carbon-source defined media and found that DCs can catabolize both short- and long-chain glucose polymers (***Figure 3 -1*****A**). The ability of DCs to generate NADH from glycogen (***Figure 3 -1*****A**) is of particular interest given its role as the predominant form of glucose macromolecule storage in normal physiology. While cells are unlikely to encounter *extracellular* glycogen *in vivo*, these assays demonstrate that DCs exhibit the capability to catabolize glycogen and are likely to express the key enzymes of glycogen metabolism.

We analyzed mRNA levels of glycogen phosphorylase (PYG) and glycogen synthase (GYS), the rate-limiting enzymes of glycogen breakdown and synthesis pathways, respectively in DCs. Glycogenolysis is executed by three different tissue-specific PYG isozymes in mice and humans: PYGL in the liver, PYGM in muscle, and PYGB in brain tissue. Glycogen synthesis is controlled by two different tissue-specific GYS isozymes: GYS1 in muscles and other peripheral tissue, and GYS2 in the liver. Both mRNA and protein analysis in BMDCs (***Figure 3 -1*B**, ***Figure 3 -1*C**) and moDCs (***Figure 3 -1*D**) showed that DCs express PYGL and GYS1 isozymes. These enzymes were not appreciably regulated following 6hr stimulation with LPS (***Figure 3 -1*B**, ***Figure 3 -1*D**). Detection of intracellular glycogen in freshly isolated human $CD14^+$ monocytes and $CD1a^+$ dendritic cells (***Figure 3 -1*E**) indicates a physiological role for glycogen in these cells. Unactivated DCs contain intracellular glycogen pools that are fully depleted when cells are cultured in glucose-free media (***Figure 3 -1*F**), and partially depleted by LPS stimulation in BMDCs (***Figure 3 -1*G**) and moDCs (***Figure 3 -1*H**). TEM images of BMDCs show distinct glycogen deposits by tannic acid stain that are absent in cells grown without glucose (***Figure 3 -1*I**) (Afzelius, 1992).

To validate the efficacy and specificity of the PYG inhibitor, CP91149 (CP), we incubated BMDCs with CP in the nutrient screening assay (as in ***Figure 3 -1*A**) and assessed inhibition of glucose or glycogen catabolism. Glycogen-dependent NADH levels were fully attenuated in the presence of CP, while glucose-dependent NADH levels were unaffected (***Figure 3 -1J***), demonstrating the specificity of this inhibitor. PYG inhibition caused a reduction in basal glycolysis rates in unactivated BMDCs (***Figure 3 -1*K**), indicating that DCs utilize intracellular glycogen to support basal

glycolytic demands. Importantly, the effect of glycolysis inhibitor 2-deoxyglucose (2DG) was non-redundant with CP, showing that free glucose and intracellular glycogen stores make distinct contributions to DC metabolism (***Figure 3 -1*K**).

PYGL inhibition impacts DC survival in hypoglycemic conditions

Glycogen metabolism supports cancer cell growth, proliferation, and cellular lifespan (Favaro et al., 2012). We tested the effect of PYG inhibition on the survival of BMDCs at early (6hrs) and late (24hrs) time points after LPS activation. PYG inhibition resulted in modest increases in cell death at early time points under low-glucose conditions (**Figure 3 -*2*A**). This phenotype was increased after 24hrs of inhibition (**Figure 3 -*2*B**). In contrast, the viability of human moDCs was not impacted at all glucose concentrations tested (**Figure 3 -*2*C**).

Glycogen metabolism preferentially supports early DC maturation

TLR-driven early glycolytic burst is a metabolic hallmark of activated DCs (O'Neill, 2014), and both lymphoid and myeloid cells depend heavily on extracellular glucose for glycolysis-dependent effector responses (Everts et al., 2012; Krawczyk et al., 2010; Pearce and Pearce, 2013; Pearce et al., 2009). This may pose a limitation on the abundance of glucose in highly inflamed tissues and secondary lymphoid organs where DCs likely experience nutrient competition with proliferating lymphocytes (Lawless et al., 2017). We hypothesized that glycogen metabolism supports early TLR-mediated glycolysis and activation in DCs by providing an intracellular source of glucose carbons.

We examined the surface expression of CD40 and CD86 in BMDCs stimulated with LPS for 6 and 24hrs in the presence or absence of PYG inhibitor over a range of glucose concentrations representing both hyper- and hypoglycemic states. CD40 and CD86 expression was attenuated by CP treatment (**Figure 3 -*2*D** and S1a), with a more pronounced effect at 6hrs and in hypoglycemic conditions (Fig 2D and S1a). PYG inhibition with an alternative inhibitor, DAB, at 6hrs after stimulation gave similar outcomes (Fig S1b). Reduced CD40 and CD86 expression was observed both in BMDCs starved of intracellular sugar (**Figure 3 -*2*E**) and in moDCs inhibited by CP (**Figure 3 -*2*F**) further suggesting a role for glycogen pools in sustaining DC maturation. In addition, PYG-targeted siRNA was used to silence PYG expression in moDCs. As mRNA expression data indicated that both PYGB and PYGL isoenzymes are expressed in human moDCs (Fig S1c), both isoforms were silenced simultaneously in moDCs using targeted siRNA (Fig S1d). Genetic silencing of PYG in moDCs resulted in attenuation of LPS-induced expression of maturation markers (**Figure 3 -*2*G**).

While the importance of GLUT1 has been conclusively delineated in both myeloid and lymphoid immune cells (Amiel et al., 2014; Freemerman et al., 2014; Macintyre et al., 2014; Wieman et al., 2007), the kinetics of GLUT1 regulation do not account for the acute glycolytic reprogramming that occurs in activated DCs. GLUT1 upregulation in activated DCs is not detected before 6 hours of LPS stimulation (Fig S1e), which correlates with the finding that extracellular glucose is depleted only after 6hrs of stimulation (**Figure 3 -*2*H**). To confirm that the cells are less dependent on imported glucose for early activation, we assessed DC maturation at 6 and 24hrs after LPS stimulation while blocking GLUT1 activity with inhibitor STF31 (**Figure 3 -*2*I** and

S1f). In contrast to PYG inhibition, GLUT1 inhibition had a significant impact on the maturation at 24hrs but not 6hrs after activation. These data provide strong evidence that cell-intrinsic glycogen metabolism plays a central role in driving DC maturation, particularly during early time points and in glucose-restricting conditions.

PYG inhibition impacts DC immune effector function

Blocking glycolysis in TLR-activated DCs impairs their ability to produce inflammatory cytokines and stimulate T cells (Amiel et al., 2014; Krawczyk et al., 2010). We tested whether these responses are also affected by PYG inhibition. Intracellular cytokine staining for TNF-α and IL-12 after 4hrs of LPS stimulation showed that PYG inhibition attenuates inflammatory cytokine production, with a larger effect in low-glucose conditions (**Figure 3 -*3*A-B**, S2a). Multiplex cytokine analysis of LPS-stimulated DCs showed reduced pro-inflammatory cytokines and chemokine production in PYG-inhibited cells compared to controls in both BMDCs (**Figure 3 -*3*C**) and moDCs (**Figure 3 -*3*D**). CP did not globally impact all LPS-mediated protein production as other cytokines were unaffected (Fig S2b-c). siRNA-mediated knockdown of PYG expression in moDCs recapitulated the inhibitor data, as LPS-driven IL-12 production was attenuated in PYG-silenced moDCs (**Figure 3 -*3*E**).

To examine the ability of DCs to take up and process antigens, we stimulated BMDCs with LPS plus OVA-AF488 or OVA-DQ for 3 hours (**Figure 3 -*3*F**). OVA-AF488 allows tracking of antigen uptake, while OVA-DQ only fluoresces upon antigen uptake and processing. PYG-inhibited DCs showed reduced antigen uptake regardless of LPS stimulation (**Figure 3 -*3*F** while antigen processing was unexpectedly enhanced by

PYG inhibition. We next tested the effect of PYG inhibition or silencing on DC ability to stimulate $CD4^+$ T cells. PYG-inhibited BMDCs exhibited significantly reduced capacity to stimulate T cells (**Figure 3 -*3*G** and **H**). PYG-silenced moDCs exhibited similar impairments in $CD4^+$ T cell stimulation (**Figure 3 -*3*I**). These data demonstrate that cell-intrinsic glycogen metabolism contributes to the regulation of the multifaceted dimensions of DC immune effector function.

Glycogen-derived carbons fuel both glycolytic reprogramming and mitochondrial respiration in activated DCs

We proposed that glycogen-derived glucose drives early glycolytic flux in TLR-activated DCs prior to GLUT1 upregulation. To test this, we performed a real-time extracellular flux analysis on BMDCs and moDCs. LPS-driven glycolytic burst was significantly attenuated by PYG inhibition in both BMDCs (**Figure 3 -*4*A**, S3a) and moDCs (**Figure 3 -*4*B**). We further tested whether cell-intrinsic glycogen metabolism also contributes to mitochondrial respiration during early activation. Concomitant with glycolysis reduction, PYG inhibition attenuated the oxygen consumption rate (OCR) in BMDCs regardless of activation (**Figure 3 -*4*C**). These data suggest that pre-existing glycogen pools contribute metabolic substrates for mitochondrial respiration. Consistent with this, PYG inhibition accelerates LPS-mediated ATP depletion during early activation in a time-dependent manner (**Figure 3 -*4*D**). The synergistic effect of combined CP and ATP-synthase inhibitor oligomycin in reducing ATP production (**Figure 3 -*4*E**) indicates that glycogen catabolism contributes to both cytosolic and mitochondrial ATP generation. These findings indicate the intriguing possibility that

there may be distinct roles for glucose and glycogen-derived carbon molecules in DC metabolism.

Since PYG inhibition resulted in reduced intracellular ATP levels (**Figure 3 -*4*D & E**), we assessed the effect of PYG inhibition on the activation of AMPK, a key metabolic sensor of intracellular nutrient and ATP levels (Hardie et al., 2012). PYG inhibition resulted in increased phosphorylation of AMPK (Fig S3b), which is reported to antagonize BMDC activation (Krawczyk et al., 2010). This is consistent with reports showing that inhibition of glycolysis induces compensatory activation of AMPK (Wang et al., 2011; Wu et al., 2015). However, PYG inhibition had no impact on LPS-mediated GLUT1 upregulation (Fig S3c), suggesting that AMPK regulation of glucose transport is not a significant mechanism at play in our model. Nevertheless, LKB1 deficient BMDCs, which are incapable of activating AMPK, show decreased sensitivity to PYG inhibition during maturation at normal glucose concentrations, suggesting that AMPK compensatory activation during PYG inhibition may be involved in regulating maturation in these conditions (Fig S3d).

Previous work has demonstrated that glucose consumed by activated DCs enters the TCA cycle to generate citrate, which is preferentially translocated from the mitochondria into the cytosol via the citrate shuttle to support *de novo* fatty acid synthesis. This process is linked to ER and Golgi membrane expansion, which is hypothesized to enhance the production of effector molecules central to DC activation (Amiel et al., 2014; Rehman et al., 2013). To examine the role of glycogenolysis in citrate metabolism explicitly, we performed metabolic tracing experiments in which BMDCs were differentiated in ^{13}C-labeled glucose to label all intracellular metabolites.

Cells were subsequently switched to normal glucose at the time of LPS stimulation in the presence or absence of CP for 1 and 3hrs. As previously published, LPS stimulation induces substantial metabolic flux through glycolysis and TCA citrate production ((Everts et al., 2014) and data not shown). PYG inhibition significantly reduced ^{13}C-labeled citrate while no statistically significant impact on hexose phosphate, pyruvate, lactate, and post-citrate metabolites fumarate and malate was observed (**Figure 3 -*4*F** and S3e). Hexose phosphate refers to any 6-carbon sugar since our metabolite tracing approach could not distinguish individual sugars among this group. These data indicate that intracellular glycogen reserves preferentially support the generation of citrate following LPS stimulation.

Glutamine can also serve as an important carbon source for the TCA cycle. However, the findings that nearly the entire glutamine pool is derived from ^{12}C-labeled sources (Fig S3f) and that CP has very little effect on glutamine levels (Fig S3f), suggests that glutamine metabolism is not directly impacted by PYG inhibition. This is further supported by observations that: 1) CP attenuates the maturation of BMDCs stimulated in the presence or absence of glutamine (Fig S3g); and 2) glutaminolysis inhibitor DON has no significant impact on glycolytic burst or OCR (Fig S3h).

To identify the role of glycogen metabolism in regulating extracellular glucose flux, the reverse metabolomics experiment was performed in which BMDCs differentiated in normal glucose were switched to ^{13}C-glucose at the time of LPS activation and analyzed at 3 and 6 hours post stimulation (**Figure 3 -*4*G**). PYG inhibition minimally affected the ^{13}C-glucose contribution to cytoplasmic hexose phosphate, lactate,

and pyruvate, while it severely attenuated both ^{12}C and ^{13}C-glucose contributions to citrate production (**Figure 3 -*4*G**).

The metabolite tracing data are consistent with previously published work (Everts et al., 2014), in which extracellular glucose contributes heavily to cytoplasmic glycolytic metabolites and citrate production from the TCA cycle (**Figure 3 -*4*F-G**). However, these data also uncover two previously unappreciated aspects of glucose metabolism in DCs: 1) glycogen-derived carbons from basal glycogen stores (CP-sensitive ^{13}C metabolites in **Figure 3 -*4*F**) preferentially support initial glycolytic intermediates and citrate synthesis; 2) a significant amount of glucose imported from the extracellular environment gets rapidly converted into glycogen (CP-sensitive ^{13}C metabolites in **Figure 3 -*4*G**). The finding that extracellular ^{13}C-glucose incorporation into citrate (**Figure 3 -*4*G**), succinate, fumarate, and malate (Fig S3i) is sensitive to PYG inhibition suggests that a significant portion of extracellular glucose destined for mitochondrial oxidation is metabolically routed via a glycogen-dependent pathway during DC activation. The routing of glucose carbons via a rapid sequence of glycogen synthesis and glycogenolysis is characteristic of a metabolic pathway described in astrocytes and muscle cells as the "glycogen shunt" (Shulman et al., 2001; Shulman and Rothman, 2001). Our metabolic profiling studies support a model where glucose processing in TLR-stimulated DCs undergoes three functionally distinct pathways: 1) the catabolism of pre-activation intracellular glycogen stores; 2) the catabolism of imported glucose directly; 3) the incorporation of imported glucose into synthesis and breakdown of glycogen via the glycogen shunt (modeled in Fig S4).

While the glycogen shunt is clearly inefficient from an energetic perspective, others have argued that glycogen breakdown and synthesis may occur in separate spatial pools within brain and muscle cells to fuel rapid bursts of metabolic activity required in these cells that override the total energetic cost of this process (Calder and Geddes, 1992; Elsner et al., 2002; Obel et al., 2012). DCs may employ a similar strategy of compartmentalized glycogen metabolism in order to fuel early immune activation. However, how this occurs and how it may be regulated in DCs remains an important question. Precedent for distinct and parallel sugar metabolism has been previously reported, whereby granulocyte phagocytic capability is driven by glycogen-derived carbons, while their chemotaxis is fueled by catabolism of free glucose carbons (Weisdorf et al., 1982). We propose that the source of carbons in activated DCs, namely whether it is glucose or glycogen –derived, may dictate differential functional responses. We speculate that spatial compartmentalization of these processes in the cytoplasm may be an important component of how glycogen metabolism is regulated.

While glycogen metabolism has been previously implicated in myeloid cells of the immune system (Maroof et al., 2005; Scott, 1968; Weisdorf et al., 1982; Yunis and Arimura, 1964, 1966), the role of glycogen metabolism in specific immune effector functions of DCs has not been previously defined. We show here a definitive role for glycogen metabolism in regulating immune effector functions of both human and mouse DCs. We further demonstrate that glucose- and glycogen-derived carbons exhibit distinct metabolic fates, a phenomenon that we suspect is not DC-specific and likely occurs in other cells that utilize cell-intrinsic glycogen metabolism. Ongoing studies are focused on elaborating the mechanistic details of how glycogen-dependent compartmentalization of

metabolic pathways occurs in response to different immune stimuli. With a growing interest in understanding how metabolic regulation controls the functional effector responses of immune cells, this work delineates an intricate and novel layer of complexity to how metabolic pathways operate at a subcellular level, which may be exploited in cell-based therapeutic applications in the future.

Author Contributions

Conceptualization, P.T. and E.A.; Methodology, P.T., A.D., B.E., and E.A.; Formal Analysis, P.T., J.R., and A.D.; Investigation, P.T., L.P, R.C., B.E., S.B., J.R., A.D., and E.A.; Writing – Original Draft, P.T. and E.A.; Writing – Review & Editing, P.T., A.D., B.E., and E.A.; Funding Acquisition, E.A.; Resources, A.D., B.E., and E.A.; Supervision, A.D., B.E., and E.A.

Acknowledgements

The authors would like to acknowledge the UVM core facilities (Flow Cytometry, Animal Resource, Microscopy Imaging, and Advance Genome Technology Cores) for services provided in support of this work. Special acknowledgement to Dr. Ralph Budd, Dr. Paula Deming, and the VCIID COBRE for extensive support. Funding Sources: AD is a recipient of the Boettcher Webb-Waring Biomedical Research - Early Career grant; Veni Fellowship NWO (BE), LUMC fellowship (BE), UVM start-up Funds (EA), P30GM118228 (EA).

Experimental Procedures

Mice

C57/Bl6J and OT-II (B6.Cg-Tg (TcraTcrb)425Cbn/J mice were purchased from Jackson Laboratory and bred in-house and maintained at the University of Vermont animals care facility under protocols approved by Institutional Animal Care and Use Committee.

Itgax^{cre} LKB1$^{fl/fl}$ mice PubMed: 21124450 were housed and bred at the LUMC, Leiden, Netherlands, under SPF conditions. All animal experiments were performed in accordance with local government regulations, and the EU Directive 2010/63EU and Recommendation 2007/526/EC regarding the protection of animals used for experimental and other scientific purposes and approved by the CCD, animal license number AVD116002015253.

Mouse DC culture and Activation

Bone marrow-derived DCs (BMDCs) were generated as follows: BM cells were flushed from femurs of 9-18-week-old mice and the cells were differentiated in GM-CSF (20ng/mL; Peprotech) in complete DC medium (CDCM), comprised of RPMI1640, 10% FCS, 2mM L-glutamine, 1IU/mL Pen-Strep, 1mM beta-mercaptoethanol, for 7 days, with a medium change every 2 days. On day 7, DCs were washed in CDCM and cultured at 2x10^5 cells per 200μL of media alone, STF31 (12.5 μM), CP91149 (75-100μM), DAB (1mM), LPS (100ng/mL), LPS plus STF31 or CP91149, or DAB, or OVA (from whole egg white) at indicated time points. Where appropriate, DCs were stimulated in CDCM containing 0mM, 1.25mM, 2.5mM, or 5mM glucose.

Glucose starvation experiment

BMDCs were starved for glucose overnight, with a non-starved group as a control. On the next day, DCs from both groups were washed with sugar free RPMI and stimulated with LPS in glucose free medium ±CP for 6hours. CD40 and CD86 expression was analyzed by Flow cytometry.

Human DC culture and activation

Human monocyte-derived DCs (moDCs) were differentiated from peripheral blood monocytes as follows: Blood filters from de-identified blood donors were provided by CVPH Medical Center Blood Bank in Plattsburgh, NY. Filters were reverse-flushed in sterile PBS, and PBMCs were prepared by Ficoll-Paque (density gradient of 1.0772) centrifugal separation using LSM media (MP biochemical; Fisher). Resulting monocytes were enriched using CD14 positive selection beads per manufacturer instructions (Miltenyi Bioscience) and cultured in complete DC medium (CDCM) supplemented with human recombinant GM-CSF (20ng/mL) plus human recombinant IL-4 (20ng/mL) (Peprotech) for 7 days. On day 7, moDC were harvested, stimulated as indicated, and analyzed by FACS for maturation and by multiplex panels (Life Technologies) for cytokine production.

Quantitative Real-time PCR of *pygl, pygm, gys1*, and *gys2* expression

RNA was isolated with an RNAeasy Kit (Qiagen) and cDNA was synthesized with an iScript cDNA Synthesis Kit (Biorad). *pygl*, *pygm*, *gys1*, *gys2*, and *slc2a1* Taqman primer probes (Applied Bioscience system) and AB7500 sequence detection system or QuantStudio 3.0 were used for relative mRNA expression. mRNA relative quantitative values were calculated based on 2(-ΔΔCT) and normalized to untreated samples.

Glycogen phosphorylase knockdown by siRNA transfection of moDC

For knockdown of glycogen phosphorylase isoforms, moDCs were generated as mentioned above. At day 4 of the culture, the cells were harvested, washed with PBS, brought to a concentration of 1x10^6 cells / 100 µL resuspension buffer, and finally, transfected by electroporation with either 10 nM anti-PGYL siRNA in combination with 10 nM anti-PYGB siRNA or 20 nM scrambled siRNA (Dharmacon). Electroporation was performed using a Neon Transfection System (Invivogen) with the following settings: 1600 V, 20 ms and one pulse. Immediately after electroporation, 1x10^6 cells were taken up in 5 mL 10% HI-FCS basal media, containing no antibiotics, and plated at 200 cells / µL. The next morning, the media was re-supplemented with penicillin, streptomycin, rGM-CSF and rIL-4. At day 6, the cells were harvested, stimulated as indicated, and analyzed by FACS for maturation and by ELISA for cytokine production. Silencing efficiency was determined by qPCR on 6 day-old cells. The transfection efficiency was routinely greater than 80%.

Antigen Uptake, Processing, and in-vitro T cell responses

BMDCs were stimulated ±LPS with OVA-AF488 (5µg/mL) and OVA-DQ (5µg/mL) for antigen uptake and processing, respectively. For *in vitro* T cell responses, T cells were generated using mouse CD4 positive selection beads from spleens of 6-10-week-old transgenic OT-II mice and age-matched Wild-type B6 mice. BMDCs were pulsed with whole Ovalbumin protein (OVA), extracted from egg white, and LPS in the presence or absence of CP for 6 hours, washed 3 times, and co-cultured with CFSE-labeled OT-II T cells at a 1:5 ratio for 72 hours. T cell proliferation (CFSE dilution) was analyzed by flow cytometry.

For alloreaction studies of siRNA transfected moDC, the cells were washed 2 times, and co-cultured with CellTrace Violet-labelled human naïve $CD4^+$ T cells, which were isolated using a naïve pan T cell isolation kit (Miltenyi) followed by negative selection using CD8 MicroBeads (Miltenyi), at a 1:4 ratio for 4 days. T cell proliferation was analyzed by flow cytometry.

Metabolism assays

Extracellular glucose and intracellular glycogen levels were measured with a Glucose assay kit (Eton Biosciences) and a Glycogen assay kit (Biovision), respectively. For Biolog assays, (Metabolic phenotypic screening assays), IFM-1 reagent, Biolog MA redox dye, and Biolog plates were purchased from Biolog Inc. Fully differentiated BMDC were plated overnight at 50,000 cells per well in specified nutrient sources in basal MC-0 medium (IFM1 media with 5%FCS, 0.3mM L-glutamine, 100I/U Pen Strep). 20μL of Biolog MA dye was added to each well the next morning plates measured at 592 nm absorbance as indicated. Data were normalized to the readings at time 0. Extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were measured with Metabolic Flux Analyzer (Seahorse Bioscience, North Billerica, MA 24XP and/or 96XP). ATP concentrations were measured with an ATP Determination Kit (Invitrogen) according to the manufacturer's instructions.

BMDC cultures and activation for metabolomics

For metabolomics tracing in Fig 4F, BMDCs were differentiated in $^{13}C_6$-glucose containing CDCM. On day 7, the cells were switched to $^{12}C_6$-glucose medium, with LPS and LPS+CP added at the time of media switch and stimulated for 1 and 3 hours. At each time point, cells were harvested, counted, pelleted, and frozen for the UHPLC-MLS

metabolomics processing below. For inverse metabolomics in Fig 4G, BMDCs normally differentiated in regular CDCM were switched to $^{13}C_6$-glucose medium at the time of stimulation, with and without CP for 3 and 6 hours. Cells were harvested and processed as above. Supernatant from the 6 hour stimulation groups was collected for Multiplex Cytokine analysis.

UHPLC-MS metabolomics

Frozen cell pellets were extracted at 2x10^6 cells/mL in ice cold lysis/extraction buffer (methanol:acetonitrile:water 5:3:2). Samples were agitated at 4 °C for 30 min followed by centrifugation at 10,000 *g* for 10 min at 4 °C. Protein and lipid pellets were discarded, and supernatants were stored at -80 °C prior to metabolomic analysis. Ten µL of extracts were injected into an UHPLC system (Vanquish, Thermo, San Jose, CA, USA) and run on a Kinetex C18 column (150 x 2.1 mm, 1.7 µm – Phenomenex, Torrance, CA, USA) at 250 µl/min (phase A: Optima H_2O, 0.1% formic acid; phase B: acetonitrile, 0.1% formic acid). The autosampler was held at 7 °C and the column compartment at 25 °C. The UHPLC system was coupled online with a Q Exactive mass spectrometer (Thermo, Bremen, Germany), scanning in Full MS mode (2 µscans) at 70,000 resolution in the 60-900 m/z range in negative and then positive ion mode (separate runs). Eluate was subjected to electrospray ionization (ESI) with 4 kV spray voltage, 15 sheath gas and 5 auxiliary gas. Metabolite assignments and isotopologue distributions were determined using the software Maven (Princeton, NJ, USA)[1], upon conversion of .raw files into .mzXML format through MassMatrix (Cleveland, OH, USA). Chromatographic and MS technical stability were assessed by determining CVs for heavy and light isotopologues in a technical mixture of extract run every 10 injections. Relative quantitation was performed by exporting the

values for integrated peak areas of light metabolites and their isotopologues into Excel (Microsoft, Redmond, CA, USA) for statistical analysis including t-test and ANOVA (significance threshold for *p-values* < 0.05).

Electron Microscopy

Samples were fixed in Karnovsky's Fixative for 1hr at 4°C, washed in 0.1M Cacodylate Buffer, and post-fixed in 1% OsO_4 for 1hr at 4°C followed by an extensive rinse with Cacodylate buffer. Samples were then dehydrated in a graded series of ethanol, and embedded in Spurr. Sections were cut with a Reichert Ultracut Microtome and stained with toluidine blue. For contrast, 1% tannic acid was added to the cut sections of the grids for 10 minutes, followed by 6 minutes of uranyl acetate and 4 minutes of lead citrate. Cells were examined with a JEM1400 transmission electron microscope (JEOL USA).

Immunoblot analysis

Cell lysates were prepared using 2X NP-40 lysis buffer. 20μg protein was loaded into each well of a 12.5% polyacrylamide gel, transferred onto activated nitrocellulose membrane (BioRad). Electrophoretic transfer was performed using Trans-Blot Turbo RTA mini Nitrocellulose transfer kit. Membranes were blocked in 2%milk in 1xTBST at RT for 1hr, and incubated in indicated antibody at 4°C overnight. Blots were washed 3x in 1xTBST at RT, probed with secondary antibodies at RT for 45-60 minutes, and washed 3-4x with 1xTBST. Proteins were visualized by SuperSignal West Pico Chemiluminescent substrate and exposed with GeneXpert System imager. Trans-Blot Turbo Transfer system and secondary antibodies for Western blots were generously

provided by Dr. Paula Deming, Medical Laboratory and Radiation Science Department, UVM.

Flow Cytometry and Cytokine measurements

The following fluorescently labeled antibodies were used for flow cytometry: anti-CD11c (N418), anti-CD40 (3/23), anti-CD86 (GL1), IA-b (AF6-120.1), anti-CD1a (HI149), anti-CD40 (5C3), anti-CD86 (IT2.2), anti-TNFα (MP6-XT22), anti-IL-12p40 (C15.6). Stimulated cells as indicated were harvested and washed in 1% FACS buffer (PBS plus 1% FBS), stained with specific antibodies, and incubated on ice for 30 minutes. All samples were acquired using a LSRII flow cytometer (BD Biosciences). For intracellular cytokine expression, cells were activated with indicated treatment groups for a total of 4 hours with an addition of Golgi plug (1:1000) (Biolegend) after the first hour of stimulation. For intracellular staining of TNF-α and IL-12 (Biolegend), cells were fixed in 4% Paraformaldehyde, permeabilized in 0.2% saponin, and stained with antibodies in FACS buffer (1%FBS in PBS). Samples were analyzed on a BD LSRII flow cytometer. For cytokine levels of BMDCs and moDCs, supernatants were collected as indicated time points and measured with Mouse Cytokine Magentic 20-Plex and Human Cytokine Magnetic 30-Plex panels (Life Technologies) per manufacture instructions using Bio-Plex array suspension system.

Statistical Analysis

Data were analyzed with GraphPad Prism software (version 6.0). Samples were analyzed using Student's *t*-test, One-way, and Two-way ANOVA where appropriate. ANOVA tests were post-calculated by Tukey's multiple comparison test or Sidak test.

Results are means +SD as indicated, and statistical values are represented significant when *p* values were equal or below 0.05.

Figures

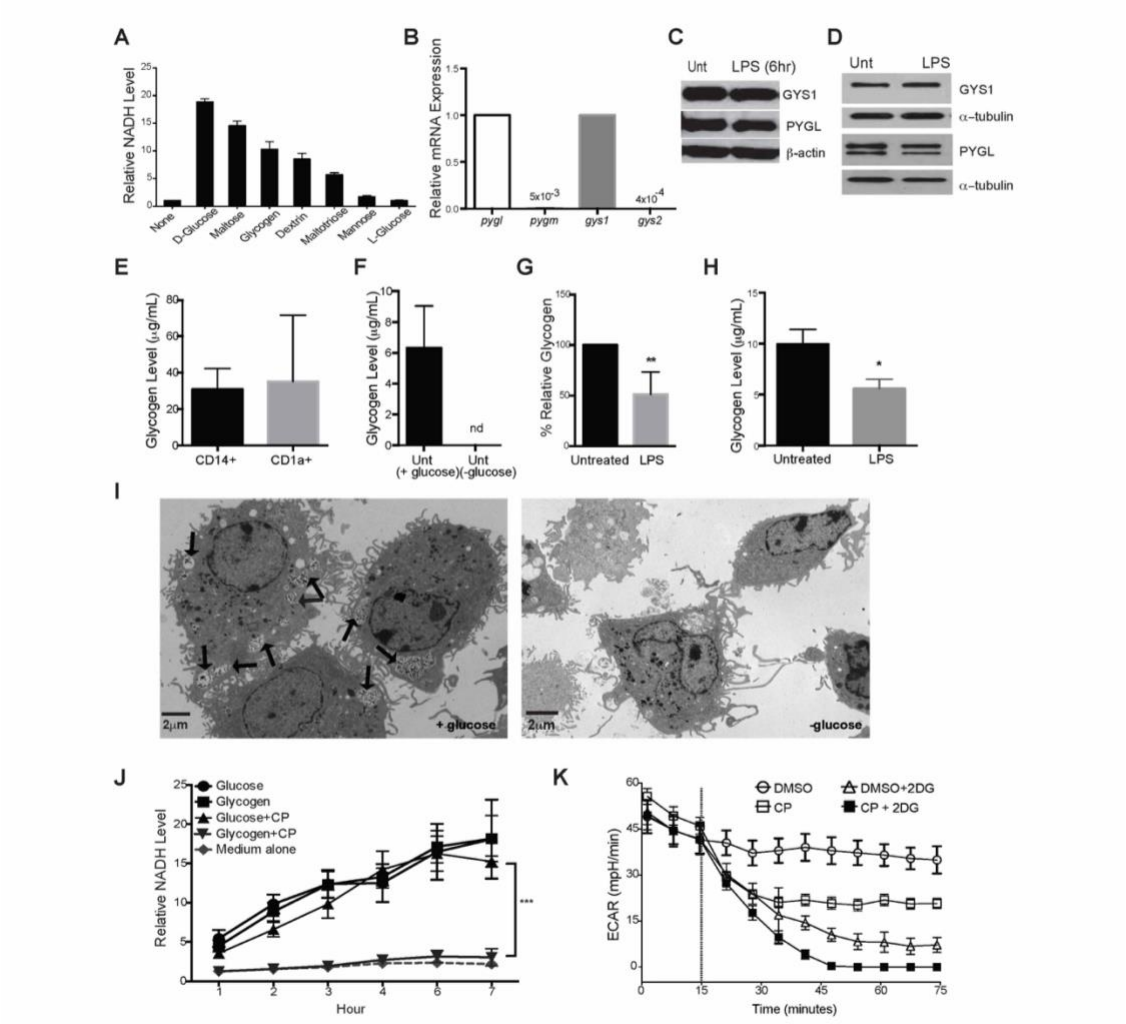


Figure 3 -1*: DCs utilize intracellular glycogen metabolism upon LPS stimulation.*
(A) BMDCs were cultured in the indicated substrates as the sole nutrient sources and measured for ability to produce NADH as described in the methods. Data indicate relative NADH production at 6 hrs normalized to no carbon source controls, n=3. (B) Relative mRNA expression of pyg and gys isoforms in naive BMDCs. (C-D) PYGL, GYS1, and β-actin protein expression in unactivated and 6hr LPS-stimulated BMDCs (C) and 24hr LPS-stimulated moDCs (D). (E-H) Intracellular glycogen levels of: human peripheral blood $CD14^+$ monocytes and $CD1a^+$ DCs (E), untreated BMDCs cultured overnight in ±glucose (F), BMDCs (G) and moDCs (H) stimulated ±LPS in 5mM glucose (n=3-6, mean ±SD, student's t-test, *P<0.05, ** P=0.0021, nd= not detected). Glycogen levels were normalized to 10^5 cells. (I) TEM images of unactivated BMDCs in 5mM glucose (left) and 0mM glucose (right), with arrows indicating intracellular glycogen deposits identified by tannic acid staining. (J) NADH levels over time in BMDCs

cultured in glucose or glycogen containing media (as in A) ±CP (n=4, mean±SD, ***P <0.0001). (K) Basal ECAR of resting BMDCs treated with CP, 2DG, or both (treatment introduced at dotted line), representative of at least 3 replicates.

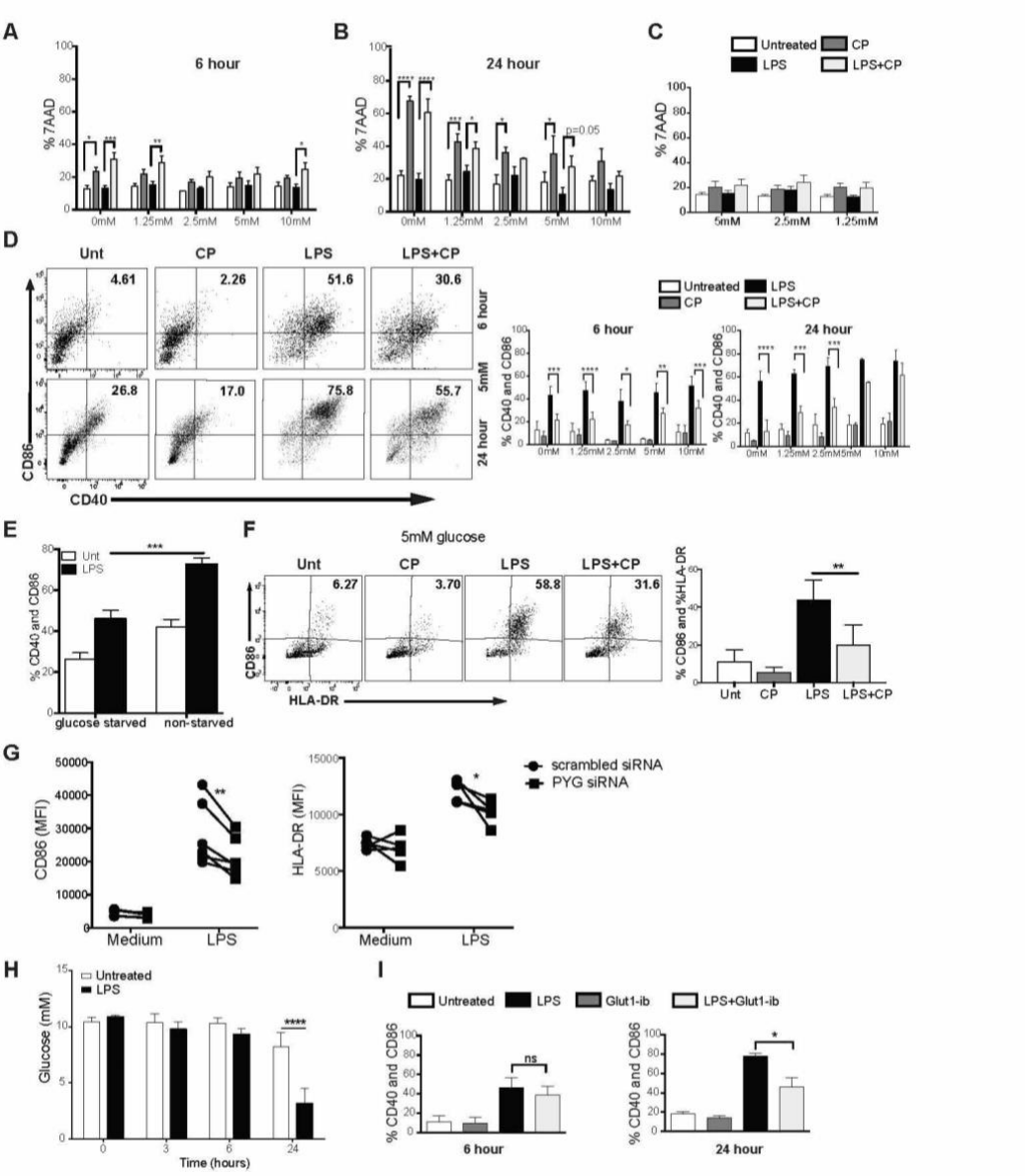


Figure 3 -2: Glycogen metabolism supports survival and early maturation of TLR-activated DCs.

(A-B) 7AAD viability staining of BMDCs stimulated with LPS ± CP for 6hrs (A) and 24hrs (B) at 5mM glucose. (C) 7AAD viability staining of moDCs stimulated with LPS ± CP for 24hrs. (D) BMDCs were stimulated for 6 and 24 hours and analyzed for CD40 and CD86 surface expression. (E) CD40 and CD86 expression of BMDCs stimulated for 6 hrs in free glucose medium with and without glucose starvation. (F) CD86 and HLA-DR expression of moDCs stimulated with LPS±CP for 24hrs in 5mM glucose. (G) CD86 and HLA-DR surface expression of 24hr LPS-stimulated moDCs silenced with control (scrambled) or PYG-targeted siRNA. (H) Glucose measurements from supernatant of

BMDCs stimulated with LPS for 3, 6, and 24 hrs. (I) CD40 and CD86 surface expression of BMDCs stimulated ±GLUT1-inhibitor in normal glucose for 6 and 24hr. (A-F, H-I) n=3-6, mean±SD, Two-way ANOVA with Tukey Post-test, *$P \leq 0.05$ ***$P = 0.0006$ ****$P < 0.0001$. (G) n=5, Paired *t*-test, *$P = 0.04$, **$P = 0.0093$.

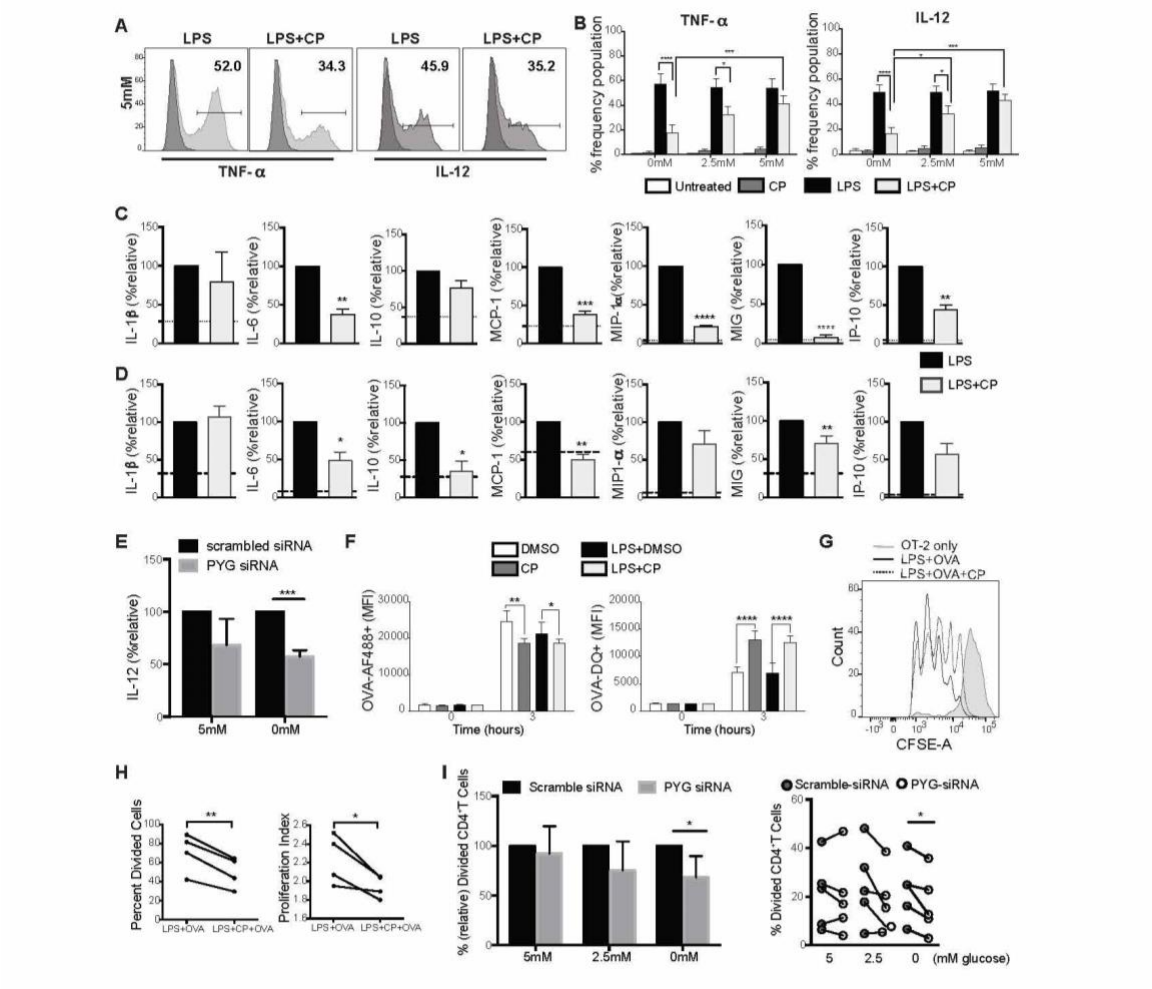


Figure 3 -3: PYGL inhibition attenuates immune effector functions of DC.

(A-B) Intracellular staining of TNF-α and IL-12 of BMDCs stimulated with LPS for 4 hrs in 5mM glucose. (C-D) Multiplex panels of cytokine and chemokine measurements from the supernatant of BMDCs (C)and moDCs (D)activated with LPS for 6 hrs. Dotted lines represent unstimulated levels. (E) Relative IL-12 production by moDCs LPS-stimulated for 24hrs transfected with control or PYG-targeted siRNA. (F) BMDCs treated with LPS ±CP plus OVA-AF488 or OVA-DQ for 3 hours and analyzed by flow cytometry for antigen uptake and processing. (G) BMDCs were pulsed for 6 hours with indicated treatments and subsequently co-cultured with CFSE-labeled OT-II Tcells. CFSE dilution was measured on day 3 post co-culture. (H) Measurements of proliferation of OT-II T cells (from (G)) stimulated by BMDCs pre-treated with indicated conditions. (I) si-RNA transfected moDCs were co-cultured with CellTrace Violet –labeled human naïve $CD4^{+}$T cells for 4 days. Data were normalized to scrambled siRNA. Proliferation was measured after 4 days. (A-I) n=3-5, mean±SD, (B, F) 2-way ANOVA Tukey Posttest. (C-E, H-I) student's *t-test*, $*P \leq 0.05$, $**P < 0.001$, $***P = 0.0004$, $****P < 0.0001$.

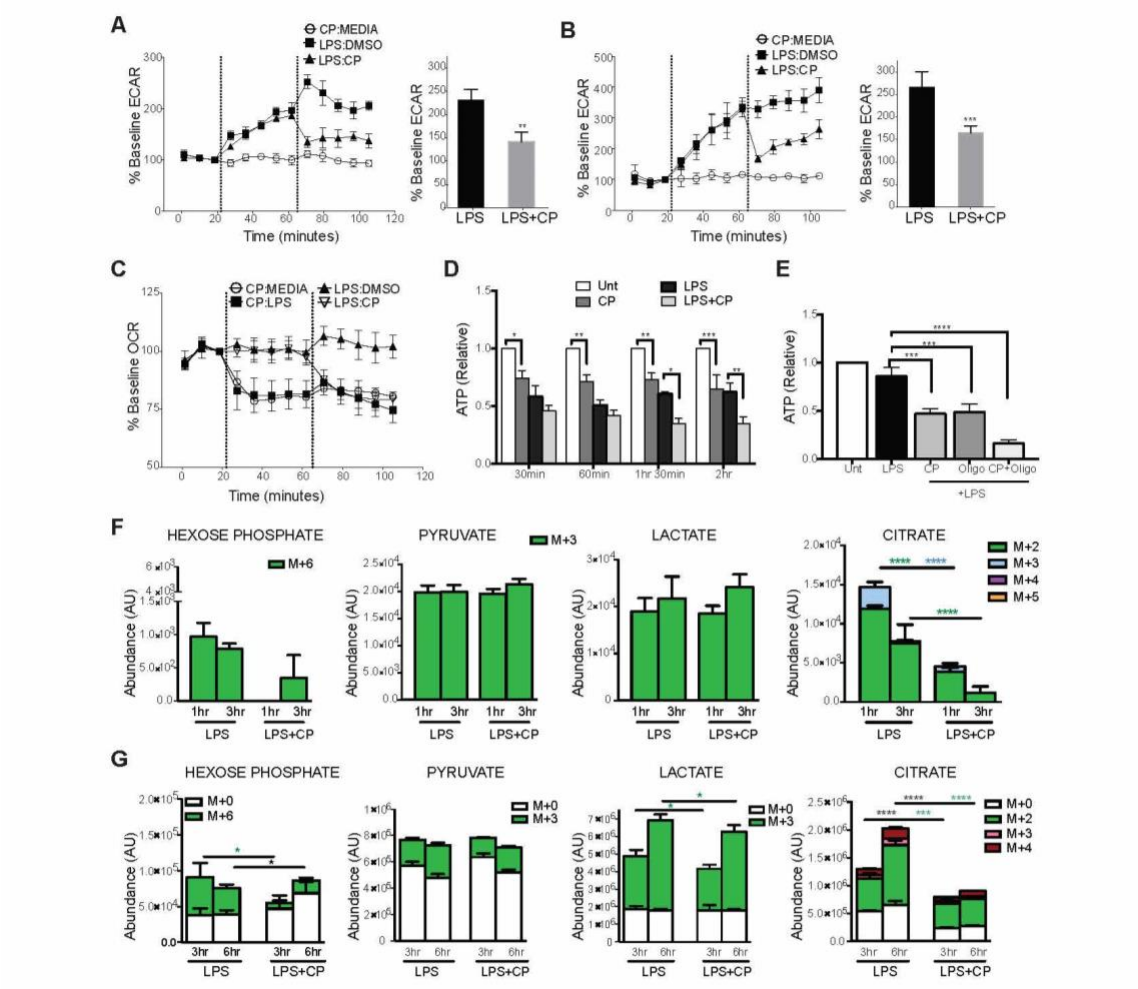
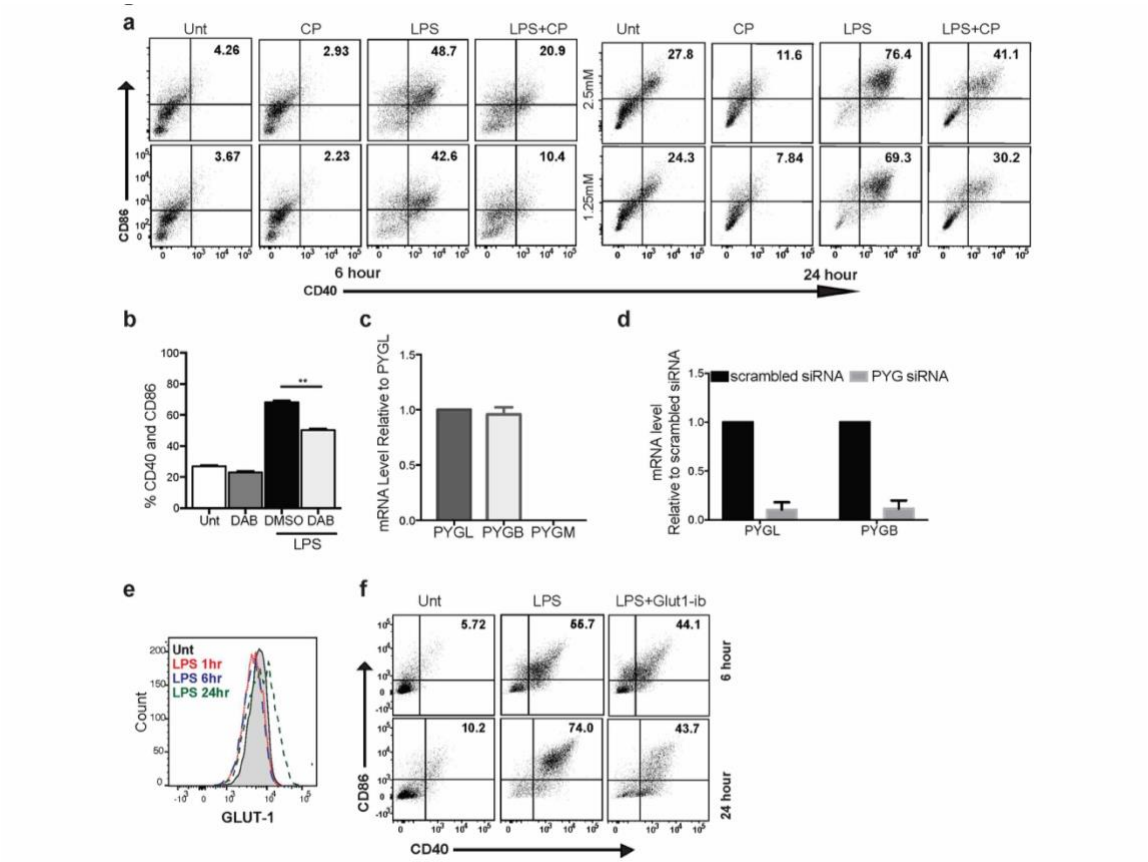


Figure 3 -4: Glycogen-derived carbons fuel early glycolytic reprogramming and mitochondrial respiration in activated DCs

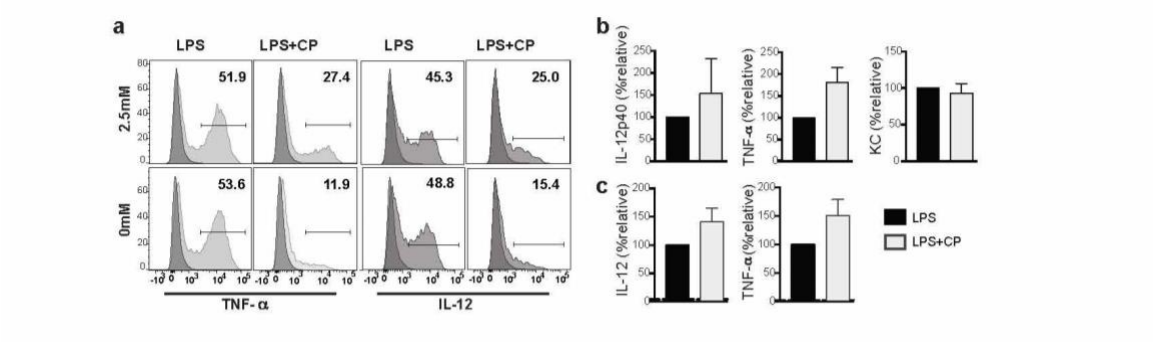
(A-B) Real-time changes in ECAR of BMDCs (A) and moDCs (B). (C) Real-time changes in OCR of BMDCs. (A-C) treatments introduced at dotted lines (1st injection: 2nd injection). (D-E) ATP levels of BMDCs in 30 minute intervals (D) and at 2 hrs (E) after stimulation with indicated treatments. (F) BMDCs cultured and differentiated in $^{13}C_6$-glucose were switched to normal glucose at the time of stimulation with LPS±CP for 1 and 3 hours and ^{13}C-labeled metabolites were detected by LC-MS spectrometry. (G) Inverse metabolomics of F, where BMDC were differentiated in normal ^{12}C glucose and switched to $^{13}C_6$-glucose at the time of stimulation with LPS±CP for 3 and 6hrs. Data represent n=4, mean ±SD, (A-B) paired students *t*-test. (D-G) n=5, Two-way ANOVA, Tukey Posttest. **P*<0.05, ***P*<0.005, ****P*<0.0005, *****P*<0.0001. (F-G) Statistical significance of each color * represents color-coded $^{13}C_6$- or black * for ^{12}C groups. White bars indicate ^{12}C-glucose and all color bars denote $^{13}C_6$-glucose.

Supplemental Figures



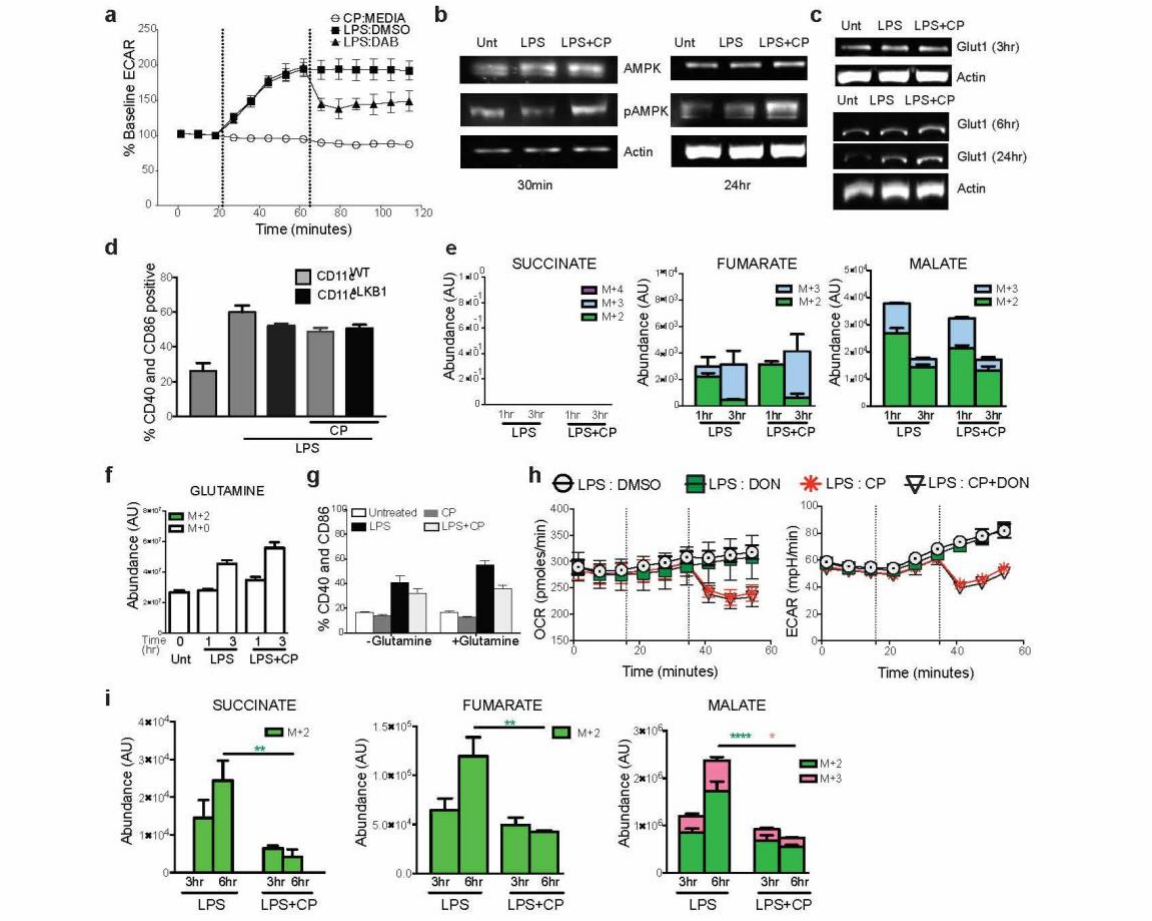
Supplemental Figure 1: (Related to Figure 3.1 and Figure 3.2)

(a) BMDCs stimulated with LPS+/-CP for 6 and 24hours in indicated glucose concentrations were analyzed for CD40 and CD86 surface expression. (b) CD40 and CD86 expression of BMDCs stimulated with LPS+/-DAB for 6hours in normal glucose concentration. (c) Intracellular stain of Glut-1 expression in BMDCs stimulated for 1, 6, and 24hrs. (d) CD40 and CD86 surface expression of BMDCs stimulated with LPSGlut1-ib for 6 and 24hours in normal glucose concentration. (e) mRNA expression of 3 PYG isoforms; data normalized to PYGL; n=6. (f) Relative mRNA level for confirmation of knockdown of pygl and pygb by si-RNA transfection of moDCs. (a, c-d) representative of more than 3 experiments. (b) n=4, mean+/-SD, One-way ANOVA with Sidak Post-test, **P=0.0038.

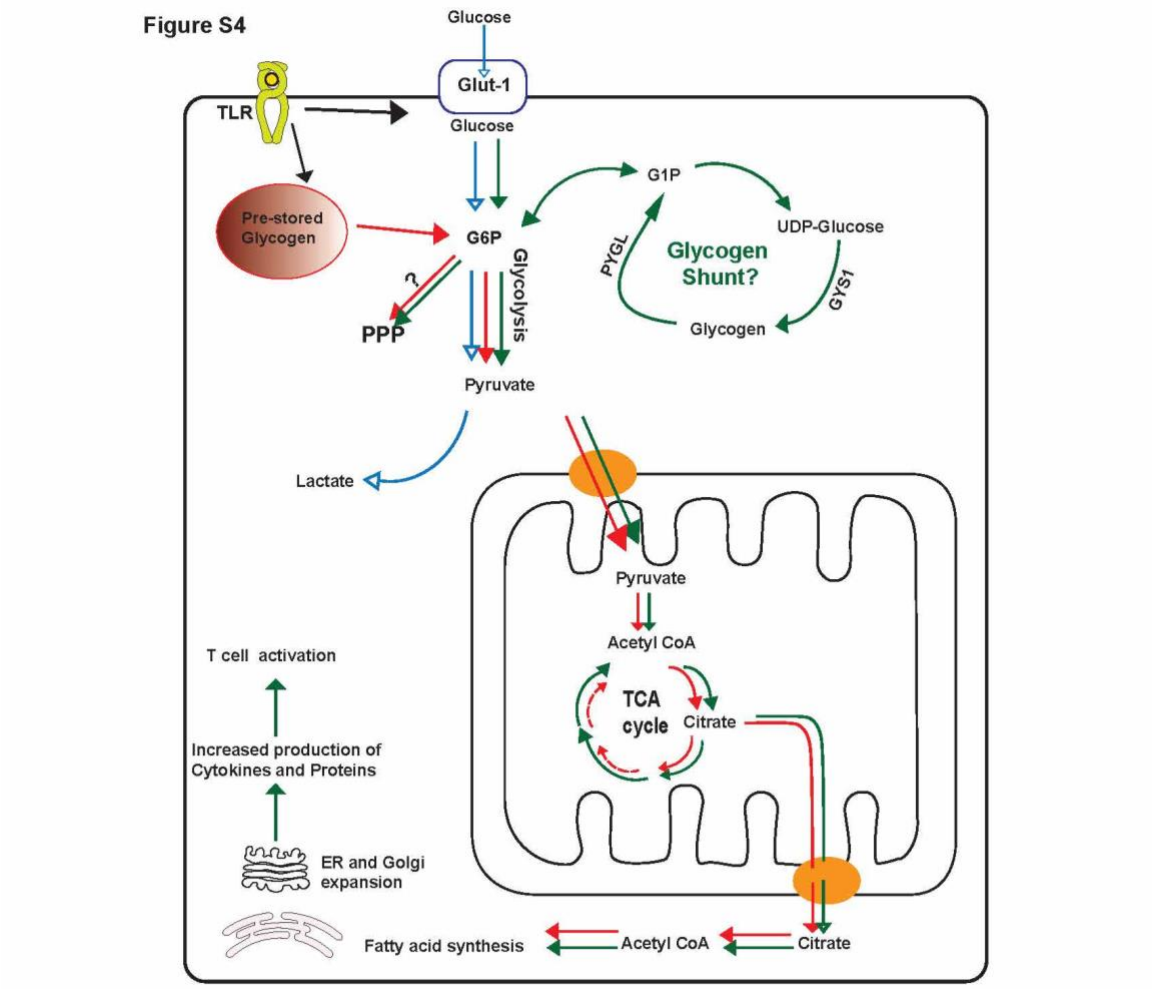


Supplemental Figure 2: Related to Figure 3.3

(a) Intracellular staining of TNF-α and IL-12 of BMDC stimulated with LPS ± CP for 4 hours, with the last 3.5 hours in Golgi plug reagent, in 2.5 and 0mM glucose. Data are from one experiment representative of four. (b-c) Multiplex panel of cytokine and chemokine measurements from the supernatant of (b) BMDC and (c)moDC activated with LPS ± CP for 6 hours. n=4, mean±SD, student's *t*-test.



Supplemental Figure 3: (Related to Figure 3.4)
(a) Real-time changes in ECAR of BMDCs using an alternate PYGL inhibitor, DAB; treatments introduced at dotted lines (1st injection: 2nd injection). Representative of 3 experiments. (b, c) BMDCs differentiated in 13C6-glucose were switched to normal glucose for stimulation with LPS+/-CP for 1 and 3hrs and detected by LC-MS spectrometry. Color bars denote 13C6-glucose whereas white bars represent light glucose. (d) CD40 and CD86 expression was analyzed in BMDCs stimulated for 6hours with LPSCP in the presence or absence of glutamine in the medium. n=6 (e) Real-time OCR and ECAR with CP, glutaminolysis inhibitor (DON), and combination; treatment injected at dotted lines (1st injection**:** 2nd injection). (f) Protein expression of AMPK and pAMPK in BMDCs stimulated with LPS+/-CP for 30 minutes and 24hours in normal glucose. (g) Protein expression of Glut1 in BMDCs stimulated with LPS CP for 3, 6, and 24hours in normal glucose. (h) Surface expression of CD40 and CD86 of BMDCs from WT or LKB1-/- mice stimulated with LPS+/-CP for 6 hrs. (i) Inverse metabolomics of b and c, where BMDCs were stimulated in $^{13}C_6$-glucose medium for 3 and 6hrs and selected TCA intermediates were shown. Data represents n=4, mean+/-SD, Two-way ANOVA with Tukey-Posttest. *P=0.019, **P<0.0039, and ****P<0.0001



Supplemental Figure 4:

Pathway 1 (red) represents the catabolism of basal glycogen stores primarily driving citrate generation. Pathway 2 (blue) represent the catabolism of free glucose which primarily supports the formation of intracellular lactate. Pathway 3 (green) represents glycogen shunt activity, which primarily supports citrate production and a full TCA cycle. PPP=Pentose phosphate pathway

References

Adeva-Andany, M.M., Gonzalez-Lucan, M., Donapetry-Garcia, C., Fernandez-Fernandez, C., and Ameneiros-Rodriguez, E. (2016). Glycogen metabolism in humans. BBA Clin *5*, 85-100.

Afzelius, B.A. (1992). Section staining for electron microscopy using tannic acid as a mordant: a simple method for visualization of glycogen and collagen. Microsc Res Tech *21*, 65-72.

Akira, S., and Takeda, K. (2004). Toll-like receptor signalling. Nat Rev Immunol *4*, 499-511.

Amati, L., Pepe, M., Passeri, M.E., Mastronardi, M.L., Jirillo, E., and Covelli, V. (2006). Toll-like receptor signaling mechanisms involved in dendritic cell activation: potential therapeutic control of T cell polarization. Curr Pharm Des *12*, 4247-4254.

Amiel, E., Everts, B., Freitas, T.C., King, I.L., Curtis, J.D., Pearce, E.L., and Pearce, E.J. (2012). Inhibition of mechanistic target of rapamycin promotes dendritic cell activation and enhances therapeutic autologous vaccination in mice. Journal of immunology *189*, 2151-2158.

Amiel, E., Everts, B., Fritz, D., Beauchamp, S., Ge, B., Pearce, E.L., and Pearce, E.J. (2014). Mechanistic target of rapamycin inhibition extends cellular lifespan in dendritic cells by preserving mitochondrial function. Journal of immunology *193*, 2821-2830.

Banchereau, J., and Steinman, R.M. (1998). Dendritic cells and the control of immunity. Nature *392*, 245-252.

Barton, G.M., and Medzhitov, R. (2002). Control of adaptive immune responses by Toll-like receptors. Curr Opin Immunol *14*, 380-383.

Calder, P.C., and Geddes, R. (1992). Heterogeneity of glycogen synthesis upon refeeding following starvation. Int J Biochem *24*, 71-77.

Elsner, P., Quistorff, B., Hansen, G.H., and Grunnet, N. (2002). Partly ordered synthesis and degradation of glycogen in cultured rat myotubes. The Journal of biological chemistry *277*, 4831-4838.

Everts, B., Amiel, E., Huang, S.C., Smith, A.M., Chang, C.H., Lam, W.Y., Redmann, V., Freitas, T.C., Blagih, J., van der Windt, G.J., et al. (2014). TLR-driven early glycolytic reprogramming via the kinases TBK1-IKKvarepsilon supports the anabolic demands of dendritic cell activation. Nature immunology *15*, 323-332.

Everts, B., Amiel, E., van der Windt, G.J., Freitas, T.C., Chott, R., Yarasheski, K.E., Pearce, E.L., and Pearce, E.J. (2012). Commitment to glycolysis sustains survival of NO-producing inflammatory dendritic cells. Blood *120*, 1422-1431.

Everts, B., and Pearce, E.J. (2014). Metabolic control of dendritic cell activation and function: recent advances and clinical implications. Frontiers in immunology *5*, 203.

Favaro, E., Bensaad, K., Chong, M.G., Tennant, D.A., Ferguson, D.J., Snell, C., Steers, G., Turley, H., Li, J.L., Gunther, U.L., et al. (2012). Glucose utilization via glycogen phosphorylase sustains proliferation and prevents premature senescence in cancer cells. Cell metabolism *16*, 751-764.

Fox, C.J., Hammerman, P.S., and Thompson, C.B. (2005). Fuel feeds function: energy metabolism and the T-cell response. Nat Rev Immunol *5*, 844-852.

Freemerman, A.J., Johnson, A.R., Sacks, G.N., Milner, J.J., Kirk, E.L., Troester, M.A., Macintyre, A.N., Goraksha-Hicks, P., Rathmell, J.C., and Makowski, L. (2014). Metabolic reprogramming of macrophages: glucose transporter 1 (GLUT1)-mediated glucose metabolism drives a proinflammatory phenotype. The Journal of biological chemistry *289*, 7884-7896.
Hardie, D.G., Ross, F.A., and Hawley, S.A. (2012). AMPK: a nutrient and energy sensor that maintains energy homeostasis. Nature reviews. Molecular cell biology *13*, 251-262.
Krawczyk, C.M., Holowka, T., Sun, J., Blagih, J., Amiel, E., DeBerardinis, R.J., Cross, J.R., Jung, E., Thompson, C.B., Jones, R.G., et al. (2010). Toll-like receptor-induced changes in glycolytic metabolism regulate dendritic cell activation. Blood *115*, 4742-4749.
Lawless, S.J., Kedia-Mehta, N., Walls, J.F., McGarrigle, R., Convery, O., Sinclair, L.V., Navarro, M.N., Murray, J., and Finlay, D.K. (2017). Glucose represses dendritic cell-induced T cell responses. Nature communications *8*, 15620.
Lee, H.K., and Iwasaki, A. (2007). Innate control of adaptive immunity: dendritic cells and beyond. Semin Immunol *19*, 48-55.
Lipscomb, M.F., and Masten, B.J. (2002). Dendritic Cells: Immune Regulators in Health and Disease.
Macintyre, A.N., Gerriets, V.A., Nichols, A.G., Michalek, R.D., Rudolph, M.C., Deoliveira, D., Anderson, S.M., Abel, E.D., Chen, B.J., Hale, L.P., et al. (2014). The glucose transporter Glut1 is selectively essential for CD4 T cell activation and effector function. Cell metabolism *20*, 61-72.
Maroof, A., English, N.R., Bedford, P.A., Gabrilovich, D.I., and Knight, S.C. (2005). Developing dendritic cells become 'lacy' cells packed with fat and glycogen. Immunology *115*, 473-483.
O'Neill, L.A. (2014). Glycolytic reprogramming by TLRs in dendritic cells. Nature immunology *15*, 314-315.
Obel, L.F., Muller, M.S., Walls, A.B., Sickmann, H.M., Bak, L.K., Waagepetersen, H.S., and Schousboe, A. (2012). Brain glycogen-new perspectives on its metabolic function and regulation at the subcellular level. Front Neuroenergetics *4*, 3.
Pearce, E.J., and Everts, B. (2015). Dendritic cell metabolism. Nature reviews. Immunology *15*, 18-29.
Pearce, E.L., and Pearce, E.J. (2013). Metabolic pathways in immune cell activation and quiescence. Immunity *38*, 633-643.
Pearce, E.L., Walsh, M.C., Cejas, P.J., Harms, G.M., Shen, H., Wang, L.S., Jones, R.G., and Choi, Y. (2009). Enhancing CD8 T-cell memory by modulating fatty acid metabolism. Nature *460*, 103-107.
Rehman, A., Hemmert, K.C., Ochi, A., Jamal, M., Henning, J.R., Barilla, R., Quesada, J.P., Zambirinis, C.P., Tang, K., Ego-Osuala, M., et al. (2013). Role of fatty-acid synthesis in dendritic cell generation and function. Journal of immunology *190*, 4640-4649.
Roach, P.J., Depaoli-Roach, A.A., Hurley, T.D., and Tagliabracci, V.S. (2012). Glycogen and its metabolism: some new developments and old themes. The Biochemical journal *441*, 763-787.

Scott, R.B. (1968). Glycogen in human peripheral blood leukocytes. I. Characteristics of the synthesis and turnover of glycogen in vitro. J Clin Invest *47*, 344-352.
Shulman, R.G., Hyder, F., and Rothman, D.L. (2001). Cerebral energetics and the glycogen shunt: neurochemical basis of functional imaging. Proc Natl Acad Sci U S A *98*, 6417-6422.
Shulman, R.G., and Rothman, D.L. (2001). The "glycogen shunt" in exercising muscle: A role for glycogen in muscle energetics and fatigue. Proc Natl Acad Sci U S A *98*, 457-461.
Voet, D., Voet, J.G., and Pratt, C.W. (2013). Fundamentals of biochemistry : life at the molecular level. (Hoboken, NJ: Wiley).
Wang, Q., Liang, B., Shirwany, N.A., and Zou, M.H. (2011). 2-Deoxy-D-glucose treatment of endothelial cells induces autophagy by reactive oxygen species-mediated activation of the AMP-activated protein kinase. PLoS One *6*, e17234.
Weisdorf, D.J., Craddock, P.R., and Jacob, H.S. (1982). Glycogenolysis versus glucose transport in human granulocytes: differential activation in phagocytosis and chemotaxis. Blood *60*, 888-893.
Wieman, H.L., Wofford, J.A., and Rathmell, J.C. (2007). Cytokine stimulation promotes glucose uptake via phosphatidylinositol-3 kinase/Akt regulation of Glut1 activity and trafficking. Molecular biology of the cell *18*, 1437-1446.
Wu, Y., Sarkissyan, M., McGhee, E., Lee, S., and Vadgama, J.V. (2015). Combined inhibition of glycolysis and AMPK induces synergistic breast cancer cell killing. Breast Cancer Res Treat *151*, 529-539.
Yunis, A.A., and Arimura, G.K. (1964). ENZYMES OF GLYCOGEN METABOLISM IN WHITE BLOOD CELLS. I. GLYCOGEN PHOSPHORYLASE IN NORMAL AND LEUKEMIC HUMAN LEUKOCYTES. Cancer Res *24*, 489-492.
Yunis, A.A., and Arimura, G.K. (1966). Enzymes of glycogen metabolism in white blood cells. II. Activation and inactivation of glycogen phosphorylase of rat chloroma. Biochim Biophys Acta *118*, 325-334.

CHAPTER 4

The role of Nitric Oxide in Metabolic Regulation of Dendritic Cell Immune Function

Abstract

Dendritic cells (DCs) are canonical antigen presenting cells of the immune system and serve as a bridge between innate and adaptive immune responses. When DCs are activated by a stimulus through toll-like receptors (TLRs), DCs undergo a process of maturation defined by cytokine & chemokine secretion, co-stimulatory molecule expression, antigen processing and presentation, and the ability to activate T cells. DC maturation is coupled with an increase in biosynthetic demand, which is fulfilled by a TLR-driven upregulation in glycolytic metabolism. Up-regulation of glycolysis in activated DCs provides these cells with molecular building blocks and cellular energy required for DC activation, and inhibition of glycolysis during initial activation impairs both the survival and effector function of activated DCs. Evidence shows that DC glycolytic upregulation is controlled by two distinct pathways, an early burst of glycolysis that is nitric oxide (NO) –independent, and a sustained commitment to glycolysis in NO-producing DC subsets. This review will address the complex role of NO in regulating DC metabolism and effector function.

Introduction

Dendritic cells (DCs) are professional antigen presenting cells of the immune system and play a central role in coordinating both innate and adaptive immune responses [1]. In their unactivated state, DCs continuously sample the tissue microenvironment for foreign material and are equipped to react to inflammatory stimuli by expressing a wide variety of innate immune receptors including the Toll-like receptor (TLR) family [2-4]. These TLRs recognize multiple forms of pathogen-associated molecules, and recognition

of cognate ligands via TLRs cause DCs to become highly activated. Activated DCs undergo a process of "maturation", which is characterized by the upregulation of co-stimulatory molecule expression, the ability *in vivo* to migrate from the site of inflammation to secondary lymphoid organs, the synthesis and secretion of immune-modulating cytokines and chemokines, and the processing and presentation of antigens to T lymphocytes. In this manner, DCs play a fundamental role in initiating and maintaining both innate and adaptive immune responses [1, 5, 6]. A number of studies in recent years have identified that DC activation is accompanied by distinct metabolic changes, highlighted by significant upregulation of aerobic glycolysis, that regulates the survival and immune effector function of both human and mouse DCs [7-13]. The microbicidal gas nitric oxide (NO) is among the activation-induced compounds synthesized and secreted by activated DCs and plays a complicated role in regulating DC immune responses as well as their cellular metabolism. TLR-mediated glycolysis induction in DCs occurs in two distinct phases (modeled in Figure 1, upper right panel). Shortly after activation, DCs experience an early phase of TLR-driven glycolytic burst that is NO-independent [8], which is subsequently followed by a sustained phase of glycolytic metabolism that is contingent upon NO production in subsets of these cells [8-10]. The focus of this review is to highlight and discuss the current understanding in the field regarding the role of NO in regulating DC immunometabolism and effector function.

NOS Expression and NO production

Cellular production of NO is catalyzed by three distinct nitric oxide synthase (NOS) enzymes. Endothelial NOS (eNOS, NOS1) and neuronal NOS (nNOS, NOS3) are

constitutively expressed and were originally named for their primary tissue distribution, although the expression of these enzymes by a wide variety of cell types is now appreciated [14-17]. Of highest relevance to this review, inducible NOS (iNOS, NOS2) is the primary NO-synthesizing enzyme expressed by immune cells and is often not constitutively expressed but is potently induced during stimulation by inflammatory signals [18, 19]. All NOS enzymes catalyze the reaction that converts substrates L-arginine, NADPH, and O_2 to L-citrulline, $NADP^+$, and NO [19]. As a membrane permeable volatile compound, NO participates in a variety of cellular processes that can extend beyond cell-intrinsic impacts on the cells that produce it [20-22]. The NO radical can influence cellular processes through a number of distinct mechanisms (reviewed in [20]), including: 1) the formation of toxic compounds such as peroxynitrite ($ONOO^-$) when combined with superoxide (O_2^-) [23]; 2) S-nitrosylation of proteins leading to altered cellular activity [24, 25]; 3) deamination of nucleic acids leading to genetic mutation [26].

Heterogeneity of DC Subsets

DCs refer to a broadly heterogeneous family of immune cells that include cells derived from both myeloid and lymphoid lineage progenitors (reviewed in [27]). These cells are specialized in their ability to acquire and process antigen, their expression of MHC-II antigen presentation machinery, their ability to travel to secondary lymphoid

organs after activation, and their capacity to initiate antigen-specific T cell activation in these compartments [27]. So called "classical DCs" found in secondary lymphoid organs can be subdivided into two major subsets: $CD11b^+$ DCs, which are thought to specialize in cytokine production and $CD4^+$ T cell activation [28, 29]; and $CD8^+$ DCs which specialize in cross-presentation of exogenous antigen and are potent activators of $CD8^+$ T cells [30-32]. In addition, there exist a number of non-classical DC subsets that play an important role in peripheral immune surveillance and infection response. These include the circulating and tissue-resident plasmacytoid DCs (pDCs) that are potent producers of Type I interferons [33, 34], skin-resident Langerhans cells [35, 36], and monocyte-derived "inflammatory DCs" (iDCs) [37-39]. With technological advances, different subsets of cDCs and pDCs in both mouse and human have been newly identified based on their tissue localization, surface markers, and ontogeny [40-42]. In the vast majority of *in vitro* – differentiated DC studies in both mouse and human systems, myeloid precursors (typically bone-marrow stem cells or circulating monocytes) are differentiated in the presence of the cytokine GM-CSF (with or without IL-4) to generate relatively pure populations of DC-like cells that are thought to most closely resemble iDCs both genetically and functionally [43, 44].

Variability of iNOS Expression in DC Subsets

In evaluating the physiological role of NO in DC biology, it is important to note that iNOS is expressed only by specific subsets of DC populations, whether these be *in vivo* subsets or *in vitro* DC models, and that there exist notable differences in iNOS regulation between mice and humans DCs. Early studies determined that LPS and IFN-γ

can induce iNOS expression in mouse GM-CSF –differentiated bone marrow-derived DCs (GM-DCs) [45]. In addition, mouse skin-derived DCs express iNOS and produce NO in response to LPS [46, 47] and a role for NO production by thymic DCs in T cell differentiation has also been reported [48]. As originally defined, monocyte-derived iDCs (originally coined TNF-a/iNOS-producing-DCs, or "TipDCs") express iNOS and are potent producers of NO that are required to control a number of different types of both bacterial and viral infections [39, 49, 50]. Nevertheless, unlike infection-associated iDCs and *in vitro* –cultivated GM-DCs, classical DC subsets in secondary lymphoid organs do not readily express iNOS nor produce detectable amounts of NO [9, 51, 52]. Conventional GM-CSF and IL-4 –differentiated monocyte-derived human DCs (moDCs) also do not readily express iNOS, which is in stark contrast to their murine GM-DC counterparts [9, 53]. Despite this, the conditions for *in vitro* differentiation of human iDCs expressing iNOS have been reported [54], suggesting that the right environmental conditions can elicit a monocyte-derived iDC population in human cells. One possible explanation for the mouse/human species difference with regard to iNOS expression is the observation that the iNOS gene promoter region in human cells is more heavily methylated than in mice and possibly controlled by negative epigenetic regulation [55]. Despite these differences, there is ample evidence to suggest that some *in vivo* human DC populations can express iNOS, ranging from blood circulating $CD1a^+$ DCs [56] to DC infiltrates in psoriatic skin lesions [57-59]. Given the significant heterogeneity in iNOS expression described above, it is critical to take into account the population of DCs being studied with regard to NO-mediated immune regulation, particularly given the

understanding that NO-production will significantly impact the duration and nature of metabolic reprogramming in these cells.

While acute induction of glycolysis has been implicated in the immune responses of most of these DC subsets, it is worth noting that tolerogenic DCs exhibit a reduced commitment to glycolysis compared to conventional DC subsets [60], highlighting the correlation between glycolysis induction and inflammation in DC populations. In addition, it has become evident that, even within a single DC subset, cellular origin may impact the different metabolic requirements of these cells [61, 62], highlighting the difficulty in fully understanding metabolic regulation in such a diverse and complex cellular family. Given the functional heterogeneity of the subsets described, it is not surprising that the production and function of NO in these cells varies as well

The Role of NO in DC Metabolic Reprogramming

The early foundation for the current interest in myeloid immune cell metabolism was established decades ago with the observation that increased glucose metabolism accompanies macrophage activation [63, 64]. Within the last decade, several studies established the existence of a similar requirement for increased glycolytic metabolism during DC activation and immune function [10, 12]. Subsequent to these studies, in the last several years researchers have intensely focused on attempting to understand the mechanisms behind metabolic regulation of DC activity with the aid of sophisticated technology and research approaches that were not available when glycolytic reprogramming was first discovered in macrophages. While a number of reports have identified a critical role for the mTOR/HIF1α/iNOS signaling axis in driving activation-

associated glycolysis induction in DCs [12, 22, 65-67], we now appreciate that metabolic reprogramming in DCs is driven by two distinct temporal events regulated by distinct signaling axes delineated below.

Early Glycolytic Reprogramming in DCs is NO-independent

Within minutes of TLR stimulation, DCs exhibit a rapid two-fold increase in their glycolytic rate that is sustained for several hours and mediated by a TBK1/IKKε/Akt signaling axis [68]. This acute "glycolytic burst" is independent of mTOR/HIF1α/iNOS signaling. It also promotes rapid translocation of Hexokinase 2 (HK2) to the outer mitochondrial membrane leading to the rapid flux of glucose metabolism, which is required for proper DC activation and immune effector function [68]. Metabolite tracing studies have shown that this early glycolytic flux supports pentose phosphate pathway (PPP) activity and the preferential generation of citrate [68, 69]. While the explicit contribution of citrate metabolism in supporting DC activation is not fully understood, citrate production supports fatty acid synthesis, which in turn contributes to endoplasmic reticulum and Golgi body expansion associated with DC maturation and DC effector function [68-71]. In addition, citrate metabolism supports acetyl-CoA production necessary for epigenetic regulation of glycolysis enzymes including, HK2 [72, 73]. While epigenetic reprogramming is not likely involved in acute glycolytic reprogramming, it may play an important role in long-term metabolic reprogramming to inflammatory stimuli, as has been notably reported in monocytes [74, 75].

In DCs that do not express iNOS as a consequence of their activation, the rapid induction of glycolysis is transient, lasting approximately 4-6 hours, after which activated

DCs return to a metabolic state that more closely resembles their pre-activation metabolism (modeled in Figure 1, upper right panel). The early and transient glycolytic reprogramming occurs in both *in vitro* –derived DC subsets and *ex vivo* populations regardless of iNOS expression [68]. Interestingly, while the conventional paradigm in the field has focused on glycolytic reprogramming as a downstream consequence of innate immune signaling, a recent study reported that hexokinase itself can serve as an innate immune receptor [76], adding a startling layer of complexity to the interplay between DC metabolism and microbial recognition by these cells.

Sustained Glycolytic Reprogramming in DCs is NO-dependent

In DCs that express iNOS, a second wave of glycolytic induction is mediated by mTOR/HIF1α/iNOS that commits these cells to glycolytic metabolism for the duration of their post-activation lifespan in an NO-dependent manner [8, 9] (modeled in **Figure 4 -*1***, upper right panel). It is this phenomenon that has led many researchers to conclude that HIF1α is critically required for DC glycolytic reprogramming [12, 22, 65-67], when in fact this is only the case for the sustained commitment to glycolysis observed in NO-producing DCs [8, 9]. While mTOR plays a multifaceted and complex role in immune cell activation (reviewed in [67]), mTOR–dependent HIF1α activity drives iNOS expression in TLR-activated DCs [22, 77]. iNOS protein expression is detectable at around 6-8 hours following DC activation and is concomitant with NO-mediated suppression of DC mitochondrial activity and oxidative phosphorylation [8, 9]. Reversible inhibition of mitochondrial cytochrome c oxidase activity [78, 79] and persistent inhibition via S-nitrosylation by NO has been well documented [79], and iNOS

inhibitors are able to rescue the mitochondrial defect in NO-producing DCs [9]. The net result of NO production in DCs is the near-complete loss of mitochondrial function and the associated catabolism of fatty acids through oxidative phosphorylation. The functional consequences of this loss of mitochondrial function is the requirement for a sustained commitment to glycolysis in these cells to meet both their bioenergetic (ATP production) and biosynthetic demands [7, 13, 68]. While glycolysis can help extend the lifespan of NO-producing DCs in the face of altered mitochondrial activity, the lifespan of these cells is still largely regulated by their NO production [8, 9, 22]. Thus, NO-production represents a critical cellular event in DC activation that initiates a chain of events that fundamentally shapes the immune activity and survival of these cells.

The Impact of NO-inhibition in DC immunometabolism

As mentioned above, iNOS-deficient DCs and DC subsets that do not upregulate iNOS engage in an early glycolytic burst followed by a return to a less-glycolytic metabolic state [9] (**Figure 4 -*1***). To try and assess the regulatory impact of NO-dependent metabolic reprogramming in DCs, a number of studies have investigated the impact of iNOS-inhibition on DC immune activation and effector function. There is a strong consensus among these studies that inhibition of NO production or its upstream signaling elements attenuates sustained glycolytic commitment and significantly enhances many DC activation parameters including costimulatory molecule upregulation, inflammatory cytokine production, T cell stimulatory capacity, and post-activation DC survival [8, 9, 22]. These effects can also be seen by perturbation of upstream regulatory elements controlling iNOS expression. For example, mTOR inhibition by rapamycin,

which reduces but does not completely ablate iNOS expression and NO production in DCs, rescues mitochondrial respiration in these cells, leading to enhanced activation and T cell stimulatory capacity [7, 8]. Additionally, a recent study reported that the mTOR/HIF1a/iNOS signaling axis serves as a glucose-sensing pathway in DCs that limits DC activation and T cell stimulatory capacity [22]. Interestingly, there appears to be significant crosstalk between NO-independent and –dependent glycolytic reprogramming in DCs. HIF1α induces upregulation of the glucose transporter Glut1, which is required for sustained commitment to glycolysis [67, 80]. In addition, citrate metabolism from early glycolytic programming generates NADPH, which is a substrate for iNOS and may contribute to NO synthesis [71, 81, 82]. These findings support a model in which NO-production critically regulates the long-term metabolic reprogramming in DCs and plays a central role in regulating the immune activity of these cells. In systems where NO production is not produced by DCs directly, exogenous sources of NO can still contribute to changes in DC metabolism [83] as well as the function of other immune cells [84, 85]. To this effect, NO from iNOS-expressing DCs or exogenous sources can impair the immune responses of DCs [7, 9, 85], and exogenous NO is reported to regulate monocyte-derived DC immune responses. In addition, NO production by macrophages can induce HIF1α expression in DCs that do not express iNOS [22], and cDCs from the spleen that do not express iNOS exhibit attenuated mitochondrial activity, possibly due to NO exposure from other NO-producing cells in the spleen such as macrophages [86].

The Role of DC NO Production in Disease States

Historically, NO has been attributed a primary role as a microbicidal compound that can directly disrupt bacterial structural proteins, lipids, and nucleic acids (reviewed in [87]). In support of this, iNOS expression is induced in immune cells during infection and contributes a protective role in variety of microbial infections [39, 88-92]. The identification of a monocyte-derived iDC subset, then termed TNF/iNOS –producing DC (TipDCs), that is induced by and controls systemic *Listeria monocytogenes* infection, helped establish a role for NO-producing iDCs in bacterial defense [39, 93]. Since their original discovery, NO-producing iDCs have been further reported to play important roles in other bacterial infections such as *Leishmania major* [21, 50] as well as viral influenza infections where they help limit viral replication but also contribute to immune-mediated pathology [49]. Interestingly, NO-producing iDCs have been shown to be both necessary and sufficient to promote T cell –dependent IgA class switch recombination in mucosa-associated lymphoid tissue, suggesting that this DC subset may have a specialized role at these sites [94]. Examples in which NO production by iDCs can also regulate non-infectious disease states are documented for atherosclerosis [95], murine models of autoimmune disease [96], and cancer. The presence of tumor-infiltrating iDCs is required for efficacy of cyototoxic T cells in eliminating tumors via a NO-dependent mechanism [97]. *In vitro* activated BMDCs have been reported to induce tumor cell death by NO and/or peroxynitrite -dependent mechanisms [98, 99]. Notably, DCs generated from cancer patients exert tumoricidal activities via peroxynitrite and enhance tumor-specific antigen presentation to T cells [100]. NO has also been shown to be released by tumor cells [101] and infection-induced granulomas [102], which may have immune-

modulatory activity on immune cells within the tumor milieu. It is probable in these systems, that NO influences on cellular metabolism regulate the types of immune responses not only of the NO-producing DCs themselves, but also the function of bystander cells in the local tissue microenvironment.

NO Regulation of DC Immune Activation

Predating the recent focus in the field on the metabolic regulation of immune cells, there exists a substantial literature delineating the complexity of NO-mediated regulation of DC immune function. While a few studies have shown a role for NO in augmenting DC immune activity from enhancing MHC-II surface expression [103, 104], promoting CCL19-directed chemotaxis [105], and driving proper activation and T cell stimulatory capacity [106], the majority of studies have reported an inhibitory role for NO on DC-mediated inflammation. iNOS inhibition during activation leads to significant enhancement of GM-DC costimulatory molecule expression and inflammatory cytokine production, indicating that NO production by these cells limits their activation [8, 9, 107]. In support of this, exogenous NO stimulation (through chemical NO-donors) significantly reduces costimulatory molecule expression and IL-12 production in human moDCs and shapes the $CD4^+$ T cell polarization by these cells [108, 109]. In addition, iNOS expression in differentiating moDCs negatively impacts both moDC differentiation and activation [25, 110]. NO additionally inhibits IL-12, IL-6, and TNFα cytokine production in human pDCs [84]. The inhibitory role of DC-produced NO is further underscored by studies showing the suppressive function of "inhibitory DCs" induced by phagocytosis of apoptotic cells is at least partially dependent on NO production in these cells [111]. In

addition, NO production suppresses the migratory capacity and limits the survival of cutaneous Langerhans cells in mice [112]. While work remains to explicitly delineate the multiple ways by which NO may be contributing to impaired DC function, it is clear that NO-mediated mitochondrial inhibition and the compensatory long-term commitment to glycolysis imparts a metabolic crisis in these cells that impacts activation and survival [8-10, 22].

DC-produced NO Limits T Cell Activation

The impact of NO on DC function is not restricted to DC cell-intrinsic effects but also negatively regulates DC-mediated T cell activation [45, 113, 114]. This is at least in part due to the membrane permeable nature of NO as NO-producing DCs are able to inhibit mitochondrial activity in co-cultured cells that do not express iNOS [8]. The presence of NO-producing DCs significantly attenuates T cell proliferation in mixed lymphocyte reactions in both mouse and human studies [51, 115]. In addition, iDCs are reported to limit antigen-specific T cell expansion through an NO-dependent mechanism [116]. We believe that the existing literature supports a model whereby DC NO production restricts T cell activity through two separate mechanisms: 1) DC stimulatory capacity is reduced in a cell-intrinsic manner by attenuation of maturation parameters as described above; 2) DC-derived NO can promote mitochondrial respiratory crisis and cell death in T cells directly [96]. Of particular relevance to both of these points, a recent study has elegantly delineated an mTOR/HIF1α/iNOS glucose-sensitive signaling axis that functionally restricts the ability of DCs to stimulate T cells [22]. This study further

showed that disruption of any component of this signaling axis can enhance T cell activation by NO-producing DCs [22].

NO and DC Cell Death

In addition to its role in regulating DC activation, NO has a well-documented role in the regulation of immune cell survival. While low-levels of NO have been reported to induce cGMP-dependent cytoprotection in a RAW264 macrophage cell line [117], most studies on this topic have reported a cytotoxic effect of NO, particularly during prolonged exposure and at high concentrations [118, 119]. One possible mechanism for this effect is the ability of NO to upregulate caspase activity [119] and to directly induce cytochrome c release from mitochondria [118]. In addition, NO derivatives such as peroxynitrite, nitrogen dioxide, and nitrosothiols can irreversibly inhibit enzymes of the electron transport chain in mitochondria at high concentrations, leading to metabolic crisis and cell death (reviewed in [120]). In DCs specifically, NO production has been identified as a principal cytotoxic factor during post-activation death in these cells [8, 9]. Furthermore, iNOS -deficient DCs exhibit greater metabolic flexibility than NO-producing cells and are able to more easily survive metabolic stress, such as culture in glucose-limiting conditions [9]. In further support of these findings, mTOR inhibitors, which reduce NO production in DCs below the threshold required for complete mitochondrial disruption, augment post-activation DC lifespan and immune function [7, 8].

Concluding Remarks

The emergent focus on the study of the metabolic regulation of DCs and the role of NO in DC metabolism has helped clarify and re-focus much of the historical literature regarding the effect of NO on DC immune function and cell survival. Because NO plays such a fundamental role in determining the long-term immunological and cellular fate of DCs (Figure 1), we anticipate that future studies in the field of DC immunometabolism will judiciously consider the source of DCs being examined, with particular focus on the iNOS expression status of the DCs and the production of NO in the tissue microenvironment. Because of the inherent complexity of both metabolic regulation in general and the pleiotropic effects of NO and its derivative compounds, further work on explicitly defining the NO-dependent and –independent aspects of DC metabolic regulation is needed. Ongoing efforts on these topics will provide new insights in how DC immune activity is controlled at the metabolic level and will help distinguish those aspects of DC immunometabolism that are universal across DC subsets (and from different species) from those that are restricted to NO-producing cells.

Figures

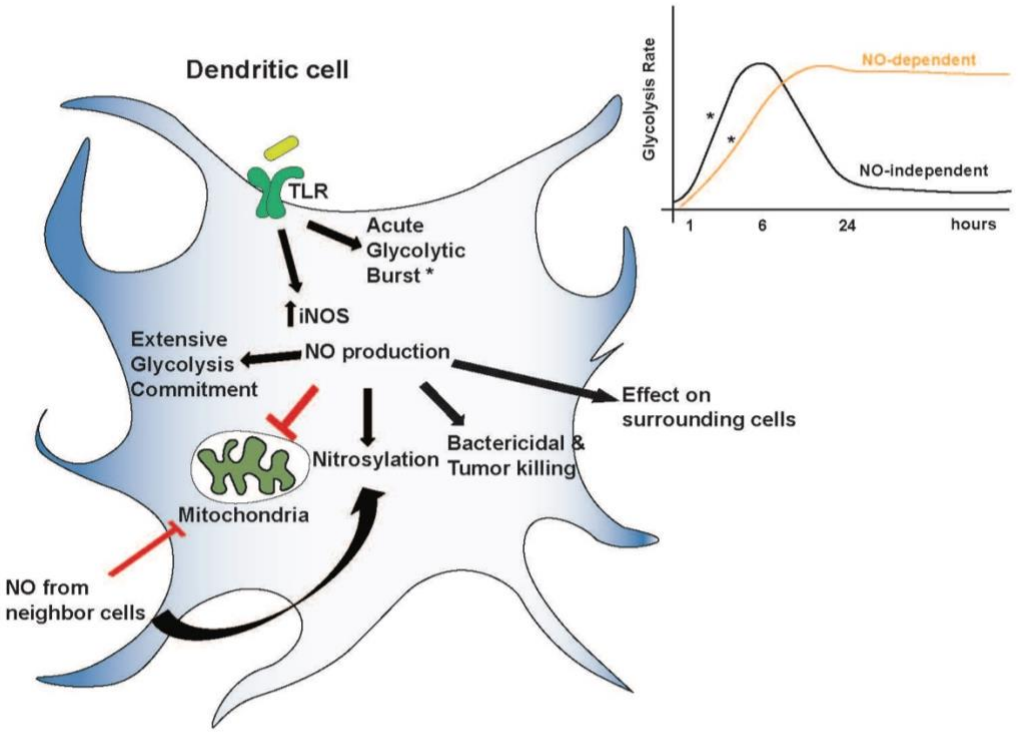


Figure 4 -1: Model of NO-mediated impacts on DC metabolism and function.

Upper right panel, kinetics of NO-dependent and –independent glycolytic induction is illustrated. Main figure, the pleiotropic effects of NO on DC metabolism and function are modeled.

References

[1] J. Banchereau, R.M. Steinman, Dendritic cells and the control of immunity, Nature, 392 (1998) 245-252.
[2] S. Akira, K. Takeda, Toll-like receptor signalling, Nat Rev Immunol, 4 (2004) 499-511.
[3] L. Amati, M. Pepe, M.E. Passeri, M.L. Mastronardi, E. Jirillo, V. Covelli, Toll-like receptor signaling mechanisms involved in dendritic cell activation: potential therapeutic control of T cell polarization, Curr Pharm Des, 12 (2006) 4247-4254.
[4] G.M. Barton, R. Medzhitov, Control of adaptive immune responses by Toll-like receptors, Curr Opin Immunol, 14 (2002) 380-383.
[5] H.K. Lee, A. Iwasaki, Innate control of adaptive immunity: dendritic cells and beyond, Seminars in immunology, 19 (2007) 48-55.
[6] M.F. Lipscomb, B.J. Masten, Dendritic Cells: Immune Regulators in Health and Disease, 2002.
[7] E. Amiel, B. Everts, T.C. Freitas, I.L. King, J.D. Curtis, E.L. Pearce, E.J. Pearce, Inhibition of mechanistic target of rapamycin promotes dendritic cell activation and enhances therapeutic autologous vaccination in mice, Journal of immunology, 189 (2012) 2151-2158.
[8] E. Amiel, B. Everts, D. Fritz, S. Beauchamp, B. Ge, E.L. Pearce, E.J. Pearce, Mechanistic target of rapamycin inhibition extends cellular lifespan in dendritic cells by preserving mitochondrial function, Journal of immunology, 193 (2014) 2821-2830.
[9] B. Everts, E. Amiel, G.J. van der Windt, T.C. Freitas, R. Chott, K.E. Yarasheski, E.L. Pearce, E.J. Pearce, Commitment to glycolysis sustains survival of NO-producing inflammatory dendritic cells, Blood, 120 (2012) 1422-1431.
[10] C.M. Krawczyk, T. Holowka, J. Sun, J. Blagih, E. Amiel, R.J. DeBerardinis, J.R. Cross, E. Jung, C.B. Thompson, R.G. Jones, E.J. Pearce, Toll-like receptor-induced changes in glycolytic metabolism regulate dendritic cell activation, Blood, 115 (2010) 4742-4749.
[11] M.M. Delmastro-Greenwood, J.D. Piganelli, Changing the energy of an immune response, Am J Clin Exp Immunol, 2 (2013) 30-54.
[12] J. Jantsch, D. Chakravortty, N. Turza, A.T. Prechtel, B. Buchholz, R.G. Gerlach, M. Volke, J. Glasner, C. Warnecke, M.S. Wiesener, K.U. Eckardt, A. Steinkasserer, M. Hensel, C. Willam, Hypoxia and hypoxia-inducible factor-1 alpha modulate lipopolysaccharide-induced dendritic cell activation and function, Journal of immunology, 180 (2008) 4697-4705.
[13] P.M. Thwe, L. Pelgrom, R. Cooper, S. Beauchamp, J.A. Reisz, A. D'Alessandro, B. Everts, E. Amiel, Cell-Intrinsic Glycogen Metabolism Supports Early Glycolytic Reprogramming Required for Dendritic Cell Immune Responses, Cell Metab, 26 (2017) 558-567 e555.
[14] D.S. Bredt, P.M. Hwang, S.H. Snyder, Localization of nitric oxide synthase indicating a neural role for nitric oxide, Nature, 347 (1990) 768-770.
[15] D.S. Bredt, S.H. Snyder, Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme, Proc Natl Acad Sci U S A, 87 (1990) 682-685.

[16] S. Lamas, P.A. Marsden, G.K. Li, P. Tempst, T. Michel, Endothelial nitric oxide synthase: molecular cloning and characterization of a distinct constitutive enzyme isoform, Proc Natl Acad Sci U S A, 89 (1992) 6348-6352.
[17] S. Lamas, T. Michel, T. Collins, B.M. Brenner, P.A. Marsden, Effects of interferon-gamma on nitric oxide synthase activity and endothelin-1 production by vascular endothelial cells, J Clin Invest, 90 (1992) 879-887.
[18] R.G. Knowles, S. Moncada, Nitric oxide synthases in mammals, Biochem J, 298 (Pt 2) (1994) 249-258.
[19] M. Lee, K. Rey, K. Besler, C. Wang, J. Choy, Immunobiology of Nitric Oxide and Regulation of Inducible Nitric Oxide Synthase, Results Probl Cell Differ, 62 (2017) 181-207.
[20] C. Bogdan, Nitric oxide synthase in innate and adaptive immunity: an update, Trends Immunol, 36 (2015) 161-178.
[21] R. Olekhnovitch, B. Ryffel, A.J. Muller, P. Bousso, Collective nitric oxide production provides tissue-wide immunity during Leishmania infection, J Clin Invest, 124 (2014) 1711-1722.
[22] S.J. Lawless, N. Kedia-Mehta, J.F. Walls, R. McGarrigle, O. Convery, L.V. Sinclair, M.N. Navarro, J. Murray, D.K. Finlay, Glucose represses dendritic cell-induced T cell responses, Nature communications, 8 (2017) 15620.
[23] R. Radi, Peroxynitrite, a stealthy biological oxidant, J Biol Chem, 288 (2013) 26464-26472.
[24] P. Anand, J.S. Stamler, Enzymatic mechanisms regulating protein S-nitrosylation: implications in health and disease, J Mol Med (Berl), 90 (2012) 233-244.
[25] V. Fernandez-Ruiz, N. Lopez-Moratalla, A. Gonzalez, Production of nitric oxide and self-nitration of proteins during monocyte differentiation to dendritic cells, J Physiol Biochem, 61 (2005) 517-525.
[26] T. Sawa, H. Ohshima, Nitrative DNA damage in inflammation and its possible role in carcinogenesis, Nitric Oxide, 14 (2006) 91-100.
[27] A. Mildner, S. Jung, Development and function of dendritic cell subsets, Immunity, 40 (2014) 642-656.
[28] D. Dudziak, A.O. Kamphorst, G.F. Heidkamp, V.R. Buchholz, C. Trumpfheller, S. Yamazaki, C. Cheong, K. Liu, H.W. Lee, C.G. Park, R.M. Steinman, M.C. Nussenzweig, Differential antigen processing by dendritic cell subsets in vivo, Science, 315 (2007) 107-111.
[29] K.L. Lewis, M.L. Caton, M. Bogunovic, M. Greter, L.T. Grajkowska, D. Ng, A. Klinakis, I.F. Charo, S. Jung, J.L. Gommerman, Ivanov, II, K. Liu, M. Merad, B. Reizis, Notch2 receptor signaling controls functional differentiation of dendritic cells in the spleen and intestine, Immunity, 35 (2011) 780-791.
[30] M. Crowley, K. Inaba, M. Witmer-Pack, R.M. Steinman, The cell surface of mouse dendritic cells: FACS analyses of dendritic cells from different tissues including thymus, Cell Immunol, 118 (1989) 108-125.
[31] J.M. den Haan, S.M. Lehar, M.J. Bevan, CD8(+) but not CD8(-) dendritic cells cross-prime cytotoxic T cells in vivo, J Exp Med, 192 (2000) 1685-1696.
[32] D. Vremec, M. Zorbas, R. Scollay, D.J. Saunders, C.F. Ardavin, L. Wu, K. Shortman, The surface phenotype of dendritic cells purified from mouse thymus and

spleen: investigation of the CD8 expression by a subpopulation of dendritic cells, J Exp Med, 176 (1992) 47-58.
[33] C. Asselin-Paturel, A. Boonstra, M. Dalod, I. Durand, N. Yessaad, C. Dezutter-Dambuyant, A. Vicari, A. O'Garra, C. Biron, F. Briere, G. Trinchieri, Mouse type I IFN-producing cells are immature APCs with plasmacytoid morphology, Nature immunology, 2 (2001) 1144-1150.
[34] F.P. Siegal, N. Kadowaki, M. Shodell, P.A. Fitzgerald-Bocarsly, K. Shah, S. Ho, S. Antonenko, Y.J. Liu, The nature of the principal type 1 interferon-producing cells in human blood, Science, 284 (1999) 1835-1837.
[35] G. Schuler, N. Romani, R.M. Steinman, A comparison of murine epidermal Langerhans cells with spleen dendritic cells, J Invest Dermatol, 85 (1985) 99s-106s.
[36] G. Schuler, R.M. Steinman, Murine epidermal Langerhans cells mature into potent immunostimulatory dendritic cells in vitro, J Exp Med, 161 (1985) 526-546.
[37] E. Segura, S. Amigorena, Inflammatory dendritic cells in mice and humans, Trends Immunol, 34 (2013) 440-445.
[38] E. Segura, S. Amigorena, Identification of human inflammatory dendritic cells, Oncoimmunology, 2 (2013) e23851.
[39] N.V. Serbina, T.P. Salazar-Mather, C.A. Biron, W.A. Kuziel, E.G. Pamer, TNF/iNOS-producing dendritic cells mediate innate immune defense against bacterial infection, Immunity, 19 (2003) 59-70.
[40] A.C. Villani, R. Satija, G. Reynolds, S. Sarkizova, K. Shekhar, J. Fletcher, M. Griesbeck, A. Butler, S. Zheng, S. Lazo, L. Jardine, D. Dixon, E. Stephenson, E. Nilsson, I. Grundberg, D. McDonald, A. Filby, W. Li, P.L. De Jager, O. Rozenblatt-Rosen, A.A. Lane, M. Haniffa, A. Regev, N. Hacohen, Single-cell RNA-seq reveals new types of human blood dendritic cells, monocytes, and progenitors, Science, 356 (2017).
[41] P. See, C.A. Dutertre, J. Chen, P. Gunther, N. McGovern, S.E. Irac, M. Gunawan, M. Beyer, K. Handler, K. Duan, H.R.B. Sumatoh, N. Ruffin, M. Jouve, E. Gea-Mallorqui, R.C.M. Hennekam, T. Lim, C.C. Yip, M. Wen, B. Malleret, I. Low, N.B. Shadan, C.F.S. Fen, A. Tay, J. Lum, F. Zolezzi, A. Larbi, M. Poidinger, J.K.Y. Chan, Q. Chen, L. Renia, M. Haniffa, P. Benaroch, A. Schlitzer, J.L. Schultze, E.W. Newell, F. Ginhoux, Mapping the human DC lineage through the integration of high-dimensional techniques, Science, 356 (2017).
[42] M. Guilliams, C.A. Dutertre, C.L. Scott, N. McGovern, D. Sichien, S. Chakarov, S. Van Gassen, J. Chen, M. Poidinger, S. De Prijck, S.J. Tavernier, I. Low, S.E. Irac, C.N. Mattar, H.R. Sumatoh, G.H. Low, T.J. Chung, D.K. Chan, K.K. Tan, T.L. Hon, E. Fossum, B. Bogen, M. Choolani, J.K. Chan, A. Larbi, H. Luche, S. Henri, Y. Saeys, E.W. Newell, B.N. Lambrecht, B. Malissen, F. Ginhoux, Unsupervised High-Dimensional Analysis Aligns Dendritic Cells across Tissues and Species, Immunity, 45 (2016) 669-684.
[43] F. Sallusto, A. Lanzavecchia, Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha, J Exp Med, 179 (1994) 1109-1118.

[44] Y. Xu, Y. Zhan, A.M. Lew, S.H. Naik, M.H. Kershaw, Differential development of murine dendritic cells by GM-CSF versus Flt3 ligand has implications for inflammation and trafficking, Journal of immunology, 179 (2007) 7577-7584.
[45] L. Lu, C.A. Bonham, F.G. Chambers, S.C. Watkins, R.A. Hoffman, R.L. Simmons, A.W. Thomson, Induction of nitric oxide synthase in mouse dendritic cells by IFN-gamma, endotoxin, and interaction with allogeneic T cells: nitric oxide production is associated with dendritic cell apoptosis, Journal of immunology, 157 (1996) 3577-3586.
[46] M.T. Cruz, C.B. Duarte, M. Goncalo, A.P. Carvalho, M.C. Lopes, Involvement of JAK2 and MAPK on type II nitric oxide synthase expression in skin-derived dendritic cells, Am J Physiol, 277 (1999) C1050-1057.
[47] M.T. Cruz, C.B. Duarte, M. Goncalo, A.P. Carvalho, M.C. Lopes, LPS induction of I kappa B-alpha degradation and iNOS expression in a skin dendritic cell line is prevented by the janus kinase 2 inhibitor, Tyrphostin b42, Nitric Oxide, 5 (2001) 53-61.
[48] S. Aiello, M. Noris, G. Piccinini, S. Tomasoni, F. Casiraghi, S. Bonazzola, M. Mister, M.H. Sayegh, G. Remuzzi, Thymic dendritic cells express inducible nitric oxide synthase and generate nitric oxide in response to self- and alloantigens, Journal of immunology, 164 (2000) 4649-4658.
[49] J.R. Aldridge, Jr., C.E. Moseley, D.A. Boltz, N.J. Negovetich, C. Reynolds, J. Franks, S.A. Brown, P.C. Doherty, R.G. Webster, P.G. Thomas, TNF/iNOS-producing dendritic cells are the necessary evil of lethal influenza virus infection, Proc Natl Acad Sci U S A, 106 (2009) 5306-5311.
[50] C. De Trez, S. Magez, S. Akira, B. Ryffel, Y. Carlier, E. Muraille, iNOS-producing inflammatory dendritic cells constitute the major infected cell type during the chronic Leishmania major infection phase of C57BL/6 resistant mice, PLoS Pathog, 5 (2009) e1000494.
[51] Y. Nishioka, H. Wen, K. Mitani, P.D. Robbins, M.T. Lotze, S. Sone, H. Tahara, Differential effects of IL-12 on the generation of alloreactive CTL mediated by murine and human dendritic cells: a critical role for nitric oxide, J Leukoc Biol, 73 (2003) 621-629.
[52] T.J. Powell, C.D. Jenkins, R. Hattori, G.G. MacPherson, Rat bone marrow-derived dendritic cells, but not ex vivo dendritic cells, secrete nitric oxide and can inhibit T-cell proliferation, Immunology, 109 (2003) 197-208.
[53] E.L. Padgett, S.B. Pruett, Evaluation of nitrite production by human monocyte-derived macrophages, Biochem Biophys Res Commun, 186 (1992) 775-781.
[54] D. Wilsmann-Theis, S. Koch, C. Mindnich, S. Bonness, S. Schnautz, D. von Bubnoff, T. Bieber, Generation and functional analysis of human TNF-alpha/iNOS-producing dendritic cells (Tip-DC), Allergy, 68 (2013) 890-898.
[55] G.C. Chan, J.E. Fish, I.A. Mawji, D.D. Leung, A.C. Rachlis, P.A. Marsden, Epigenetic basis for the transcriptional hyporesponsiveness of the human inducible nitric oxide synthase gene in vascular endothelial cells, Journal of immunology, 175 (2005) 3846-3861.
[56] Y.M. Huang, B.G. Xiao, I. Westerlund, H. Link, Phenotypic and functional properties of dendritic cells isolated from human peripheral blood in comparison with mononuclear cells and T cells, Scand J Immunol, 49 (1999) 177-183.

[57] M.A. Lowes, F. Chamian, M.V. Abello, J. Fuentes-Duculan, S.L. Lin, R. Nussbaum, I. Novitskaya, H. Carbonaro, I. Cardinale, T. Kikuchi, P. Gilleaudeau, M. Sullivan-Whalen, K.M. Wittkowski, K. Papp, M. Garovoy, W. Dummer, R.M. Steinman, J.G. Krueger, Increase in TNF-alpha and inducible nitric oxide synthase-expressing dendritic cells in psoriasis and reduction with efalizumab (anti-CD11a), Proc Natl Acad Sci U S A, 102 (2005) 19057-19062.
[58] A.S. Haider, M.A. Lowes, M. Suarez-Farinas, L.C. Zaba, I. Cardinale, A. Khatcherian, I. Novitskaya, K.M. Wittkowski, J.G. Krueger, Identification of cellular pathways of "type 1," Th17 T cells, and TNF- and inducible nitric oxide synthase-producing dendritic cells in autoimmune inflammation through pharmacogenomic study of cyclosporine A in psoriasis, Journal of immunology, 180 (2008) 1913-1920.
[59] L.C. Zaba, I. Cardinale, P. Gilleaudeau, M. Sullivan-Whalen, M. Suarez-Farinas, J. Fuentes-Duculan, I. Novitskaya, A. Khatcherian, M.J. Bluth, M.A. Lowes, J.G. Krueger, Amelioration of epidermal hyperplasia by TNF inhibition is associated with reduced Th17 responses, J Exp Med, 204 (2007) 3183-3194.
[60] F. Malinarich, K. Duan, R.A. Hamid, A. Bijin, W.X. Lin, M. Poidinger, A.M. Fairhurst, J.E. Connolly, High mitochondrial respiration and glycolytic capacity represent a metabolic phenotype of human tolerogenic dendritic cells, Journal of immunology, 194 (2015) 5174-5186.
[61] P. Saas, A. Varin, S. Perruche, A. Ceroi, Recent insights into the implications of metabolism in plasmacytoid dendritic cell innate functions: Potential ways to control these functions, F1000Res, 6 (2017) 456.
[62] D. Wu, D.E. Sanin, B. Everts, Q. Chen, J. Qiu, M.D. Buck, A. Patterson, A.M. Smith, C.H. Chang, Z. Liu, M.N. Artyomov, E.L. Pearce, M. Cella, E.J. Pearce, Type 1 Interferons Induce Changes in Core Metabolism that Are Critical for Immune Function, Immunity, 44 (2016) 1325-1336.
[63] P. Newsholme, R. Curi, S. Gordon, E.A. Newsholme, Metabolism of glucose, glutamine, long-chain fatty acids and ketone bodies by murine macrophages, Biochem J, 239 (1986) 121-125.
[64] P. Newsholme, S. Gordon, E.A. Newsholme, Rates of utilization and fates of glucose, glutamine, pyruvate, fatty acids and ketone bodies by mouse macrophages, Biochem J, 242 (1987) 631-636.
[65] M. Fliesser, C.O. Morton, M. Bonin, F. Ebel, K. Hunniger, O. Kurzai, H. Einsele, J. Loffler, Hypoxia-inducible factor 1alpha modulates metabolic activity and cytokine release in anti-Aspergillus fumigatus immune responses initiated by human dendritic cells, Int J Med Microbiol, 305 (2015) 865-873.
[66] J. Jantsch, M. Wiese, J. Schodel, K. Castiglione, J. Glasner, S. Kolbe, D. Mole, U. Schleicher, K.U. Eckardt, M. Hensel, R. Lang, C. Bogdan, M. Schnare, C. Willam, Toll-like receptor activation and hypoxia use distinct signaling pathways to stabilize hypoxia-inducible factor 1alpha (HIF1A) and result in differential HIF1A-dependent gene expression, J Leukoc Biol, 90 (2011) 551-562.
[67] M. Linke, S.D. Fritsch, N. Sukhbaatar, M. Hengstschlager, T. Weichhart, mTORC1 and mTORC2 as regulators of cell metabolism in immunity, FEBS Lett, (2017).
[68] B. Everts, E. Amiel, S.C. Huang, A.M. Smith, C.H. Chang, W.Y. Lam, V. Redmann, T.C. Freitas, J. Blagih, G.J. van der Windt, M.N. Artyomov, R.G. Jones, E.L. Pearce, E.J.

Pearce, TLR-driven early glycolytic reprogramming via the kinases TBK1-IKKvarepsilon supports the anabolic demands of dendritic cell activation, Nature immunology, 15 (2014) 323-332.
[69] B. Everts, E.J. Pearce, Metabolic control of dendritic cell activation and function: recent advances and clinical implications, Frontiers in immunology, 5 (2014) 203.
[70] A. Rehman, K.C. Hemmert, A. Ochi, M. Jamal, J.R. Henning, R. Barilla, J.P. Quesada, C.P. Zambirinis, K. Tang, M. Ego-Osuala, R.S. Rao, S. Greco, M. Deutsch, S. Narayan, H.L. Pachter, C.S. Graffeo, D. Acehan, G. Miller, Role of fatty-acid synthesis in dendritic cell generation and function, Journal of immunology, 190 (2013) 4640-4649.
[71] L.A. O'Neill, E.J. Pearce, Immunometabolism governs dendritic cell and macrophage function, J Exp Med, 213 (2016) 15-23.
[72] B. Kelly, L.A. O'Neill, Metabolic reprogramming in macrophages and dendritic cells in innate immunity, Cell Res, 25 (2015) 771-784.
[73] K.E. Wellen, G. Hatzivassiliou, U.M. Sachdeva, T.V. Bui, J.R. Cross, C.B. Thompson, ATP-citrate lyase links cellular metabolism to histone acetylation, Science, 324 (2009) 1076-1080.
[74] R.J. Arts, B. Novakovic, R. Ter Horst, A. Carvalho, S. Bekkering, E. Lachmandas, F. Rodrigues, R. Silvestre, S.C. Cheng, S.Y. Wang, E. Habibi, L.G. Goncalves, I. Mesquita, C. Cunha, A. van Laarhoven, F.L. van de Veerdonk, D.L. Williams, J.W. van der Meer, C. Logie, L.A. O'Neill, C.A. Dinarello, N.P. Riksen, R. van Crevel, C. Clish, R.A. Notebaart, L.A. Joosten, H.G. Stunnenberg, R.J. Xavier, M.G. Netea, Glutaminolysis and Fumarate Accumulation Integrate Immunometabolic and Epigenetic Programs in Trained Immunity, Cell Metab, 24 (2016) 807-819.
[75] S.C. Cheng, J. Quintin, R.A. Cramer, K.M. Shepardson, S. Saeed, V. Kumar, E.J. Giamarellos-Bourboulis, J.H. Martens, N.A. Rao, A. Aghajanirefah, G.R. Manjeri, Y. Li, D.C. Ifrim, R.J. Arts, B.M. van der Veer, P.M. Deen, C. Logie, L.A. O'Neill, P. Willems, F.L. van de Veerdonk, J.W. van der Meer, A. Ng, L.A. Joosten, C. Wijmenga, H.G. Stunnenberg, R.J. Xavier, M.G. Netea, mTOR- and HIF-1alpha-mediated aerobic glycolysis as metabolic basis for trained immunity, Science, 345 (2014) 1250684.
[76] A.J. Wolf, C.N. Reyes, W. Liang, C. Becker, K. Shimada, M.L. Wheeler, H.C. Cho, N.I. Popescu, K.M. Coggeshall, M. Arditi, D.M. Underhill, Hexokinase Is an Innate Immune Receptor for the Detection of Bacterial Peptidoglycan, Cell, 166 (2016) 624-636.
[77] A.J. Harris, A.R. Thompson, M.K. Whyte, S.R. Walmsley, HIF-mediated innate immune responses: cell signaling and therapeutic implications, Hypoxia (Auckl), 2 (2014) 47-58.
[78] M.C. Barone, V.M. Darley-Usmar, P.S. Brookes, Reversible inhibition of cytochrome c oxidase by peroxynitrite proceeds through ascorbate-dependent generation of nitric oxide, J Biol Chem, 278 (2003) 27520-27524.
[79] E. Clementi, G.C. Brown, M. Feelisch, S. Moncada, Persistent inhibition of cell respiration by nitric oxide: crucial role of S-nitrosylation of mitochondrial complex I and protective action of glutathione, Proc Natl Acad Sci U S A, 95 (1998) 7631-7636.
[80] C. Chen, N. Pore, A. Behrooz, F. Ismail-Beigi, A. Maity, Regulation of glut1 mRNA by hypoxia-inducible factor-1. Interaction between H-ras and hypoxia, J Biol Chem, 276 (2001) 9519-9525.

[81] V. Infantino, P. Convertini, L. Cucci, M.A. Panaro, M.A. Di Noia, R. Calvello, F. Palmieri, V. Iacobazzi, The mitochondrial citrate carrier: a new player in inflammation, Biochem J, 438 (2011) 433-436.
[82] A.K. Jha, S.C. Huang, A. Sergushichev, V. Lampropoulou, Y. Ivanova, E. Loginicheva, K. Chmielewski, K.M. Stewart, J. Ashall, B. Everts, E.J. Pearce, E.M. Driggers, M.N. Artyomov, Network integration of parallel metabolic and transcriptional data reveals metabolic modules that regulate macrophage polarization, Immunity, 42 (2015) 419-430.
[83] B. Beltran, A. Mathur, M.R. Duchen, J.D. Erusalimsky, S. Moncada, The effect of nitric oxide on cell respiration: A key to understanding its role in cell survival or death, Proc Natl Acad Sci U S A, 97 (2000) 14602-14607.
[84] R. Morita, T. Uchiyama, T. Hori, Nitric oxide inhibits IFN-alpha production of human plasmacytoid dendritic cells partly via a guanosine 3',5'-cyclic monophosphate-dependent pathway, Journal of immunology, 175 (2005) 806-812.
[85] C. Paolucci, P. Rovere, C. De Nadai, A.A. Manfredi, E. Clementi, Nitric oxide inhibits the tumor necrosis factor alpha -regulated endocytosis of human dendritic cells in a cyclic GMP-dependent way, The Journal of biological chemistry, 275 (2000) 19638-19644.
[86] A. Pantel, A. Teixeira, E. Haddad, E.G. Wood, R.M. Steinman, M.P. Longhi, Direct type I IFN but not MDA5/TLR3 activation of dendritic cells is required for maturation and metabolic shift to glycolysis after poly IC stimulation, PLoS Biol, 12 (2014) e1001759.
[87] F.C. Fang, Antimicrobial reactive oxygen and nitrogen species: concepts and controversies, Nat Rev Microbiol, 2 (2004) 820-832.
[88] M.S. Alam, T. Akaike, S. Okamoto, T. Kubota, J. Yoshitake, T. Sawa, Y. Miyamoto, F. Tamura, H. Maeda, Role of nitric oxide in host defense in murine salmonellosis as a function of its antibacterial and antiapoptotic activities, Infect Immun, 70 (2002) 3130-3142.
[89] J.P. Cobb, R.S. Hotchkiss, P.E. Swanson, K. Chang, Y. Qiu, V.E. Laubach, I.E. Karl, T.G. Buchman, Inducible nitric oxide synthase (iNOS) gene deficiency increases the mortality of sepsis in mice, Surgery, 126 (1999) 438-442.
[90] J.D. MacMicking, R.J. North, R. LaCourse, J.S. Mudgett, S.K. Shah, C.F. Nathan, Identification of nitric oxide synthase as a protective locus against tuberculosis, Proc Natl Acad Sci U S A, 94 (1997) 5243-5248.
[91] B.B. Mishra, R.R. Lovewell, A.J. Olive, G. Zhang, W. Wang, E. Eugenin, C.M. Smith, J.Y. Phuah, J.E. Long, M.L. Dubuke, S.G. Palace, J.D. Goguen, R.E. Baker, S. Nambi, R. Mishra, M.G. Booty, C.E. Baer, S.A. Shaffer, V. Dartois, B.A. McCormick, X. Chen, C.M. Sassetti, Nitric oxide prevents a pathogen-permissive granulocytic inflammation during tuberculosis, Nat Microbiol, 2 (2017) 17072.
[92] G. Bajwa, R.J. DeBerardinis, B. Shao, B. Hall, J.D. Farrar, M.A. Gill, Cutting Edge: Critical Role of Glycolysis in Human Plasmacytoid Dendritic Cell Antiviral Responses, Journal of immunology, 196 (2016) 2004-2009.
[93] P.M. Dominguez, M. Lopez-Bravo, U. Kalinke, C. Ardavin, Statins inhibit iNOS-mediated microbicidal potential of activated monocyte-derived dendritic cells by an IFN-beta-dependent mechanism, Eur J Immunol, 41 (2011) 3330-3339.

[94] H. Tezuka, Y. Abe, M. Iwata, H. Takeuchi, H. Ishikawa, M. Matsushita, T. Shiohara, S. Akira, T. Ohteki, Regulation of IgA production by naturally occurring TNF/iNOS-producing dendritic cells, Nature, 448 (2007) 929-933.
[95] S. Bacci, L. Pieri, A.M. Buccoliero, A. Bonelli, G. Taddei, P. Romagnoli, Smooth muscle cells, dendritic cells and mast cells are sources of TNFalpha and nitric oxide in human carotid artery atherosclerosis, Thromb Res, 122 (2008) 657-667.
[96] L. Xu, Y. Huang, J. Yang, P.H. Van Der Meide, M. Levi, B. Wahren, H. Link, B. Xiao, Dendritic cell-derived nitric oxide is involved in IL-4-induced suppression of experimental allergic encephalomyelitis (EAE) in Lewis rats, Clin Exp Immunol, 118 (1999) 115-121.
[97] I. Marigo, S. Zilio, G. Desantis, B. Mlecnik, A.H. Agnellini, S. Ugel, M.S. Sasso, J.E. Qualls, F. Kratochvill, P. Zanovello, B. Molon, C.H. Ries, V. Runza, S. Hoves, A.M. Bilocq, G. Bindea, E.M. Mazza, S. Bicciato, J. Galon, P.J. Murray, V. Bronte, T Cell Cancer Therapy Requires CD40-CD40L Activation of Tumor Necrosis Factor and Inducible Nitric-Oxide-Synthase-Producing Dendritic Cells, Cancer Cell, 30 (2016) 651.
[98] J. Fraszczak, M. Trad, N. Janikashvili, D. Cathelin, D. Lakomy, V. Granci, A. Morizot, S. Audia, O. Micheau, L. Lagrost, E. Katsanis, E. Solary, N. Larmonier, B. Bonnotte, Peroxynitrite-dependent killing of cancer cells and presentation of released tumor antigens by activated dendritic cells, Journal of immunology, 184 (2010) 1876-1884.
[99] A. Nicolas, D. Cathelin, N. Larmonier, J. Fraszczak, P.-E. Puig, A. Bouchot, A. Bateman, E. Solary, B. Bonnotte, Dendritic Cells Trigger Tumor Cell Death by a Nitric Oxide-Dependent Mechanism, The Journal of Immunology, 179 (2007) 812-818.
[100] D. Lakomy, N. Janikashvili, J. Fraszczak, M. Trad, S. Audia, M. Samson, M. Ciudad, J. Vinit, C. Vergely, D. Caillot, P. Foucher, L. Lagrost, S. Chouaib, E. Katsanis, N. Larmonier, B. Bonnotte, Cytotoxic dendritic cells generated from cancer patients, Journal of immunology, 187 (2011) 2775-2782.
[101] S.A. Glynn, B.J. Boersma, T.H. Dorsey, M. Yi, H.G. Yfantis, L.A. Ridnour, D.N. Martin, C.H. Switzer, R.S. Hudson, D.A. Wink, D.H. Lee, R.M. Stephens, S. Ambs, Increased NOS2 predicts poor survival in estrogen receptor-negative breast cancer patients, The Journal of clinical investigation, 120 (2010) 3843-3854.
[102] F. Facchetti, W. Vermi, S. Fiorentini, M. Chilosi, A. Caruso, M. Duse, L.D. Notarangelo, R. Badolato, Expression of Inducible Nitric Oxide Synthase in Human Granulomas and Histiocytic Reactions, The American Journal of Pathology, 154 (1999) 145-152.
[103] D. Huang, D.T. Cai, R.Y. Chua, D.M. Kemeny, S.H. Wong, Nitric-oxide synthase 2 interacts with CD74 and inhibits its cleavage by caspase during dendritic cell development, J Biol Chem, 283 (2008) 1713-1722.
[104] S.H. Wong, L. Santambrogio, J.L. Strominger, Caspases and nitric oxide broadly regulate dendritic cell maturation and surface expression of class II MHC proteins, Proc Natl Acad Sci U S A, 101 (2004) 17783-17788.
[105] D. Giordano, D.M. Magaletti, E.A. Clark, Nitric oxide and cGMP protein kinase (cGK) regulate dendritic-cell migration toward the lymph-node-directing chemokine CCL19, Blood, 107 (2006) 1537-1545.

[106] H.S. Adler, A. Simon, E. Graulich, A. Habermeier, N. Bacher, A. Friebe, E.I. Closs, K. Steinbrink, Neuronal nitric oxide synthase modulates maturation of human dendritic cells, Journal of immunology, 184 (2010) 6025-6034.
[107] P.U. Simioni, L.G. Fernandes, W.M. Tamashiro, Downregulation of L-arginine metabolism in dendritic cells induces tolerance to exogenous antigen, Int J Immunopathol Pharmacol, 30 (2017) 44-57.
[108] S. Corinti, S. Pastore, F. Mascia, G. Girolomoni, Regulatory role of nitric oxide on monocyte-derived dendritic cell functions, J Interferon Cytokine Res, 23 (2003) 423-431.
[109] C. Obregon, L. Graf, K.F. Chung, V. Cesson, L.P. Nicod, Nitric oxide sustains IL-1beta expression in human dendritic cells enhancing their capacity to induce IL-17-producing T-cells, PLoS One, 10 (2015) e0120134.
[110] V. Fernandez-Ruiz, A. Gonzalez, N. Lopez-Moratalla, Effect of nitric oxide in the differentiation of human monocytes to dendritic cells, Immunol Lett, 93 (2004) 87-95.
[111] K. Zhong, W. Song, Q. Wang, C. Wang, X. Liu, D. Chen, Z. Zhu, Y. Wu, W. Zhang, M. Zhang, Murine myeloid dendritic cells that phagocytose apoptotic T cells inhibit the immune response via NO, PLoS One, 7 (2012) e49378.
[112] K. Sugita, K. Kabashima, R. Yoshiki, A. Ikenouchi-Sugita, M. Tsutsui, J. Nakamura, N. Yanagihara, Y. Tokura, Inducible nitric oxide synthase downmodulates contact hypersensitivity by suppressing dendritic cell migration and survival, J Invest Dermatol, 130 (2010) 464-471.
[113] C.A. Bonham, L. Lu, R.A. Hoffman, R.L. Simmons, A.W. Thomson, Nitric oxide production by dendritic cells is associated with impairment of T cell responses, Transplant Proc, 29 (1997) 1116-1117.
[114] C.A. Bonham, L. Lu, Y. Li, R.A. Hoffman, R.L. Simmons, A.W. Thomson, Nitric oxide production by mouse bone marrow-derived dendritic cells: implications for the regulation of allogeneic T cell responses, Transplantation, 62 (1996) 1871-1877.
[115] K. Yamamoto, S.M. Akbar, T. Masumoto, M. Onji, Increased nitric oxide (NO) production by antigen-presenting dendritic cells is responsible for low allogeneic mixed leucocyte reaction (MLR) in primary biliary cirrhosis (PBC), Clin Exp Immunol, 114 (1998) 94-101.
[116] G. Kania, S. Siegert, S. Behnke, R. Prados-Rosales, A. Casadevall, T.F. Luscher, S.A. Luther, M. Kopf, U. Eriksson, P. Blyszczuk, Innate signaling promotes formation of regulatory nitric oxide-producing dendritic cells limiting T-cell expansion in experimental autoimmune myocarditis, Circulation, 127 (2013) 2285-2294.
[117] Y. Yoshioka, A. Yamamuro, S. Maeda, Nitric oxide at a low concentration protects murine macrophage RAW264 cells against nitric oxide-induced death via cGMP signaling pathway, Br J Pharmacol, 139 (2003) 28-34.
[118] P. Ghafourifar, U. Schenk, S.D. Klein, C. Richter, Mitochondrial nitric-oxide synthase stimulation causes cytochrome c release from isolated mitochondria. Evidence for intramitochondrial peroxynitrite formation, J Biol Chem, 274 (1999) 31185-31188.
[119] A. Stanford, Y. Chen, X.R. Zhang, R. Hoffman, R. Zamora, H.R. Ford, Nitric oxide mediates dendritic cell apoptosis by downregulating inhibitors of apoptosis proteins and upregulating effector caspase activity, Surgery, 130 (2001) 326-332.
[120] G.C. Brown, Regulation of mitochondrial respiration by nitric oxide inhibition of cytochrome c oxidase, Biochim Biophys Acta, 1504 (2001) 46-57

CHAPTER 5

Nitric oxide regulates glycogen metabolism utilization in LPS-activated dendritic cells.

Introduction

Dendritic cells (DCs) are canonical antigen presenting cells (APCs) of the immune system and play a crucial role in orchestrating the innate and adaptive immune responses. Through the expression of a broad array of pattern recognition receptors (PRRs), such as toll-like receptors (TLRs), DCs recognize a variety of microbial pathogens and inflammatory stimuli (*1, 2*). TLR stimulation of DCs initiates a series of activation events characterized by the upregulation of a number of genes and proteins required for surface co-stimulatory molecule expression, pro-inflammatory cytokine and chemokine secretion, the ability to migrate to secondary lymphoid tissues, and present antigens to T lymphocytes via antigen presentation by MHC molecules (*1*). These events are collectively termed "maturation" and coupled with a rapid change in metabolism, featured by the upregulation of aerobic glycolysis. TLR-driven aerobic glycolysis is essential in supporting DC effector function and survival (*3-6*), and inhibition of glycolysis has detrimental effects on the immune outcome.

Nitric oxide (NO) is a functionally versatile gas implicated in many physiological and patho-physiological processes. NO is generated from amino acid arginine by a catalytic reaction of three distinct enzymes – endothelial nitric oxide synthase (eNOS, NOS1), inducible NOS (iNOS), and neuronal NOS (nNOS, NOS3). eNOS and nNOS are constitutively expressed in a broad number of cells, whereas iNOS expression is induced upon exposure to infection or inflammatory stimuli (Bogdan, 2001; Bogdan, 2015; Förstermann and Sessa, 2012; Thwe and Amiel, 2018). Although iNOS is expressed in many different cell types, its role is widely appreciated in the context of infection responses in immune cells, such as DCs and macrophages. While NO has anti-microbial

properties (*7-10*), it can also alter cellular functions by a number of distinct mechanisms, including: formation of toxic compounds, such as superoxide and peroxy nitrite (*11*); post translational modification of proteins and nucleic acids via S-nitrosylation (*12-19*) or deamination (*20*), respectively. One of the deleterious effects of NO on the metabolism of activated DCs is impaired mitochondrial respiratory function due to S-nitrosylation of cytochrome C oxidase of the electron transport chain (ETC) (*21*), thus forcing cells to switch to aerobic glycolysis.

A large body of work has reported that GM-CSF cultured bone marrow-derived DCs (BMDCs), which is a classic mouse model of inflammatory DCs, and a few subsets of mouse tissue resident DCs express iNOS and produce NO. On the other hand, neither iNOS expression nor NO production is observed in GM-CSF and IL-4 cultured monocyte-derived human DCs (moDCs), a conventional model of human DCs commonly used in the field. Regardless of the functional similarities between BMDCs and moDCs, fundamental differences in NO-mediated immuno-metabolic effects between these two cell types are not clearly understood.

Previous studies have shown that murine DCs experience TLR-mediated glycolytic reprogramming in two distinct phases: an early glycolytic burst that is independent of nitric oxide (NO) and a later phase of activation that is supported by a long-term commitment to aerobic glycolysis in NO-producing DCs (*4, 6, 10*). The two phases of glycolytic reprogramming are also regulated by two different signaling mechanisms: the early phase, which happens immediately after TLR stimulation, is regulated by TBK1/IKKe/Akt signaling (*4*); and the NO-dependent glycolysis is largely controlled by the PI3k/mTOR/HIF-1a/iNOS axis (*5, 22*). DCs that do not make NO, such

as moDCs, engage in a TLR-driven early glycolytic burst for a brief period, lasting for about 4-6 hours, followed by a metabolic reversion back to pre-activation metabolism (*4*). In NO-producing DCs, activation of the PI3k/mTOR/HIF-1/iNOS axis forces the cells to commit to aerobic glycolysis that is sustained throughout the post-stimulation lifespan (*4*, *5*).

While several studies have demonstrated the effect of NO on glucose metabolism, including the aforementioned findings of NO on DCs, such as NO-mediated inhibition of aerobic respiration, little is known about the implication of NO on cellular glycogen metabolism. In liver glycogen metabolism, iNOS expression and NO production has been shown to induce insulin resistance by reducing glycogen synthase activity (*23*, *24*). On the other hand, iNOS expression is coupled with a rise in glycogen levels in stimulated astrocytes (*25*). Given that NO has a substantial impact on immune cell metabolism, we hypothesized that NO is likely involved in regulating glycogen utilization in DCs.

Here, we show that NO influences glycogen utilization in BMDCs and moDCs via distinct mechanisms and that PI3k-mTOR signaling is most likely regulating this process in mouse DCs. Our data altogether suggest a potential regulatory mechanism of DC glycogen utilization, by which NO affects TLR-activated DCs via modulating their glycogen metabolic pathways.

Materials and Method

Mouse DC culture and activation

Bone marrow-derived DCs (BMDCs) were generated from 6-12-week-old C57/Bl6J mice as follows: bone marrow cells were flushed from femurs and the cells

were differentiated in murine recombinant GM-CSF (20ng/mL; Peprotech) in complete DC medium (CDCM), comprised of RPMI1640, 10%FBS, 2mM L-glutamine, 1IU/mL Pen-Strep, 1mM beta-mercaptoethanol, for 7 days, with a medium change every 2 days. On Day 7, DCs were harvested, washed in CDCM and cultured at 2x10^5 cells/well (96-well plate) per 200μL of total volume per well in indicated treatment conditions.

Reagents

These reagents were purchased from given companies: SEITU (iNOS inhibitor) (500μM): Tocris; NO donor, SNAP: Cayman chemical; LPS (100ng/mL): Invivogen; Oligomycin (1μM): Sigma-Aldrich; and Rotenone (100nM): Sigma-Aldrich.

In vitro differentiation and isolation of human monocyte-derived DCs

Leukocyte Filters of de-identified donors were provided by CVPH medical center blood bank in Plattsburgh, NY. Filters were reverse-flushed with sterile 1xDPBS (without calcium and magnesium) and peripheral blood monocytes (PBMCs) were extracted by Ficoll-plaque centrifugal separation (density gradient of 1.0772) using LSM (lymphocyte separation medium, MP Biochemical). Monocytes were enriched using CD14 positive magnetic selection beads per manufacturer protocols (Miltenyi Bioscience) and resulting monocytes were cultured in complete DC medium containing human recombinant GM-CSF (20ng/mL) and human recombinant IL-4 (20ng/mL) (Peprotech) for 7 days. On day 7, monocyte-derived dendritic cells (moDCs) were harvested, stimulated as indicated, and intracellular glycogen levels were assessed from prepared lysates, using Biovision hydrolysis enzyme and Eton Glucose reagent kit.

BMDC and moDC co-culture

BMDCs were pre-stimulated with LPS, with and without SEITU (500μM), at 0.5x10^6 cells per well for the first 6 hours in Transwell inserts. moDCs were stimulated for 24 hours with and without LPS at 0.5x10^6 cells/well in lower chambers of a transwell plate. After 24hours total, moDCs were harvested for analysis of intracellular glycogen contents.

Western blot

Cell lysates were prepared using 2X NP-40 lysis buffer. 20μg protein was loaded into each well of a 12.5% polyacrylamide gel, transferred onto activated nitrocellulose membrane (BioRad). Electrophoretic transfer was performed using Trans-Blot Turbo RTA mini Nitrocellulose transfer kit. Membranes were blocked in 2% milk in 1xTBST at RT for 1hr, and incubated in indicated antibody at 4°C overnight. Blots were washed 3x in 1xTBST at RT, probed with secondary antibodies at RT for 45-60 minutes, and washed 3-4x with 1x TBST. Proteins were visualized by SuperSignal West Pico Chemiluminescent substrate and exposed with GeneXpert System imager. Trans-Blot Turbo Transfer system and secondary antibodies for Western blots were generously provided by Dr. Paula Deming, Medical Laboratory and Radiation Science Department, UVM.

Metabolism assays

Glycogen assay

Intracellular glycogen was measured using a modified protocol that consists of the use of hydrolysis enzyme from Biovision Inc. and glucose assay reagents from Eton Bioscience as briefly described: Diluted cell lysates were incubated with and without hydrolysis enzyme (Biovision Inc.) for 30 minutes at room temperature, followed by addition of glucose assay reagents (Eton Bioscience) and subsequent incubation for 15 minutes at 37^0-C in a non-CO2 incubator. Color development was measured at 490nm.

Nitrite assay

Supernatants collected from indicated culture conditions were assayed for nitrite using Griess nitrite assay kit (Life Technology Inc.) per manufacturer instructions.

Statistical analysis

Data were analyzed using Graphpad Prism (version 7) and statistical analyses were calculated using paired student's *t* test and one-way ANOVA test.

Results

BMDCs but not human monocyte-derived DCs accumulate more glycogen within 24hours of LPS stimulation

We demonstrated that the stimulation of BMDCs and moDCs with LPS for 6 hours results in a partial depletion of intracellular glycogen in BMDCs (**Figure 5 -1A),** a finding consistent with our previously published work (*26*), and a minimal change in the glycogen levels of moDCs (**Figure 5 -1B**). We also showed that DCs utilize intracellular glycogen to support the TLR-driven early effector responses and that glycogen-carbons

contribute directly to fuel acute glycolytic reprogramming required for DC activation (*26*). Although our recent work has shown that DCs require glycogen for the expression of key maturation-associated surface proteins within 24 hours of LPS stimulation, their glycogen metabolic utilization during this late activation stage has not yet been elucidated.

Thus, we were interested in examining DC utilization of glycogen metabolism beyond 6 hours of activation. To test this, we stimulated BMDCs and moDCs for 24 hours and measured the intracellular glycogen content. Because DCs partially utilize their stored glycogen within the first 6 hours of LPS activation (*26*), we predicted that stimulation with LPS for 24 hours would deplete the entire stock of intracellular glycogen in both cell types. As expected, the glycogen level in moDCs is significantly reduced although it is not completely exhausted (**Figure 5 -1C**). To our surprise, LPS-stimulated BMDCs accumulate glycogen to levels higher than their resting counterparts (**Figure 5 -1D**), and this is not affected by the *in vitro* culture differentiation in cytokine GM-CSF, the glycogen levels of which were compared with those of splenic tissue-resident DCs (data not shown). This rise in glycogen content is observed only in murine BMDCs, but not in human moDCs, and raises two major questions: 1) why do BMDCs utilize glycogen in early activation but increase their storage in late activation? and 2) why is there a difference in glycogen utilization between mouse and human DCs in the late activation phase?

Previous work by us and others has shown that mouse GM-CSF cultured BMDCs are capable of upregulating inducible nitric oxide synthase (iNOS) and generating nitric oxide (NO) a few hours after TLR-activation, and that human DCs do not express nor

produce NO (*6*). Therefore, it is plausible that NO is a common solution to both questions described above. Because iNOS upregulation in BMDCs only occurs a few hours after TLR activation prompted us to examine the effect of NO on BMDC glycogen metabolism during the late activation phase. Therefore, we hypothesized that TLR-driven glycogen accumulation in 24 hour-activated BMDCs is mediated by NO production in these cells. Additionally, the fact that moDCs did not display a similar phenotype might be attributed to their lack of NO production. Therefore, we next set out to test the hypothesis that NO is the underlying cause of glycogen accumulation in BMDCs during their later activation stages.

Glycogen accumulation in BMDCs is mediated by NO

To determine whether iNOS upregulation and NO induction in BMDCs elevates intracellular glycogen contents, we stimulated BMDCs with LPS for 24 hours in the presence or absence of the potent and highly specific iNOS inhibitor, SEITU, and measured nitrite and intracellular glycogen levels. Consistent with our previously published data (*6*), nitrite production from activated BMDCs was attenuated to baseline levels in the presence of SEITU (**Figure 5 -2A**). In support of our hypothesis, SEITU also abolished the intracellular glycogen accumulation in activated BMDCs (**Figure 5 -2B**), suggesting that NO is potentially at play in glycogen accumulation beyond 6 hours of activation in the murine DC model, thereby resulting in the increased synthesis or decreased consumption of glycogen by these cells.

Next, we questioned whether we would observe a similar NO-induced glycogen buildup in moDCs. Because moDCs do not make NO in response to LPS stimulation, we

introduced exogenous NO to the cultures. We specifically employed two different approaches to induce NO into the moDC culture media. First, to attain a constant level of NO from the NO-donor SNAP, we added SNAP into the culture medium every 5 hours for a total of 15 hours. Contrary to our expectation, glycogen utilization in moDCs was accelerated in the presence of the NO-donor in both resting and LPS-activated conditions, with a more pronounced effect in the resting cells (**Figure 5 -2C**). In parallel, we co-cultured BMDCs, that were pre-stimulated with LPS for the first 6 hours to serve as a source of NO, with LPS-activated moDCs in a transwell assay plate and examined the glycogen content of moDCs (**Figure 5 -2D**). NO generated from the LPS-activated BMDCs caused no effect on the intracellular glycogen level of moDCs (**Figure 5 -2D**). Interestingly, the acute induction of exogenous NO to moDCs for 6 hours caused a much faster depletion of glycogen (**Figure 5 -2E**) than the prolonged exposure to NO shown in **Figure 5 -2C**. Despite a relative difference in the rate of glycogen depletion in the NO-containing media in **Figure 5 -2C** and **E**, the NO-mediated rapid depletion of glycogen in moDCs was consistent across any time course in both unstimulated and LPS-activated conditions. The fact that NO-exposed moDCs display a different phenotype in consuming their intracellular glycogen, a finding opposite from BMDC glycogen utilization, indicates that the glycogen metabolism is differentially regulated by NO in BMDCs and moDCs.

PYGL/GYS1 Protein expression by iNOS inhibitor

Glycogen accumulation observed in activated BMDCs (**Figure 5 -1C** and **Figure 5 -2B**) is potentially due to the inhibitory effect of NO on the rate-limiting enzyme

glycogen phosphorylase (PYG), perhaps via S-nitrosylation reaction of NO, thereby preventing the cells from utilizing intracellular stores of glycogen. It is also plausible that NO can promote the activity of glycogen synthase (GYS), rate-limiting enzyme of glycogen synthesis, resulting in a net buildup of glycogen in BMDCs. Or in the case of moDCs, it is also likely that the activity of GYS is inhibited by NO, possibly resulting in a metabolic imbalance towards over-utilization of glycogen. To determine the impact of NO on the expression of these two enzymes, we determined the protein expression levels of GYS1 and PYGL of BMDCs stimulated with LPS for 24 hours in the presence or absence of SEITU. GYS1 expression is increased with LPS stimulation but its level is relatively similar in the presence of the iNOS inhibitor (**Figure 5 -3A**). In contrast to GYS1 expression, PYG expression does not change regardless of LPS activation (**Figure 5 -3B**). To our surprise, GYS1 expression in moDCs showed no differences regardless of any treatment conditions (data not shown).

Glycogen accumulation/depletion is due to NO-induced mitochondria toxicity

Among the multifaceted effects of NO on cellular physiological functions, the detrimental impact on cellular metabolism is its ability to block oxidative phosphorylation (OXPHOS) by reversible S-nitrosylation of cytochrome C oxidase of the electron transport chain (ETC), resulting in a collapse of mitochondrial function (*13*). Therefore, we were interested in examining whether the NO-mediated mitochondrial impairment induces the cells to accumulate intracellular glycogen in DCs. To test this, we stimulated moDCs with LPS for 24 hours in the presence or absence of mitochondrial toxins, Oligomycin (ATP synthase inhibitor) and Rotenone (ETC transport inhibitor) and

measured intracellular glycogen content. Intriguingly, the inhibition of mitochondrial function in resting moDCs forced the cells to rapidly deplete their intracellular glycogen stores, a phenomenon not observed in the LPS-activated cells (**Figure 5 -4A**). Forcing the cells to switch to aerobic glycolysis via the introduction of mitochondrial toxins, Oligomycin and Rotenone, accelerates the moDC utilization of intracellular glycogen, which is likely to fuel glucose-carbons directly to the glycolytic pathway. While these effects shown here were not mediated by NO, the fact that these phenotypes were similar to their exogenous NO-mediated effects shown in **Figure 5 -2C** and **E** suggested a possible link between NO and the mitochondrial dysfunction, both of which result in the increased glycogen utilization in resting moDCs.

NO-mediated glycogen accumulation is regulated by PI3k-mTOR axis

TLR-driven metabolic changes in BMDCs have been demonstrated to be mediated by two distinct temporally regulated signaling axes: the NO-independent early activation phase mediated by TBK1/IKK-Akt; and the NO-dependent late activation stage mediated by PI3k/Akt/mTOR signaling. Based on this, we were interested in finding out whether the observed increase in DC glycogen level is under mTOR regulation. We first measured the total glycogen levels of BMDCs stimulated with LPS for 24 hours in the presence of PI3k inhibitor (Ly), mTORC1 inhibitor (Rapamycin) and iNOS inhibitor (SEITU). Inhibition of mTORC1 or PI3k dramatically attenuated the glycogen levels regardless of activation (**Figure 5 -5A**). iNOS inhibitor, SEITU, also knocked down the glycogen content, even much more dramatically than the former two inhibitors, suggesting the PI3k-mTOR-iNOS axis, the same signaling pathway for NO

production, is implicated in the regulation of glycogen metabolism in activated BMDCs. As expected, iNOS inhibition of activated BMDCs resulted in the attenuation of nitrite levels to the baseline (**Figure 5 -5B**), a phenotype similar to the reduced glycogen levels upon iNOS-inhibition shown in **Figure 5 -5A**. Interestingly, PI3k or mTORC1 inhibition resulted in the minimal reduction of nitrite levels (**Figure 5 -5B**) as opposed to the dramatic decrease in glycogen content observed in **Figure 5 -5A**. Of note, the fact that PI3k inhibitor, Rapamycin, and iNOS-inhibitor reduced the glycogen levels in untreated cells suggested the basal signaling activity of the PI3k/mTOR axis. These data implied that the regulation of BMDC glycogen utilization is not necessarily dependent on the presence of NO although it appears to be mediated by iNOS upregulation via the PI3k-mTOR-iNOS axis.

Next, we were interested in determining whether the master regulatory protein GSK3β, which is also a regulatory kinase protein of canonical glycogen synthesis pathway, regulates TLR-driven BMDC utilization of glycogen metabolism. GSK3β canonically acts as a negative regulator of glycogen synthase GYS and inhibition of GSK3β generally results in active glycogen synthesis. Interestingly, GSK3β inhibition had very little impact on glycogen accumulation in BMDCs (**Figure 5 -5A**). The protein expression of GYS1 dramatically increased in the un-stimulated BMDCs compared to that of activated counterparts (**Figure 5 -5C**), suggesting that the GSK3β-mediated signaling pathway for glycogen synthesis in BMDCs seems to be different from the canonical glycogen metabolic regulation.

Discussion

Multiple lines of evidence have shown the pleotropic effects of iNOS-derived NO on immune responses. NO originated from tissue macrophages has potent anti-microbial activity against intracellular pathogens, such as *Salmonella* (*27*), *Mycobacterium tuberculosis* (*8*) and *Leishmania major* (*28-30*). In line with its anti-microbial effects, NO plays an important role in promoting TH-1 polarization by mature DCs (*31*), enhancing pro-inflammatory cytokine responsiveness, and increasing NK cell cytotoxic ability (*32*). In contrast to its ability in enhancing immune responses and clearing microbial pathogens, inhibition of iNOS activity in NO-generating cells is associated with reduced responsiveness to IL-12 in inflammatory environments, increased pathogen burdens, and impaired NK-cell activity (*28, 32*). Other studies have also reported the immuno-suppressive effects of NO, such as reduced proliferation of T cells that are exposed to NO from NO-producing cells (*33, 34*). While NO is shown to induce inflammatory cytokine responses of innate immune cells (*28, 35*), certain cytokines such as IFN-γ and/or LPS stimulation of DCs can induce the expression of iNOS and generation of NO (*33, 34, 36*), indicating that NO production can be induced by non-microbial stimuli.

Despite its protective actions against invading pathogens, NO is known to cause detrimental effects on immune cell longevity and certain aspects of immune responses (*6, 28, 35, 37*). A significant body of work has demonstrated that NO significantly impairs the antigen presentation ability of TLR-stimulated NO-producing DCs and that inhibition of iNOS rescues their effector function (*6, 33, 34*). iNOS suppression in LPS-activated macrophages is also shown to inhibit the polarization of macrophages to pro-inflammatory M1 macrophages (*38*).

DCs are highly specialized in antigen recognition, capture, and stimulation of T cells, thus serving as a critical link between innate and adaptive immune responses (*1, 39*). Activation of GM-CSF cultured murine BMDCs is coupled with a rapid metabolic reprogramming, which is characterized as the initial NO-independent burst of glycolysis, lasting for a few hours, followed by the NO-mediated sustained aerobic glycolysis. Although these NO-driven metabolic effects in DCs were shown to arise from an endogenous source of NO, a recent study has established that NO originated from other cells impairs the mitochondria activity of iNOS deficient DCs (*22*). NO-mediated impaired effector responses are also observed in non-NO-producing immune cells that are exposed to exogenous NO present in tissue micro-environments (*33, 34, 36, 40*), suggesting the serious functional outcomes inflicted by NO can still happen regardless of the cell's ability to produce NO.

Previous studies have identified the implication of NO in cellular ability to utilize glucose and mitochondria in DCs (*3, 5, 6, 41*). In addition, the impact of NO production and iNOS expression in regard to glycogen metabolism has been widely investigated in non-immune systems (*23-25, 42*). In this study, we have demonstrated the link between NO and glycogen utilization of activated DCs. We have also attempted to establish a fundamental difference in glycogen utilization between mouse BMDCs and human moDCs and found that these two types of DCs respond differently to iNOS upregulation or exogenous NO. Here, we show the increased intracellular glycogen levels in iNOS-upregulated BMDCs and the NO-driven acceleration of glycogen consumption in moDCs (**Figure 5 -2**). These findings reveal that iNOS upregulation and exogenous NO can differentially regulate the glycogen utilization of BMDCs and moDCs, respectively.

NO production in BMDCs is regulated by the canonical signaling axis, PI3k-mTOR-iNOS (*5, 6, 43, 44*), and our findings in **Figure 5 -5A&B** suggest that BMDCs undertake the similar signal transduction mechanism in regulation of their glycogen metabolism. Since PI3k-mTOR signaling is responsible for glycolytic upregulation in TLR-activated BMDCs, inhibition of this axis would force the cells to depend heavily on the mitochondrial respiration for bioenergetics purposes (*5, 43*). On this account, the reduction of intracellular glycogen in the PI3k- and mTOR-inhibited BMDCs is possibly due to the direct contribution of glycogen into mitochondrial functions. This notion is further supported by the increased glycogen levels upon iNOS upregulation in these cells (**Figure 5 -1** and **Figure 5 -2**), strongly suggesting that there is a potential link between NO-induced mitochondrial toxicity and increased glycogen accumulation in BMDCs.

As the long-term commitment to aerobic glycolysis in TLR-activated BMDCs is a survival and metabolic adaptation mechanism due to NO-induced mitochondrial dysfunction (*5, 10, 43, 44*), we have tried to understand our data on NO-mediated regulation of glycogen metabolism within this framework. We reason that a large pool of glycogen induced by NO would provide a continuous supply of glucose carbons for aerobic glycolysis induced in conditions of NO-mediated mitochondrial stress in BMDCs. While we have not formally tested this concept in the murine model of BMDCs, we examined the effect of mitochondrial inhibition on moDC glycogen utilization (**Figure 5 -4A**). Inhibition of mitochondrial OXPHOS induced by the mitochondrial toxins forces the cells to switch to aerobic glycolysis. This metabolic switch is coupled with the acceleration of glycogen utilization in moDCs, suggesting that glycolysis is directly fueled by the glucose-carbon derived from glycogen. In this framework, we have

yet to establish this phenotype in correlation with NO-mediated regulatory mechanisms in moDCs. In light of this, it appears that both mouse and human models of DCs in this study differentially respond to the induction of NO in the inflammatory environments.

A major question still remains as to how these TLR-stimulated BMDCs can continuously build their glycogen stock during the time of metabolic crisis because glycogen synthesis is an energetically costly process. One possibility is that those glycogen pools are not static in nature, but constantly turning over by engaging in the "glycogen shunt," as shown in our recent publication and other work in brain and muscle (*26, 45-52*). Because G6P derived from the glycogen shunt can theoretically enter the hexose monophosphate shunt, which is also known as pentose phosphate pathway (PPP), and generate energy in the form of NADPH, DC participation in the glycogen shunt can potentially protect the cells from energetic crisis. On the other hand, continued expenditure on building intracellular glycogen stocks can possibly trigger "metabolic exhaustion." Similar to the concept of "exhaustion" in T effector cell function (*53*), metabolic exhaustion can arise from bioenergetic insufficiencies and prolonged metabolic stress (*54*). One of the contributing factors for metabolic exhaustion is shown to be directly associated with chronic mTOR signaling during T cell receptor activation (*55*). While our data suggest the TLR-mediated accumulation of glycogen is under regulation of PI3k-mTOR signaling, we suspect these DCs would undergo metabolic exhaustion perhaps due to two reasons. The first is that unlike the early and prolonged activation of mTOR in T cells, mTOR-mediated transcriptional and translational events in TLR-activated DCs can take several hours (*55*). Secondly, it is likely that the cellular energy sensor AMP kinase (AMPK) could become activated at a much later stage of TLR

stimulation presumably due to the fact that the cellular energetic payloads for synthesizing such a large pool of glycogen in BMDCs can trigger AMPK activation that antagonizes mTOR activities.

One of the potential mechanisms for a rise in intracellular glycogen in activated BMDCs and the opposite phenotype observed in moDCs is post-translational modification (PTM) of NO on glycogen phosphorylase enzyme. NO has been historically known to modulate the activities of a majority of metabolic proteins and enzymes through the reversible S-nitrosylation at their cysteine residues (*12, 14, 56, 57*), and glycogen phosphorylase is one of those enzymes susceptible to S-nitrosylation reaction (*15, 58-60*). Although the activity of PYG in brain and hepatocytes is shown to be enhanced upon reversible S-nitrosylation, further aspects of S-nitrosylated PYG function has not been identified in any other systems (*58, 61*). In light of this, it is likely that the regulatory activities of S-nitrosylated PYG may be opposite in NO-producing BMDCs and NO-non-producing, thereby resulting in their differential utilization of intracellular glycogen in these two models. The information acquired from identification of PYG S-nitrosylation in DCs would be immensely helpful in better understanding of regulatory and signaling mechanisms implicated in cellular functional aspects. This necessitates further work including the investigation of whether or not PYG is S-nitrosylated in TLR-activated DCs.

Overall, we believe that we have discovered an intriguing behavior of glycogen metabolism in both mouse and human models of TLR-activated DCs, which is potentially regulated by NO (a proposed model illustrating this mechanism shown in **Figure 5-6**). Despite the fact that moDCs do not express iNOS nor produce NO, a number of studies

demonstrated that NO is generated in human patients of tuberculous granuloma, granulomatous infections, and psoriasis (*62-64*), strongly implying the tremendous side-effects of NO-mediated metabolic regulation on infiltrating immune cells, such as inflammatory monocyte derived DCs from the peripheral circulation. Given that NO functions as a "double-edged sword" in its ability to both enhance and suppress immune responses during infections, elucidating this novel regulatory mechanism in the near future will definitely provide valuable insights into how we can harness the desired effects mediated by NO, without harming the host cells, to elicit optimal immune outcomes.

Figures

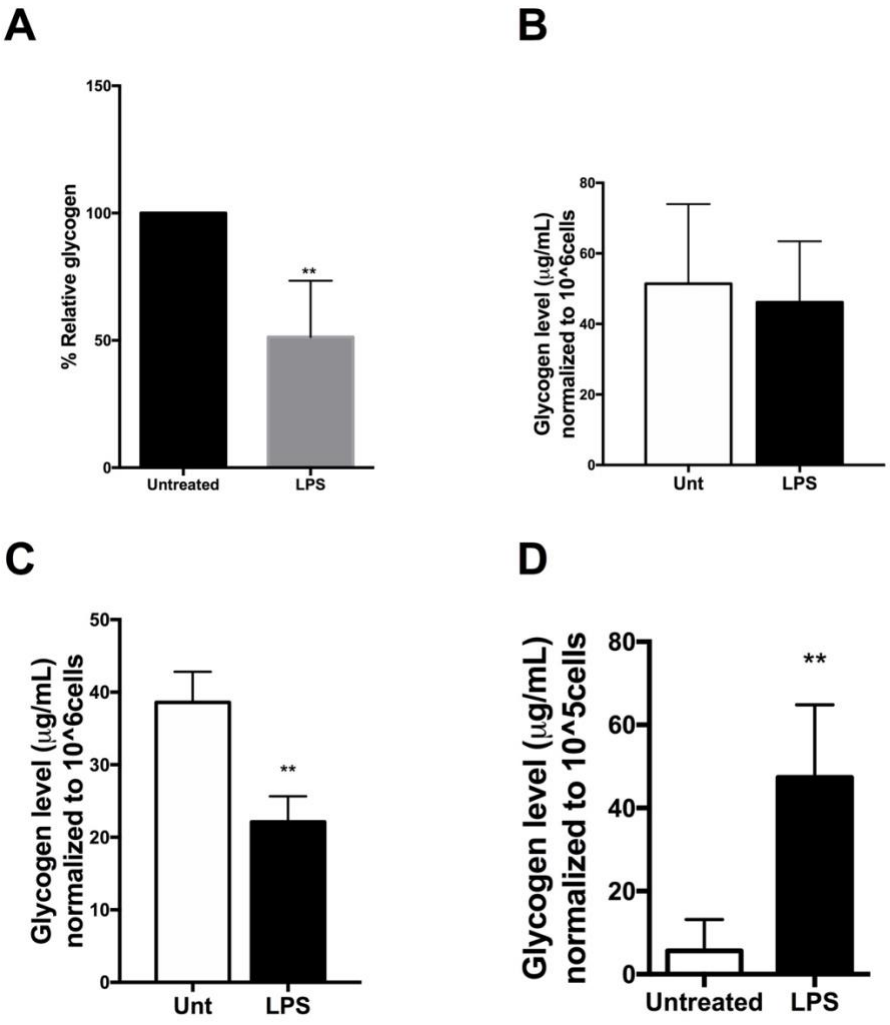


Figure 5 -1: BMDCs, but not human monocyte-derived DCs (moDCs) accumulate more glycogen.

(A and B) BMDCs (A) and moDCs (B) were stimulated with LPS for 6 hours. Intracellular glycogen levels were then measured from the cellular lysates. For (A), n=5 and p **<0.005 for. For B, n=3. (C and D) moDCs (C) and BMDCs (D) were stimulated with LPS for 24 hours, and then intracellular glycogen levels were measured from cell lysates. For C, n=3 and **p <0.005. For D, n=3 and **p<0.005. Data were analyzed using student's t test.

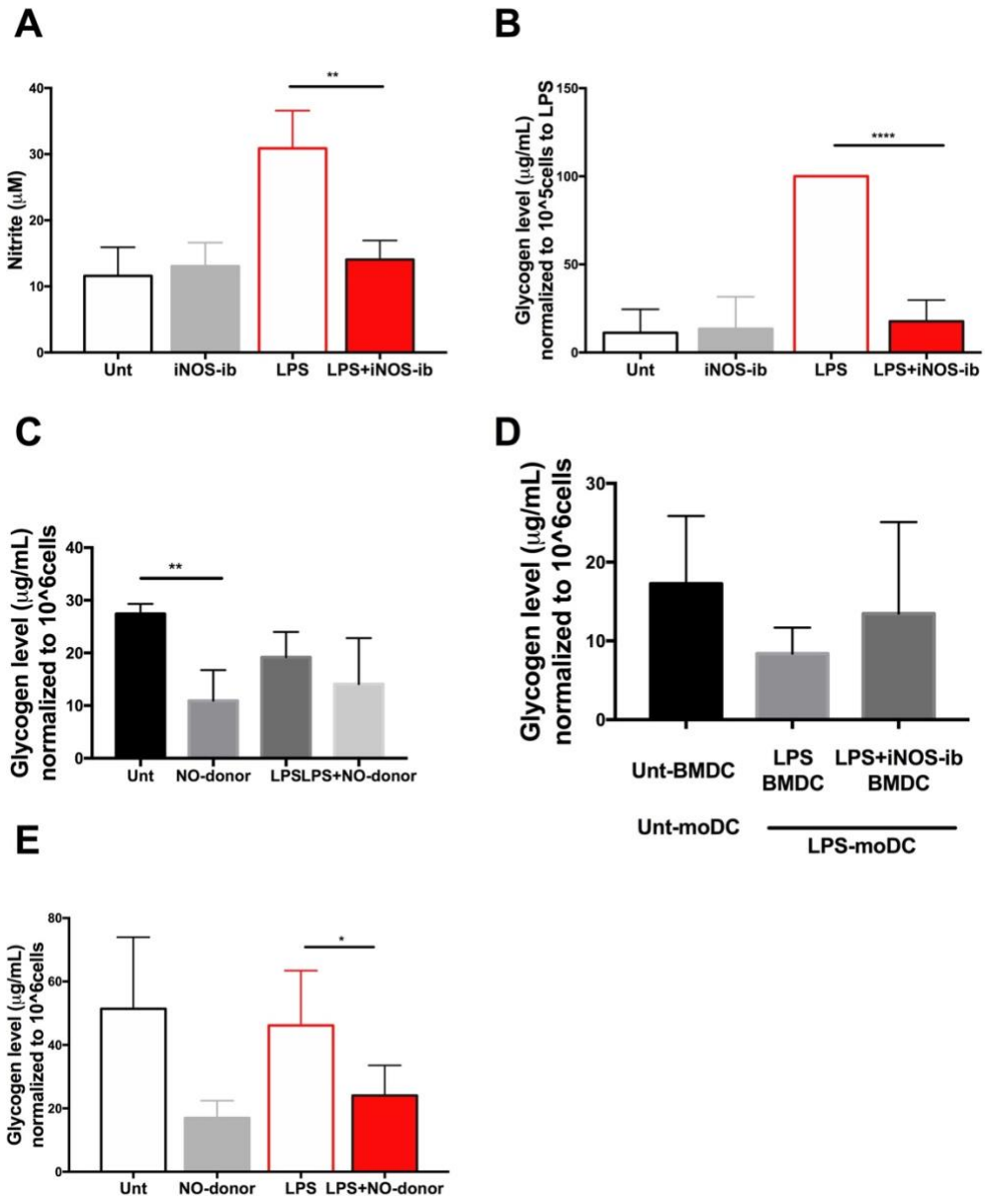


Figure 5 -2: Glycogen accumulation in LPS-activated BMDCs is mediated by NO

(A and B) BMDCs were stimulated with and without LPS for 24 hours in the presence or absence of iNOS inhibitor (iNOS-ib). Unt=untreated (A) Supernatants were collected from the culture media and Griess Nitrite assay was performed. n=4; **p<0.005. (B) Cell lysates were prepared from the media and glycogen levels were measured. Data were normalized to LPS-only group; n=4; and ***p<0.0005. (C) moDCs were stimulated with LPS and NO-donor (SNAP) was added to the media every 5 hours, for a total of 15 hours. Cell lysates were then prepared and glycogen levels were measured. n=3; **p=0.0095. (D) BMDCs were pre-activated with LPS, with and without iNOS inhibitor

(SEITU) for 6 hours before co-culturing with moDCs for the next 18 hours. At the time of co-culture, moDCs were stimulated with and without LPS in the lower chambers of a transwell plate, and pre-activated BMDCs were incubated in transwell inserts. Supernatants were collected for Griess nitrite assay, and glycogen levels were measured from the cell lysates of moDCs. n=3. (E) moDCs were stimulated with and without LPS in the presence or absence of NO-donor for 6 hours, and glycogen levels were measured from the lysates. n=3; *p=0.05. Statistical analyses were calculated with *student's t* test.

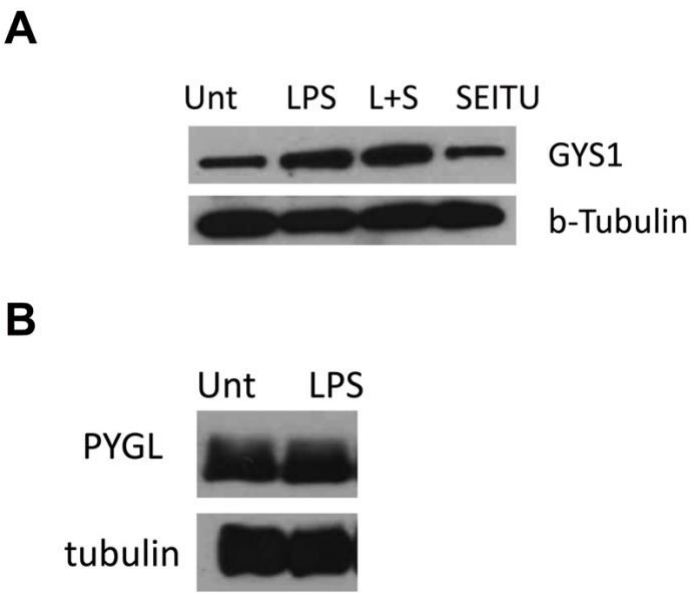


Figure 5 -3: PYG and GYS1 expression in iNOS-inhibited BMDCs.

(A) Glycogen synthase 1 (GYS1) expression of BMDCs stimulated with and without LPS for 24 hours in the presence or absence of iNOS-inhibitor (SEITU). β-tubulin was used as loading controls. (B) Liver Glycogen phosphorylase (PYGL) protein expression of BMDCs stimulated with LPS for 24 hours. β-tubulin was used as loading controls. (A and B) Representative of n=5.

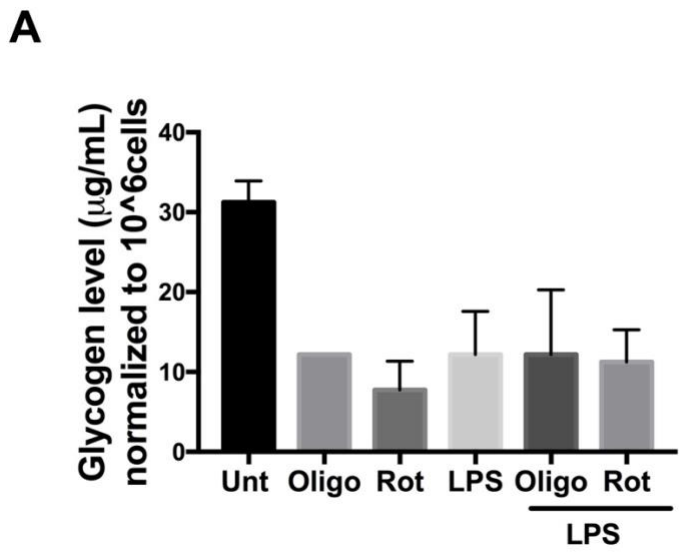


Figure 5 -4: Mitochondrial toxicity induces glycogen depletion in moDCs.

(A) moDCs were stimulated with and without LPS for 24 hours in the presence or absence of Oligomycin and Rotenone and glycogen levels were assessed from cellular lysates.

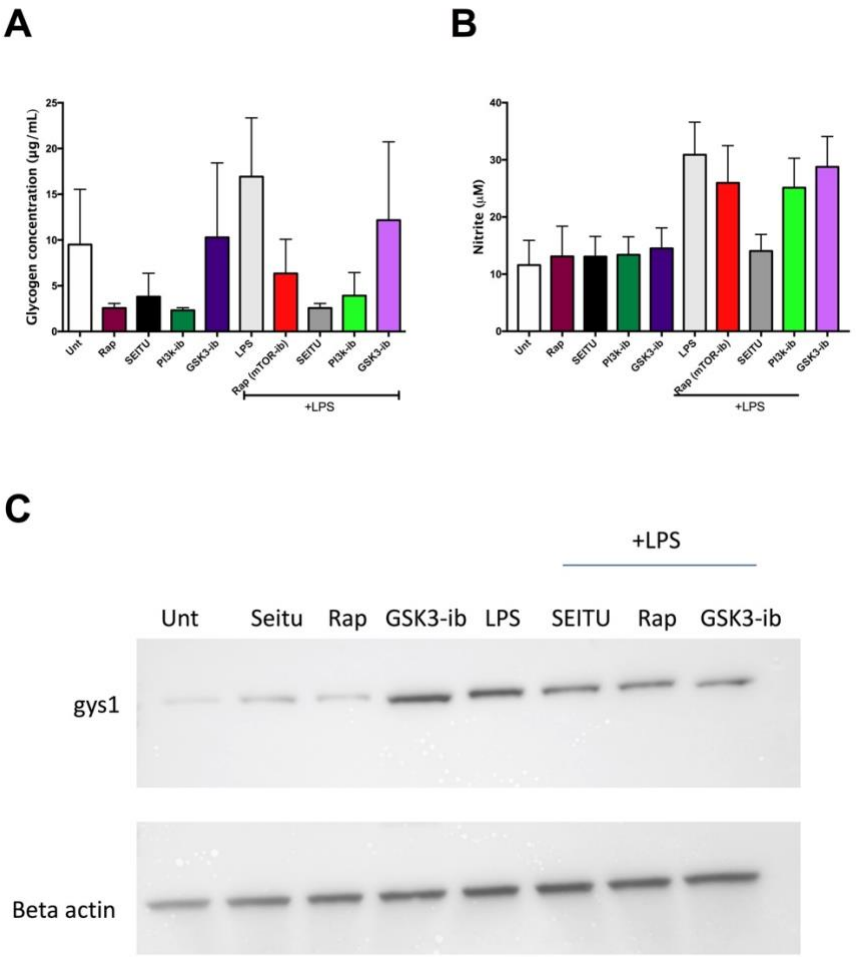


Figure 5 -5: NO-mediated BMDC glycogen accumulation is mediated by PI3k-mTOR axis.

(A and B) BMDCs were stimulated with and without LPS for 24 hours in the presence or absence of mTOR inhibitor (Rapamycin), PI3k-inhibitor, iNOS inhibitor (SEITU), and GSK3β-inhibitor. (A) Griess nitrite assay from culture supernatant; n=4. (B) Cell lysates were prepared and glycogen content was assessed. n=3. (C) Expression of GYS1 from cell lysates of BMDCs stimulated similarly as in A. β-actin was used as loading controls.

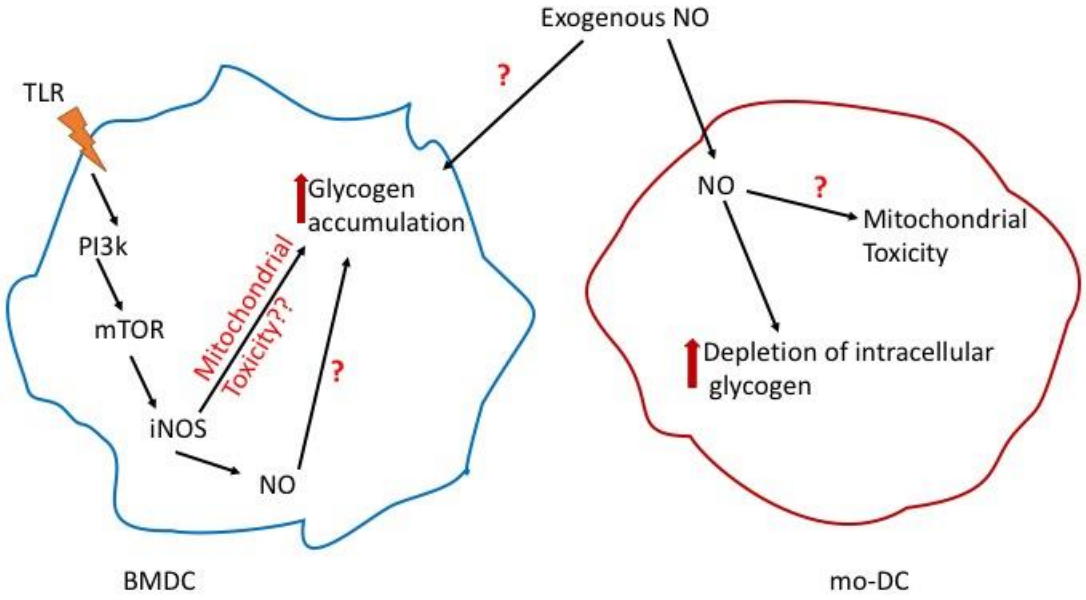


Figure 5-6: Model Illustration of NO/iNOS regulation in BMDC and moDC

References

1. J. Banchereau, R. M. Steinman, Dendritic cells and the control of immunity. *Nature* **392**, 245-252 (1998).
2. R. M. Steinman, Decisions about dendritic cells: past, present, and future. *Annual review of immunology* **30**, 1-22 (2012).
3. E. Amiel *et al.*, Inhibition of mechanistic target of rapamycin promotes dendritic cell activation and enhances therapeutic autologous vaccination in mice. *Journal of immunology (Baltimore, Md. : 1950)* **189**, 2151-2158 (2012).
4. B. Everts *et al.*, TLR-driven early glycolytic reprogramming via the kinases TBK1-IKKvarepsilon supports the anabolic demands of dendritic cell activation. *Nature immunology* **15**, 323-332 (2014).
5. C. M. Krawczyk *et al.*, Toll-like receptor-induced changes in glycolytic metabolism regulate dendritic cell activation. *Blood* **115**, 4742-4749 (2010).
6. B. Everts *et al.*, Commitment to glycolysis sustains survival of NO-producing inflammatory dendritic cells. *Blood* **120**, 1422-1431 (2012).
7. R. Olekhnovitch, B. Ryffel, A. J. Müller, P. Bousso, Collective nitric oxide production provides tissue-wide immunity during Leishmania infection. *The Journal of clinical investigation* **124**, 1711-1722 (2014).
8. J. E. Qualls *et al.*, Sustained generation of nitric oxide and control of mycobacterial infection requires argininosuccinate synthase 1. *Cell Host Microbe* **12**, 313-323 (2012).
9. A. P. Ralph *et al.*, Impaired pulmonary nitric oxide bioavailability in pulmonary tuberculosis: association with disease severity and delayed mycobacterial clearance with treatment. *The Journal of infectious diseases* **208**, 616-626 (2013).
10. P. M. Thwe, E. Amiel, The role of nitric oxide in metabolic regulation of Dendritic cell immune function. *Cancer letters* **412**, 236-242 (2018).
11. R. Radi, Peroxynitrite, a Stealthy Biological Oxidant. *Journal of Biological Chemistry* **288**, 26464-26472 (2013).
12. O. A. Bizzozero, J. Zheng, Identification of major S-nitrosylated proteins in murine experimental autoimmune encephalomyelitis. *Journal of neuroscience research* **87**, 2881-2889 (2009).
13. E. Clementi, G. C. Brown, M. Feelisch, S. Moncada, Persistent inhibition of cell respiration by nitric oxide: crucial role of S-nitrosylation of mitochondrial complex I and protective action of glutathione. *Proc Natl Acad Sci U S A* **95**, 7631-7636 (1998).
14. M. W. Foster, T. J. McMahon, J. S. Stamler, S-nitrosylation in health and disease. *Trends in molecular medicine* **9**, 160-168 (2003).
15. D. T. Hess, A. Matsumoto, S. O. Kim, H. E. Marshall, J. S. Stamler, Protein S-nitrosylation: purview and parameters. *Nature reviews. Molecular cell biology* **6**, 150-166 (2005).
16. T. Into *et al.*, Regulation of MyD88-dependent signaling events by S nitrosylation retards toll-like receptor signal transduction and initiation of acute-phase immune responses. *Molecular and cellular biology* **28**, 1338-1347 (2008).

17. S. Shinozaki *et al.*, Liver-specific inducible nitric-oxide synthase expression is sufficient to cause hepatic insulin resistance and mild hyperglycemia in mice. *The Journal of biological chemistry* **286**, 34959-34975 (2011).
18. J. Sun, M. Morgan, R.-F. Shen, C. Steenbergen, E. Murphy, Preconditioning Results in S-Nitrosylation of Proteins Involved in Regulation of Mitochondrial Energetics and Calcium Transport. *Circulation Research* **101**, 1155-1163 (2007).
19. T. Yasukawa *et al.*, S-nitrosylation-dependent inactivation of Akt/protein kinase B in insulin resistance. *The Journal of biological chemistry* **280**, 7511-7518 (2005).
20. J. L. Caulfield, J. S. Wishnok, S. R. Tannenbaum, Nitric Oxide-induced Deamination of Cytosine and Guanine in Deoxynucleosides and Oligonucleotides. *Journal of Biological Chemistry* **273**, 12689-12695 (1998).
21. B. Beltran, A. Mathur, M. R. Duchen, J. D. Erusalimsky, S. Moncada, The effect of nitric oxide on cell respiration: A key to understanding its role in cell survival or death. *Proc Natl Acad Sci U S A* **97**, 14602-14607 (2000).
22. S. J. Lawless *et al.*, Glucose represses dendritic cell-induced T cell responses. *Nat Commun* **8**, 15620 (2017).
23. S. Shinozaki *et al.*, Liver-specific Inducible Nitric-oxide Synthase Expression Is Sufficient to Cause Hepatic Insulin Resistance and Mild Hyperglycemia in Mice. *Journal of Biological Chemistry* **286**, 34959-34975 (2011).
24. F. Sprangers, H. P. Sauerwein, J. A. Romijn, G. M. van Woerkom, A. J. Meijer, Nitric oxide inhibits glycogen synthesis in isolated rat hepatocytes. *The Biochemical journal* **330 (Pt 2)**, 1045-1049 (1998).
25. N. F. Cruz, G. A. Dienel, High glycogen levels in brains of rats with minimal environmental stimuli: implications for metabolic contributions of working astrocytes. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism* **22**, 1476-1489 (2002).
26. P. M. Thwe *et al.*, Cell-Intrinsic Glycogen Metabolism Supports Early Glycolytic Reprogramming Required for Dendritic Cell Immune Responses. *Cell metabolism* **26**, 558-567.e555.
27. S. Eriksson, B. J. Chambers, M. Rhen, Nitric Oxide Produced by Murine Dendritic Cells is Cytotoxic for Intracellular Salmonella enterica sv. Typhimurium. *Scandinavian Journal of Immunology* **58**, 493-502 (2003).
28. C. Bogdan, M. Rollinghoff, A. Diefenbach, The role of nitric oxide in innate immunity. *Immunological reviews* **173**, 17-26 (2000).
29. T. G. Evans, L. Thai, D. L. Granger, J. B. Hibbs, Jr., Effect of in vivo inhibition of nitric oxide production in murine leishmaniasis. *Journal of immunology (Baltimore, Md. : 1950)* **151**, 907-915 (1993).
30. F. Y. Liew, S. Millott, C. Parkinson, R. M. Palmer, S. Moncada, Macrophage killing of Leishmania parasite in vivo is mediated by nitric oxide from L-arginine. *Journal of immunology (Baltimore, Md. : 1950)* **144**, 4794-4797 (1990).
31. S. Z. Chong *et al.*, Human CD8(+) T cells drive Th1 responses through the differentiation of TNF/iNOS-producing dendritic cells. *Eur J Immunol* **41**, 1639-1651 (2011).

32. A. Diefenbach, H. Schindler, M. Rollinghoff, W. M. Yokoyama, C. Bogdan, Requirement for type 2 NO synthase for IL-12 signaling in innate immunity. *Science* **284**, 951-955 (1999).
33. C. A. Bonham, L. Lu, R. A. Hoffman, R. L. Simmons, A. W. Thomson, Generation of nitric oxide by mouse dendritic cells and its implications for immune response regulation. *Advances in experimental medicine and biology* **417**, 283-290 (1997).
34. C. A. Bonham *et al.*, Nitric oxide production by mouse bone marrow-derived dendritic cells: implications for the regulation of allogeneic T cell responses. *Transplantation* **62**, 1871-1877 (1996).
35. C. Bogdan, Nitric oxide synthase in innate and adaptive immunity: an update. *Trends in immunology* **36**, 161-178 (2015).
36. L. Lu *et al.*, Induction of nitric oxide synthase in mouse dendritic cells by IFN-gamma, endotoxin, and interaction with allogeneic T cells: nitric oxide production is associated with dendritic cell apoptosis. *Journal of immunology (Baltimore, Md. : 1950)* **157**, 3577-3586 (1996).
37. C. Bogdan, Nitric oxide and the immune response. *Nature immunology* **2**, 907-916 (2001).
38. G. Lu *et al.*, Myeloid cell-derived inducible nitric oxide synthase suppresses M1 macrophage polarization. *Nat Commun* **6**, 6676 (2015).
39. R. M. Steinman, J. Banchereau, Taking dendritic cells into medicine. *Nature* **449**, 419-426 (2007).
40. S. Corinti, S. Pastore, F. Mascia, G. Girolomoni, Regulatory role of nitric oxide on monocyte-derived dendritic cell functions. *J Interferon Cytokine Res* **23**, 423-431 (2003).
41. E. Amiel *et al.*, Mechanistic target of rapamycin inhibition extends cellular lifespan in dendritic cells by preserving mitochondrial function. *Journal of immunology (Baltimore, Md. : 1950)* **193**, 2821-2830 (2014).
42. H. Sugita *et al.*, Inducible nitric oxide synthase plays a role in LPS-induced hyperglycemia and insulin resistance. *American journal of physiology. Endocrinology and metabolism* **282**, E386-394 (2002).
43. E. J. Pearce, B. Everts, Dendritic cell metabolism. *Nature reviews. Immunology* **15**, 18-29 (2015).
44. E. L. Pearce, E. J. Pearce, Metabolic pathways in immune cell activation and quiescence. *Immunity* **38**, 633-643 (2013).
45. E. Jakobsen *et al.*, Glycogen Shunt Activity and Glycolytic Supercompensation in Astrocytes May Be Distinctly Mediated via the Muscle Form of Glycogen Phosphorylase. *Neurochemical Research*, (2017).
46. L. F. Obel *et al.*, Brain glycogen-new perspectives on its metabolic function and regulation at the subcellular level. *Front Neuroenergetics* **4**, 3 (2012).
47. A. Schousboe, H. M. Sickmann, A. B. Walls, L. K. Bak, H. S. Waagepetersen, Functional importance of the astrocytic glycogen-shunt and glycolysis for maintenance of an intact intra/extracellular glutamate gradient. *Neurotox Res* **18**, 94-99 (2010).

48. R. G. Shulman, F. Hyder, D. L. Rothman, Cerebral energetics and the glycogen shunt: neurochemical basis of functional imaging. *Proc Natl Acad Sci U S A* **98**, 6417-6422 (2001).
49. R. G. Shulman, D. L. Rothman, The "glycogen shunt" in exercising muscle: A role for glycogen in muscle energetics and fatigue. *Proc Natl Acad Sci U S A* **98**, 457-461 (2001).
50. R. G. Shulman, D. L. Rothman, The Glycogen Shunt Maintains Glycolytic Homeostasis and the Warburg Effect in Cancer. *Trends in Cancer* **3**, 761-767 (2017).
51. A. B. Walls, C. M. Heimburger, S. D. Bouman, A. Schousboe, H. S. Waagepetersen, Robust glycogen shunt activity in astrocytes: Effects of glutamatergic and adrenergic agents. *Neuroscience* **158**, 284-292 (2009).
52. R. Ma *et al.*, A Pck1-directed glycogen metabolic program regulates formation and maintenance of memory CD8(+) T cells. *Nat Cell Biol* **20**, 21-27 (2018).
53. E. F. McKinney, K. G. Smith, T-cell exhaustion: understanding the interface of chronic viral and autoinflammatory diseases. *Immunology and cell biology* **94**, 935-942 (2016).
54. E. F. McKinney, K. G. C. Smith, Metabolic exhaustion in infection, cancer and autoimmunity. *Nature immunology* **19**, 213-221 (2018).
55. B. Bengsch *et al.*, Bioenergetic Insufficiencies Due to Metabolic Alterations Regulated by the Inhibitory Receptor PD-1 Are an Early Driver of CD8(+) T Cell Exhaustion. *Immunity* **45**, 358-373 (2016).
56. Y. W. Lam *et al.*, Comprehensive identification and modified-site mapping of S-nitrosylated targets in prostate epithelial cells. *PLoS One* **5**, e9075 (2010).
57. Y. Wang, T. Liu, C. Wu, H. Li, A Strategy for Direct Identification of Protein S-Nitrosylation Sites by Quadrupole Time-of-Flight Mass Spectrometry. *Journal of the American Society for Mass Spectrometry* **19**, 1353-1360 (2008).
58. S. R. Jaffrey, H. Erdjument-Bromage, C. D. Ferris, P. Tempst, S. H. Snyder, Protein S-nitrosylation: a physiological signal for neuronal nitric oxide. *Nat Cell Biol* **3**, 193-197 (2001).
59. B. Derakhshan, P. C. Wille, S. S. Gross, Unbiased identification of cysteine S-nitrosylation sites on proteins. *Nat. Protocols* **2**, 1685-1691 (2007).
60. M. T. Forrester, M. W. Foster, M. Benhar, J. S. Stamler, Detection of protein S-nitrosylation with the biotin-switch technique. *Free radical biology & medicine* **46**, 119-126 (2009).
61. M. Borgs *et al.*, Modulation of basal hepatic glycogenolysis by nitric oxide. *Hepatology (Baltimore, Md.)* **23**, 1564-1571 (1996).
62. M. A. Lowes *et al.*, Increase in TNF-α and inducible nitric oxide synthase-expressing dendritic cells in psoriasis and reduction with efalizumab (anti-CD11a). *P Natl Acad Sci USA* **102**, 19057-19062 (2005).
63. F. Facchetti *et al.*, Expression of Inducible Nitric Oxide Synthase in Human Granulomas and Histiocytic Reactions. *The American Journal of Pathology* **154**, 145-152 (1999).
64. J. T. Mattila *et al.*, Microenvironments in Tuberculous Granulomas Are Delineated by Distinct Populations of Macrophage Subsets and Expression of

Nitric Oxide Synthase and Arginase Isoforms. *The Journal of Immunology* **191**, 773-784 (2013).

CHAPTER 6

CONCLUDING REMARKS AND FUTURE DIRECTIONS

The discovery of glycogen metabolism by Claude Bernard in the 1800's opened up many avenues for investigation into cellular utilization of glucose at different levels. Since then, glycogen metabolism has been studied sporadically in immune cells, particularly in total mixed leukocyte populations, with very few studies in isolated or purified phagocytes specifically. Prior to this work, the functional aspects of glycogen metabolism in DCs has not been clearly defined. Preceding knowledge on the importance of glycogen metabolism in maintaining energy and metabolite homeostasis in metabolically active tissues, such as brain, liver, and muscle, coupled with the already established importance of glucose metabolism in supporting DC immune responses, prompted us to elucidate the roles of glycogen metabolism in DC effector function.

While the bulk of this work primarily focuses on DC-intrinsic glycogen metabolism in the TLR4-activation model discussed in Chapter 3 and chapter 5, the work in Chapter 2 compares the metabolic regulation between TLR and non-TLR activation by fungal ligand Dectin-1 stimulation. We have demonstrated the fundamental link between cellular glycolytic reprogramming responses and Dectin-1-ligand specific activation of Syk-dependent NLRP3 inflammasome formation. Because Syk signaling has been shown to be coordinated with another signaling molecule, CARD9, in many instances (Gross et al., 2006; Jia et al., 2014), we think that examining the potential implication of CARD9 in association with Syk regulation may better elucidate whether CARD9 is also required for Dectin-1 specific activation of glycolytic reprogramming. This is an important area of investigation because of the complexity of fungal cell wall composition that can trigger distinct metabolic responses and diverse signaling mechanisms. On an important note, future work worthy of further exploration is the role for glycogen metabolism in

regulating fungal ligand-mediated immune responses, in line with the overarching theme of this dissertation.

In Chapter 3, we have examined the role and metabolic behavior of DC glycogen metabolism in 3 different parts: 1) establishing the presence of DC-intrinsic glycogen metabolic machinery; 2) defining its role in supporting DC effector function; and 3) defining the contribution of glycogen to the activation-associated DC "metabolome". One interesting feature of DC expression of glycogen metabolic enzymes, demonstrated in this Chapter, is that DCs express both liver and brain isozymes of glycogen phosphorylase (PYG). However, one recent report showed that T lymphocytes express the muscle isoform of PYG and that the stimulation of PYGM is required for IL-2-stimulated T cell proliferation (Arrizabalaga et al., 2012). These data imply that the immune cells of myeloid and lymphoid origins seem to differentially express distinct PYG isoforms. More interestingly, PYG activity appears to be more complex than its canonical function of glycogen degradation. Studies in NIH3T3 cell lines demonstrate that PYG activity contributes to caspase-independent cellular necroptosis (Zhang et al., 2009). In contrast, PYG is shown to be essential in cancer cell proliferation and survival (Favaro et al., 2012; Iida et al., 2012). The pleiotropic functions of PYG in these scenarios suggest additional roles in which PYG can be implicated in DC metabolism. One situation would be its potential role as an energy and metabolic sensor in coordination with GYS in DC glycogen shunt described in Chapter 3, given that the continuous breakdown and synthesis of glycogen in the shunt is a metabolically costly process that could upset the overall energy balance of the cells. The implications of this were described in Chapter 3, in which we showed a drop in ATP and crucial metabolites

upon inhibition of PYG. In addition, the fact that inhibition of PYG in low glucose conditions impairs BMDC survival plus maturation as well as induces AMPK activation suggests an interesting mechanism by which PYG perhaps can act in parallel with both mTOR and AMPK -mediated pathways, particularly during nutrient starvation. A need for the comprehensive understanding of how these molecules work together in maintaining cellular energy and metabolite homeostasis, particularly during active inflammatory reactions, necessitates further work in the future. Further questions also remain as to whether PYG and/or the overall glycogen metabolism is involved in DC longevity.

One prominent metabolic phenomenon that our work demonstrated in Chapter 3 is the ability of DCs to engage in a relatively obscure metabolic pathway termed the glycogen shunt. Compared to the glycogen shunt observed in neuronal and muscle systems, several questions arise in regard to the biological relevance and the purpose of this shunt in DCs. As this continuous cycle of glycogen turnover is a seemingly wasteful process from an energetic standpoint, one key question is how this process is sustained or regulated in activated DCs without disturbing the cellular metabolic balance. One possible explanation could be through the geospatial localization of the two parallel pathways of glycogen metabolism. A few early studies suggested the potential existence of spatial regulation of glycogen metabolism in immune cells (Scott, 1968; Scott and Still, 1968). A few pieces of recent evidence, such as the rapid translocation of hexokinase-II (HK-II) to the mitochondrial outer membrane in TLR-activated DCs, and the observation of clusters of "vacuole-like" glycogen-containing compartments shown in Chapter 3, corroborate the notion of spatially compartmentalized localization of glycogen

and thus perhaps also glycogen metabolic pathways (Everts et al., 2014; Thwe et al.). It is highly plausible that some of the glycogen clusters that are potentially designated for degradation can locate in close proximity to mitochondria so that those glycogen molecules can easily funnel the metabolic intermediates such as G6P into the glycolytic pathway and the TCA cycle. This hypothetical projection strongly correlates with the previous finding of HK-II translocation to mitochondria (Everts et al., 2014) and our metabolomics data in Chapter 3, suggesting a need to explore this aspect of DC cellular biology for a better understanding of the nature of spatial compartmentalized regulation of glycogen metabolism that supports the downstream metabolic pathways.

Another question with respect to the glycogen shunt is the exact purpose(s) of DCs for participating in the glycogen shunt activity. As we have learned from the non-immune system, the glycogen shunt in astrocytes and neurons helps support their action potential firing activities and glutamate production (Obel et al., 2012; Schousboe et al., 2010; Shulman et al., 2001; Shulman and Rothman, 2017; Walls et al., 2009; Walls et al., 2008). Similarly, muscle cells take advantage of the glycogen shunt for rapid contractile functions (Jakobsen et al., 2017; Shulman and Rothman, 2001). As demonstrated by a previous study, in which phagocytes differentially utilize G6P derived from intracellular glycogen breakdown for phagocytic activities and from free glucose for chemotactic function (Weisdorf et al., 1982), it is likely that the carbons derived from the glycogen shunt may support differential functional activities in activated DCs. This area will be of significant value for pursuit in our laboratory because a comprehensive understanding of nutrient sourcing from a specific carbon pool for differential effector function would be a powerful tool in the future to fine-tune the immune responses in these cells.

Our metabolomics findings on the glycogen shunt activity and the differential utilization of glycogen and free glucose metabolism by DCs suggest the possibility that these phenotypes are not DC-specific and are likely to be observed in other cell types. For example, a recent study following our publication of this work demonstrated that a constant turnover of glycogen metabolic reprogramming is essential in formation and maintenance of the memory $CD8^+$ T cell population (Ma et al., 2018), thus corroborating our opinion that examination of these pathways in other lineages of the immune system would be of significant interest.

One of the fundamental metabolites of interest that is frequently mentioned throughout this dissertation is citrate, and Chapter 3 describes the generation of citrate from DC glycogen shunt. Citrate plays a crucial role in anabolic processes during the activation responses of both myeloid and lymphoid immune cells. Our previous work, delineating the extracellular glucose-driven early glycolytic reprogramming of TLR-activated DCs, as well as our current work focusing on DC-intrinsic glycogen metabolism, have demonstrated that both free glucose-derived and glycogen-derived-glycolytic pathways are primarily enriched in the citrate pool (Everts et al., 2014; Thwe et al.). In this regard, one of the metabolites worthy of further investigation is acetyl-CoA. Acetyl-CoA is generated from citrate for *de novo* fatty acid synthesis to support the TLR-driven DC maturation. This metabolic process, in which citrate is transported into the cytosol and converted to acetyl-CoA for the generation of downstream metabolites and proteins, has been broadly studied in other immune cell types, such as macrophages and effector T cells (Everts et al., 2014; Galván-Peña and O'Neill, 2014; Kelly and O'Neill, 2015; O'Neill, 2011) . Given the fact that trained monocytes, primed to recognize

specific pathogens in their secondary infections, elicit innate immune memory responses upon experiencing epigenetic reprogramming events (Cheng et al., 2014), it is also possible that DCs may undergo epigenetic rewiring directly relevant to the glycogen shunt activity, thereby modulating the immune outcome. Among the several mechanisms of epigenetic reprogramming, histone modification by acetylation and/or deacetylation is one of the common mechanisms cells undertake to regulate protein and gene expressions, and the cellular availability of acetyl-CoA has been proposed to be directly associated with epigenetic changes (Cameron et al., 2016). While acetyl-CoA is not included in our metabolomics data, it would be relevant to begin by examining the levels of acetyl-CoA indirectly generated from the glycogen shunt. In light of a growing appreciation of epigenetic regulatory mechanisms in immune cell function, identifying the possibility of histone modification via glycogen shunt-derived acetyl-CoA is one of the fundamental questions to be answered in the near future.

Since the work presented in Chapter 5 is not yet ready for publication, more studies are needed to fully comprehend the NO-mediated regulatory mechanisms and signaling pathways involved in the glycogen metabolism of both mouse and human models of DCs. Intriguingly, we identified that NO-producing TLR-activated BMDCs accumulate more glycogen than their resting counterparts, a phenomenon found to be opposite in the human monocyte-derived DCs (moDCs), which deplete their intracellular stores of glycogen more rapidly upon exogenous NO exposure. While the NO-induced glycogen accumulation in NO-producing mouse DCs is an interesting phenotype, the physiologically relevant findings on the NO-mediated glycogen depletion in moDCs are substantiated by a few studies stating that the endotoxin-challenged hepatocytes display a

decrease in synthase activity and an increase in phosphorylase (PYG) activity in response to iNOS upregulation in those cells (Shinozaki et al., 2011; Sugita et al., 2002).

Given that PYG can be reversibly S-nitrosylated by NO, which is one of the post-translational mechanisms of NO in modulating cellular function, we were interested in whether PYG in our experimental models undergoes the S-nitrosylation reaction. Although we have not had the opportunity to test this in Chapter 5 yet, it is possible that an increased level of glycogen in BMDCs is likely due to the inhibitory effect of NO on PYG. Similarly, increased glycogen utilization in moDCs could be also mediated by the activity of nitrosylated-PYG. Considering NO as a "double-edged sword" in its effects on immune responses, we would gain valuable insights into how we can harness the NO-mediated positive immune outcomes without harming the host cells.

Importantly, there are a few major points to consider in an attempt to elucidate the NO regulation of glycogen metabolism in both mouse and human DCs. The first question is whether the rapid depletion of glycogen in moDCs is due to the universal effect of exogenous NO observed only in non-NO-producer human DCs, but not in NO-producing BMDCs. Because there are several distinct subsets of tissue-resident DCs in humans that have been claimed to upregulate iNOS, which we described in Chapter 4 (Haider et al., 2008; Lowes et al., 2005; Wilsmann-Theis et al., 2013), how do these distinct DC subsets utilize their glycogen metabolism in response to their autocrine production of NO? Will they behave as the NO-producing mouse model of BMDCs or the monocyte-derived *in vitro* model of DCs that are induced by exogenous NO? Overall, these details are definitively worthy of further pursuits for a better understanding of DC glycogen

utilization that would have potentially consequent effects on the functional activities of immune cells localized in the NO-enriched environment in most microbial infections.

One of the interesting aspects of glycogen metabolism in Chapter 5 is the signaling pathway involving PI3k-mTOR-iNOS, the regulatory pathway for induction of NO in BMDCs, which we believe is also possibly regulating the glycogen utilization in NO-producing BMDCs. Our data showing the activity of GYS1 that is modulated upon the activation of PI3k-mTOR-iNOS in BMDCs are relatively preliminary. However, one of the critiques is the lack of information on other effector proteins downstream to PI3k-mTOR that are likely to be impacted by this signaling axis. Therefore, in addition to the expression of total GYS1 we have shown in Chapter 5, we acknowledge the need to identify the expression of PYG and phosphorylated GYS1, which is the inactive form of GYS1, so that it would help us fully understand this regulatory mechanism. Of interest, another major signaling molecule highly relevant to this pathway is hypoxia inducible factor-1α (HIF-1α), which is one of the regulatory proteins directly downstream of mTOR. Because HIF-1α canonically induces the upregulation of aerobic glycolysis and iNOS expression in immune cells and also modulates glycogen synthesis in cancer cells (Corcoran and O'Neill, 2016; Iida et al., 2012; Jantsch et al., 2008; Pelletier et al., 2012; Pescador et al., 2010), it is of great relevance to examine the implication of HIF-1α in this signaling mechanism.

Following our studies on the elucidation of TLR versus Dectin-1-driven glucose metabolism, we uncovered a novel understanding of intracellular glucose reserves in DCs. Using a pharmacological and genetic approach in Chapter 3, we established the glycogen-driven early glycolytic reprogramming responses. In particular, we have

acquired a more profound understanding of the role of DC-intrinsic glycogen storage in supporting the TLR-driven early metabolic responses associated with their effector function, and DC utilization of differential metabolic pathways driven by free glucose- and glycogen-derived carbons. We have also shown that inhibition of DC glycogen metabolism causes detrimental effects on their activation responses. With respect to the regulatory mechanisms of glycogen metabolism utilization in DCs, our preliminary reports strongly implicate nitric oxide in this process. We found a drastic difference between NO-mediated effects on the mouse and human DC models, explicitly illustrated in Chapter 5. Namely, mouse DCs stockpile their intracellular glycogen upon iNOS upregulation whereas human DCs accelerate their glycogen usage upon exposure to exogenous NO. This intriguing phenotype opens up many questions to be answered in the future, especially because NO is frequently involved in a variety of microbial infections, in addition to its versatile regulatory roles in many physiological and metabolic processes.

With a rising appreciation of the fundamental roles of cellular metabolism in regulating immune cell responses to various inflammatory stimuli, there has also been an increasing interest in targeting immune cell metabolism for immuno-therapeutic applications. In light of this, a growing effort has been contributed towards the development of metabolism-targeted approaches to enhance the immune functional outcomes. In the studies described in this dissertation, we have established how DCs maintain nutrient homeostasis to support their metabolic requirements during activation responses, giving particular attention towards DC utilization of nutrients from their intracellular glycogen stores, and the potential regulatory mechanisms governing these

processes. We believe that the experimental evidence exhibited in this dissertation has significantly contributed to the field for an enhanced understanding of the intricacies of carbohydrate metabolism in DCs of the immune system, which may be further exploited in metabolic interventions towards the development of cell-based therapeutic applications in the future.

COMPREHENSIVE BIBLIOGRAPHY

Abbas, A.K., and Janeway, C.A., Jr. (2000). Immunology: improving on nature in the twenty-first century. Cell *100*, 129-138.

Adeva-Andany, M.M., Gonzalez-Lucan, M., Donapetry-Garcia, C., Fernandez-Fernandez, C., and Ameneiros-Rodriguez, E. (2016). Glycogen metabolism in humans. BBA Clin *5*, 85-100.

Akira, S. (2003). Toll-like receptor signaling. The Journal of biological chemistry *278*, 38105-38108.

Akira, S., and Takeda, K. (2004). Toll-like receptor signalling. Nature reviews. Immunology *4*, 499-511.

Alexopoulou, L., Holt, A.C., Medzhitov, R., and Flavell, R.A. (2001). Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature *413*, 732-738.

Alvarez, Y., Rodriguez, M., Municio, C., Hugo, E., Alonso, S., Ibarrola, N., Fernandez, N., and Crespo, M.S. (2012). Sirtuin 1 is a key regulator of the interleukin-12 p70/interleukin-23 balance in human dendritic cells. The Journal of biological chemistry *287*, 35689-35701.

Alvarez-Errico, D., Vento-Tormo, R., Sieweke, M., and Ballestar, E. (2015). Epigenetic control of myeloid cell differentiation, identity and function. Nature reviews. Immunology *15*, 7-17.

Amiel, E., Everts, B., Freitas, T.C., King, I.L., Curtis, J.D., Pearce, E.L., and Pearce, E.J. (2012). Inhibition of mechanistic target of rapamycin promotes dendritic cell activation and enhances therapeutic autologous vaccination in mice. Journal of immunology (Baltimore, Md. : 1950) *189*, 2151-2158.

Amiel, E., Everts, B., Fritz, D., Beauchamp, S., Ge, B., Pearce, E.L., and Pearce, E.J. (2014). Mechanistic target of rapamycin inhibition extends cellular lifespan in dendritic cells by preserving mitochondrial function. Journal of immunology (Baltimore, Md. : 1950) *193*, 2821-2830.

Andrade, Warrison A., Souza, Maria do C., Ramos-Martinez, E., Nagpal, K., Dutra, Miriam S., Melo, Mariane B., Bartholomeu, Daniella C., Ghosh, S., Golenbock, Douglas T., and Gazzinelli, Ricardo T. Combined Action of Nucleic Acid-Sensing Toll-like Receptors and TLR11/TLR12 Heterodimers Imparts Resistance to Toxoplasma gondii in Mice. Cell Host & Microbe *13*, 42-53.

Ariizumi, K., Shen, G.L., Shikano, S., Xu, S., Ritter, R., 3rd, Kumamoto, T., Edelbaum, D., Morita, A., Bergstresser, P.R., and Takashima, A. (2000). Identification of a novel, dendritic cell-associated molecule, dectin-1, by subtractive cDNA cloning. The Journal of biological chemistry *275*, 20157-20167.

Arrizabalaga, O., Lacerda, H.M., Zubiaga, A.M., and Zugaza, J.L. (2012). Rac1 protein regulates glycogen phosphorylase activation and controls interleukin (IL)-2-dependent T cell proliferation. The Journal of biological chemistry *287*, 11878-11890.

Arts, R.J., Novakovic, B., Ter Horst, R., Carvalho, A., Bekkering, S., Lachmandas, E., Rodrigues, F., Silvestre, R., Cheng, S.C., Wang, S.Y., et al. (2016). Glutaminolysis and Fumarate Accumulation Integrate Immunometabolic and Epigenetic Programs in Trained Immunity. Cell metabolism *24*, 807-819.

Auron, P.E., Webb, A.C., Rosenwasser, L.J., Mucci, S.F., Rich, A., Wolff, S.M., and Dinarello, C.A. (1984). Nucleotide sequence of human monocyte interleukin 1 precursor cDNA. Proc Natl Acad Sci U S A *81*, 7907-7911.

Baker, D.J., Timmons, J.A., and Greenhaff, P.L. (2005). Glycogen Phosphorylase Inhibition in Type 2 Diabetes Therapy: A Systematic Evaluation of Metabolic and Functional Effects in Rat Skeletal Muscle. Diabetes *54*, 2453-2459.

Banchereau, J., and Steinman, R.M. (1998a). Dendritic cells and the control of immunity. Nature *392*, 245-252.

Banchereau, J., and Steinman, R.M. (1998b). Dendritic cells and the control of immunity. Nature. *6673*.

Batbayar, S., Lee, D.H., and Kim, H.W. (2012). Immunomodulation of Fungal beta-Glucan in Host Defense Signaling by Dectin-1. Biomol Ther (Seoul) *20*, 433-445.

Bernard, N.J., and O'Neill, L.A. (2013). Mal, more than a bridge to MyD88. IUBMB life *65*, 777-786.

Beutler, B. (2004). Toll-like receptors and their place in immunology. Where does the immune response to infection begin? Nature reviews. Immunology *4*, 498.

Bogdan, C. (2001). Nitric oxide and the immune response. Nature immunology *2*, 907-916.

Bogdan, C. (2015). Nitric oxide synthase in innate and adaptive immunity: an update. Trends in immunology *36*, 161-178.

Bogdan, C., Rollinghoff, M., and Diefenbach, A. (2000). The role of nitric oxide in innate immunity. Immunological reviews *173*, 17-26.

Bonham, C.A., Lu, L., Hoffman, R.A., Simmons, R.L., and Thomson, A.W. (1997). Generation of nitric oxide by mouse dendritic cells and its implications for immune response regulation. Advances in experimental medicine and biology *417*, 283-290.

Bonham, C.A., Lu, L., Li, Y., Hoffman, R.A., Simmons, R.L., and Thomson, A.W. (1996). Nitric oxide production by mouse bone marrow-derived dendritic cells: implications for the regulation of allogeneic T cell responses. Transplantation *62*, 1871-1877.

Bonnert, T.P., Garka, K.E., Parnet, P., Sonoda, G., Testa, J.R., and Sims, J.E. (1997). The cloning and characterization of human MyD88: a member of an IL-1 receptor related family. FEBS letters *402*, 81-84.

Borregaard, N., and Herlin, T. (1982). Energy metabolism of human neutrophils during phagocytosis. The Journal of clinical investigation *70*, 550-557.

Bossaller, L., Chiang, P.I., Schmidt-Lauber, C., Ganesan, S., Kaiser, W.J., Rathinam, V.A., Mocarski, E.S., Subramanian, D., Green, D.R., Silverman, N., et al. (2012). Cutting edge: FAS (CD95) mediates noncanonical IL-1beta and IL-18 maturation via caspase-8 in an RIP3-independent manner. Journal of immunology (Baltimore, Md. : 1950) *189*, 5508-5512.

Bourke, E., Bosisio, D., Golay, J., Polentarutti, N., and Mantovani, A. (2003). The toll-like receptor repertoire of human B lymphocytes: inducible and selective expression of TLR9 and TLR10 in normal and transformed cells. Blood *102*, 956-963.

Brown, A.M., Sickmann, H.M., Fosgerau, K., Lund, T.M., Schousboe, A., Waagepetersen, H.S., and Ransom, B.R. (2005). Astrocyte glycogen metabolism is required for neural activity during aglycemia or intense stimulation in mouse white matter. Journal of neuroscience research *79*, 74-80.

Brown, A.M., Tekkok, S.B., and Ransom, B.R. (2003a). Glycogen regulation and functional role in mouse white matter. The Journal of physiology *549*, 501-512.

Brown, G.D. (2006). Dectin-1: a signalling non-TLR pattern-recognition receptor. Nature reviews. Immunology *6*, 33-43.

Brown, G.D., Herre, J., Williams, D.L., Willment, J.A., Marshall, A.S., and Gordon, S. (2003b). Dectin-1 mediates the biological effects of beta-glucans. The Journal of experimental medicine *197*, 1119-1124.

Brown, J., Wang, H., Hajishengallis, G.N., and Martin, M. (2011). TLR-signaling networks: an integration of adaptor molecules, kinases, and cross-talk. Journal of dental research *90*, 417-427.

Brubaker, S.W., Bonham, K.S., Zanoni, I., and Kagan, J.C. (2015). Innate Immune Pattern Recognition: A Cell Biological Perspective. Annual review of immunology *33*, 257-290.

Cameron, A.M., Lawless, S.J., and Pearce, E.J. (2016). Metabolism and acetylation in innate immune cell function and fate. Seminars in immunology *28*, 408-416.

Cao, Y., Feng, Y., Zhang, Y., Zhu, X., and Jin, F. (2016). L-Arginine supplementation inhibits the growth of breast cancer by enhancing innate and adaptive immune responses mediated by suppression of MDSCs in vivo. BMC Cancer *16*, 343.

Carlson, G.M., Dienel, G.A., and Colbran, R.J. (2018). Novel insights into brain glycogen metabolism. The Journal of biological chemistry.

Cheng, S.C., Quintin, J., Cramer, R.A., Shepardson, K.M., Saeed, S., Kumar, V., Giamarellos-Bourboulis, E.J., Martens, J.H., Rao, N.A., Aghajanirefah, A., et al. (2014). mTOR- and HIF-1alpha-mediated aerobic glycolysis as metabolic basis for trained immunity. Science *345*, 1250684.

Cheng, S.C., van de Veerdonk, F.L., Lenardon, M., Stoffels, M., Plantinga, T., Smeekens, S., Rizzetto, L., Mukaremera, L., Preechasuth, K., Cavalieri, D., et al. (2011). The dectin-1/inflammasome pathway is responsible for the induction of protective T-helper 17 responses that discriminate between yeasts and hyphae of Candida albicans. Journal of leukocyte biology *90*, 357-366.

Chistiakov, D.A., Sobenin, I.A., Orekhov, A.N., and Bobryshev, Y.V. (2015). Myeloid dendritic cells: Development, functions, and role in atherosclerotic inflammation. Immunobiology *220*, 833-844.

Chow, K.V., Sutherland, R.M., Zhan, Y., and Lew, A.M. (2017). Heterogeneity, functional specialization and differentiation of monocyte-derived dendritic cells. Immunology and cell biology *95*, 244-251.

Clementi, E., Brown, G.C., Feelisch, M., and Moncada, S. (1998). Persistent inhibition of cell respiration by nitric oxide: crucial role of S-nitrosylation of mitochondrial complex I and protective action of glutathione. Proc Natl Acad Sci U S A *95*, 7631-7636.

Collin, M., McGovern, N., and Haniffa, M. (2013). Human dendritic cell subsets. Immunology *140*, 22-30.

Condeelis, J., and Pollard, J.W. (2006). Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. Cell. *2*.

Corcoran, S.E., and O'Neill, L.A. (2016). HIF1alpha and metabolic reprogramming in inflammation. The Journal of clinical investigation *126*, 3699-3707.

Corinti, S., Pastore, S., Mascia, F., and Girolomoni, G. (2003). Regulatory role of nitric oxide on monocyte-derived dendritic cell functions. J Interferon Cytokine Res *23*, 423-431.

Corraliza, I.M., Soler, G., Eichmann, K., and Modolell, M. (1995). Arginase Induction by Suppressors of Nitric Oxide Synthesis (IL-4, IL-10 and PGE2) in Murine Bone-Marrow-Derived Macrophages. Biochemical and Biophysical Research Communications *206*, 667-673.

Crowley, M., Inaba, K., Witmer-Pack, M., and Steinman, R.M. (1989). The cell surface of mouse dendritic cells: FACS analyses of dendritic cells from different tissues including thymus. Cellular Immunology *118*, 108-125.

Cunliffe, V., and Trowsdale, J. (1987). The molecular genetics of human chromosome 6. Journal of Medical Genetics *24*, 649-658.

Cusso, R., Domingo, A., and Rosell-Perez, M. (1975). Studies on glycogen metabolism in human leukemic cells. I. Glycogen content, glycogen synthetase forms and their metabolic interconversion. Revista espanola de fisiologia *31*, 309-316.

da Silva Correia, J., Soldau, K., Christen, U., Tobias, P.S., and Ulevitch, R.J. (2001). Lipopolysaccharide Is in Close Proximity to Each of the Proteins in Its Membrane Receptor Complex: TRANSFER FROM CD14 TO TLR4 AND MD-2. Journal of Biological Chemistry *276*, 21129-21135.

Dambuza, I.M., and Brown, G.D. (2015). C-type lectins in immunity: recent developments. Current Opinion in Immunology *32*, 21-27.

Dashty, M. (2013). A quick look at biochemistry: carbohydrate metabolism. Clinical biochemistry *46*, 1339-1352.

Deberardinis, R.J., Lum, J.J., and Thompson, C.B. (2006). Phosphatidylinositol 3-kinase-dependent modulation of carnitine palmitoyltransferase 1A expression regulates lipid metabolism during hematopoietic cell growth. The Journal of biological chemistry *281*, 37372-37380.

Deguine, J., and Barton, G.M. (2014). MyD88: a central player in innate immune signaling. F1000prime reports *6*, 97.

den Haan, J.M., Lehar, S.M., and Bevan, M.J. (2000). CD8(+) but not CD8(-) dendritic cells cross-prime cytotoxic T cells in vivo. The Journal of experimental medicine *192*, 1685-1696.

Dennehy, K.M., and Brown, G.D. (2007). The role of the β-glucan receptor Dectin-1 in control of fungal infection. Journal of leukocyte biology *82*, 253-258.

Diebold, S.S., Kaisho, T., Hemmi, H., Akira, S., and Reis e Sousa, C. (2004). Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. Science *303*, 1529-1531.

Dominy, J.E., Jr., Lee, Y., Gerhart-Hines, Z., and Puigserver, P. (2010). Nutrient-dependent regulation of PGC-1alpha's acetylation state and metabolic function through the enzymatic activities of Sirt1/GCN5. Biochimica et biophysica acta *1804*, 1676-1683.

Drummond, R.A., and Brown, G.D. (2011). The role of Dectin-1 in the host defence against fungal infections. Current opinion in microbiology *14*, 392-399.

Duvel, K., Yecies, J.L., Menon, S., Raman, P., Lipovsky, A.I., Souza, A.L., Triantafellow, E., Ma, Q., Gorski, R., Cleaver, S., et al. (2010). Activation of a metabolic gene regulatory network downstream of mTOR complex 1. Molecular cell *39*, 171-183.

Eder, C. (2009). Mechanisms of interleukin-1β release. Immunobiology *214*, 543-553.

Elhelu, M.A. (1983). The role of macrophages in immunology. J Natl Med Assoc *75*, 314-317.

Equils, O., Madak, Z., Liu, C., Michelsen, K.S., Bulut, Y., and Lu, D. (2004). Rac1 and Toll-IL-1 receptor domain-containing adapter protein mediate Toll-like receptor 4 induction of HIV-long terminal repeat. Journal of immunology (Baltimore, Md. : 1950) *172*, 7642-7646.

Everts, B., Amiel, E., Huang, S.C., Smith, A.M., Chang, C.H., Lam, W.Y., Redmann, V., Freitas, T.C., Blagih, J., van der Windt, G.J., et al. (2014). TLR-driven early glycolytic reprogramming via the kinases TBK1-IKKvarepsilon supports the anabolic demands of dendritic cell activation. Nature immunology *15*, 323-332.

Everts, B., Amiel, E., van der Windt, G.J., Freitas, T.C., Chott, R., Yarasheski, K.E., Pearce, E.L., and Pearce, E.J. (2012). Commitment to glycolysis sustains survival of NO-producing inflammatory dendritic cells. Blood *120*, 1422-1431.

Everts, B., and Pearce, E.J. (2014). Metabolic control of dendritic cell activation and function: recent advances and clinical implications. Frontiers in immunology *5*, 203.

Fang, T.C., Schaefer, U., Mecklenbrauker, I., Stienen, A., Dewell, S., Chen, M.S., Rioja, I., Parravicini, V., Prinjha, R.K., Chandwani, R., et al. (2012a). Histone H3 lysine 9 di-methylation as an epigenetic signature of the interferon response. The Journal of experimental medicine *209*, 661-669.

Fang, T.C., Schaefer, U., Mecklenbrauker, I., Stienen, A., Dewell, S., Chen, M.S., Rioja, I., Parravicini, V., Prinjha, R.K., Chandwani, R., et al. (2012b). Histone H3 lysine 9 di-methylation as an epigenetic signature of the interferon response. The Journal of experimental medicine *209*, 661-669.

Favaro, E., Bensaad, K., Chong, M.G., Tennant, D.A., Ferguson, D.J., Snell, C., Steers, G., Turley, H., Li, J.L., Gunther, U.L., et al. (2012). Glucose utilization via glycogen phosphorylase sustains proliferation and prevents premature senescence in cancer cells. Cell metabolism *16*, 751-764.

Fernandes, P., O'Donnell, C., Lyons, C., Keane, J., Regan, T., O'Brien, S., Fallon, P., Brint, E., and Houston, A. (2014). Intestinal expression of Fas and Fas ligand is upregulated by bacterial signaling through TLR4 and TLR5, with activation of Fas modulating intestinal TLR-mediated inflammation. Journal of immunology (Baltimore, Md. : 1950) *193*, 6103-6113.

Fernandez-Novell, J.M., Arino, J., and Guinovart, J.J. (1994). Effects of glucose on the activation and translocation of glycogen synthase in diabetic rat hepatocytes. European journal of biochemistry *226*, 665-671.

Fernandez-Novell, J.M., Arino, J., Vilaro, S., and Guinovart, J.J. (1992). Glucose induces the translocation and the aggregation of glycogen synthase in rat hepatocytes. The Biochemical journal *281 (Pt 2)*, 443-448.

Fernandez-Novell, J.M., Roca, A., Bellido, D., Vilaro, S., and Guinovart, J.J. (1996). Translocation and aggregation of hepatic glycogen synthase during the fasted-to-refed transition in rats. European journal of biochemistry *238*, 570-575.

Ferwerda, G., Meyer-Wentrup, F., Kullberg, B.-J., Netea, M.G., and Adema, G.J. (2008). Dectin-1 synergizes with TLR2 and TLR4 for cytokine production in human primary monocytes and macrophages. Cellular Microbiology *10*, 2058-2066.

Fitzgerald, K.A., Palsson-McDermott, E.M., Bowie, A.G., Jefferies, C.A., Mansell, A.S., Brady, G., Brint, E., Dunne, A., Gray, P., Harte, M.T., et al. (2001). Mal (MyD88-adapter-like) is required for Toll-like receptor-4 signal transduction. Nature *413*, 78-83.

Förstermann, U., and Sessa, W.C. (2012). Nitric oxide synthases: regulation and function. European Heart Journal *33*, 829-837.

Fossati, G., Moulding, D.A., Spiller, D.G., Moots, R.J., White, M.R., and Edwards, S.W. (2003). The mitochondrial network of human neutrophils: role in chemotaxis, phagocytosis, respiratory burst activation, and commitment to apoptosis. Journal of immunology (Baltimore, Md. : 1950) *170*, 1964-1972.

Freemerman, A.J., Johnson, A.R., Sacks, G.N., Milner, J.J., Kirk, E.L., Troester, M.A., Macintyre, A.N., Goraksha-Hicks, P., Rathmell, J.C., and Makowski, L. (2014). Metabolic reprogramming of macrophages: glucose transporter 1 (GLUT1)-mediated glucose metabolism drives a proinflammatory phenotype. The Journal of biological chemistry *289*, 7884-7896.

Fu, Y., Maianu, L., Melbert, B.R., and Garvey, W.T. (2004). Facilitative glucose transporter gene expression in human lymphocytes, monocytes, and macrophages: a role for GLUT isoforms 1, 3, and 5 in the immune response and foam cell formation. Blood cells, molecules & diseases *32*, 182-190.

Fuller, G.L., Williams, J.A., Tomlinson, M.G., Eble, J.A., Hanna, S.L., Pohlmann, S., Suzuki-Inoue, K., Ozaki, Y., Watson, S.P., and Pearce, A.C. (2007). The C-type lectin receptors CLEC-2 and Dectin-1, but not DC-SIGN, signal via a novel YXXL-dependent signaling cascade. The Journal of biological chemistry *282*, 12397-12409.

Galván-Peña, S., and O'Neill, L.A.J. (2014). Metabolic Reprograming in Macrophage Polarization. Frontiers in immunology *5*, 420.

Garcia-Rocha, M., Roca, A., De La Iglesia, N., Baba, O., Fernandez-Novell, J.M., Ferrer, J.C., and Guinovart, J.J. (2001). Intracellular distribution of glycogen synthase and glycogen in primary cultured rat hepatocytes. The Biochemical journal *357*, 17-24.

Garcia-Vallejo, J.J., and van Kooyk, Y. (2013). The physiological role of DC-SIGN: a tale of mice and men. Trends in immunology *34*, 482-486.

Gay, N.J., Gangloff, M., and O'Neill, L.A. (2011). What the Myddosome structure tells us about the initiation of innate immunity. Trends in immunology *32*, 104-109.

Gella, F.J., Cusso, R., Domingo, A., and Rosell-Perez, M. (1978). Glycogen phosphorylase from human normal and leukemic leucocytes: activities and interconversions between active and inactive forms. Int J Biochem *9*, 701-707.

Ghosh, S., May, M.J., and Kopp, E.B. (1998). NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. Annual review of immunology *16*, 225-260.

Gibb, R.P., and Stowell, R.E. (1949). Glycogen in human blood cells. Blood *4*, 569-579.

Gorden, K.K., Qiu, X.X., Binsfeld, C.C., Vasilakos, J.P., and Alkan, S.S. (2006). Cutting edge: activation of murine TLR8 by a combination of imidazoquinoline immune response modifiers and polyT oligodeoxynucleotides. Journal of immunology (Baltimore, Md. : 1950) *177*, 6584-6587.

Gringhuis, S.I., den Dunnen, J., Litjens, M., van der Vlist, M., Wevers, B., Bruijns, S.C., and Geijtenbeek, T.B. (2009). Dectin-1 directs T helper cell differentiation by controlling noncanonical NF-kappaB activation through Raf-1 and Syk. Nature immunology *10*, 203-213.

Gringhuis, S.I., Kaptein, T.M., Wevers, B.A., Theelen, B., van der Vlist, M., Boekhout, T., and Geijtenbeek, T.B. (2012). Dectin-1 is an extracellular pathogen sensor for the induction and processing of IL-1beta via a noncanonical caspase-8 inflammasome. Nature immunology *13*, 246-254.

Gross, O., Gewies, A., Finger, K., Schafer, M., Sparwasser, T., Peschel, C., Forster, I., and Ruland, J. (2006). Card9 controls a non-TLR signalling pathway for innate anti-fungal immunity. Nature *442*, 651-656.

Gross, O., Poeck, H., Bscheider, M., Dostert, C., Hannesschlager, N., Endres, S., Hartmann, G., Tardivel, A., Schweighoffer, E., Tybulewicz, V., et al. (2009). Syk kinase signalling couples to the Nlrp3 inflammasome for anti-fungal host defence. Nature *459*, 433-436.

Gudewicz, P.W., and Filkins, J.P. (1974). Glycogen metabolism in macrophages: effect of exogenous glycogen on glucogenesis in inflammatory exudate leukocytes and macrophages. Journal of the Reticuloendothelial Society *16*, 1-8.

Gudewicz, P.W., and Filkins, J.P. (1976). Glycogen metabolism in inflammatory macrophages. Journal of the Reticuloendothelial Society *20*, 147-157.

Guo, X., Zhou, Y., Wu, T., Zhu, X., Lai, W., and Wu, L. (2016). Generation of mouse and human dendritic cells in vitro. Journal of Immunological Methods *432*, 24-29.

Hacker, H., Redecke, V., Blagoev, B., Kratchmarova, I., Hsu, L.C., Wang, G.G., Kamps, M.P., Raz, E., Wagner, H., Hacker, G., et al. (2006). Specificity in Toll-like receptor signalling through distinct effector functions of TRAF3 and TRAF6. Nature *439*, 204-207.

Haider, A.S., Lowes, M.A., Suárez-Fariñas, M., Zaba, L.C., Cardinale, I., Khatcherian, A., Novitskaya, I., Wittkowski, K.M., and Krueger, J.G. (2008). Identification of Cellular Pathways of "Type 1," Th17 T Cells, and TNF- and Inducible Nitric Oxide Synthase-Producing Dendritic Cells in Autoimmune Inflammation through Pharmacogenomic Study of Cyclosporine A in Psoriasis. The Journal of Immunology *180*, 1913-1920.

Haidinger, M., Poglitsch, M., Geyeregger, R., Kasturi, S., Zeyda, M., Zlabinger, G.J., Pulendran, B., Hörl, W.H., Säemann, M.D., and Weichhart, T. (2010). A Versatile Role of Mammalian Target of Rapamycin in Human Dendritic Cell Function and Differentiation. The Journal of Immunology *185*, 3919-3931.

Hammer, G.E., and Ma, A. (2013). Molecular control of steady-state dendritic cell maturation and immune homeostasis. Annual review of immunology *31*, 743-791.

Haniffa, M., Bigley, V., and Collin, M. (2015). Human mononuclear phagocyte system reunited. Seminars in cell & developmental biology *41*, 59-69.

Hansen, P.A., Gulve, E.A., Marshall, B.A., Gao, J., Pessin, J.E., Holloszy, J.O., and Mueckler, M. (1995). Skeletal muscle glucose transport and metabolism are enhanced in transgenic mice overexpressing the Glut4 glucose transporter. The Journal of biological chemistry *270*, 1679-1684.

Hard, G.C. (1970). Some biochemical aspects of the immune macrophage. British journal of experimental pathology *51*, 97-105.

Hardie, D.G., Ross, F.A., and Hawley, S.A. (2012). AMPK: a nutrient and energy sensor that maintains energy homeostasis. Nature reviews. Molecular cell biology *13*, 251-262.

Hasan, U., Chaffois, C., Gaillard, C., Saulnier, V., Merck, E., Tancredi, S., Guiet, C., Brière, F., Vlach, J., Lebecque, S., et al. (2005). Human TLR10 Is a Functional Receptor, Expressed by B Cells and Plasmacytoid Dendritic Cells, Which Activates Gene Transcription through MyD88. The Journal of Immunology *174*, 2942-2950.

Hashimoto, D., Miller, J., and Merad, M. Dendritic Cell and Macrophage Heterogeneity In Vivo. Immunity *35*, 323-335.

Hashimoto, D., Miller, J., and Merad, M. (2011). Dendritic cell and macrophage heterogeneity in vivo. Immunity *35*, 323-335.

Hawn, T.R., Verbon, A., Lettinga, K.D., Zhao, L.P., Li, S.S., Laws, R.J., Skerrett, S.J., Beutler, B., Schroeder, L., Nachman, A., et al. (2003). A common dominant TLR5 stop codon polymorphism abolishes flagellin signaling and is associated with susceptibility to legionnaires' disease. The Journal of experimental medicine *198*, 1563-1572.

Hayashi, F., Smith, K.D., Ozinsky, A., Hawn, T.R., Yi, E.C., Goodlett, D.R., Eng, J.K., Akira, S., Underhill, D.M., and Aderem, A. (2001). The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature *410*, 1099-1103.

He, Y., Hara, H., and Núñez, G. Mechanism and Regulation of NLRP3 Inflammasome Activation. Trends in Biochemical Sciences *41*, 1012-1021.

Heil, F., Hemmi, H., Hochrein, H., Ampenberger, F., Kirschning, C., Akira, S., Lipford, G., Wagner, H., and Bauer, S. (2004). Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science *303*, 1526-1529.

Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K., et al. (2000). A Toll-like receptor recognizes bacterial DNA. Nature *408*, 740.

Hertz, L. (2004). Intercellular metabolic compartmentation in the brain: past, present and future. Neurochemistry international *45*, 285-296.

Hertz, L., and Dienel, G.A. (2002). Energy metabolism in the brain. International review of neurobiology *51*, 1-102.

Hertz, L., Peng, L., and Dienel, G.A. (2007). Energy metabolism in astrocytes: high rate of oxidative metabolism and spatiotemporal dependence on glycolysis/glycogenolysis. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism *27*, 219-249.

Hise, A.G., Tomalka, J., Ganesan, S., Patel, K., Hall, B.A., Brown, G.D., and Fitzgerald, K.A. An Essential Role for the NLRP3 Inflammasome in Host Defense against the Human Fungal Pathogen Candida albicans. Cell Host & Microbe *5*, 487-497.

Hoebe, K., Janssen, E.M., Kim, S.O., Alexopoulou, L., Flavell, R.A., Han, J., and Beutler, B. (2003). Upregulation of costimulatory molecules induced by lipopolysaccharide and double-stranded RNA occurs by Trif-dependent and Trif-independent pathways. Nature immunology *4*, 1223-1229.

Hoffman, W., Lakkis, F.G., and Chalasani, G. (2016). B Cells, Antibodies, and More. Clinical Journal of the American Society of Nephrology : CJASN *11*, 137-154.

Hoshino, K., Takeuchi, O., Kawai, T., Sanjo, H., Ogawa, T., Takeda, Y., Takeda, K., and Akira, S. (2016). Pillars Article: Cutting Edge: Toll-Like Receptor 4 (TLR4)-Deficient Mice Are Hyporesponsive to Lipopolysaccharide: Evidence for TLR4 as the Lps Gene Product. J. Immunol. 1999. 162: 3749-3752. Journal of immunology (Baltimore, Md. : 1950) *197*, 2563-2566.

Hu, L., Yu, Y., Huang, H., Fan, H., Hu, L., Yin, C., Li, K., Fulton, D.J., and Chen, F. (2016). Epigenetic Regulation of Interleukin 6 by Histone Acetylation in Macrophages and Its Role in Paraquat-Induced Pulmonary Fibrosis. Frontiers in immunology *7*, 696.

Huang, K.P., Chen, C.H., and Robinson, J.C. (1978). Glycogen synthesis by choriocarcinoma cells in vitro. Active synthesis in the presence of the glucose 6-phosphate-dependent form of glycogen synthase. The Journal of biological chemistry *253*, 2596-2603.

Huang, S.C.-C., Everts, B., Ivanova, Y., O'Sullivan, D., Nascimento, M., Smith, A.M., Beatty, W., Love-Gregory, L., Lam, W.Y., O'Neill, C.M., et al. (2014). Cell-intrinsic lysosomal lipolysis is essential for alternative activation of macrophages. Nature immunology *15*, 846.

Hume, D.A. (2006). The mononuclear phagocyte system. Current Opinion in Immunology *18*, 49-53.

Hume, D.A. (2008). Macrophages as APC and the Dendritic Cell Myth. The Journal of Immunology *181*, 5829-5835.

Iida, Y., Aoki, K., Asakura, T., Ueda, K., Yanaihara, N., Takakura, S., Yamada, K., Okamoto, A., Tanaka, T., and Ohkawa, K. (2012). Hypoxia promotes glycogen synthesis and accumulation in human ovarian clear cell carcinoma. International journal of oncology *40*, 2122-2130.

Infantino, V., Convertini, P., Cucci, L., Panaro, M.A., Di Noia, M.A., Calvello, R., Palmieri, F., and Iacobazzi, V. (2011). The mitochondrial citrate carrier: a new player in inflammation. The Biochemical journal *438*, 433-436.

Jakobsen, E., Bak, L.K., Walls, A.B., Reuschlein, A.-K., Schousboe, A., and Waagepetersen, H.S. (2017). Glycogen Shunt Activity and Glycolytic Supercompensation in Astrocytes May Be Distinctly Mediated via the Muscle Form of Glycogen Phosphorylase. Neurochemical Research.

Janeway, C. (2005). Immunobiology : the immune system in health and disease. (New York: Garland Science).

Janeway, C.A., Jr., and Medzhitov, R. (2002). Innate immune recognition. Annual review of immunology *20*, 197-216.

Jantsch, J., Chakravortty, D., Turza, N., Prechtel, A.T., Buchholz, B., Gerlach, R.G., Volke, M., Glasner, J., Warnecke, C., Wiesener, M.S., et al. (2008). Hypoxia and hypoxia-inducible factor-1 alpha modulate lipopolysaccharide-induced dendritic cell activation and function. Journal of immunology (Baltimore, Md. : 1950) *180*, 4697-4705.

Jha, A.K., Huang, S.C., Sergushichev, A., Lampropoulou, V., Ivanova, Y., Loginicheva, E., Chmielewski, K., Stewart, K.M., Ashall, J., Everts, B., et al. (2015). Network integration of parallel metabolic and transcriptional data reveals metabolic modules that regulate macrophage polarization. Immunity *42*, 419-430.

Jia, X.M., Tang, B., Zhu, L.L., Liu, Y.H., Zhao, X.Q., Gorjestani, S., Hsu, Y.M., Yang, L., Guan, J.H., Xu, G.T., et al. (2014). CARD9 mediates Dectin-1-induced ERK activation by linking Ras-GRF1 to H-Ras for antifungal immunity. The Journal of experimental medicine *211*, 2307-2321.

Juliana, C., Fernandes-Alnemri, T., Kang, S., Farias, A., Qin, F., and Alnemri, E.S. (2012). Non-transcriptional Priming and Deubiquitination Regulate NLRP3 Inflammasome Activation. Journal of Biological Chemistry *287*, 36617-36622.

Jurk, M., Heil, F., Vollmer, J., Schetter, C., Krieg, A.M., Wagner, H., Lipford, G., and Bauer, S. (2002). Human TLR7 or TLR8 independently confer responsiveness to the antiviral compound R-848. Nature immunology *3*, 499.

Kambayashi, T., and Laufer, T.M. (2014). Atypical MHC class II-expressing antigen-presenting cells: can anything replace a dendritic cell? Nature reviews. Immunology *14*, 719-730.

Kankkunen, P., Teirila, L., Rintahaka, J., Alenius, H., Wolff, H., and Matikainen, S. (2010). (1,3)-beta-glucans activate both dectin-1 and NLRP3 inflammasome in human macrophages. Journal of immunology (Baltimore, Md. : 1950) *184*, 6335-6342.

Kapteyn, J.C., Hoyer, L.L., Hecht, J.E., Muller, W.H., Andel, A., Verkleij, A.J., Makarow, M., Van Den Ende, H., and Klis, F.M. (2000). The cell wall architecture of Candida albicans wild-type cells and cell wall-defective mutants. Molecular microbiology *35*, 601-611.

Kawai, T., Adachi, O., Ogawa, T., Takeda, K., and Akira, S. (1999). Unresponsiveness of MyD88-deficient mice to endotoxin. Immunity *11*, 115-122.

Kayagaki, N., Wong, M.T., Stowe, I.B., Ramani, S.R., Gonzalez, L.C., Akashi-Takamura, S., Miyake, K., Zhang, J., Lee, W.P., Muszynski, A., et al. (2013). Noncanonical inflammasome activation by intracellular LPS independent of TLR4. Science *341*, 1246-1249.

Kelly, B., and O'Neill, L.A. (2015). Metabolic reprogramming in macrophages and dendritic cells in innate immunity. Cell research *25*, 771-784.

Kerrigan, A.M., and Brown, G.D. (2010). Syk-coupled C-type lectin receptors that mediate cellular activation via single tyrosine based activation motifs. Immunological reviews *234*, 335-352.

Kim, Y.B., Peroni, O.D., Aschenbach, W.G., Minokoshi, Y., Kotani, K., Zisman, A., Kahn, C.R., Goodyear, L.J., and Kahn, B.B. (2005). Muscle-specific deletion of the Glut4 glucose transporter alters multiple regulatory steps in glycogen metabolism. Molecular and cellular biology *25*, 9713-9723.

Kimura, H. (2013). Histone modifications for human epigenome analysis. Journal of human genetics *58*, 439-445.

Kingeter, L.M., and Lin, X. (2012). C-type lectin receptor-induced NF-kappaB activation in innate immune and inflammatory responses. Cellular & molecular immunology *9*, 105-112.

Kingston, D., Schmid, M.A., Onai, N., Obata-Onai, A., Baumjohann, D., and Manz, M.G. (2009). The concerted action of GM-CSF and Flt3-ligand on in vivo dendritic cell homeostasis. Blood *114*, 835-843.

Kondo, M. (2010). Lymphoid and myeloid lineage commitment in multipotent hematopoietic progenitors. Immunological reviews *238*, 37-46.

Krawczyk, C.M., Holowka, T., Sun, J., Blagih, J., Amiel, E., DeBerardinis, R.J., Cross, J.R., Jung, E., Thompson, C.B., Jones, R.G., et al. (2010). Toll-like receptor-induced changes in glycolytic metabolism regulate dendritic cell activation. Blood *115*, 4742-4749.

Kreft, M., Bak, L.K., Waagepetersen, H.S., and Schousboe, A. (2012). Aspects of astrocyte energy metabolism, amino acid neurotransmitter homoeostasis and metabolic compartmentation. ASN neuro *4*.

Krug, A., French, A.R., Barchet, W., Fischer, J.A., Dzionek, A., Pingel, J.T., Orihuela, M.M., Akira, S., Yokoyama, W.M., and Colonna, M. (2004a). TLR9-dependent recognition of MCMV by IPC and DC generates coordinated cytokine responses that activate antiviral NK cell function. Immunity *21*, 107-119.

Krug, A., Luker, G.D., Barchet, W., Leib, D.A., Akira, S., and Colonna, M. (2004b). Herpes simplex virus type 1 activates murine natural interferon-producing cells through toll-like receptor 9. Blood *103*, 1433-1437.

Kumar, V. (2014). Innate lymphoid cells: new paradigm in immunology of inflammation. Immunol Lett *157*, 23-37.

Lamkanfi, M., and Dixit, V.M. (2014). Mechanisms and functions of inflammasomes. Cell *157*, 1013-1022.

Lampropoulou, V., Sergushichev, A., Bambouskova, M., Nair, S., Vincent, E.E., Loginicheva, E., Cervantes-Barragan, L., Ma, X., Huang, S.C., Griss, T., et al. (2016). Itaconate Links Inhibition of Succinate Dehydrogenase with Macrophage Metabolic Remodeling and Regulation of Inflammation. Cell metabolism *24*, 158-166.

Lassila, O., Vainio, O., and Matzinger, P. (1988). Can B cells turn on virgin T cells? Nature *334*, 253-255.

Latham, T., Mackay, L., Sproul, D., Karim, M., Culley, J., Harrison, D.J., Hayward, L., Langridge-Smith, P., Gilbert, N., and Ramsahoye, B.H. (2012). Lactate, a product of glycolytic metabolism, inhibits histone deacetylase activity and promotes changes in gene expression. Nucleic acids research *40*, 4794-4803.

Lawless, S.J., Kedia-Mehta, N., Walls, J.F., McGarrigle, R., Convery, O., Sinclair, L.V., Navarro, M.N., Murray, J., and Finlay, D.K. (2017). Glucose represses dendritic cell-induced T cell responses. Nat Commun *8*, 15620.

LeBien, T.W., and Tedder, T.F. (2008). B lymphocytes: how they develop and function. Blood *112*, 1570-1580.

Lee, H.K., and Iwasaki, A. (2007). Innate control of adaptive immunity: dendritic cells and beyond. Seminars in immunology *19*, 48-55.

Lee, M.S., and Kim, Y.-J. (2007). Signaling Pathways Downstream of Pattern-Recognition Receptors and Their Cross Talk. Annual Review of Biochemistry *76*, 447-480.

Lee, S.M.Y., Kok, K.-H., Jaume, M., Cheung, T.K.W., Yip, T.-F., Lai, J.C.C., Guan, Y., Webster, R.G., Jin, D.-Y., and Peiris, J.S.M. (2014). Toll-like receptor 10 is involved in induction of innate immune responses to influenza virus infection. Proceedings of the National Academy of Sciences *111*, 3793-3798.

LeibundGut-Landmann, S., Gross, O., Robinson, M.J., Osorio, F., Slack, E.C., Tsoni, S.V., Schweighoffer, E., Tybulewicz, V., Brown, G.D., Ruland, J., et al. (2007). Syk- and CARD9-dependent coupling of innate immunity to the induction of T helper cells that produce interleukin 17. Nature immunology *8*, 630-638.

Levene, P.A., and Meyer, G.M. (1912). THE ACTION OF LEUCOCYTES ON GLUCOSE. Journal of Biological Chemistry *11*, 361-370.

Lin, K.-M., Hu, W., Troutman, T.D., Jennings, M., Brewer, T., Li, X., Nanda, S., Cohen, P., Thomas, J.A., and Pasare, C. (2014). IRAK-1 bypasses priming and directly links TLRs to rapid NLRP3 inflammasome activation. Proceedings of the National Academy of Sciences *111*, 775-780.

Lin, S.C., and Hardie, D.G. (2018). AMPK: Sensing Glucose as well as Cellular Energy Status. Cell metabolism *27*, 299-313.

Lipscomb, M.F., and Masten, B.J. (2002). Dendritic Cells: Immune Regulators in Health and Disease.

Lopez-Castejon, G., and Brough, D. (2011). Understanding the mechanism of IL-1β secretion. Cytokine & growth factor reviews *22*, 189-195.

Lord, K.A., Hoffman-Liebermann, B., and Liebermann, D.A. (1990). Nucleotide sequence and expression of a cDNA encoding MyD88, a novel myeloid differentiation primary response gene induced by IL6. Oncogene *5*, 1095-1097.

Lowes, M.A., Chamian, F., Abello, M.V., Fuentes-Duculan, J., Lin, S.-L., Nussbaum, R., Novitskaya, I., Carbonaro, H., Cardinale, I., Kikuchi, T., et al. (2005). Increase in TNF-α and inducible nitric oxide synthase-expressing dendritic cells in psoriasis and reduction with efalizumab (anti-CD11a). P Natl Acad Sci USA *102*, 19057-19062.

Lund, J., Sato, A., Akira, S., Medzhitov, R., and Iwasaki, A. (2003). Toll-like receptor 9-mediated recognition of Herpes simplex virus-2 by plasmacytoid dendritic cells. The Journal of experimental medicine *198*, 513-520.

Lutz, M.B., Kukutsch, N., Ogilvie, A.L., Rossner, S., Koch, F., Romani, N., and Schuler, G. (1999). An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. J Immunol Methods *223*, 77-92.

Lv, L., Li, D., Zhao, D., Lin, R., Chu, Y., Zhang, H., Zha, Z., Liu, Y., Li, Z., Xu, Y., et al. (2011). Acetylation targets the M2 isoform of pyruvate kinase for degradation through chaperone-mediated autophagy and promotes tumor growth. Molecular cell *42*, 719-730.

Ma, R., Ji, T., Zhang, H., Dong, W., Chen, X., Xu, P., Chen, D., Liang, X., Yin, X., Liu, Y., et al. (2018). A Pck1-directed glycogen metabolic program regulates formation and maintenance of memory CD8(+) T cells. Nat Cell Biol *20*, 21-27.

Maianski, N.A., Geissler, J., Srinivasula, S.M., Alnemri, E.S., Roos, D., and Kuijpers, T.W. (2004). Functional characterization of mitochondria in neutrophils: a role restricted to apoptosis. Cell Death Differ *11*, 143-153.

Maroof, A., English, N.R., Bedford, P.A., Gabrilovich, D.I., and Knight, S.C. (2005). Developing dendritic cells become 'lacy' cells packed with fat and glycogen. Immunology *115*, 473-483.

Martinon, F., Burns, K., and Tschopp, J. (2002). The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. Molecular cell *10*, 417-426.

Matarese, G., Colamatteo, A., and De Rosa, V. (2014). Metabolic fuelling of proper T cell functions. Immunol Lett *161*, 174-178.

Matzaraki, V., Kumar, V., Wijmenga, C., and Zhernakova, A. (2017). The MHC locus and genetic susceptibility to autoimmune and infectious diseases. Genome Biology *18*, 76.

Mayadas, T.N., Cullere, X., and Lowell, C.A. (2014). The Multifaceted Functions of Neutrophils. Annual review of pathology *9*, 181-218.

Mc, A.B. (1951). Myopathy due to a defect in muscle glycogen breakdown. Clinical science *10*, 13-35.

Medzhitov, R. (2007). TLR-mediated innate immune recognition. Seminars in immunology *19*, 1-2.

Medzhitov, R., Preston-Hurlburt, P., Kopp, E., Stadlen, A., Chen, C., Ghosh, S., and Janeway, C.A., Jr. (1998). MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways. Molecular cell *2*, 253-258.

Merad, M., Sathe, P., Helft, J., Miller, J., and Mortha, A. (2013). The Dendritic Cell Lineage: Ontogeny and Function of Dendritic Cells and Their Subsets in the Steady State and the Inflamed Setting. Annual review of immunology *31*, 10.1146/annurev-immunol-020711-074950.

Michels, P.A.M., Bringaud, F., Herman, M., and Hannaert, V. (2006). Metabolic functions of glycosomes in trypanosomatids. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research *1763*, 1463-1477.

Michelucci, A., Cordes, T., Ghelfi, J., Pailot, A., Reiling, N., Goldmann, O., Binz, T., Wegner, A., Tallam, A., Rausell, A., et al. (2013). Immune-responsive gene 1 protein links metabolism to immunity by catalyzing itaconic acid production. Proc Natl Acad Sci U S A *110*, 7820-7825.

Mildner, A., and Jung, S. (2014). Development and function of dendritic cell subsets. Immunity *40*, 642-656.

Mocsai, A., Ruland, J., and Tybulewicz, V.L. (2010). The SYK tyrosine kinase: a crucial player in diverse biological functions. Nature reviews. Immunology *10*, 387-402.

Mogensen, T.H. (2009). Pathogen recognition and inflammatory signaling in innate immune defenses. Clinical microbiology reviews *22*, 240-273, Table of Contents.

Moon, J.S., Hisata, S., Park, M.A., DeNicola, G.M., Ryter, S.W., Nakahira, K., and Choi, A.M. (2015). mTORC1-Induced HK1-Dependent Glycolysis Regulates NLRP3 Inflammasome Activation. Cell reports *12*, 102-115.

Muzio, M., Bosisio, D., Polentarutti, N., D'Amico, G., Stoppacciaro, A., Mancinelli, R., van't Veer, C., Penton-Rol, G., Ruco, L.P., Allavena, P., et al. (2000). Differential expression and regulation of toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells. Journal of immunology (Baltimore, Md. : 1950) *164*, 5998-6004.

Muzio, M., Natoli, G., Saccani, S., Levrero, M., and Mantovani, A. (1998). The human toll signaling pathway: divergence of nuclear factor kappaB and JNK/SAPK activation upstream of tumor necrosis factor receptor-associated factor 6 (TRAF6). The Journal of experimental medicine *187*, 2097-2101.

Muzio, M., Ni, J., Feng, P., and Dixit, V.M. (1997). IRAK (Pelle) family member IRAK-2 and MyD88 as proximal mediators of IL-1 signaling. Science *278*, 1612-1615.

Nagy, C., and Haschemi, A. (2015). Time and Demand are Two Critical Dimensions of Immunometabolism: The Process of Macrophage Activation and the Pentose Phosphate Pathway. Frontiers in immunology *6*, 164.

Nakano, H., Yanagita, M., and Gunn, M.D. (2001). CD11c(+)B220(+)Gr-1(+) cells in mouse lymph nodes and spleen display characteristics of plasmacytoid dendritic cells. The Journal of experimental medicine *194*, 1171-1178.

Nathan, C. (2002). Points of control in inflammation. Nature *420*, 846-852.

Naujoks, J., Tabeling, C., Dill, B.D., Hoffmann, C., Brown, A.S., Kunze, M., Kempa, S., Peter, A., Mollenkopf, H.J., Dorhoi, A., et al. (2016). IFNs Modify the Proteome of Legionella-Containing Vacuoles and Restrict Infection Via IRG1-Derived Itaconic Acid. PLoS pathogens *12*, e1005408.

Neefjes, J., Jongsma, M.L., Paul, P., and Bakke, O. (2011). Towards a systems understanding of MHC class I and MHC class II antigen presentation. Nature reviews. Immunology *11*, 823-836.

Netea, M.G., Brown, G.D., Kullberg, B.J., and Gow, N.A. (2008). An integrated model of the recognition of Candida albicans by the innate immune system. Nature reviews. Microbiology *6*, 67-78.

Netea, M.G., Gow, N.A., Munro, C.A., Bates, S., Collins, C., Ferwerda, G., Hobson, R.P., Bertram, G., Hughes, H.B., Jansen, T., et al. (2006). Immune sensing of Candida albicans requires cooperative recognition of mannans and glucans by lectin and Toll-like receptors. The Journal of clinical investigation *116*, 1642-1650.

Netea, M.G., Simon, A., van de Veerdonk, F., Kullberg, B.-J., Van der Meer, J.W.M., and Joosten, L.A.B. (2010). IL-1β Processing in Host Defense: Beyond the Inflammasomes. PLoS pathogens *6*, e1000661.

Newsholme, P., Curi, R., Gordon, S., and Newsholme, E.A. (1986). Metabolism of glucose, glutamine, long-chain fatty acids and ketone bodies by murine macrophages. The Biochemical journal *239*, 121-125.

Newsholme, P., and Newsholme, E.A. (1989). Rates of utilization of glucose, glutamine and oleate and formation of end-products by mouse peritoneal macrophages in culture. The Biochemical journal *261*, 211-218.

Nielsen, J., Holmberg, H.C., Schroder, H.D., Saltin, B., and Ortenblad, N. (2011). Human skeletal muscle glycogen utilization in exhaustive exercise: role of subcellular localization and fibre type. The Journal of physiology *589*, 2871-2885.

Nijman, H.W., Kleijmeer, M.J., Ossevoort, M.A., Oorschot, V.M., Vierboom, M.P., van de Keur, M., Kenemans, P., Kast, W.M., Geuze, H.J., and Melief, C.J. (1995). Antigen capture and major histocompatibility class II compartments of freshly isolated and

cultured human blood dendritic cells. The Journal of experimental medicine *182*, 163-174.

Norata, G.D., Caligiuri, G., Chavakis, T., Matarese, G., Netea, M.G., Nicoletti, A., O'Neill, L.A., and Marelli-Berg, F.M. (2015). The Cellular and Molecular Basis of Translational Immunometabolism. Immunity *43*, 421-434.

Nowarski, R., Gagliani, N., Huber, S., and Flavell, R.A. (2013). Innate immune cells in inflammation and cancer. Cancer Immunol Res *1*, 77-84.

O'Neill, L.A. (2011). A critical role for citrate metabolism in LPS signalling. The Biochemical journal *438*, e5-6.

O'Neill, L.A. (2014). Glycolytic reprogramming by TLRs in dendritic cells. Nature immunology *15*, 314-315.

O'Neill, L.A., Golenbock, D., and Bowie, A.G. (2013). The history of Toll-like receptors - redefining innate immunity. Nature reviews. Immunology *13*, 453-460.

O'Neill, L.A., and Hardie, D.G. (2013). Metabolism of inflammation limited by AMPK and pseudo-starvation. Nature *493*, 346-355.

Obel, L.F., Muller, M.S., Walls, A.B., Sickmann, H.M., Bak, L.K., Waagepetersen, H.S., and Schousboe, A. (2012). Brain glycogen-new perspectives on its metabolic function and regulation at the subcellular level. Front Neuroenergetics *4*, 3.

Odegaard, J.I., and Chawla, A. (2011). Alternative Macrophage Activation and Metabolism. Annual Review of Pathology: Mechanisms of Disease *6*, 275-297.

Oganesyan, G., Saha, S.K., Guo, B., He, J.Q., Shahangian, A., Zarnegar, B., Perry, A., and Cheng, G. (2006). Critical role of TRAF3 in the Toll-like receptor-dependent and -independent antiviral response. Nature *439*, 208-211.

Oosting, M., Cheng, S.C., Bolscher, J.M., Vestering-Stenger, R., Plantinga, T.S., Verschueren, I.C., Arts, P., Garritsen, A., van Eenennaam, H., Sturm, P., et al. (2014). Human TLR10 is an anti-inflammatory pattern-recognition receptor. Proc Natl Acad Sci U S A *111*, E4478-4484.

Oshiumi, H., Matsumoto, M., Funami, K., Akazawa, T., and Seya, T. (2003). TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-beta induction. Nature immunology *4*, 161-167.

Ozinsky, A., Underhill, D.M., Fontenot, J.D., Hajjar, A.M., Smith, K.D., Wilson, C.B., Schroeder, L., and Aderem, A. (2000). The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. Proc Natl Acad Sci U S A *97*, 13766-13771.

Palsson-McDermott, E.M., and O'Neill, L.A. (2013). The Warburg effect then and now: from cancer to inflammatory diseases. BioEssays : news and reviews in molecular, cellular and developmental biology *35*, 965-973.

Parsons, M. (2004). Glycosomes: parasites and the divergence of peroxisomal purpose. Molecular microbiology *53*, 717-724.

Pavlova, N.N., and Thompson, C.B. (2016). The Emerging Hallmarks of Cancer Metabolism. Cell metabolism *23*, 27-47.

Pearce, E.J., and Everts, B. (2015). Dendritic cell metabolism. Nature reviews. Immunology *15*, 18-29.

Pearce, E.L., and Pearce, E.J. (2013). Metabolic pathways in immune cell activation and quiescence. Immunity *38*, 633-643.

Pelletier, J., Bellot, G., Gounon, P., Lacas-Gervais, S., Pouyssegur, J., and Mazure, N.M. (2012). Glycogen Synthesis is Induced in Hypoxia by the Hypoxia-Inducible Factor and Promotes Cancer Cell Survival. Frontiers in oncology *2*, 18.

Peng, M., Yin, N., Chhangawala, S., Xu, K., Leslie, C.S., and Li, M.O. (2016). Aerobic glycolysis promotes T helper 1 cell differentiation through an epigenetic mechanism. Science *354*, 481-484.

Pescador, N., Villar, D., Cifuentes, D., Garcia-Rocha, M., Ortiz-Barahona, A., Vazquez, S., Ordonez, A., Cuevas, Y., Saez-Morales, D., Garcia-Bermejo, M.L., et al. (2010). Hypoxia promotes glycogen accumulation through hypoxia inducible factor (HIF)-mediated induction of glycogen synthase 1. PLoS One *5*, e9644.

Pierre, P., Turley, S.J., Gatti, E., Hull, M., Meltzer, J., Mirza, A., Inaba, K., Steinman, R.M., and Mellman, I. (1997). Developmental regulation of MHC class II transport in mouse dendritic cells. Nature *388*, 787-792.

Plato, A., Willment, J.A., and Brown, G.D. (2013). C-Type Lectin-Like Receptors of the Dectin-1 Cluster: Ligands and Signaling Pathways. International Reviews of Immunology *32*, 134-156.

Poltorak, A., He, X., Smirnova, I., Liu, M.-Y., Huffel, C.V., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., et al. (1998). Defective LPS Signaling in C3H/HeJ and C57BL/10ScCr Mice: Mutations in Tlr4 Gene. Science *282*, 2085-2088.

Porgador, A., Yewdell, J.W., Deng, Y., Bennink, J.R., and Germain, R.N. (1997). Localization, Quantitation, and In Situ Detection of Specific Peptide–MHC Class I Complexes Using a Monoclonal Antibody. Immunity *6*, 715-726.

Prats, C., Cadefau, J.A., Cusso, R., Qvortrup, K., Nielsen, J.N., Wojtaszewski, J.F., Hardie, D.G., Stewart, G., Hansen, B.F., and Ploug, T. (2005). Phosphorylation-dependent translocation of glycogen synthase to a novel structure during glycogen resynthesis. The Journal of biological chemistry *280*, 23165-23172.

Prats, C., Gomez-Cabello, A., and Hansen, A.V. (2011). Intracellular compartmentalization of skeletal muscle glycogen metabolism and insulin signalling. Experimental physiology *96*, 385-390.

Prats, C., Helge, J.W., Nordby, P., Qvortrup, K., Ploug, T., Dela, F., and Wojtaszewski, J.F. (2009). Dual regulation of muscle glycogen synthase during exercise by activation and compartmentalization. The Journal of biological chemistry *284*, 15692-15700.

Próchnicki, T., and Latz, E. (2017). Inflammasomes on the Crossroads of Innate Immune Recognition and Metabolic Control. Cell metabolism *26*, 71-93.

Prochnicki, T., Mangan, M.S., and Latz, E. (2016). Recent insights into the molecular mechanisms of the NLRP3 inflammasome activation. F1000Research *5*.

Qiu, Z., Lei, S., Zhao, B., Wu, Y., Su, W., Liu, M., Meng, Q., Zhou, B., Leng, Y., and Xia, Z.-y. (2017). NLRP3 Inflammasome Activation-Mediated Pyroptosis Aggravates Myocardial Ischemia/Reperfusion Injury in Diabetic Rats. Oxidative Medicine and Cellular Longevity *2017*, 17.

Quaglino, D., and Hayhoe, F.G. (1959). Observations on the periodic acid-Schiff reaction in lymphoproliferative diseases. The Journal of pathology and bacteriology *78*, 521-532.

Qualls, J.E., Subramanian, C., Rafi, W., Smith, A.M., Balouzian, L., DeFreitas, A.A., Shirey, K.A., Reutterer, B., Kernbauer, E., Stockinger, S., et al. (2012). Sustained generation of nitric oxide and control of mycobacterial infection requires argininosuccinate synthase 1. Cell Host Microbe *12*, 313-323.

Quiniou, S.M., Boudinot, P., and Bengten, E. (2013). Comprehensive survey and genomic characterization of Toll-like receptors (TLRs) in channel catfish, Ictalurus punctatus: identification of novel fish TLRs. Immunogenetics *65*, 511-530.

Ren, J.M., Marshall, B.A., Gulve, E.A., Gao, J., Johnson, D.W., Holloszy, J.O., and Mueckler, M. (1993). Evidence from transgenic mice that glucose transport is rate-limiting for glycogen deposition and glycolysis in skeletal muscle. The Journal of biological chemistry *268*, 16113-16115.

Roach, P.J. (2002). Glycogen and its metabolism. Current molecular medicine *2*, 101-120.

Roach, P.J., Cheng, C., Huang, D., Lin, A., Mu, J., Skurat, A.V., Wilson, W., and Zhai, L. (1998). Novel aspects of the regulation of glycogen storage. Journal of basic and clinical physiology and pharmacology *9*, 139-151.

Roach, P.J., Depaoli-Roach, A.A., Hurley, T.D., and Tagliabracci, V.S. (2012). Glycogen and its metabolism: some new developments and old themes. The Biochemical journal *441*, 763-787.

Roche, P.A., and Furuta, K. (2015). The ins and outs of MHC class II-mediated antigen processing and presentation. Nature reviews. Immunology *15*, 203-216.

Rock, K.L., Reits, E., and Neefjes, J. (2016). Present Yourself! By MHC Class I and MHC Class II Molecules. Trends in immunology *37*, 724-737.

Rodriguez, P.C., Ochoa, A.C., and Al-Khami, A.A. (2017). Arginine Metabolism in Myeloid Cells Shapes Innate and Adaptive Immunity. Frontiers in immunology *8*, 93.

Rodriguez-Espinosa, O., Rojas-Espinosa, O., Moreno-Altamirano, M.M., Lopez-Villegas, E.O., and Sanchez-Garcia, F.J. (2015). Metabolic requirements for neutrophil extracellular traps formation. Immunology *145*, 213-224.

Rogers, N.C., Slack, E.C., Edwards, A.D., Nolte, M.A., Schulz, O., Schweighoffer, E., Williams, D.L., Gordon, S., Tybulewicz, V.L., Brown, G.D., et al. (2005). Syk-Dependent Cytokine Induction by Dectin-1 Reveals a Novel Pattern Recognition Pathway for C Type Lectins. Immunity *22*, 507-517.

Ronchese, F., and Hausmann, B. (1993). B lymphocytes in vivo fail to prime naive T cells but can stimulate antigen-experienced T lymphocytes. The Journal of experimental medicine *177*, 679-690.

Rosenfeld, E.L. (1975). Alpha-glucosidases (gamma-amylases) in human and animal organisms. Pathologie-biologie *23*, 71-84.

Rosenstreich, D.L., Glode, L.M., and Mergenhagen, S.E. (1977). Action of endotoxin on lymphoid cells. The Journal of infectious diseases *136 Suppl*, S239-245.

Rossetti, L., Stenbit, A.E., Chen, W., Hu, M., Barzilai, N., Katz, E.B., and Charron, M.J. (1997). Peripheral but not hepatic insulin resistance in mice with one disrupted allele of the glucose transporter type 4 (GLUT4) gene. The Journal of clinical investigation *100*, 1831-1839.

Rousset, M., Chevalier, G., Rousset, J.P., Dussaulx, E., and Zweibaum, A. (1979). Presence and cell growth-related variations of glycogen in human colorectal adenocarcinoma cell lines in culture. Cancer research *39*, 531-534.

Rousset, M., Zweibaum, A., and Fogh, J. (1981). Presence of glycogen and growth-related variations in 58 cultured human tumor cell lines of various tissue origins. Cancer research *41*, 1165-1170.

Rybicka, K.K. (1996). Glycosomes--the organelles of glycogen metabolism. Tissue & cell *28*, 253-265.

Saas, P., Varin, A., Perruche, S., and Ceroi, A. (2017). Recent insights into the implications of metabolism in plasmacytoid dendritic cell innate functions: Potential ways to control these functions [version 2; referees: 3 approved].

Saeed, S., Quintin, J., Kerstens, H.H., Rao, N.A., Aghajanirefah, A., Matarese, F., Cheng, S.C., Ratter, J., Berentsen, K., van der Ent, M.A., et al. (2014). Epigenetic programming of monocyte-to-macrophage differentiation and trained innate immunity. Science *345*, 1251086.

Sallusto, F., and Lanzavecchia, A. (1994). Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. The Journal of experimental medicine *179*, 1109-1118.

Sallusto, F., and Lanzavecchia, A. (2018). Pillars Article: Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. J. Exp. Med. 1994. 179:1109-1118. Journal of immunology (Baltimore, Md. : 1950) *200*, 887-896.

Santos-Sierra, S., Deshmukh, S.D., Kalnitski, J., Kuenzi, P., Wymann, M.P., Golenbock, D.T., and Henneke, P. (2009). Mal connects TLR2 to PI3Kinase activation and phagocyte polarization. The EMBO journal *28*, 2018-2027.

Sato, S., Sugiyama, M., Yamamoto, M., Watanabe, Y., Kawai, T., Takeda, K., and Akira, S. (2003). Toll/IL-1 receptor domain-containing adaptor inducing IFN-beta (TRIF) associates with TNF receptor-associated factor 6 and TANK-binding kinase 1, and activates two distinct transcription factors, NF-kappa B and IFN-regulatory factor-3, in the Toll-like receptor signaling. Journal of immunology (Baltimore, Md. : 1950) *171*, 4304-4310.

Sbarra, A.J., and Karnovsky, M.L. (1959). The biochemical basis of phagocytosis. I. Metabolic changes during the ingestion of particles by polymorphonuclear leukocytes. The Journal of biological chemistry *234*, 1355-1362.

Schousboe, A., Sickmann, H.M., Walls, A.B., Bak, L.K., and Waagepetersen, H.S. (2010). Functional importance of the astrocytic glycogen-shunt and glycolysis for maintenance of an intact intra/extracellular glutamate gradient. Neurotox Res *18*, 94-99.

Schwartz, A.L. (1976). The metabolism of carbohydrate. Major problems in clinical pediatrics *3*, 3-28.

Scott, R.B. (1968). Glycogen in human peripheral blood leukocytes. I. Characteristics of the synthesis and turnover of glycogen in vitro. The Journal of clinical investigation *47*, 344-352.

Scott, R.B., and Cooper, L.W. (1974). Leucocyte glycogen response in inflammatory exudates. British journal of haematology *26*, 485-496.

Scott, R.B., and Still, W.J. (1968). Glycogen in human peripheral blood leukocytes. II. The macromolecular state of leukocyte glycogen. The Journal of clinical investigation *47*, 353-359.

See, P., Dutertre, C.A., Chen, J., Gunther, P., McGovern, N., Irac, S.E., Gunawan, M., Beyer, M., Handler, K., Duan, K., et al. (2017). Mapping the human DC lineage through the integration of high-dimensional techniques. Science *356*.

Segal, A.W. (2005). How neutrophils kill microbes. Annual review of immunology *23*, 197-223.

Seitz, I.F. (1965). Biochemistry of normal and leukemic leucocytes, thrombocytes, and bone marrow cells. Advances in cancer research *9*, 303-410.

Shen, Y., Kawamura, I., Nomura, T., Tsuchiya, K., Hara, H., Dewamitta, S.R., Sakai, S., Qu, H., Daim, S., Yamamoto, T., et al. (2010). Toll-Like Receptor 2- and MyD88-Dependent Phosphatidylinositol 3-Kinase and Rac1 Activation Facilitates the Phagocytosis of Listeria monocytogenes by Murine Macrophages. Infection and Immunity *78*, 2857-2867.

Shepherd, M.G. (1987). Cell envelope of Candida albicans. Critical reviews in microbiology *15*, 7-25.

Shinozaki, S., Choi, C.S., Shimizu, N., Yamada, M., Kim, M., Zhang, T., Dong, H.H., Kim, Y.-B., and Kaneki, M. (2011). Liver-specific Inducible Nitric-oxide Synthase Expression Is Sufficient to Cause Hepatic Insulin Resistance and Mild Hyperglycemia in Mice. Journal of Biological Chemistry *286*, 34959-34975.

Shulman, R.G., Hyder, F., and Rothman, D.L. (2001). Cerebral energetics and the glycogen shunt: neurochemical basis of functional imaging. Proc Natl Acad Sci U S A *98*, 6417-6422.

Shulman, R.G., and Rothman, D.L. (2001). The "glycogen shunt" in exercising muscle: A role for glycogen in muscle energetics and fatigue. Proc Natl Acad Sci U S A *98*, 457-461.

Shulman, R.G., and Rothman, D.L. (2017). The Glycogen Shunt Maintains Glycolytic Homeostasis and the Warburg Effect in Cancer. Trends in Cancer *3*, 761-767.

Sichien, D., Lambrecht, B.N., Guilliams, M., and Scott, C.L. (2017). Development of conventional dendritic cells: from common bone marrow progenitors to multiple subsets in peripheral tissues. Mucosal Immunol *10*, 831-844.

Sickmann, H.M., Waagepetersen, H.S., Schousboe, A., Benie, A.J., and Bouman, S.D. (2012). Brain glycogen and its role in supporting glutamate and GABA homeostasis in a type 2 diabetes rat model. Neurochemistry international *60*, 267-275.

Siegal, F.P., Kadowaki, N., Shodell, M., Fitzgerald-Bocarsly, P.A., Shah, K., Ho, S., Antonenko, S., and Liu, Y.J. (1999). The nature of the principal type 1 interferon-producing cells in human blood. Science *284*, 1835-1837.

Silvano, A. (2014). Dendritic cells: phenotypic and functional heterogeneity. Ital J Anat Embryol *119*, 304-330.

Siracusa, M.C., Kim, B.S., Spergel, J.M., and Artis, D. (2013). Basophils and allergic inflammation. The Journal of allergy and clinical immunology *132*, 789-801; quiz 788.

Spiro, R.G. (1958). The effect of N-acetylglucosamine and glucosamine on carbohydrate metabolism in rat liver slices. The Journal of biological chemistry *233*, 546-550.

Sprent, J. Antigen-Presenting Cells: Professionals and amateurs. Current Biology *5*, 1095-1097.

Steinman, R.M. (2001). Dendritic cells and the control of immunity: enhancing the efficiency of antigen presentation. Mt Sinai J Med. *3*.

Steinman, R.M. (2012). Decisions about dendritic cells: past, present, and future. Annual review of immunology *30*, 1-22.

Steinman, R.M., and Banchereau, J. (2007). Taking dendritic cells into medicine. Nature *449*, 419-426.

Steinman, R.M., and Hemmi, H. (2006). Dendritic cells: translating innate to adaptive immunity. Curr Top Microbiol Immunol *311*, 17-58.

Stossel, T.P., Murad, F., Mason, R.J., and Vaughan, M. (1970). Regulation of glycogen metabolism in polymorphonuclear leukocytes. The Journal of biological chemistry *245*, 6228-6234.

Strelko, C.L., Lu, W., Dufort, F.J., Seyfried, T.N., Chiles, T.C., Rabinowitz, J.D., and Roberts, M.F. (2011). Itaconic acid is a mammalian metabolite induced during macrophage activation. Journal of the American Chemical Society *133*, 16386-16389.

Sugita, H., Kaneki, M., Tokunaga, E., Sugita, M., Koike, C., Yasuhara, S., Tompkins, R.G., and Martyn, J.A. (2002). Inducible nitric oxide synthase plays a role in LPS-induced hyperglycemia and insulin resistance. American journal of physiology. Endocrinology and metabolism *282*, E386-394.

Sukhbaatar, N., Hengstschlager, M., and Weichhart, T. (2016). mTOR-Mediated Regulation of Dendritic Cell Differentiation and Function. Trends in immunology *37*, 778-789.

Sutterwala, F.S., Haasken, S., and Cassel, S.L. (2014). Mechanism of NLRP3 inflammasome activation. Annals of the New York Academy of Sciences *1319*, 82-95.

Swanson, R.A. (1992). Physiologic coupling of glial glycogen metabolism to neuronal activity in brain. Canadian journal of physiology and pharmacology *70 Suppl*, S138-144.

Takeda, K., and Akira, S. (2004). TLR signaling pathways. Seminars in immunology *16*, 3-9.

Takeda, K., and Akira, S. (2005). Toll-like receptors in innate immunity. Int Immunol *17*, 1-14.

Takeuchi, O., and Akira, S. Pattern Recognition Receptors and Inflammation. Cell *140*, 805-820.

Takeuchi, O., and Akira, S. (2004). [Roles of Toll-like receptor in host defense and the mechanism of its signal transduction]. Nihon Saikingaku Zasshi *59*, 523-529.

Tannahill, G.M., Curtis, A.M., Adamik, J., Palsson-McDermott, E.M., McGettrick, A.F., Goel, G., Frezza, C., Bernard, N.J., Kelly, B., Foley, N.H., et al. (2013). Succinate is an inflammatory signal that induces IL-1beta through HIF-1alpha. Nature *496*, 238-242.

Tavakoli, S., Zamora, D., Ullevig, S., and Asmis, R. (2013). Bioenergetic Profiles Diverge During Macrophage Polarization: Implications for the Interpretation of 18F-FDG PET Imaging of Atherosclerosis. Journal of Nuclear Medicine *54*, 1661-1667.

Taylor, P.R., Brown, G.D., Reid, D.M., Willment, J.A., Martinez-Pomares, L., Gordon, S., and Wong, S.Y. (2002). The beta-glucan receptor, dectin-1, is predominantly expressed on the surface of cells of the monocyte/macrophage and neutrophil lineages. Journal of immunology (Baltimore, Md. : 1950) *169*, 3876-3882.

Thwe, P.M., and Amiel, E. (2018). The role of nitric oxide in metabolic regulation of Dendritic cell immune function. Cancer letters *412*, 236-242.

Thwe, P.M., Pelgrom, L., Cooper, R., Beauchamp, S., Reisz, J.A., D'Alessandro, A., Everts, B., and Amiel, E. Cell-Intrinsic Glycogen Metabolism Supports Early Glycolytic Reprogramming Required for Dendritic Cell Immune Responses. Cell metabolism *26*, 558-567.e555.

Ting, J.P., Lovering, R.C., Alnemri, E.S., Bertin, J., Boss, J.M., Davis, B.K., Flavell, R.A., Girardin, S.E., Godzik, A., Harton, J.A., et al. (2008). The NLR gene family: a standard nomenclature. Immunity *28*, 285-287.

Uematsu, S., and Akira, S. (2007). [Toll-like receptor and innate immunity]. Seikagaku *79*, 769-776.

Uematsu, S., Fujimoto, K., Jang, M.H., Yang, B.G., Jung, Y.J., Nishiyama, M., Sato, S., Tsujimura, T., Yamamoto, M., Yokota, Y., et al. (2008). Regulation of humoral and cellular gut immunity by lamina propria dendritic cells expressing Toll-like receptor 5. Nature immunology *9*, 769-776.

Uematsu, S., Jang, M.H., Chevrier, N., Guo, Z., Kumagai, Y., Yamamoto, M., Kato, H., Sougawa, N., Matsui, H., Kuwata, H., et al. (2006). Detection of pathogenic intestinal

bacteria by Toll-like receptor 5 on intestinal CD11c+ lamina propria cells. Nature immunology *7*, 868-874.

Unanue, E.R. (1984). Antigen-presenting function of the macrophage. Annual review of immunology *2*, 395-428.

Underhill, D.M., and Goodridge, H.S. (2007). The many faces of ITAMs. Trends in immunology *28*, 66-73.

Underhill, D.M., Rossnagle, E., Lowell, C.A., and Simmons, R.M. (2005). Dectin-1 activates Syk tyrosine kinase in a dynamic subset of macrophages for reactive oxygen production. Blood *106*, 2543-2550.

Valentine, W.N., Follette, J.H., and Lawrence, J.S. (1953). The glycogen content of human leukocytes in health and in various disease states. The Journal of clinical investigation *32*, 251-257.

van de Laar, L., van den Bosch, A., Boonstra, A., Binda, R.S., Buitenhuis, M., Janssen, H.L.A., Coffer, P.J., and Woltman, A.M. (2012). PI3K-PKB hyperactivation augments human plasmacytoid dendritic cell development and function. Blood *120*, 4982-4991.

Van den Bossche, J., O'Neill, L.A., and Menon, D. (2017). Macrophage Immunometabolism: Where Are We (Going)? Trends in immunology *38*, 395-406.

Vanhaesebroeck, B., Guillermet-Guibert, J., Graupera, M., and Bilanges, B. (2010). The emerging mechanisms of isoform-specific PI3K signalling. Nature reviews. Molecular cell biology *11*, 329-341.

Vats, D., Mukundan, L., Odegaard, J.I., Zhang, L., Smith, K.L., Morel, C.R., Greaves, D.R., Murray, P.J., and Chawla, A. (2006). Oxidative metabolism and PGC-1β attenuate macrophage-mediated inflammation. Cell metabolism *4*, 13-24.

Villani, A.C., Satija, R., Reynolds, G., Sarkizova, S., Shekhar, K., Fletcher, J., Griesbeck, M., Butler, A., Zheng, S., Lazo, S., et al. (2017). Single-cell RNA-seq reveals new types of human blood dendritic cells, monocytes, and progenitors. Science *356*.

Voehringer, D. (2013). Protective and pathological roles of mast cells and basophils. Nature reviews. Immunology *13*, 362-375.

Voet, D., Voet, J.G., and Pratt, C.W. (2013). Fundamentals of biochemistry : life at the molecular level. (Hoboken, NJ: Wiley).

Vremec, D., Zorbas, M., Scollay, R., Saunders, D.J., Ardavin, C.F., Wu, L., and Shortman, K. (1992). The surface phenotype of dendritic cells purified from mouse thymus and spleen: investigation of the CD8 expression by a subpopulation of dendritic cells. The Journal of experimental medicine *176*, 47-58.

Wagner, R. (1946). The estimation of glycogen in whole blood and white blood cells. Archives of biochemistry *11*, 249-258.

Wagner, R. (1947). Studies on the physiology of the white blood cell; the glycogen content of leukocytes in leukemia and polycythemia. Blood *2*, 235-243.

Walls, A.B., Heimburger, C.M., Bouman, S.D., Schousboe, A., and Waagepetersen, H.S. (2009). Robust glycogen shunt activity in astrocytes: Effects of glutamatergic and adrenergic agents. Neuroscience *158*, 284-292.

Walls, A.B., Sickmann, H.M., Brown, A., Bouman, S.D., Ransom, B., Schousboe, A., and Waagepetersen, H.S. (2008). Characterization of 1,4-dideoxy-1,4-imino-d-arabinitol (DAB) as an inhibitor of brain glycogen shunt activity. J Neurochem *105*, 1462-1470.

Wang, C., Deng, L., Hong, M., Akkaraju, G.R., Inoue, J., and Chen, Z.J. (2001). TAK1 is a ubiquitin-dependent kinase of MKK and IKK. Nature *412*, 346-351.

Warburg, O. (1956). On the origin of cancer cells. Science *123*, 309-314.

Warburg, O., Wind, F., and Negelein, E. (1927). THE METABOLISM OF TUMORS IN THE BODY. The Journal of general physiology *8*, 519-530.

Weber, A.N., Moncrieffe, M.C., Gangloff, M., Imler, J.L., and Gay, N.J. (2005). Ligand-receptor and receptor-receptor interactions act in concert to activate signaling in the Drosophila toll pathway. The Journal of biological chemistry *280*, 22793-22799.

Weichhart, T., and Saemann, M.D. (2008). The PI3K/Akt/mTOR pathway in innate immune cells: emerging therapeutic applications. Annals of the rheumatic diseases *67 Suppl 3*, iii70-74.

Weisdorf, D.J., Craddock, P.R., and Jacob, H.S. (1982). Glycogenolysis versus glucose transport in human granulocytes: differential activation in phagocytosis and chemotaxis. Blood *60*, 888-893.

Weller, P.F., and Spencer, L.A. (2017). Functions of tissue-resident eosinophils. Nature reviews. Immunology *17*, 746-760.

Wellington, M., Koselny, K., Sutterwala, F.S., and Krysan, D.J. (2014). Candida albicans Triggers NLRP3-Mediated Pyroptosis in Macrophages. Eukaryotic Cell *13*, 329-340.

Wilsmann-Theis, D., Koch, S., Mindnich, C., Bonness, S., Schnautz, S., von Bubnoff, D., and Bieber, T. (2013). Generation and functional analysis of human TNF-alpha/iNOS-producing dendritic cells (Tip-DC). Allergy *68*, 890-898.

Wilson, W.A., Roach, P.J., Montero, M., Baroja-Fernandez, E., Munoz, F.J., Eydallin, G., Viale, A.M., and Pozueta-Romero, J. (2010). Regulation of glycogen metabolism in yeast and bacteria. FEMS microbiology reviews *34*, 952-985.

Wilson, W.A., Wang, Z., and Roach, P.J. (2002). Systematic identification of the genes affecting glycogen storage in the yeast Saccharomyces cerevisiae: implication of the vacuole as a determinant of glycogen level. Molecular & cellular proteomics : MCP *1*, 232-242.

Wolf, A.J., Reyes, C.N., Liang, W., Becker, C., Shimada, K., Wheeler, M.L., Cho, H.C., Popescu, N.I., Coggeshall, K.M., Arditi, M., et al. (2016). Hexokinase Is an Innate Immune Receptor for the Detection of Bacterial Peptidoglycan. Cell *166*, 624-636.

Wulff, H.R. (1962). Histochemical studies of leukocytes from an inflammatory exudate. Glycogen and phosphorylase. Acta haematologica *28*, 86-94.

Xu, n., Huang, n., Yang, n., Van Der Meide, n., Levi, n., Wahren, n., Link, n., and Xiao, n. (2001). Dendritic cell-derived nitric oxide is involved in IL-4-induced suppression of experimental allergic encephalomyelitis (EAE) in Lewis rats. Clinical & Experimental Immunology *118*, 115-121.

Xu, X., Ye, L., Araki, K., and Ahmed, R. (2012). mTOR, linking metabolism and immunity. Seminars in immunology *24*, 429-435.

Yamamoto, M., Sato, S., Hemmi, H., Hoshino, K., Kaisho, T., Sanjo, H., Takeuchi, O., Sugiyama, M., Okabe, M., Takeda, K., et al. (2003a). Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. Science *301*, 640-643.

Yamamoto, M., Sato, S., Hemmi, H., Uematsu, S., Hoshino, K., Kaisho, T., Takeuchi, O., Takeda, K., and Akira, S. (2003b). TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway. Nature immunology *4*, 1144-1150.

Yang, D., He, Y., Muñoz-Planillo, R., Liu, Q., and Núñez, G. (2015). Caspase-11 Requires the Pannexin-1 Channel and the Purinergic P2X7 Pore to Mediate Pyroptosis and Endotoxic Shock. Immunity *43*, 923-932.

Yang, H., Lee, S.M., Gao, B., Zhang, J., and Fang, D. (2013). Histone deacetylase sirtuin 1 deacetylates IRF1 protein and programs dendritic cells to control Th17 protein differentiation during autoimmune inflammation. The Journal of biological chemistry *288*, 37256-37266.

Yoon, S.I., Kurnasov, O., Natarajan, V., Hong, M., Gudkov, A.V., Osterman, A.L., and Wilson, I.A. (2012). Structural basis of TLR5-flagellin recognition and signaling. Science *335*, 859-864.

Young, F.G. (1957a). Claude Bernard and the Discovery of Glycogen. British Medical Journal *1*, 1431-1437.

Young, F.G. (1957b). Claude Bernard and the discovery of glycogen; a century of retrospect. British medical journal *1*, 1431-1437.

Young, J.W. (2012). Human Dendritic Cell Heterogeneity: Opportunities and Challenges for the Control of Immunity. Blood *120*, SCI-21-SCI-21.

Yu, J., Nagasu, H., Murakami, T., Hoang, H., Broderick, L., Hoffman, H.M., and Horng, T. (2014). Inflammasome activation leads to Caspase-1-dependent mitochondrial damage and block of mitophagy. Proc Natl Acad Sci U S A *111*, 15514-15519.

Yu, J.S.L., and Cui, W. (2016). Proliferation, survival and metabolism: the role of PI3K/AKT/mTOR signalling in pluripotency and cell fate determination. Development *143*, 3050-3060.

Yunis, A.A., and Arimura, G.K. (1964). ENZYMES OF GLYCOGEN METABOLISM IN WHITE BLOOD CELLS. I. GLYCOGEN PHOSPHORYLASE IN NORMAL AND LEUKEMIC HUMAN LEUKOCYTES. Cancer research *24*, 489-492.

Yunis, A.A., and Arimura, G.K. (1968). Isoenzymes of glycogen phosphorylase in human leukocytes and platelets: Relation to muscle phosphorylase. Biochemical and Biophysical Research Communications *33*, 119-128.

Zandi, E., Rothwarf, D.M., Delhase, M., Hayakawa, M., and Karin, M. (1997). The IkappaB kinase complex (IKK) contains two kinase subunits, IKKalpha and IKKbeta, necessary for IkappaB phosphorylation and NF-kappaB activation. Cell *91*, 243-252.

Zhang, D.-W., Shao, J., Lin, J., Zhang, N., Lu, B.-J., Lin, S.-C., Dong, M.-Q., and Han, J. (2009). RIP3, an Energy Metabolism Regulator That Switches TNF-Induced Cell Death from Apoptosis to Necrosis. Science *325*, 332-336.

Zhou, R., Tardivel, A., Thorens, B., Choi, I., and Tschopp, J. (2010). Thioredoxin-interacting protein links oxidative stress to inflammasome activation. Nature immunology *11*, 136-140.

Zois, C.E., Favaro, E., and Harris, A.L. (2014). Glycogen metabolism in cancer. Biochemical pharmacology *92*, 3-11.

APPENDIX A

Analysis of Glycogen Metabolic Pathway Utilization by Dendritic Cells and T Cells Using Custom Phenotype Metabolic Assays

Abstract

In the field of immunology, there is an increasing interest in cellular energy metabolism and its outcome on immune cell effector function. Activation of immune cells leads to rapid metabolic changes that are central to cellular biology in order to support the effector responses. Therefore, the need for user-friendly and dependable assay technologies to address metabolic regulation and nutrient utilization in immune cells is an important need in this field. Redox-dye reduction-based Phenotype MicroArray (PM) assays, which measure NADH reduction as a readout, developed by Biolog Inc., provide a wide screening of metabolites both in bacteria and mammalian cells. In this study, we delineate a detailed protocol of a customized Biolog assay for investigation of a specific metabolic pathway of interest. The option to be able to easily customize this technology offers researchers with a convenient assay platform to methodically examine specific nutrient substrates or metabolic pathways of interest in a rapid and cost-effective manner.

Introduction

There is a growing appreciation in the field of immunology that immune cell activation is accompanied by dramatic shifts in cellular metabolism, often highlighted by increased cellular uptake of glucose (1-9). Cellular utilization of specific metabolic pathways has traditionally been assessed by radioisotope labeling of metabolites and/or indirect measurements of enzymatic activities. While the techniques like carbon isotopic labeling, such as LC-MS approaches, provide reliable snapshots of cellular metabolic fluxes, they demand specialized expertise from the execution of experiments to data

analysis. In this work, we outline an experimental method using Biolog Inc. Phenotype MicroArray (PM) technology to perform simple and rapid screenings of cellular energy-producing metabolic pathways that provides fast, cost effective, and clear results.

The Biolog PM assay technology was originally developed for microbial metabolic screening, based on assaying the ability of microorganisms to metabolize distinct nutrient substrates. This assay platform was subsequently developed for a variety of eukaryotic cell applications including assays for evaluating cancer cell drug targets, metabolic disorders, cancer cell metabolism, drug toxicity testing, and general metabolic pathway analyses. For these assays, cells are plated in a 96-well format where each well is pre-loaded with a single distinct metabolic substrate. After allowing cells to equilibrate to their nutrient environment and metabolism to occur, cells are then co-incubated with a colorimetric dye that is reduced in the presence of NADH. If cells are capable of metabolizing a substrate, NADH will be produced resulting in proportional reduction of the dye. This assay is based on the premise that the amount of NADH produced directly correlates to the cell's ability to utilize a particular substrate as an energy source (10). Although it is similar to the principle of MTT assays, the Biolog tetrazolium redox dye mix is specifically optimized to measure cellular NADH production. It is also water soluble and readily adapted for metabolic rate measurements. This eliminates the solubility issues and the precipitation of formazan, thereby reducing the high background noise that is a typical obstacle for data interpretation in standard MTT methodologies (10). The intensity of color formation that results from dye reduction directly correlates with the amount of cellular NADH production driven by metabolism of a given substrate. In addition, cells can be assayed over time to provide kinetic analysis of substrate usage.

Figure A-1 provides a simple illustration of NADH production during glycolytic and mitochondrial metabolism, which is read as dye reduction of the Biolog Inc. Dye Mix in these assays.

One of the major advantages of this assay, from the perspective of larger-scale screening assays, is that it can be accomplished with relatively few cells in a total volume of 100 μL (see procedure for details) in each well of a 96-microwell plate. Cells are originally plated in a minimal nutrient media (MC-0) for overnight incubation. During this incubation, the cells will utilize residual glucose provided in MC-0 before they adapt and switch to the saturating single nutrient source in each well of a PM plate. Cellular utilization of metabolic substrates and their kinetics can be simply measured by colorimetric readings of purple formazan formation from Biolog redox dye reduction. The advantage of this technology is that it not only allows an individual to perform a broad screening of multiple metabolites using pre-designed PM plates, but also provides the flexibility to customize the assay for a thorough investigation of a single metabolite/metabolic pathway of interest, specifically tailored to individual's research needs. Below, we delineate an illustrative example of how researchers can adapt this technology to screen for the efficacy of metabolic-inhibiting drugs for a specific research question.

In a recently published research article, we originally identified that dendritic cells (DCs) of the immune system can use glycogen as an energy substrate from screening a wide number of carbon substrates using Biolog Inc. PM assay plates (11). In this screen, we identified that DCs can utilize not only single units of glucose but also various lengths of glucose polymers including the long-chain glucose polymer known as glycogen (11).

To systematically examine glycogen usage by DCs, we customized this technology to allow us to specifically screen for the efficacy of drugs that targeted glycogen metabolism in our assay system (Figure A-2). In this case, we built our custom assays using our substrates of interest, glycogen and glucose. Because of the extreme flexibility of the assay, we were also able to use this approach to simultaneously examine cellular nutrient utilization in both resting and activated DCs. With this customized approach, we have successfully identified a metabolite of our interest, glycogen, and validated the specificity of multiple inhibitors of glycogen metabolism in DCs as well as other immune cell types at different activation stages. Because of a growing interest in the metabolic pathway utilization of immune cells in response to stimuli, we believe that this assay platform will be attractive to researchers in the field based on the flexibility to customize this technology to query a wide range of metabolic parameters in a quick and cost-effective way.

Materials

1. *[a]96-well micro plates (Biolog Inc.); Other 96-well flat bottom plates can be substituted. Please see discussion for more information.
2. *[b]Biolog dye mix MA (6x) (Biolog Inc.) (for some cell types dye mix MB may work better)
3. *[c]IFM-1 (Biolog Inc.); This can be substituted with phenol red free, glucose and glutamine free RPMI.
4. Glutamine 2mM

5. 5% Fetal Bovine Serum (FBS) (or dialyzed FBS if limiting exogenous glucose is desired)
6. Bovine pancreatic glycogen, 5mg/mL concentration (Sigma aldrich)
7. CP91149 (CP) glycogen phosphorylase inhibitor I (Selleckham)
8. DAB, 1,4-Dideoxy-1,4-imino-D-arabinitol hydrochloride, glycogen phosphorylase inhibitor II (Santa Cruz)
9. Glucose 10mM
10. Multi-channel pipette and reservoir (optional)
11. Plate reader at 590nm wavelength

*These reagents/supplies are obtained from Biolog Inc.; however, options and limitations to substitute *[a] and *[c] with alternative sources are mentioned in discussion.

Procedure

Murine Bone-marrow -derived Dendritic Cells (BMDCs) are used as an example in this protocol.

I. Reagent preparation:

1. Assay Media (MC-0)
    a. MC-0 assay media supplemented with 5%FBS is prepared with the following reagents: for 25mL of MC-0 medium, add 1.25mL of FBS, 500μL of Pen Strep, and 50μL of 200mM stock of glutamine into 23.5mL of IF-M1 medium. (NOTE 1)

b. Glycogen containing MC-0 medium is prepared from the MC-0 medium from 1a to get a 10mg/mL (2x concentration). Glucose containing MC-0 medium with a final concentration 10mM (2x concentration) is prepared similarly by using the MC-0 medium from 1a.

c. For dose titrations of pharmacological inhibitors, for example glycogen phosphorylase inhibitor CP and DAB, inhibitor dilutions are prepared in glycogen containing MC-0 medium from Ia.

2. Assay Media (IFM-1)

a. For testing a certain pharmacological inhibitor such as glucose transporter-1 inhibitor (STF31), it is best to use IF-M1, without the addition of serum to limit the contribution of exogenous glucose by serum. In this case, glucose containing IF-M1 medium can be prepared by a certain concentration of glucose in IF-M1 medium with a range of concentration of the inhibitor.

II. Preparation of cells

1. BMDCs are generated as previously described (1). Briefly, bone marrow cells are flushed from femurs of 6-10-week-old C57/Bl6J mice, and cultured in CDCM, which is composed of RPMI, 10%fetal bovine serum, 5mM glutamine, 1mM beta-mercaptoethanol, and10IU Pen/Strep, supplemented in 20ng/μL of murine GM-CSF. The cells are cultured for 7 days, with a medium change every other day.

2. Day 7 BMDCs, differentiated in GM-CSF, are washed 2x in PBS to eliminate any trace of culture medium (See NOTE 2). After the final wash, the cells are re-

suspended in MC-0 basal medium from 1a at 1x10^6/mL concentration. Proceed to III-1 to seed the cells.

3. If using other cell types such as T or B lymphocytes, the cell number must be adjusted (See Note 2) as mentioned previously. If using human monocyte-derived DCs, cell numbers must also be adjusted due to differences in cell sizes. Furthermore, the redox dye mixes MA and MB should be compared for each cell type to determine which works best.

III. Setting up the experiment

1. BMDCs are seeded at 50,000 cells per well in technical duplicates or triplicates per condition. To do this, add 50μL of 1x10^6/mL BMDCs prepared in II-1 to each well of a 96-microwell Biolog plate (NOTE 3). Then add 50 μL of the prepared medium (at 2x concentration) from I-1 (containing carbon substrates of interest with and without the inhibitors). The total volume in each well will be 100 μL. Note that the basal media alone, either MC-0 or IF-M1, without the cells should be included for background correction.
2. Incubate the plate at 37^0C in 5%CO2 overnight.
3. The next morning, add 20 μL of Biolog dye Mix MA (6x) to each well containing 100 μL total medium volume (NOTE 4). This gives a final concentration of tetrazolium at 500 μM. Then, immediately measure the tetrazolium reduction at 590nm using a spectrophotometric plate reader (NOTE 2). This will be a time zero "0", baseline, reading. Return the plate to the incubator and continue reading the assay at every 15 minutes for the first hour. After the first hour, continue the assay with hourly readings for 6 hours or till the color change/the value of the

kinetic reading is plateaued. This will provide the kinetics of formazan production that is linear with time (NOTE 5)

4. The same day analysis (incubation) can also be performed using IF-M1 as a base medium without addition of serum (See Note 1 and the Protocol for IF-M1 based assay media). Then allow the cells for an hour or two of incubation time before adding the Biolog dye MA for kinetics readings. Upon dye addition, continue reading the assay using a colorimetric plate reader as mentioned above.

Data Analysis

Data are analyzed by normalizing to hour "0" (baseline) readings of each corresponding media:

a. Check raw readings to see if the readouts from replicates are approximately the same.
b. Average the replicates; the readings of technical replicates should be in a close range to each other.
c. For background correction, subtract the readings of MC-0 (no cells) from each group.
d. Next, normalize all the kinetic values to the baseline hour "0" by dividing the readings of each time point to the hour "0" readings of corresponding groups.
e. Graph the normalized values, with the baseline hour "0" as a starting point to represent relative increase (fold change) over baseline.

An example graph of glycogen utilization in mouse BMDCs with an inhibitor dose titration are shown in Figure 3A. A dose titration for glycogen phosphorylase pharmacological inhibitor, CP, in activated T cells is shown in Figure 3B.

Results

To validate the ability of DCs to metabolize glycogen from the PM screening assay in our recently published article (11), we modified the PM assay technology, in which untreated BMDCs were seeded in minimal nutrient medium (MC-0), MC-0 that has glycogen or glucose (control), and a range of dilutions of glycogen phosphorylase inhibitor, CP, in MC-0+glycogen. MC-0 medium alone with no cells was also included for background subtraction. Workflow schematics is shown in Figure A-2. As shown in Figure A-3A, NADH levels from the MC-0+glycogen group are comparable to those of MC-0+glucose. Based on our previously published findings (2, 11), it is not surprising that the cells release such a high level of NADH from MC-0+glucose as glucose is considered a primary fuel source in these cells. To confirm that DCs can generate NADH from glycogen, we titrated CP in a range of doses. Optimal dosage of the inhibitor is determined by comparing the NADH of inhibitor-containing groups to that of MC-0 only group. We identified that a 100μM dosage of CP provides optimal inhibition of glycogen phosphorylase.

Since BMDCs are partially adherent cells, we are interested in whether we could conduct similar approaches in non-adherent immune cells. Thus, we set up a similar assay using activated T cells as in Fig. A-3B and measured the kinetics of NADH production. Although T cells can generate NADH, the optimal inhibitory dose of CP is

much lower than that of BMDCs, which, we believe, is most likely due to a difference in cell size.

Discussion

We have previously utilized pre-designed Biolog Inc. PM assay plates to investigate the effect of inhibition of mechanistic target of rapamycin (mTOR) in various nutrient sources (2). In our recent publication, we also utilized PM-M1 plates to screen for potential nutrient sources that can serve as an intracellular carbon storage (11). While the pre-designed PM assays are extremely beneficial in screening a mass number of nutrient sources, the idea of utilizing this technology to conveniently investigate a specific carbon source or metabolic pathway prompted us to modify this assay by using a combination of in-house reagents and Biolog Inc. products.

Although the assay adaptation and procedure are relatively simple, there are several steps that require attention in order to achieve optimal results. In this paper, we examined and validated glycogen utilization in BMDCs and activated T cells. The seeding density for both types of cells are different due to differences in cell size. It is best to optimize the cell number for each cell type of interest in order to obtain optimal assay signal. Too few or too many cells will result in inconsistent assay signal and high variability among technical replicates. It is recommended that the cells be in a monolayer (if adherent). If the wells are too crowded, nutrients in the basal medium can be exhausted quickly even before dye addition, and this can result in inaccurate readings. **Table 3** describes the approximate cell numbers for each immune cell type we tested, for a 96-well microplate from Biolog Inc. Other types of 96-well flat bottom plates can be

used, in which case, the cell number and total medium volume per well should be adjusted. Extreme deviation from a monolayer of cells can cause adverse effects on nutrient utilization and impact the rate of NADH production.

In our assays, we incubated the cells overnight in MC-0 medium to allow the cells to utilize minimal nutrients present in MC-0 before they switch to the saturating single nutrient source. This incubation time can be adjusted to the same day or a longer period by varying the serum concentration (usually from a range of 0 to 20% FBS) in IF-M1 media. In some cases, an extended incubation period might be necessary after the addition of reduction dye to be able to observe distinct purple color formation. This is likely due to slow metabolic responses in some cells. These adjustments depend on the metabolic needs or activities of an individual cell type of interest. Serum can also be omitted in IF-M1 medium if the same day analysis is performed or if testing for a certain substrate or pharmacological inhibitor. Regardless of serum concentration, the end goal is to obtain a linear reading of NADH over a certain period of time.

The choice of Biolog dye mix MA or MB depends on the cell type, and in this work, we characterize the use of Dye Mix MA for myeloid (2) and lymphoid immune cells (Fig A-3). For other cell types, extensive information about which dye to use can be found at:http://biolog.com/pdf/pmmlit/00P%20133rC%20Redox%20Dye%20Mix%20Brochure%20JUL07.pdf. While other commercial formazan dyes are commercially available, we found that, based on our experience, Biolog Dye Mix provides the most reliable results, likely due to the fact that 1) a wide range of serum concentration does not impact the spectral absorbance of the dye and 2) colored chemical inhibitors cause very little

interference in absorption. Of note, these Dye Mixes should be protected from light as discoloration can occur and create a higher background, thus potentially skewing the data collected.

While we found this assay platform extremely useful and intuitive, one major drawback could be found in rapidly proliferating cells, such as activated T cells. The rate of proliferation could impact the results due to a varying degree of final cell numbers over a period of kinetic readings or during overnight incubation. Such a problem can be potentially solved by performing the same day assay and multiple replicates of experiments. Another point to consider is that one has to choose the right type of base medium, IF-M1 or IF-M2, depending on the type of metabolic pathways or nutrients of interest. For instance, if there is interest in examining the metabolism of a specific amino acid, it is best to substitute IF-M2, which lacks all 20 amino acids, for IF-M1, and subsequently add back the amino acid/s of choice.

We measured dye reduction kinetically, according to the guidelines by Biolog Inc. until the kinetics readings reach linear, which varies from one cell type to another depending on their metabolic activities. For our purpose, we typically normalize the data to baseline readings at time point "0" after dye addition so that our baseline is a value of "1" and kinetic production of NADH reflects a fold-change of signal above baseline. One of the real strengths of the Biolog assay platform is the ability to couple specific nutrient conditions to energetic production over a dynamic kinetic range. This is in distinct contrast to many other platforms in the field where researchers are forced to choose between either nutrient or energetic constraints. In addition, other kinetic assays (such as the Extracellular Flux Analysis from Agilent/Seahorse Biociences) are not directly

coupled to the production of reducing equivalents. Overall, the benefits of this assay outweigh its limitations, and this customizable assay platform presents a powerful tool to better understand complex metabolic networks at the cellular level.

Acknowledgement

The authors would like to acknowledge Dr. Ralph Budd, Dr. Paula Deming, and the VCIID COBRE for extensive support; Dr. Barry Bochner for technical advice and manuscript proofreading; Michael Secinaro for providing activated T cells for some of the described assays. Funding sources: 2016 AAI Careers in Immunology Fellowship (P.T. and E.A.), UVM College of Nursing and Health Sciences Incentive Grant (E.A.), UVM start-up Funds (E.A.), P30GM118228 (E.A.), 1R21AI135385-01A1 (E.A.).

Figures and Tables

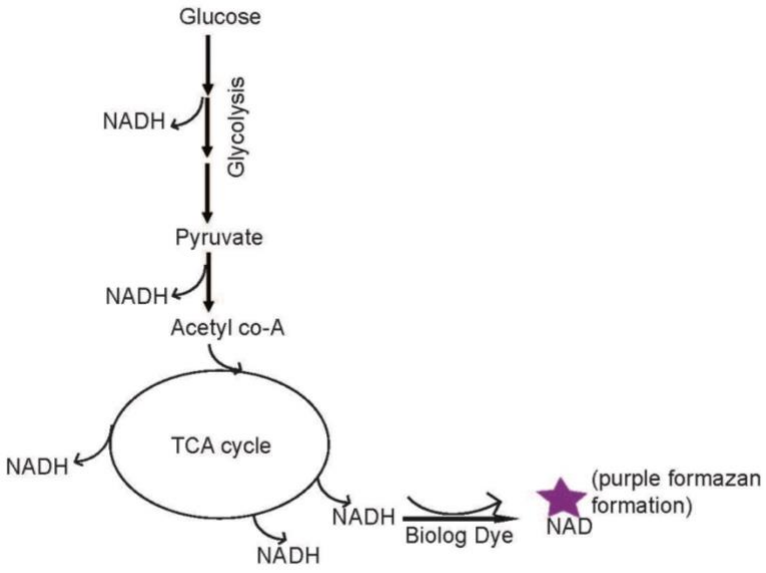


Figure A-1: Illustration of NADH production (with dye reduction) from glucose metabolism

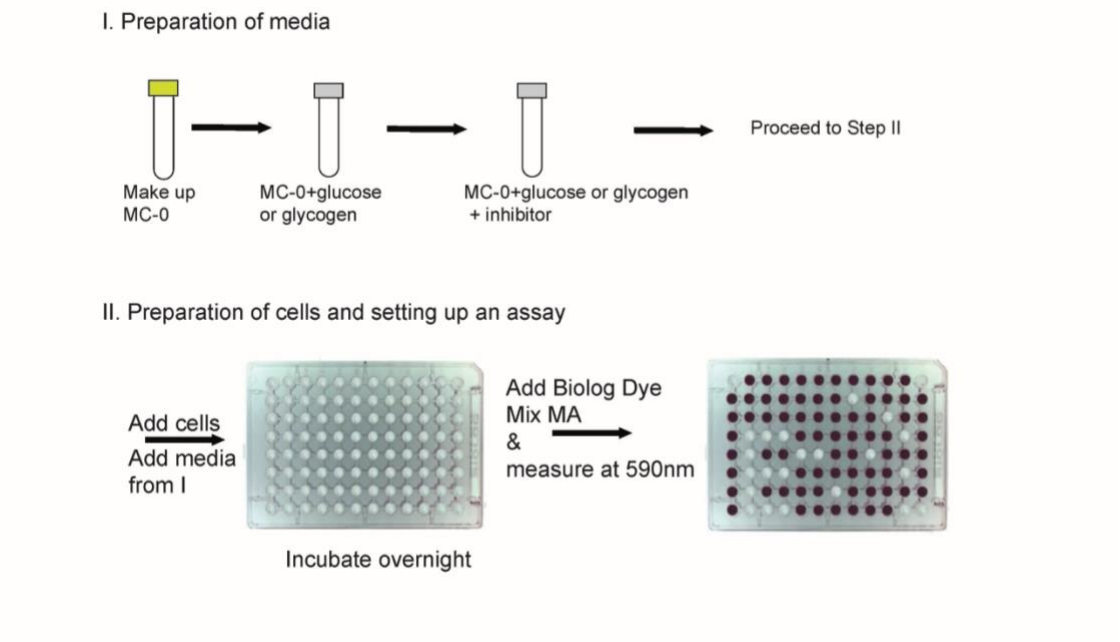


Figure A-2: Workflow for a customized Biolog assay set up (96-well plate picture courtesy of Biolog Inc.)

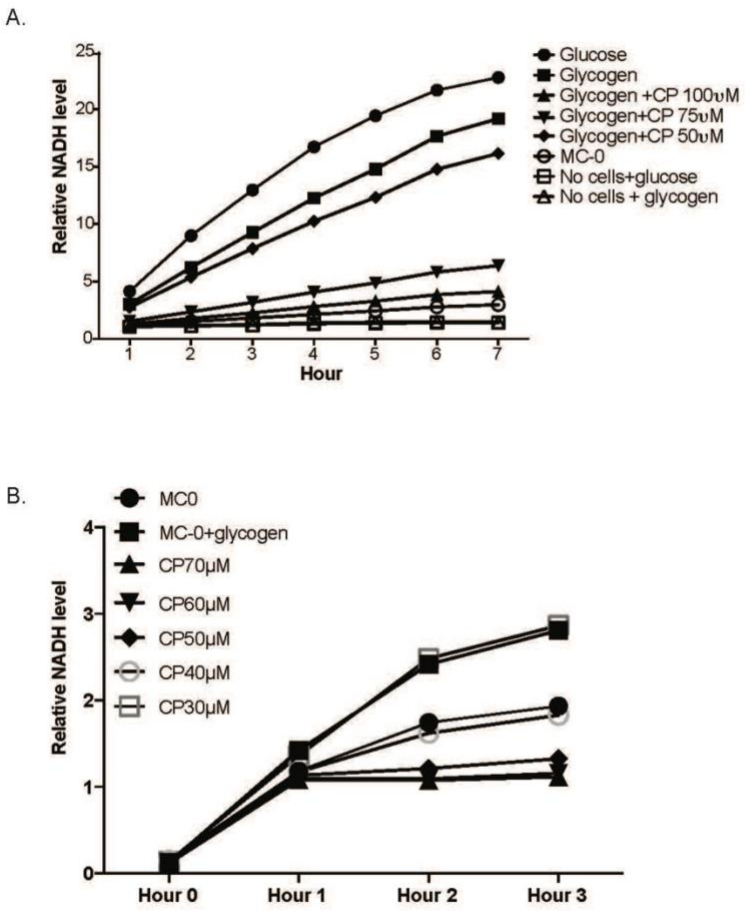


Figure A-3: (A) Titration of CP in BMDCs. BMDCs differentiated in GM-CSF were seeded at 50,000 cells/well in a 96-well Biolog microplate plate in a total volume of 100μL corresponding media and incubated overnight. 20μL of Biolog Redox Dye Mix MA was added the next morning. The assay was read hourly from basal time 0 (right after dye addition) up to 7 hours. Data were analyzed by dividing the average readings of each time point by basal time "0" of the corresponding medium. A representative of at least 3independent experiments. (B) Titration of CP in activated T cells. Activated T cells were seeded at 100,000 cells/well in a 96-well Biolog microplate in a total volume of 100μL of corresponding media and incubated overnight. CP were titrated in MC-0+glycogen medium. 20μL of Biolog Redox Dye Mix MA was added to each well the next morning. The assay was read hourly from basal time "0" through hour 3. Data were analyzed by dividing the average readings of each time point by basal time "0" of the corresponding medium. A representative of 3 independent experiments.

	MC-0 (no cells)	MC-0 only (with cells)	glucose	Glycogen	Glucose+ PYG inhibitor	Glycogen + PYG inhibitor
Triplicate		BMDCs	BMDCs	BMDCs	BMDCs	BMDCs
		BMDCs	BMDCs	BMDCs	BMDCs	BMDCs
		BMDCs	BMDCs	BMDCs	BMDCs	BMDCs

Table 2: An example of customized layout for Biolog plate

An example of customized plate layout for validating DC utilization of glucose and glycogen metabolic pathways with Biolog plates and the Biolog assay platform with Bone-marrow -derived Dendritic Cells (BMDCs) and Glycogen Phosphorylase (PYG) inhibitor.

Cell Type	Cell # per well of a 96-well Biolog microplate
BMDCs (Murine Bone marrow-derived DCs)	50,000
Human monocyte-derived DCs	50,000 – 80,000
Lymphocytes – naïve or activated B and T cells	100,000

Table 3: Guidelines for different immune cell numbers for a 96-well microplate set-up.

References

1. Amiel E, Everts B, Freitas TC, King IL, Curtis JD, Pearce EL, Pearce EJ. Inhibition of mechanistic target of rapamycin promotes dendritic cell activation and enhances therapeutic autologous vaccination in mice. Journal of immunology (Baltimore, Md : 1950). 2012;189(5):2151-8. Epub 2012/07/25. doi: 10.4049/jimmunol.1103741. PubMed PMID: 22826320; PMCID: 3424310.
2. Amiel E, Everts B, Fritz D, Beauchamp S, Ge B, Pearce EL, Pearce EJ. Mechanistic target of rapamycin inhibition extends cellular lifespan in dendritic cells by preserving mitochondrial function. Journal of immunology (Baltimore, Md : 1950). 2014;193(6):2821-30. Epub 2014/08/12. doi: 10.4049/jimmunol.1302498. PubMed PMID: 25108022; PMCID: Pmc4302759.
3. Everts B, Amiel E, Huang SC, Smith AM, Chang CH, Lam WY, Redmann V, Freitas TC, Blagih J, van der Windt GJ, Artyomov MN, Jones RG, Pearce EL, Pearce EJ. TLR-driven early glycolytic reprogramming via the kinases TBK1-IKKvarepsilon supports the anabolic demands of dendritic cell activation. Nature immunology. 2014;15(4):323-32. Epub 2014/02/25. doi: 10.1038/ni.2833. PubMed PMID: 24562310; PMCID: 4358322.
4. Everts B, Amiel E, van der Windt GJ, Freitas TC, Chott R, Yarasheski KE, Pearce EL, Pearce EJ. Commitment to glycolysis sustains survival of NO-producing inflammatory dendritic cells. Blood. 2012;120(7):1422-31. Epub 2012/07/13. doi: 10.1182/blood-2012-03-419747. PubMed PMID: 22786879; PMCID: 3423780.
5. Krawczyk CM, Holowka T, Sun J, Blagih J, Amiel E, DeBerardinis RJ, Cross JR, Jung E, Thompson CB, Jones RG, Pearce EJ. Toll-like receptor-induced changes in glycolytic metabolism regulate dendritic cell activation. Blood. 2010;115(23):4742-9. Epub 2010/03/31. doi: 10.1182/blood-2009-10-249540. PubMed PMID: 20351312; PMCID: 2890190.
6. Frauwirth KA, Riley JL, Harris MH, Parry RV, Rathmell JC, Plas DR, Elstrom RL, June CH, Thompson CB. The CD28 signaling pathway regulates glucose metabolism. Immunity. 2002;16(6):769-77. PubMed PMID: 12121659.
7. Freemerman AJ, Johnson AR, Sacks GN, Milner JJ, Kirk EL, Troester MA, Macintyre AN, Goraksha-Hicks P, Rathmell JC, Makowski L. Metabolic reprogramming of macrophages: glucose transporter 1 (GLUT1)-mediated glucose metabolism drives a proinflammatory phenotype. The Journal of biological chemistry. 2014;289(11):7884-96. doi: 10.1074/jbc.M113.522037. PubMed PMID: 24492615; PMCID: PMC3953299.
8. Macintyre AN, Gerriets VA, Nichols AG, Michalek RD, Rudolph MC, Deoliveira D, Anderson SM, Abel ED, Chen BJ, Hale LP, Rathmell JC. The glucose transporter Glut1 is selectively essential for CD4 T cell activation and effector function. Cell metabolism. 2014;20(1):61-72. doi: 10.1016/j.cmet.2014.05.004. PubMed PMID: 24930970; PMCID: PMC4079750.
9. Maciver NJ, Jacobs SR, Wieman HL, Wofford JA, Coloff JL, Rathmell JC. Glucose metabolism in lymphocytes is a regulated process with significant effects on immune cell function and survival. Journal of leukocyte biology. 2008;84(4):949-57. doi: 10.1189/jlb.0108024. PubMed PMID: 18577716; PMCID: PMC2638731.

10. Bochner BR, Siri M, Huang RH, Noble S, Lei XH, Clemons PA, Wagner BK. Assay of the multiple energy-producing pathways of mammalian cells. PLoS One. 2011;6(3):e18147. doi: 10.1371/journal.pone.0018147. PubMed PMID: 21455318; PMCID: PMC3063803.
11. Thwe PM, Pelgrom L, Cooper R, Beauchamp S, Reisz JA, D'Alessandro A, Everts B, Amiel E. Cell-Intrinsic Glycogen Metabolism Supports Early Glycolytic Reprogramming Required for Dendritic Cell Immune Responses. Cell metabolism.26(3):558-67.e5. doi: 10.1016/j.cmet.2017.08.012.