2020

Pharmacological Inhibition Of Hif-1 Alpha And Its Effects On Dendritic Cell Metabolic Reprogramming

Warrick Sahene
University of Vermont

PHARMACOLOGICAL INHIBITION OF HIF-1 ALPHA AND ITS EFFECTS ON DENDRITIC CELL METABOLIC REPROGRAMMING

A Thesis Presented

by

Warrick Sahene

to

The Faculty of the Graduate College

of

The University of Vermont

In Partial Fulfillment of the Requirements
for the Degree of Masters of Science
Specializing in Pharmacology

January, 2020

Defense Date: November 15, 2019
Thesis Examination Committee:

Eyal Amiel, Ph.D., Advisor
Alicia Ebert, Ph.D., Chairperson
Karen Lounsbury, Ph.D.
Anthony Morelli, Ph.D.
Cynthia J. Forehand, Ph.D., Dean of the Graduate College

ABSTRACT

Dendritic cells (DCs) are antigen presenting cells (APCs), a subtype of immune cells that present cellular information to T cells in the immune system. Hypoxia inducible factor 1 alpha (HIF-1 alpha) is an important transcription factor that facilitates dendritic cell metabolism by upregulating glycolysis in activated DCs. In this project, we examined the effects of HIF-1 alpha inhibition on metabolic processes of dendritic cells. Using techniques such as flow cytometry, western blotting, and extracellular flux analyzers, we used a selective inhibitor of HIF-1 alpha to test the hypothesis that HIF-1 alpha promotes glycolytic dependent processes such as glucose production, survival, and maturation. The results revealed that HIF-1 alpha impacts oxygen consumption rates in DCs, but does not affect survival, maturation rates, and glycolytic rates under the conditions studied. Dendritic cell secretion of IL-12, a proinflammatory cytokine upregulated during metabolism, decreased in a dose dependent manner under HIF-1 alpha inhibition. Understanding the effects of HIF-1 alpha can provide insight on how dendritic cells utilize their fuel source to facilitate immunological tasks and how in the future, we can optimize these sources to improve immune system functionality.

ACKNOWLEDGMENTS

I would like to thank my thesis committee Dr. Alicia Ebert and Dr. Karen Lounsbury for the support they provided as I prepared for my defense. I would like express my gratitude to Dr. Eyal Amiel for providing a nurturing environment and great mentorship throughout my research experience. Lastly, I would like to thank Dr. Anthony Morielli for serving as an excellent advisor and providing guidance throughout my entire graduate school experience.

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CHAPTER 1: Literature Review

1.1. Innate Immune System Overview

The innate immune system is responsible for an array of defenses used to resist infection (Steinman & Hemmi, 2006). These defenses include barriers that protect the body from foreign particles. These barriers include physical barriers such as the skin, defense mechanisms such as secretions, and generalized immune responses such as inflammation (Steinman & Hemmi, 2006). Dendritic cells (DCs) are immune cells that have multiple immune functions to bridge the adaptive and innate immune systems. The innate immune system is responsible for nonspecific responses when pathogens invade the body while the adaptive immune system is responsible for specific coordinated immune responses through T and B cells. The innate immune system contains antimicrobial agents such as lysosomes, which digest foreign pathogens. DCs mobilize lymphocytes and possess some adaptive immunity functionality, such as activating $CD8^+$ and $CD4^+$ T memory cells to facilitate specific responses to microbial infections (Medzhitov & Janeway, 1997). Adaptive immunity is only found in vertebrates whom can differentiate between self and nonself. The adaptive immune system can produce and recognize antigens which are important in immune cell memory (Medzhitov & Janeway, 1997). Key functionality provided by DCs allows stimulus-dependent irreversible differentiation called maturation that modulates the response of these cells and the cells

with which they interact (Steinman & Hemmi, 2006). DC maturation is initiated in response to a broad range of stimuli and can facilitate adaptive immunity to infection, tumors, and allergens (Medzhitov & Janeway, 1997). Through activation of toll like receptors (TLR) in DCs, DCs release cytokines that communicate with other immune cells. Primarily, DCs are one of the main cell types responsible for linking innate and adaptive immunity, specifically T-cell mediated immunity (Steinman & Hemmi, 2006). Antigen presenting cells like DCs utilize major histocompatibility complex (MHC), which allows antigen presenting cells to communicate with T lymphocytes using communicatory markers on the cell surface.

1.2 MHC Molecules and Antigen Producing Cells (APCs)

Major histocompatibility complex (MHC) class I and class II play an important role in the immune system (Wieczorek et al., 2017). These cell surface proteins present antigen peptides (acquired from phagocytosed pathogenic proteins) at the DC cell surface to $CD8^+$ and $CD4^+$ T cells respectively (Neefjes, Jongsma, Paul, & Bakke, 2011). MHC Class I and Class II molecules arise from different genes. Class I peptide antigens originate intracellularly, while antigens originate exogenously for MHC class II molecules (Neefjes et al., 2011). These classes share a similar fold comprised of a single alpha chain for MHC class I and two chains for class II, an alpha and beta chain (Wieczorek et al., 2017). The structure of these molecules allows the presentation of a broader range of peptides rather than a single protein to a single chain. The structures of these MHC molecules are especially important in DC biology, as only “professional antigen presenting cells” like

DCs express class II molecules and present antigens to $CD4^+$ T cells (Wieczorek et al., 2017).

Class II molecules are mainly found on antigen presenting cells like dendritic cells, macrophages, and B cells. Class II molecules are comprised of two alpha and two beta domains which are encoded by chromosome 6 (Cella, Engering, Pinet, Pieters, & Lanzavecchia, 1997). These class II molecules are important in stimulating adaptive T-cell mediated responses to extracellular infection.

All nucleated cells present class I molecules and protein fragments at their cell surfaces. These protein fragments are antigens degraded by nuclear and cytosolic proteasomes (Neefjes et al., 2011). The remaining peptides are translocated into the endoplasmic reticulum (ER) and assembled into a light chain complex (beta 2 microglobulin). Without these peptides, ER chaperone proteins like ERp57 promote peptide translocation in the ER (Neefjes et al., 2011). After peptides bind to the class I molecules, the class I complexes leave the ER and present at the cell surface, but interference in binding results in peptidic degradation (Neefjes et al., 2011). MHC- I molecules are expressed by virtually all cells to monitor intracellular infection. Class I molecules are also monitored by killer T cells, which survey the immune system. If killer T cells detect pathogenic or unfamiliar antigens, these infected cells are destroyed.

1.3. Cells of Innate Immunity

The innate immune system contains hemopoietic cells which develop into phenotypically and functionally distinct subclasses. These differentiations occur when progenitor cell populations are exposed to the right amount of signals and the correct

conditions. For example, exposing adult somatic cells to gain pluripotent stem cell functionality requires an expression of specific transcription factors. These factors allow upregulation of expression when hemopoietic stem cells are exposed to certain environmental cues. They differentiate into different cell types with an array of functionality (Galli, Borregaard, & Wynn, 2011). There are three main cell types that are present in the peripheral tissues that contribute to both innate and adaptive immunity: macrophages, mast cells, neutrophils and dendritic cells. (Galli et al., 2011).

1.3.1. Macrophages

Common myeloid progenitor cells in bone marrow are responsible for generating red blood cells, neutrophils, megakaryocytes, macrophages and DCs (Galli et al., 2011). Macrophages have long life spans, lasting anywhere from hours to years, and also express CD11b. The major functional difference between macrophages and DCs is that DCs primarily induce T cell responses, while macrophages are more skilled in digesting pathogens using phagocytosis, tissue remodeling, and clearing cellular debris (Galli et al., 2011). Although these macrophages reside in different locations (osteoclasts, in the alveolar membranes, histiocytes, and liver cells), their functionality is similar when stimulated (Galli et al., 2011). Distinguishing between the different subpopulations is difficult due to similar results using phenotypic markers, thus macrophage functionality has been used as an alternative approach. M1 macrophages, for example, are classically activated which mediate host defense while M2 macrophages are responsible for wound recovery through the secretion of IL-10 (Galli et al., 2011). Although there are

differences between the two classes of macrophages, they both induce host defense and inflammatory responses (Galli et al., 2011).

One of the main roles of macrophages is to maintain homeostasis without using special stimuli, like dendritic cell activation, to keep its functions activated (Galli et al., 2011). Macrophages remove pathogens and debris from circulation but can also up or downregulate stimuli for modulation of immune response (Wynn, Chawla, & Pollard, 2013). These cells express pattern recognition receptors that recognize microbial pathogens or cellular stress, which promote inflammation through downstream effects (Gordon, 2003). Macrophages have two main phenotypes: M1 and M2 (Wynn et al., 2013). These two states respond to cytokine interferon- gamma, interleukins, and Toll-like receptors (TLRs) (Wynn et al., 2013). The M1 phenotype is characterized by production of reactive oxygen species responsible for killing pathogens (Mosser & Edwards, 2008). The M2 specifically uses interleukin 4 (IL-4) and CD4+ type 2 helper T cells to induce M2 macrophages (Galli et al., 2011). Jmid3 is the epigenetic control center for M2 polarization through transcription factor IRF4, which inhibits many of the M1 genes (Satoh et al., 2010).

Regulatory macrophages are the third main class of macrophages that are responsible for the inhibition of M2 cells (Mosser & Edwards, 2008). The induction of regulatory macrophages are carried out by TLRs and produce immunosuppressive cytokines such as IL-10 and TGF-B (Mosser & Edwards, 2008). This counterregulatory mechanism is thought to counteract the regulatory macrophage ability to induce CD4+

type 2 helper T cells which can further suppress chronic inflammation and antitumor responses (Galli et al., 2011).

Specifically in the innate immune system, macrophages use their different receptors to destroy foreign materials through the M1 phenotype (Mosser & Edwards, 2008). They are also important in wound repair responses to platelet degradation factor from injury (Galli et al., 2011). These macrophages that initiate wound response are the M2 like phenotypes, which are induced through the PPAR-gamma receptor (Lumeng, Bodzin, & Saltiel, 2007). PPAR-gamma important in adipocyte function, glucose tolerance, and controls for diet induced obesity. As obesity progresses, these M2 macrophages switch to an M1 like state with proinflammatory activity (Lumeng et al., 2007).

1.3.2 Mast Cell Biology and Innate Immunity

Mast cells in humans develop from CD34+/CD117+ pluripotent progenitor cells in the bone marrow (Kirshenbaum et al., 1999). Cell maturation is dependent on KIT activation due to stem cell factor (SCF) induced KIT dimerization (Gilfillan, Austin, & Metcalfe, 2011). Tsai, Grimbaldeston, & Galli's research has shown that defective KIT catalytic activity causes a decrease in total mast cell numbers (2011). In contrast to human mast cells which require SCF for growth, mouse mast cell growth can be modulated by IL-3 in the absence of SCF (Kirshenbaum et al., 1999). The migration of mast cells are controlled in a tissue specific manner where specific regulatory chemokines are responsible for mast cells in a specific location (Gilfillan et al., 2011). For example, the expression of alpha 4 beta 7 is required for mast cell migration into the lungs (Gilfillan et al., 2011). Just like

macrophages, terminally differentiated mast cells have a long lifespan. Their lifespan is dependent on the presence of SCF in humans, but also the KIT catalytic activity (Gilfillan et al., 2011). Using tyrosine kinase inhibitors to block KIT catalytic activity by blocking dimerization, causes apoptosis of human mast cells (Jensen, Akin, & Gilfillan, 2008). In rodent and human models, inhibition of TNF receptors like Fas/CD95R, triggers apoptosis in both human and rodent mast cells (Jensen et al., 2008).

Mast cells serve to increase or decrease immune responses through a wide secretion of products that are proinflammatory, anti-inflammatory, and or immunosuppressive (Galli et al., 2011). The immediate response of mass cell is degranulation; removal of cytoplasmic granule contents into the extracellular matrix (Gilfillan et al., 2011). The response of mast cells to their stimuli are dependent on IgE receptor density on cell surfaces as well as their environmental influences (Galli et al., 2005).

Mast cells can boost innate immunity by increasing the amount of neutrophils locally, which is important in host resistance (Galli et al., 2005). In mice, mast cells release large amounts of proteases and proteoglycans that can limit the toxicity of toxins (Maurer et al., 2004). Mast cells proteases such as mMCP-6 can recruit neutrophils to bacterial infections which can enhance the host resistance to toxins (Gilfillan et al., 2011). Human mast cell protease release is similar to protease release in mice, with the exception that chymase and trypase are responsible for regulating secretory factors (Caughey, 2007). In vitro studies reveal that human mast cells incubated with SCF in media release more chymase after incubation with IL-4, which can strengthen host resistance (Shalit, Pickholz, & Levi-Shaffer, 1993).

1.3.3. Neutrophil Biology and Innate Immunity

Neutrophils are produced in the bone marrow, but their primary functions are launching immune responses, healing and destroying tissues, and providing instruction to the degree of specificity of lymphocyte response (Nathan, 2006). Neutrophils are responsible for the integration of information as well as the modulation of the inflammatory response (Nathan, 2006). These cells are the primary defense line against pathogens. Neutrophils phagocytose pathogens use granule proteins which are thought to be activated through NADPH oxidase (Segal, 2005). There are two theories that help explain immunology specifically in neutrophils. Danger theory is the understanding that immune cells release signals that activate antigen presenting cells which create a cascade response in other immune cell components (Aickelin & Cayzer, 2008). The pattern recognition theory differentiates between the self and the microbial non self, which induces the innate response, which activates the adaptive immune response (Medzhitov & Janeway, 1997). Both of these theories still do not explain complete neutrophil physiology, such as how certain injuries destroy a large number of host cells but does not promote reactions to foreign compounds (Nathan, 2006). A newer developing theory involves constant modulation of molecular signals to determine the initiation of an immune response (Galli et al., 2005). Neutrophils become activated by endothelial cells at sites of inflammation which allow transcription factors to produce cytokines responsible for the modulation of the inflammatory response (Galli et al., 2005). These neutrophils are short lived and provide local responses to inflammation. (Nathan, 2006).

1.4. Dendric Cell Functionality and Location

Dendritic cells are the forefront of innate immunity. They sense and respond to pathogens using pattern recognition receptors (PRRs) (Kelly & O'Neill, 2015). Activation of these PRRs causes intracellular signaling leading to the release of anti-microbial agents such as chemokines and proinflammatory cytokines which leads to the induction of the adaptive immune response (Kelly & O'Neill, 2015). These cells are mediated by T and B lymphocytes. There are many categories of PRRs but the most researched are Toll-like receptors (TLRs) and NOD-like receptors (NLRs) (Kelly & O'Neill, 2015). The downstream outcome of PRR activation is the induction of gene expression that is essential for initiating cellular inflammatory responses. Dendritic cells were discovered as a separate leucocyte that exhibit a strong ability to stimulate T lymphocytes (Steinman & Hemmi, 2006). Dendritic cells can be identified by their high levels of antigen presenting MHC class II molecules, their ability to present different properties than macrophages such as activation with CD86, that they can include a stellate cell shape with processes extending in multiple directions (Steinman & Hemmi, 2006). DCs are found in many locations such as body surfaces, afferent lymph, blood, peripheral lymphoid tissues, and mucosal lymphoid tissues (Steinman & Hemmi, 2006).

In body surfaces, DCs cycle antigens and move them to lymphoid organs to initiate immunity (Steinman & Hemmi, 2006). DCs form a network that comprises part of the epithelium which allows communication of processes through the lumen (Steinman & Hemmi, 2006). DCs also provide antigen capture which allows tolerance to self-damage as well as triggering response during stress.

In the afferent lymph, DCs migrate throughout the lymph from tissues to organs, following a similar path to lymphocytes (Steinman & Hemmi, 2006). DC migration into the afferent lymph can increase due to response. DCs in the blood express CD11c in humans and may be derived directly from the marrow in steady state.

In the lymphoid organ, the DCs are found in T cell and B cell areas (Steinman & Hemmi, 2006). DC precursors have been identified in mouse spleen and skin which contributes to the regeneration of DCs in local areas (Steinman & Hemmi, 2006). In mucosal associated lymphoid tissues, DCs ready for antigen capture are at the internal or mucosal body surfaces with their traffic area draining into the lymph nodes (Steinman & Hemmi, 2006). Injection of TNF-alpha or TLR ligands mobilize DCs from the intestine to the mesenteric afferent lymph (Steinman & Hemmi, 2006). DCs at mucosal surfaces are likely to be important for studying protective immunity and tolerance responses against chronic inflammation and allergy (Steinman & Hemmi, 2006).

1.4.1. DC Maturation

DC maturation was first used to describe the development that is necessary for the induction of immunity (Schuler & Steinman, 1985). The initial experiments of DC maturation took place in Langerhans cells (LCs), a subset of skin-resident DCs, which were weak stimulators of mixed leukocytes and other T cell mitogens (Sallusto et al., 1998). LCs became stronger stimulatory cells after being incubated with granulocyte-macrophage colony stimulating factor (GM-CSF) and this maturation was accompanied by the loss of dendritic cell markers with the replacement of highly dendritic cell morphology (Sallusto et al., 1998). This early research paved the way for understanding

the role of costimulatory markers and differentiating between the requirements of immunity (Schuler & Steinman, 1985). Immature DC phenotypic identification begins with antigen capture and post maturation functionality. Immature DCs are endocytic and bring extracellular information into the cells. Immature DCs also have lower levels of costimulatory molecules such as CD40, CD86, and CD86. Post maturation, DCs increase their costimulatory molecule production through TLRs. (Sallusto et al., 1998). Following the understanding of maturation, CD86 antibodies became available allowing researchers to recognize matured DCs could upregulate their expression of CD86 rapidly and to higher levels than other cells (Inaba et al., 1994). Many scientists consider increased CD86 expression to be synonymous with maturation as well as CD40, which are phenotypic indicators we used in our flow cytometry panel (Schuler & Steinman, 1985).

1.4.2. Distinct Maturation in Dendritic Cells

Myeloid DCs found in the blood mature through different maturation factors and different pathways. Thymic stromal lymphopoietin (TSLP) or CD40L both cause cellular differentiation that makes mature DCs look very similar phenotypically (Soumelis et al., 2002). Maturation in DCs increases MHC class II and CD86 expression which changes dendritic morphology. The TSLP naïve DCs cause T cells to differentiate into inflammatory cells that produce TNF and lead to other cytokine production (Soumelis et al., 2002). When DCs are incubated with CD40L, they fail to make inflammatory cytokines such as IL-1, 6, and 12 (Schuler & Steinman, 1985). While continued research is necessary to understand the differences in maturation, it is important to understand that distinct maturation responses are attributed to different maturation stimuli.

1.4.3. Pattern Recognition Receptors and Inflammation

The inflammatory response serves as a protective response by the body to protect tissue while allowing damaged tissue to be removed (Takeuchi & Akira, 2010). The classic inflammation symptoms involve redness, swelling, heat, pain, and loss of tissue function (Takeuchi & Akira, 2010). The inflammatory response is fueled by cytokine production, but overproduction of cytokines in response to pathogens can be fatal (Takeuchi & Akira, 2010). Innate immunity is a major contributor to the acute inflammation specifically induced by macrophages and dendritic cells. PRRs are responsible for sensing the presence of organisms by recognizing pathogen associated molecular patterns (PAMPs), which are conserved among microbial species (Takeuchi & Akira, 2010). These PRRs then activate genes that encode for proinflammatory cytokines, type I interferons, chemokines, and proteins that modulate PRR signaling (Takeuchi & Akira, 2010). Cytokines such as TNF, IL-1, IL-12, and IL-6 are responsible for cell death regulation, blood cell recruitment, and modification of vascular endothelial permeability (Takeuchi & Akira, 2010). IL-1 beta is another cytokine that plays an important role during the early initiation of tissue inflammation. IL-1 beta is regulated by a two-step mechanism rather than transcriptional and translational levels of TNF and IL-6. The first step in IL-1 beta production involves the IL-1 beta zymogen, pro IL-1 beta, which is synthesized in a TLR signal dependent manner (Takeuchi & Akira, 2010). To activate the zymogen (step 2), IL-1 beta must be cleaved by caspase 1 which is activated independently of TLR signaling. Caspase 1 is activated by a complex called inflammasome .

1.5. TLRs and Ligands

TLRs have dual functionality in sensing the extracellular environment as well as intracellular functionality (Spirig et al., 2010). TLRs are transmembrane proteins with each TLR being able to differentiate between molecular patterns of microorganisms (Takeuchi & Akira, 2010). There have been 12 TLRs discovered in mice and 10 discovered in humans (Takeuchi & Akira, 2010).

Cells	TLR Expression	Roles
Neutrophils(PMN)	1,2,4,6,8	Pattern recognition
Mast Cells	2,4	Pattern recognition
Monocytes	1,2,4,5,6,8	Pattern recognition
Macrophages	1,2,3,4,6,7,9	Pattern recognition (Ag presentation)
NK cells	1,(7,9)	Pattern recognition
Resting B cells	(1,4),6,7,(8,)9,10	Pattern recognition
Germinal c B cells	1,2,6,7,8,9,10	Pattern recognition
preDC(Monocytes)	1,2,4,5,(6,)8	Pattern recognition
iDC ($CD11c^{+}$iDC)	1,2,3,(5,6,8,10)	Pattern recognition Ag presentation (Ag crosspresentation)
plasmacytoid DC(preDC2)	(1,6,)7,9	Pattern recognition (Ag presentation)(Ag crosspresentation)

Table 1. TLR expression in different cell types

TLR4 recognizes lipopolysaccharide (LPS) with myeloid differentiation factor 2 (MD2) on the cell surface (Takeuchi & Akira, 2010). LPS is a component on the outer membrane of gram negative bacteria that forms a TLR4 homodimer. In our studies, we have used LPS as a canonical TLR ligand that induces DC maturation and activation.

1.5.1. TLR Ligation on DCs

DCs respond rapidly to different TLRs, specifically TLR7 and 9, which responds to nucleic acids and produce the largest amounts of type I interferons (Gilliet et al., 2002). For example, TLR3 is expressed highly in mouse lymphoid tissues when acted on with flt-3L instead of GM-CSF. This leads to plasmacytoid DCs which express distinct TLRs

(Gilliet et al., 2002). The production of a TLR response is primarily through the (NF)-kB pathway leading to upregulation of inflammatory cytokines like TNF alpha, IL-1, IL-12, and IL-6. The importance of these inflammatory cytokines on adaptive immunity require more research, but it is thought that DCs use cytokines to overcome suppression while communicating to suppressor T cells (Tarbell, Yamazaki, & Steinman, 2006). TLR ligands act on many antigen presenting cells. Therefore, it is important to understand the degree that each TLR is interacting with each DC subtype in vivo to continue to improve vaccine development (Schuler & Steinman, 1985).

1.5.2. Negative Feedback of DC Maturation and Immune Responses

Although DCs respond positively to external stimuli for maturation, DCs can also respond to negative molecules to suppress maturation and immune responses. Specific negative molecules that can interact with TLR and cytokine receptor signaling pathways are suppressors of cytokine signaling (SOCS) proteins (Tarbell et al., 2006). SOCS proteins primarily work in innate cytokine production rather than adaptive immunity. For example, CD47 and TSP suppresses IL-12 production and enhances IL-10 production (Yao, Li, Kaplan, & Chang, 2005). Similarly, IL-4 can knock down IL-10 levels while increasing IL-12 production in DCs. The amount of inhibition can also depend on cellular development. Even though LPS can induce differentiation of immature DCs classically, LPS can inhibit the formation and differentiation of DCs from monocytes. (Tarbell et al., 2006). DCs can also expand different types of suppressive pathways through the different stages of maturation (Steinman & Hemmi, 2006). Some of these negative regulators include CD25+ CD4+ foxp3+ (Schuler & Steinman, 1985). The indoleamine

dioxygenase pathway also leads to suppression of immune responses by DCs, but activation of this suppressive pathway can be toxic to lymphocytes (Frumento et al., 2002).

1.6.1. Metabolism Overview

Metabolism can be broken down into catabolism and anabolism. These are the processes of synthesis and degradation of carbohydrates, fatty acids, nucleic acids, and amino acids, respectively. The purpose of these metabolic processes are to either break down complex carbohydrates into monomeric molecules to produce adenosine triphosphate (ATP) or to create complex molecules for energy storage. ATP serves as a key regulator of these metabolic processes and cellular functionality. Metabolism at all levels is regulated by key rate limiting enzymes, systemic, and cellular regulation. Metabolic processes are altered in immune cells, which will be explored in this thesis.

The major metabolic processes are glycolysis, tricarboxylic acid pathway (TCA), electron transport chain (ETC), the pentose phosphate pathway, fatty acid biosynthesis, beta oxidation, and nucleotide synthesis. Glucose, the primary carbon source of glycolysis enters the cell and begins a stepwise process of phosphorylations and isomerizations (Rodrigues, Ludovico, & Leão, 2006). These enzymatic processes require an input of ATP to begin, making glycolysis not energetically favorable as aerobic respiration. Once glucose is converted into pyruvate, it is shuffled into the pyruvate dehydrogenase (PDH) pathway to form acetyl-CoA, a high energy molecule that will be used in the TCA pathway (Rodrigues et al., 2006). Oxaloacetate and acetyl-CoA condense to begin a series of oxidation-reduction reactions that generate high electron

carrier molecules that will be used in the generation of ATP in the ETC. Oxidative phosphorylation occurs in the mitochondrial inner membrane where protons are pumped into the inner membrane space where they pass through ATP synthase to generate molecules of ATP (Rodrigues et al., 2006). Carbohydrate metabolism is not the only metabolic process that produces ATP. Beta oxidation of palmitate produces about 106 molecules of ATP, but requires the decarboxylation of a fatty acid.

Anaerobic metabolism, or metabolism that occurs without the requirement for oxygen still converts glucose to pyruvate. To maintain redox balance, NAD+ is regenerated and lactic acid fermentation occurs (Rodrigues et al., 2006). Buildup of lactic acid results in acidosis, where the blood pH becomes too acidic. The interplay of anaerobic metabolism in immune cells will be explored in this project as well.

1.6.2 iNOS Metabolic Reprogramming in DCs

The Warburg effect is important for understanding the effects of metabolic changes post activation in immune cells (House, Warburg, Burk, & Schade, 1956). Under normoxic conditions, tumor cells use glycolysis instead of oxidative phosphorylation despite oxygen being available. Instead of pyruvate being decarboxylated into acetyl-CoA, it is converted into lactate (House et al., 1956). In the later 1950s, this paradigm appeared in neutrophils in which glucose consumption was high, but oxygen consumption was low (House et al., 1956). Activation of TLR4 by LPS increases glucose consumption in neutrophils but also increases peroxide production, which is important for neutrophil mediated killing. Similar results were observed during macrophage activation where glucose was converted to lactate with little oxidative phosphorylation usage

(House et al., 1956). Glycolytic enzymes such as glucose 6 phosphate dehydrogenase and hexokinase were upregulated indicating increased glycolytic activity in these cells. Using a wide range of stimuli including LPS, macrophages, or DCs switch from oxidative phosphorylation to glycolysis characterized by a decrease in TCA activity and an increase in lactic acid production. This effect is observed in mature activated immune cells and is largely thought to be a rapid production source of ATP (Kelly & O'neill, 2015). LPS activation on TLR4 has many downstream activation pathways such as nitric oxide synthase (iNOS), mTOR, u-PFK2, AMPK. In the context of this project, we will be primarily focusing on iNOS and mTOR activation.

LPS stimulated activation leads to increased expression of the inducible iNOS which generates nitric oxide (NO) (Kelly & O'neill, 2015). NO plays various roles in smooth muscle vasodilation, but in immune cells nitrogen species can inhibit mitochondrial respiration. NO inhibits ETC complexes as well as cytochrome c oxidase (Kelly & O'neill, 2015). Inhibition of iNOS after LPS stimulation restores normal mitochondrial respiration while knockouts of iNOS in DCs fail to switch from oxidative phosphorylation. NO is derived from the amino acid arginine which is metabolized to NO and citrulline by iNOS. Citrulline is used when arginine levels are depleted but is important in controlling for mycobacterial infections. In Helicobacter pylori, inhibition of NO production makes macrophages less effective to killing bacteria (Kelly & O'neill, 2015). In summary, it is important to note that NO facilitates the inhibition of oxidative phosphorylation but has other target sites to induce its effects.

1.7. HIF-1 Alpha Structure

HIF transcription factors are regulators of the cellular response to hypoxia and manifest pathways to ensure cellular functionality despite oxygen shortages (Palazon, Goldrath, Nizet, & Johnson, 2014). HIF-1 is expressed broadly across innate and adaptive cell types including neutrophils, dendritic cells, and macrophages (Jantsch et al., 2008). HIF 2 alpha, an isoform, is expressed in certain immune cells such as CD8+ T cells in response to hypoxia (Jantsch et al., 2008). The role of HIF 2 alpha is still largely unexplored but the differing expression patterns of HIF-1 and HIF-2 are dependent on intrinsic and extrinsic factors that activate distinct sets of target genes (Palazon et al., 2014).

HIF is a basic loop protein that forms a heterodimeric complex which acts as a regulator of promoters containing hypoxia response consensus sequences (HREs) (Wang, Jiang, Rue, & Semenza, 1995). The regulatory complex contains HIF -1 Beta, and either HIF-1 alpha or HIF-2 alpha. The stability of HIF 1 alpha post-transcriptionally is regulated by oxygen availability through iron dependent enzymes. When oxygen availability decreases, prolyl-hydroxylases (PHDs) become inactivated resulting in an increase of HIF-1 alpha (Palazon et al., 2014). Another way of inhibiting HIF transcription is through factor inhibiting HIF (FIH). Hydroxylating asparaginyl residues in HIF-1 alpha and HIF- 2 alpha blocks interactions between HIF-1 alpha transactivation domain and coactivators that form a transcriptional complex (Palazon et al., 2014).

HIF-1 stabilization in immune cells occurs in an oxygen independent manner. In macrophages and dendritic cells, stimulation of TLR4 under normoxic conditions

stabilizes HIF-1. (Kelly & O'neill, 2015). Enterobacterial siderophores, iron chelating agents can lead to the stabilization of HIF-1 alpha oxygen independently (Palazon et al., 2014). LPS stimulation induces HIF-1 alpha protein accumulation in macrophages through translational activation (Blouin, Pagé, Soucy, & Richard, 2004). The NF-kB pathway which is important for regulating immune responses is also responsible for bacteria-induced HIF-1 transcriptional response in macrophages (Rius et al., 2008). HIF-1 alpha stabilization in dendritic cells is still not completely understood. DCs cultured under hypoxic conditions have less costimulatory and maturation markers leading to decreased T cell proliferation (Palazon et al., 2014). Hypoxic conditions induce the production of proinflammatory cytokines such as IL- 1 beta and TNF alpha (Sica et al., 2008).

DCs stabilize HIF-1 alpha through TLR ligation during the metabolic shift from aerobic respiration to glycolysis. Pharmacological stabilization of HIF-1 alpha using a PHD inhibitor leads to increases in costimulatory molecules (Palazon et al., 2014). However, the hypoxia pathway has yet to be fully understood and understanding HIF-1 alpha's role is necessary for the interplay between the innate and adaptive immune systems.

1.7.2. HIF-1 alpha and Glycolysis in DCs

HIF-1 alpha promotes the metabolic switch to glycolysis since oxygen limited conditions induces this transcription factor. HIF-1 alpha facilitates this switch by binding to HREs in target genes such as glucose transporter 1 (GLUT1) (Kelly & O'neill, 2015). HIF-1 alpha also induce lactate dehydrogenase (LDH) expression, which facilitates lactate production

from pyruvate decreasing the amount of acetyl- CoA available to enter the TCA cycle (Kelly & O'neill, 2015). HIF-1 alpha also inhibits pyruvate dehydrogenase which decarboxylates pyruvate into acetyl- CoA, thus promoting the glycolytic switch (Kim, Tchernyshyov, Semenza, & Dang, 2006).

1.7.3. HIF-1 alpha Activation through mTOR

HIF-1 alpha activation by LPS involves the mammalian target of rapamycin (mTOR), a serine/threonine protein kinase that is abundant in proliferating cells after TLR stimulation (Byles et al., 2013). mTOR helps cells reach a high metabolic demand by having cells increase the expression of HIF-1 alpha (Byles et al., 2013). mTOR promotes translation of signaling motifs that are present in HIF-1 alpha mRNA.

Inhibition of glycolysis leads to blocked maturation, leading to a decrease in costimulatory molecules such as CD80 and CD86 (Kim et al., 2006). Similarly, mTOR activation by LPS is inhibited by Rapamycin, which removes LPS induced IL-10 and IL-12 levels (Ohtani et al., 2008). HIF-1 alpha and mTOR are important in regulatory mechanisms in glycolytic switching and could impact survival and maturation in DCs. Exploring these pathways and their target effects will help us understand signaling in inflammatory situations (Kelly & O'neill, 2015).

1.8 Project Hypothesis

Dendritic cells are antigen presenting cells that play an important response in activating immune responses through T cell activation. In this thesis, we aim to study the role of HIF-1 alpha inhibitor in DC survival and immune function using pharmacological inhibitor of HIF-1 alpha BAY 87-2243. Since HIF-1 alpha expression is increased during DC activation, we hypothesize that HIF-1 alpha inhibition will decrease metabolic processes such as glycolysis and decrease survival and maturation rates (Figure 1. Proinflammatory cytokines are upregulated during DC activation, we expected a decrease in cytokines like IL-12 during inhibition of HIF-1 alpha. We utilized western blotting to look at HIF-1 alpha protein expression levels in response to the inhibitor. We also utilized ELIZA techniques to test the requirement for HIF-1 alpha in regulating inflammatory cytokine production by DCs in response to LPS. Finally, we used an extracellular flux metabolic analysis, to examine the impact of HIF-1 alpha inhibition on basal and post-activation metabolic profiles of DCs. Below is a model of the hypothesis.

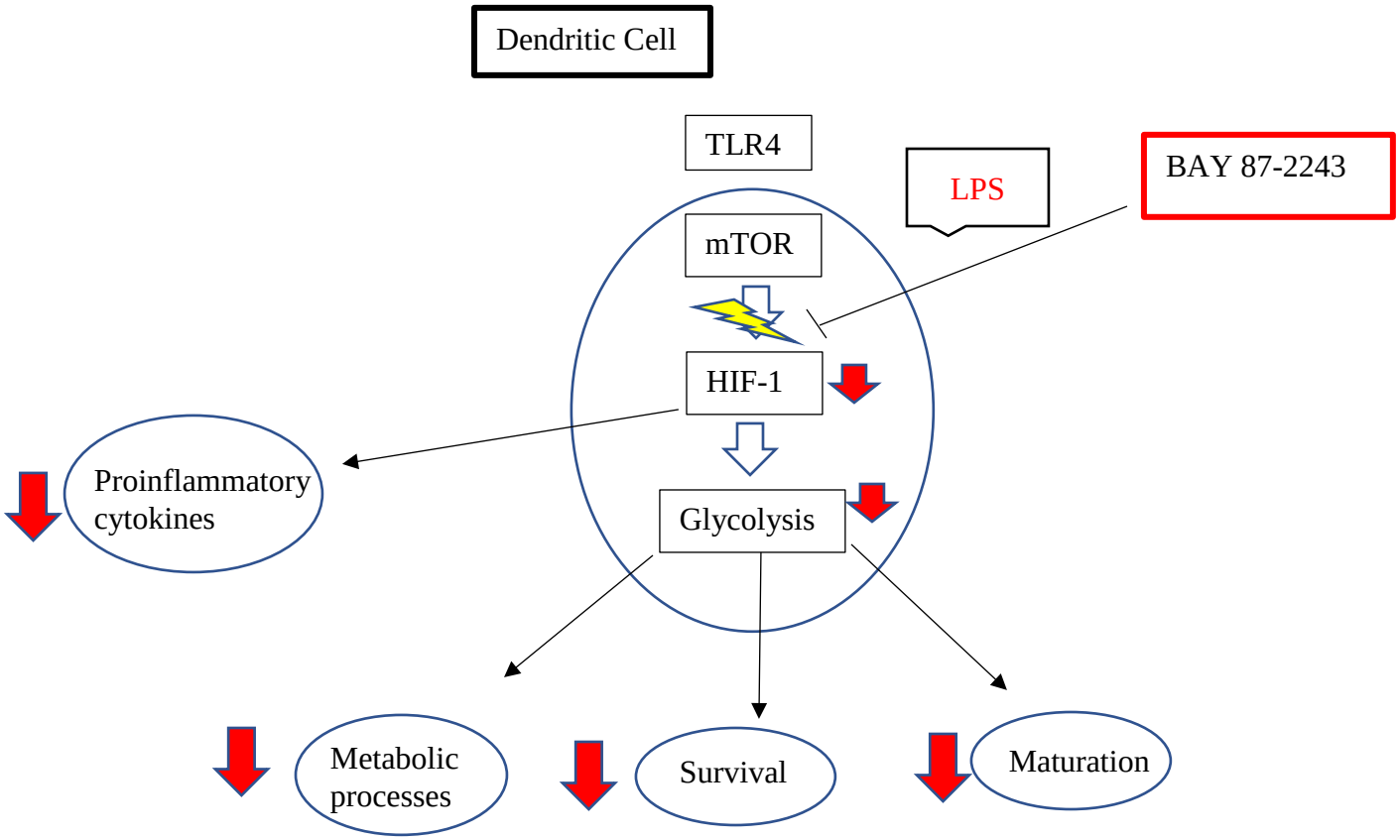


Figure 1: Model of role of HIF-1 alpha in DC biology and predicted impact of HIF-1 alpha inhibition

CHAPTER 2: 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 and pharmacological inhibtors BAY 87-2243 were purchased from Selleckchem. BAY 87-2243 has many proposed mechanisms such as inhibition of mitochondrial production of reactive oxygen species (ROS) leading to degradation of HIF. The inhibitor also works by inhibiting HIF-1 target genes such as CA-9, leading to decreased transcription of HIF.

Mouse DC culture and Activation

Bone marrow derived DCs were generated as described in Lutz *et al (41)*. 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 200mL of media 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.

Flow Cytometry and Cytokine measurements

Above mentioned antibodies were used for flow cytometry. Samples were 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).

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.

Seahorse Assay Protocol

The Oxygen Consumption Rate (OCR) and Extracellular Acidification Rate (ECAR) were measured using the extracellular flux analyzer (XF24, Seahorse Bioscience)

according to the manufacturers protocol. Probe plate was calibrated with 200 μL XF Calibrant Solution 24 hours to running the Seahorse plate. Cells were loaded using a two-step method. Step 1 required resuspension of all groups with 5% FCS and 10 mM Glucose (XF Running media) and an hour in a NON-CO_2 37°C incubator. Step 2 required the addition of another 150 μL of XF Running media and incubated until ready for loading. Mitochondrial drugs were loaded into appropriate ports of the probe plates. The final concentrations of each drug was: Oligomycin 1 μM, FCCP 1.5 μM, Rotenone 100 nM, Antimycin A 1 μM. After probe plate was loaded, Seahorse plate was placed into extracellular flux analyzer and software was programmed to run the mitochondrial stress test.

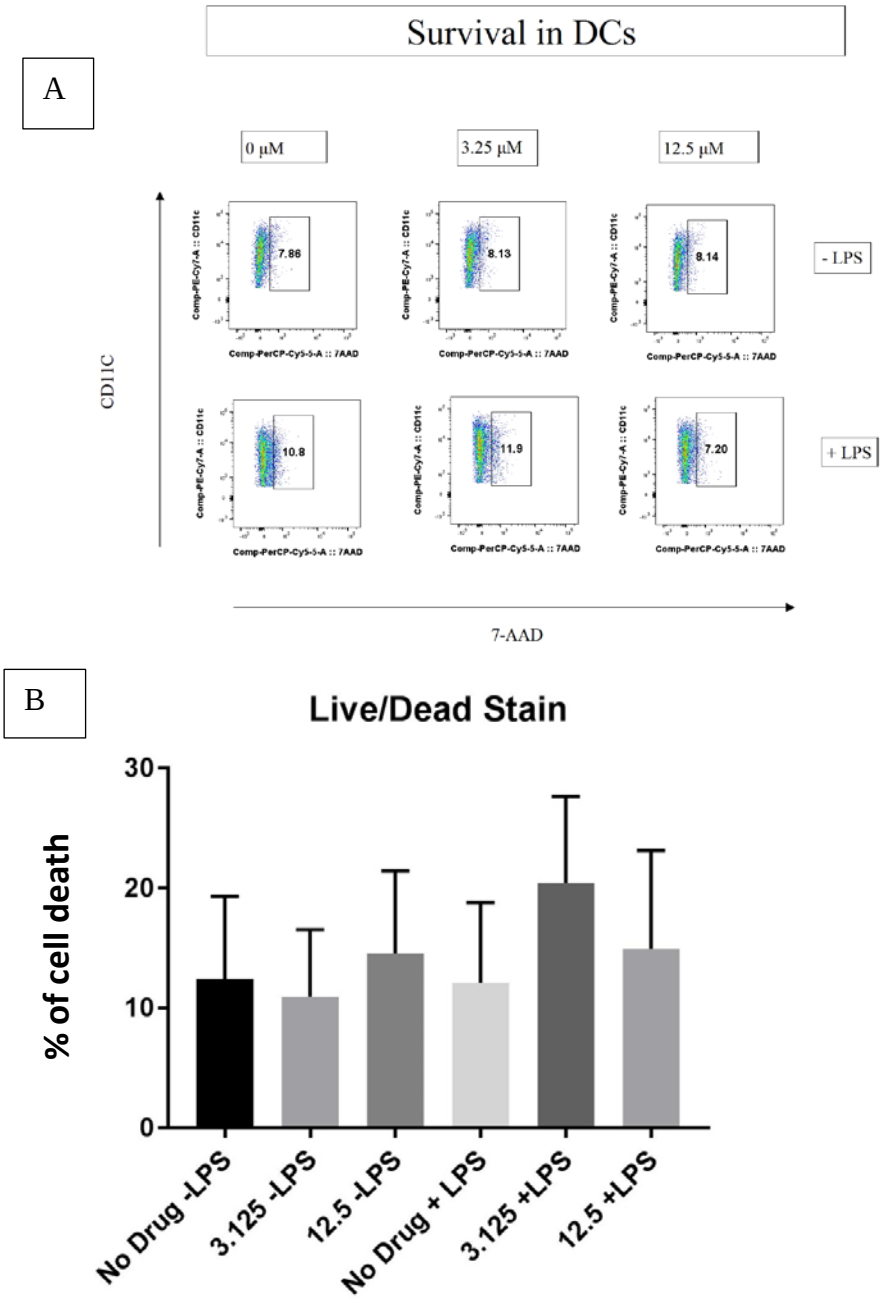


Figure 2: HIF-1 inhibitor does not induce DC death in unstimulated or stimulated conditions.
(A and B) 7AAD viability of CD11c+ -gated DCs incubated stimulated with LPS for 24hrs in the presence or absence of HIF1-alpha inhibitor does not impact survival of DCs.

We were interested in establishing the non-toxic dose of inhibitor that still inhibited HIF-1 alpha in DCs. BAY 87-2243 leads to decreased nuclear HIF-1 alpha expression by inhibiting mitochondrial production of reactive oxygen species (ROS) which leads to decreased HIF-1 alpha activity. In previous studies, ROS has shown to increase HIF-1 alpha inductions, so inhibiting mitochondrial ROS production, can lead to the destabilization of HIF-1 alpha (Jung et al., 2008). We hypothesized DC survival was dependent on inhibitor concentration. The cells were stained using 7AAD, a live dead marker and CD11C, a glycoprotein expressed by dendritic cells. Cells expressing both markers were windowed in the flow cytometry data. The initial survival experiments (data not shown) showed concentrations of 50 micro molar were more toxic to cells, establishing an upper limit of 50 micromolars. As we decreased inhibitor concentration, cellular survival rates increased and plateaued at 3.25 micromolar, establishing our lower limit. Therefore, cells were cultured with an inhibitor concentration of 12.5 micromolar to 3.125 micromolar for 24 hours. They were then treated with 7AAD and CD11C stains to identify DCs. Levels of CD11C in live cells were measured using flow cytometry. The results shown in Figure 2 are a collection of cells from 3 different mice stimulated and non-stimulated with LPS. The results in Figure 2 show there is no difference in survival of DCs between the LPS and non-LPS groups with the varying inhibitor concentrations. DCs have an internal energy stores, like glycogen, which can contribute to overall cell function. During early activation of DCs, glycogen stores are utilized initially to power internal DC functionality. We have shown that even the highest concentration (12.5

micromolar) of inhibitor are non-toxic to DCs. Therefore, we felt comfortable deducing that any functional differences observed between each group at these doses may be attributed to the downstream inhibitory effects of the inhibitor and not due to inhibitor toxicity in DCs.

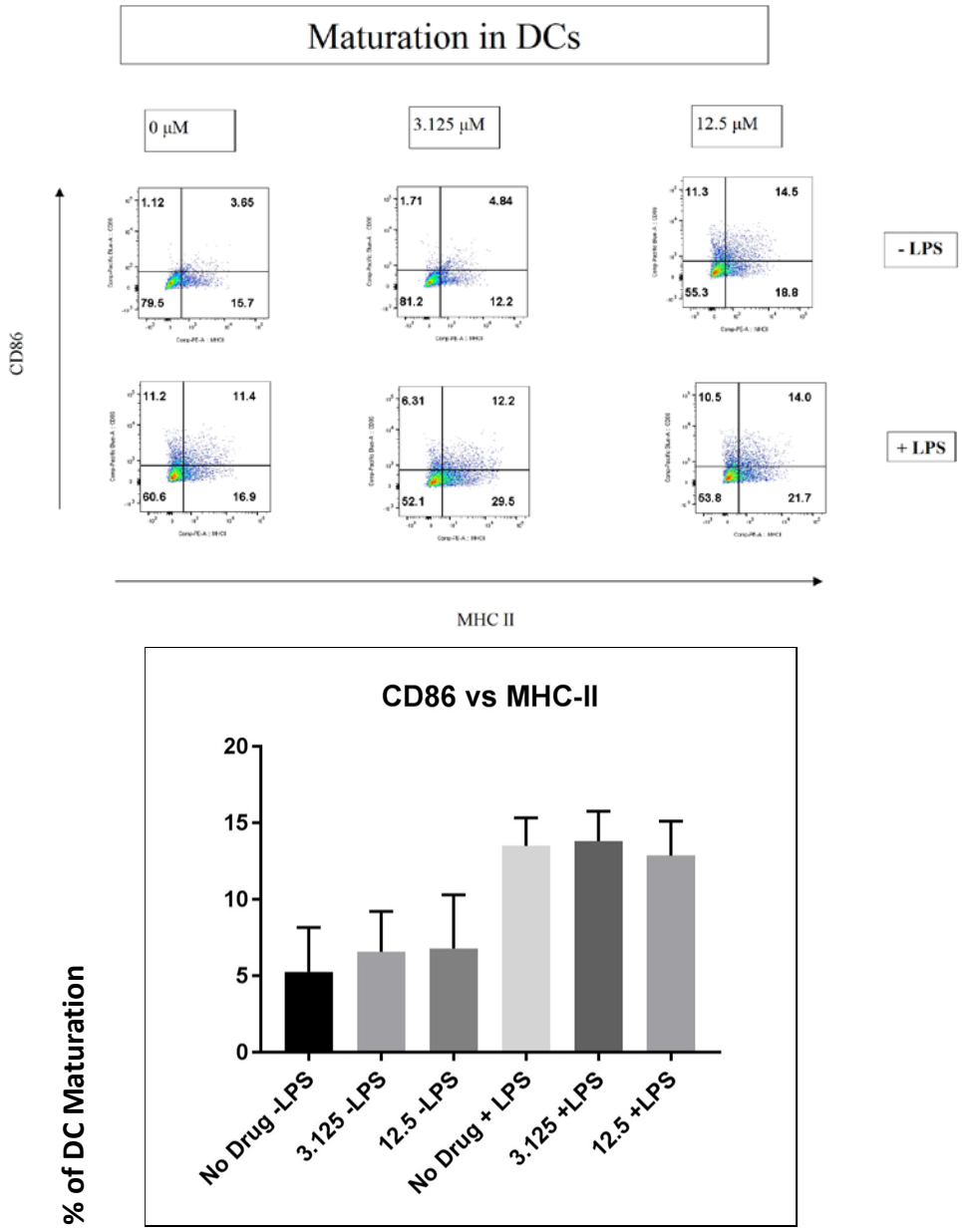


Figure 3: Flow cytometric staining of DC stimulated for 24 hours in media or LPS in the presence/absence of 12.5 and 3.125 micromolar of inhibitor
(A & B):Increased CD40 & CD86 costimulatory molecule expression in stimulated DCs after 24 hours of LPS

After establishing a non-toxic inhibitor concentration range, we investigated the role of the inhibitor on DC maturation. DCs were incubated for 24 hours with the same 12.5 6.25, and 3.25 micromolars of inhibitor used in the survival experiments. Since HIF1-alpha regulates glycolysis in most cellular system, and glycolysis has been shown to be required for DC activation, we expected to see a dose dependent decrease in cellular maturation as concentrations of inhibitor increased. DCs were analyzed using flow cytometry and were stained with antibodies to analyze surface expression of MHCII and CD86, a costimulatory molecule upregulated during DC maturation. There is a statistically significant difference between the LPS group and non-LPS group incubated with the inhibitor (Figure 3). The percentage of cells expressing MHCII and CD86 molecules remained constant across groups. We hypothesized an increased maturation in the LPS group due to LPS induction of co-stimulatory molecules during maturation. However, there was no significant difference between inhibitor concentrations of both the LPS and non-LPS groups. There was an increased amount of costimulatory molecules in the LPS group. LPS stimulation of DCs leads to increased costimulatory molecules, but under different inhibitor concentrations, the lack of difference could be due to cells being stimulated with LPS first. Although LPS was added around the same time frame, maturation cascades could have begun before the inhibitor could block HIF-1 alpha downstream in the mTOR pathway. The mTOR pathway could be inhibited using rapamycin, which could be co-incubated with our cells after DC maturation to explore survival and maturation rates. Because mTOR can be independently inhibited using rapamycin, we could study the effects of the inhibitor without mTOR activation. In a

separate experiment to explore the long term effects of the HIF-1 alpha inhibitor, cells could be incubated overnight with the inhibitor and then stimulated with LPS. Although this could provide the longer term effects on inhibitor concentration, examining the effects of the HIF-1 alpha inhibitor on maturation can be combined with prolyl hydroxylases (PHDs) inhibitors. These PHDs are responsible for the expression and degradation of HIF-1 alpha in the mTOR pathway, so by inhibiting their expression, HIF-1 alpha could be inhibited in another way that may affect maturation. These effects could also be studied by the same experimental techniques used in our experiments. LPS induced DC maturation during hypoxia is increased, which can be marked by increased HIF-1 alpha levels. Our inhibitor, should decrease HIF-1 alpha protein levels, leading to decreased glycolytic rates, leading to decreased costimulatory molecule expression. In our non-LPS group, the DCs had not been stimulated with LPS, which would not lead to downstream activation of glycolysis, thus increasing the amount of costimulatory molecules. If HIF-1 alpha is being inhibited downstream, without the activation of DCs, our flow cytometry experiments would not be able to measure downstream readouts.

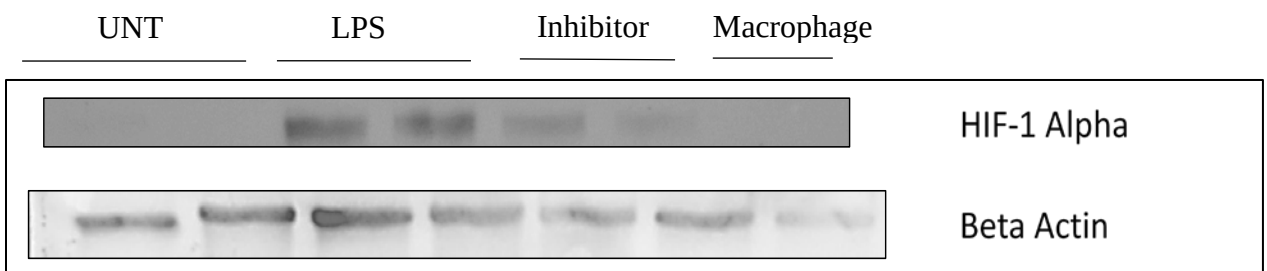


Figure 4: HIF-1 Alpha Protein Expression after 48 hours in the presence or absence of various concentrations of inhibitor (2.5 and 0.5 micromolar) N= 3

Our next step was to ensure the inhibitor was inhibiting HIF-1 alpha protein levels. BAY 87-2243 inhibits the expression of HIF-1 alpha target gene CA9 leading to an inhibition of protein expression. To assess the expression of HIF-1 alpha, we analyzed protein levels by western blot. We hypothesized decreased HIF-1 alpha protein expression as inhibitor concentration increased. Cells were incubated for 24 hours with varying inhibitor concentrations and their cell lysates were collected the day of gel analysis in the presence or absence of LPS stimulation. A beta actin loading control was used in conjunction with our experimental samples. The results in Figure 4 show knockdown of HIF 1 protein levels at the 2.5 micromolar and 0.5 micromolar. The levels of beta actin were not quantified in our experiment. It can be observed that increased density of beta actin show increased protein expression. In our LPS group, we observed increased protein expression compared to our untreated group due to activation. Quantification levels and ratio levels of beta actin could prove knockdown of HIF-1 alpha since we would know the ratio of decrease of protein relative to the sample loaded amongst all groups. We found the minimal effective dose of inhibitor was 0.5 micromolar, so we did not need to utilize a maximal dose of 12 micromolar used in our flow cytometry experiments. There was little to no expression of HIF 1 alpha in our untreated groups since HIF-1 alpha is produced after the induction of LPS through the mTOR pathway. Reduction of HIF-1 alpha increased as the concentration of inhibitor increased creating a dose dependent response in DCs. These data established that our inhibitor is effective at reducing LPS-induced HIF-1 alpha expression.

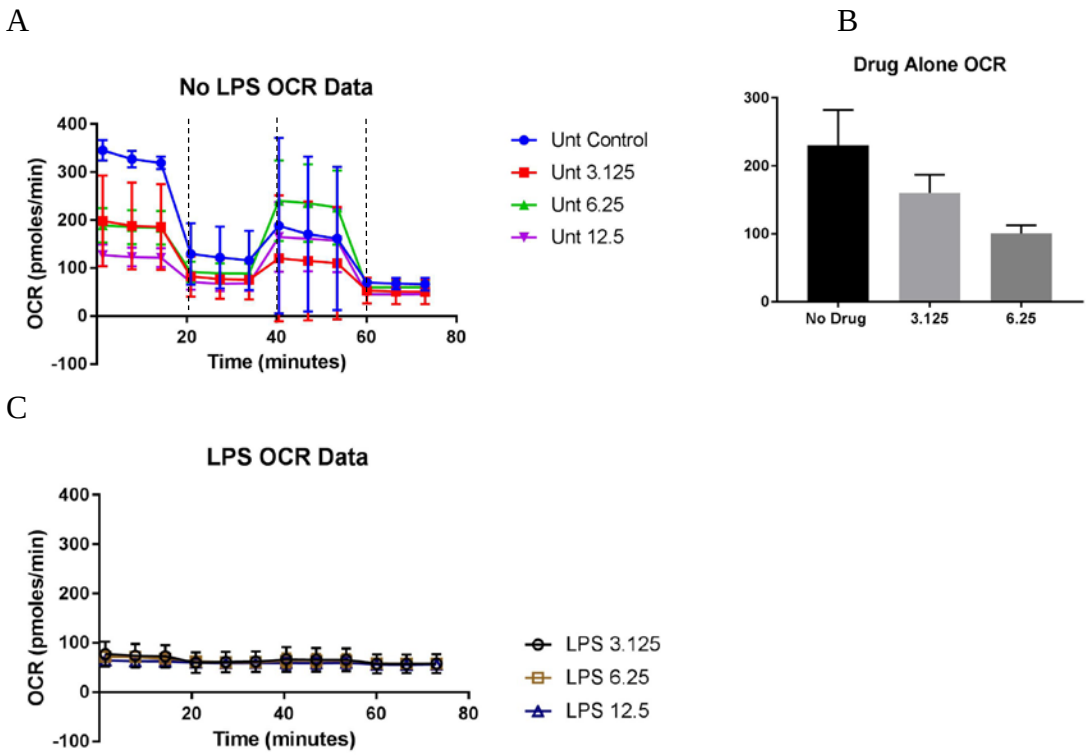
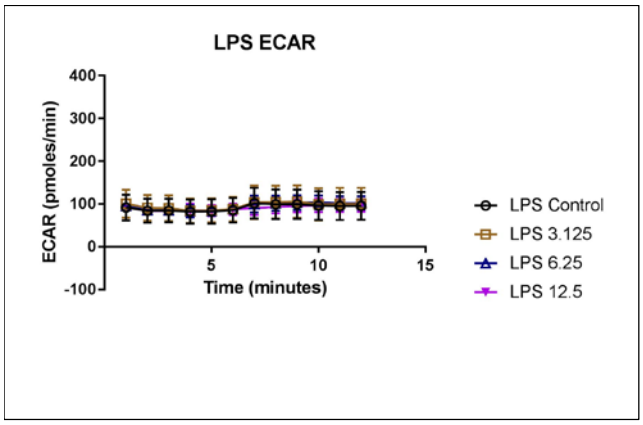


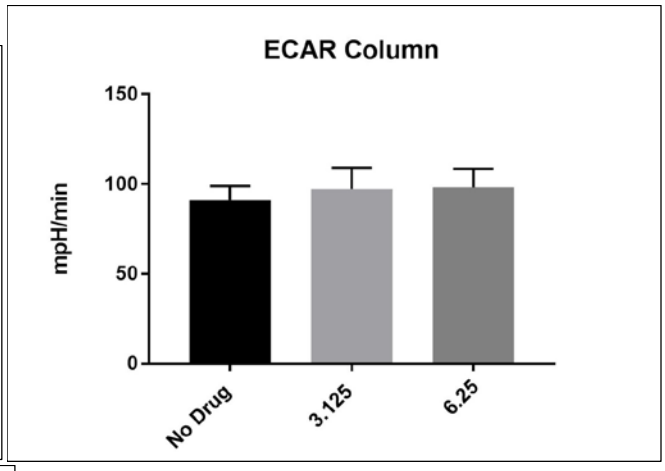
Figure 5: HIF1-alpha inhibitor impacts mitochondrial respiration in unstimulated DCs, but fails to rescue activation-associated suppression of mitochondrial function. Cells were pretreated for 24 hours with LPS and drug concentrations. Mitochondrial stress test used 3 drugs to analyze mitochondrial function in 20 minute intervals.

A) Real-time changes in OCR in response to various concentrations of HIF1-alpha inhibitor alone.

B) Quantified results from Figure 4A, data represents variability from 3 biological replicates (insert correct number here).

C) Real-time changes in OCR in response to various concentrations of HIF1-alpha inhibitor in the presence of LPS.





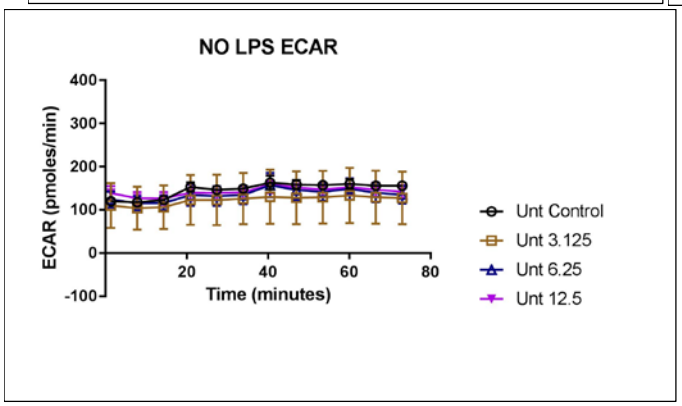


Figure 6: Real time glycolytic rates in activated DCs remains constant in stimulated and unstimulated DCs despite decreased oxygen consumption rate. Cells were pretreated with LPS and drug concentrations for 24 hours prior to mitochondrial stress test.

To study the effects of our inhibitor on glucose metabolism in DCs, we studied the effects of real time oxygen consumption rates and acidification rates in the cells. Using a mitochondrial stress test analyzed by an extracellular flux analyzer, we hypothesized that there would be a decrease in oxygen consumption rate (OCR) when DCs are stimulated by LPS (Amiel et al., 2014). This stimulation facilitates a switch from aerobic respiration to anaerobic respiration in DCs, leading to an increase in glycolysis. Using our mitochondrial stress test, 3 drugs were injected at 20 minute intervals. At the first 20 minutes, oligomycin inhibited ATP synthase causing a decrease in oxygen consumption. At time 40, FCCP, an uncoupling agent that collapses the proton gradient leads to increased OCR. Lastly, at time 60, Rotenone/Antimycin A inhibits complex 1 and complex 3 of the electron transport chain shutting down mitochondrial respiration leading to decreased OCR. Figure 5 shows a statistically significant dose dependent decrease in OCR with increasing inhibitor concentrations in unstimulated cells. This is consistent with the inhibitor's reported effect on mitochondrial Complex I activity. In the LPS group, OCR was significantly reduced across the board, regardless of varying concentrations of inhibitor indicating a switch to glycolysis and inhibition of mitochondrial respiration (presumably due to nitric oxide production as published by our group previously) (Amiel et al., 2014) . We also measured the acidification rate (ECAR) in DCs. The conversion of glucose to lactic acid causes the release of protons into the extracellular membrane, which can be measured using the extracellular flux analyzer telling us the acidity levels. According to Figure 6, there is no significant dose effect of HIF1-alpha inhibition on glycolytic rate in the non-LPS group with increasing drug

concentrations. We expected that as inhibitor concentration increased, glycolytic rates would decrease. It is possible that the inhibitor is blocking HIF-1 alpha through its mechanism, but HIF-1 alpha has off target effects through other cascades that aren't inhibited by our inhibitor. It is also possible that HIF-1 alpha inhibition decreased glycolytic rates, but activation of DCs are also trying to upregulate glycolysis because of the metabolic switch. We observed the metabolic switch with decreased oxygen consumption rates in our OCR data, but expected an opposing increase in ECAR. Due to this opposing effect, we could be examining a neutralizing effect in glycolytic rates. In the LPS group, ECAR remains low and consistent. We hypothesize that an iNOS inhibitor in future studies will provide a rescue effect to glycolysis in the LPS group, as we have previously published (Amiel et al., 2014).

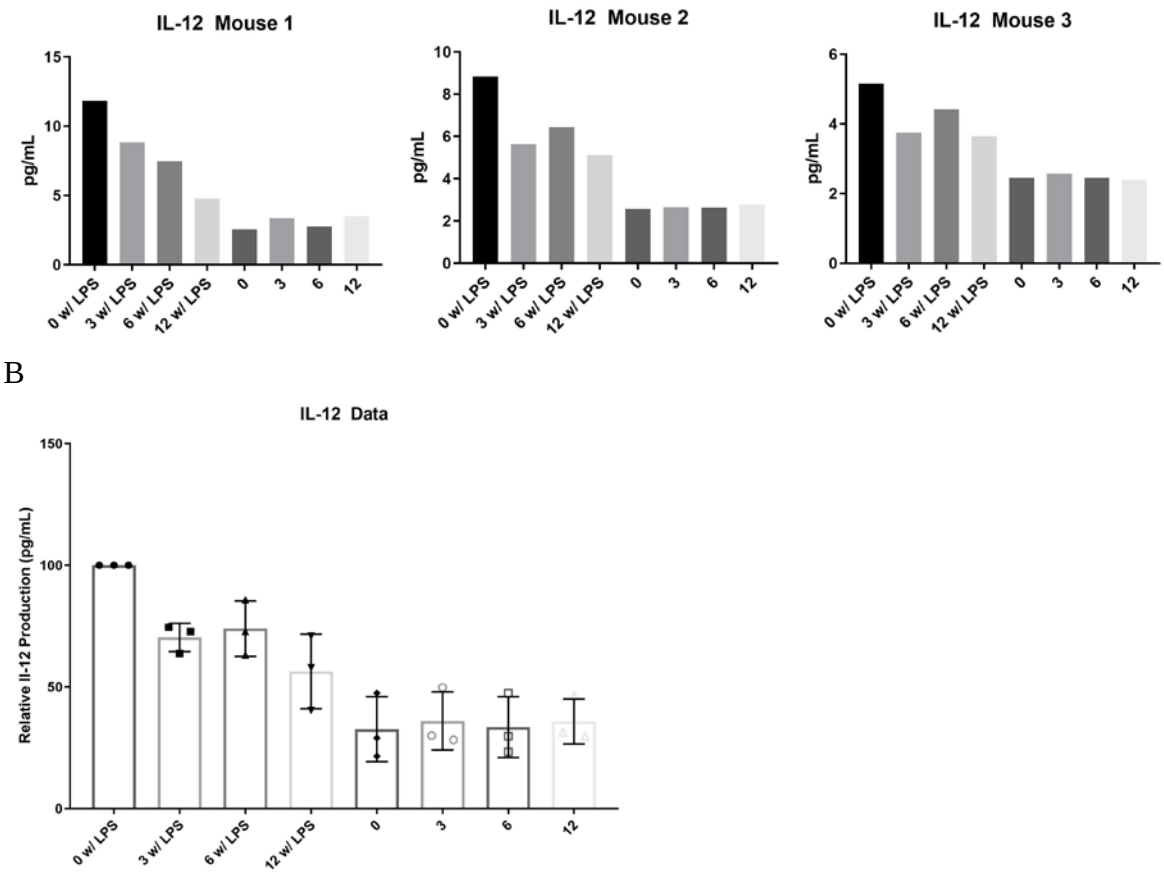


Figure 7: HIF1-alpha inhibitor decreases LPS-mediated IL-12 production.
A) ELISA IL-12 data of DCs stimulated for 24 hours with LPS in the presence or absence of HIF1-alpha inhibitor from 3 biological replicate mice
B) Normalized pooled IL-12 ELISA data from 3 biological replicates for IL-12 production.

We were interested in the role of HIF-1 alpha on the production of pro-inflammatory cytokines such as IL-12 and IL-1 Beta. Elevated levels of IL-1 beta increase transcriptional levels of HIF-1 alpha. IL-12 secretion increases after dendritic cell activation. We hypothesized a decrease in proinflammatory cytokines with increasing HIF-1 alpha inhibitor concentrations. These pro-inflammatory cytokines induce NFKB activation which is activated by low oxygen concentrations showing the importance of

these cytokines in the inflammatory response. Compromised expression of IL-1 beta and IL-12 can indicate functional abnormalities in HIF-1 protein expression. Using ELISA techniques, we cultured DCs using various HIF1-alpha inhibitor concentrations and LPS. Figure 7 shows an elevation of IL-12 in the LPS group and a dose dependent decrease in IL-12 in the LPS group. We can attribute elevated cytokine levels to LPS stimulation. When DCs are stimulated by LPS, we get increased HIF-1 alpha levels which through nF-kappa B can increase proinflammatory cytokines. As our inhibitor concentration increases, HIF-1 alpha is blocked and we see downstream cytokines were decreased due to HIF-1 inhibition. Figure 7 also shows three different animals all with the same trend of lower cytokine output as inhibitor concentration increases. We attribute these results to HIF-1 alpha inhibition decreasing cytokine output.

In conclusion, HIF-1 alpha is an important regulator in glycolytic reprogramming of DCs. Activated DCs go through internal cascade changes that lead to increased communicatory molecules for communication with T-cells. These increased metabolic changes can be dependent on the stability of HIF-1 alpha. BAY 87-2243 affected protein expression of HIF-1 alpha leading to changes in oxygen consumption and cytokine production. Our results may contribute to furthering the understanding of DC physiology and the mechanisms behind metabolic changes to survival, maturation, and implications in immune responses.

In DCs, we observed changes in oxygen consumption that are important to the metabolic demands of activated cells. Pharmacological inhibition of HIF-1 alpha may decrease metabolic stress in DCs. Although glycolytic energy production is less

productive than oxidative phosphorylation, DCs are protected from total oxygen depletion and cell death by upregulation of glycolysis after their activation. We were able to confirm BAY 87-2243 impacts HIF-1 alpha protein expression levels. With this, we can attribute molecular effects to the inhibitor impacting metabolic processes. Activated dendritic cells in the presence of BAY 87-2243 leads to significantly decreased mitochondrial respiration. The effects of BAY 87-2243 shows that metabolism in DCs require oxygen restoration to bring oxygen levels to normal. Inhibition of HIF-1 alpha was confirmed through western blot, but inhibition did not induce significant changes in survival and maturation. It is possible that inhibition of HIF-1 alpha by BAY 87-2243 does have inhibitory off target effects that lead to global HIF-1 alpha inhibition which would require other inhibition methods. Overall, HIF-1 alpha expression is negatively affected by BAY 87-2243 and destabilizes metabolic processes that could impact T- cell communication and energy consumption. HIF-1 alpha inhibition could be a strategy that increases the lifespan and decreases metabolic stress in DCs.

CHAPTER 4: FUTURE DIRECTIONS

Overall, our HIF 1 alpha inhibitor impacted maturation at the non-toxic range we used and also worked to knockdown HIF-1 alpha expression levels which were confirmed by western blot data. Using our extracellular flux analyzer, glycolysis rates decreased with increasing concentrations of our inhibitor but glycolytic rates remained low in stimulated DCs regardless of inhibitor concentration. The findings from our research shows the complex interactions between HIF-1 alpha and dendritic cell biology. The interplay between stimulated DCs and HIF-1 alpha through the mTOR pathway has many proponents that contribute to its activation and inactivation. HIF 1 alpha serves as the bridge between glycolysis and oxidative phosphorylation by mediating glucose metabolism through the above processes. The way glucose is metabolized in stimulated DCs determines its effectiveness in coordinating successful immune responses. The upregulation or destabilization of HIF-1 alpha could negatively impact glucose metabolism, thus impacting glycolytic rates, which could decrease T-cell activation.

mTOR regulation may not be the only pathway responsible for glucose metabolism. Induction of GLUT1 transporters is upregulated by the expression of HIF-1 alpha. It is possible that HIF-1 inhibition may affect glucose levels in dendritic cells. Decreased induction of GLUT1 transporters may affect glycolytic rates and metabolic processes like survival in DCs. Future studies could investigate the role of glucose transporters in dendritic cells and the role they play after activation.

Future studies of DC glucose metabolism could involve iNOS inhibitor studies. iNOS is responsible for NO production, which inhibits oxidative phosphorylation, the

primary energy production source in unstimulated DCs. iNOS inhibitors could potentially increase glycolytic rates (ECAR) after cells have been incubated with a HIF-1 alpha inhibitor post stimulation. The use of other inhibitors could support our initial findings, although other HIF-1 alpha inhibitors could inhibit transcripts differently, which could also provide new findings about the way HIF-1 alpha modulates glycolytic rates. HIF-1 knockout models would also help us confirm some of the results we found by providing another route of protein inhibition. Through our experimentation, glycolytic reprogramming was only regarded at 24 hour incubation times. Understanding which fuel source DCs use in early DC activation can allow us to know whether HIF-1 alpha is responsible for the initial anabolic processes that occur during activation and how that process could be optimized. Finally, through this project, maturation was the only DC function that was studied with the inhibitor. Future studies to identify off target effects of our inhibitor can investigate induction of other downstream glycolytic enzymes of HIF. Researching the effects of the inhibitor on T-cell activation, phagocytosis, antigen presenting, and antigen processing are important effectors of DCs that are not fully researched under HIF-1 alpha inhibition. Further studies can help us understand the multifaceted role of HIF-1 alpha on DC physiology.

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