Dendritic Cell Response during Chlamydia Infection: A Role for Alpha Enolase

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ABSTRACT

BIOLOGICAL SCIENCES

RYANS, KHAMIA 
B.S. TRINITY WASHINGTON UNIVERSITY, 2011

DENDRITIC CELL RESPONSE DURING CHLAMYDIA INFECTION: A ROLE FOR ALPHA ENOLASE

Committee Chair: Nathan Bowen, Ph.D.

Dissertation dated July 2016

Interleukin-10 (IL-10) deficient dendritic cells (DCs) are potent antigen presenting cells during Chlamydia infection. To further understand the mechanism underlying this protective property in IL-10 DCs, we combined two proteomic techniques: 2D DIGE and liquid chromatography mass spectrometry. We then performed western blotting on proteins from Chlamydia infected wild type (WT) and IL-10 knock out (KO) DCs. The results showed that alpha enolase (ENO1), a metabolic enzyme involved in the ninth step of glycolysis, was significantly upregulated in Chlamydia infected IL-10KO DCs compared to WT DCs. We further studied the role of ENO1 by silencing ENO1 gene using lentiviral siRNA technology. Flow cytometry, confocal microscopy, cytokine analysis, infectivity analysis and T-cell proliferation analysis were
also used to determine DC function. We then analyzed the effect of the ENO1 knockdown on DC metabolism during Chlamydia stimulation.

The results showed that DC maturation and activation were decreased in ENO1 knockdown DCs that were stimulated with Chlamydia compared to the Chlamydia stimulated WT DCs. In addition, pyruvate concentration decreased significantly in ENO1 knockdown DCs stimulated with Chlamydia. The mitochondrial structure of Chlamydia stimulated ENO1 knockdown DCs appeared damaged compared to the Chlamydia stimulated WT DCs. The function of ENO1 in the immune response to Chlamydia trachomatis infection is unknown. However, the results from this study indicates that ENO1 may contribute to DC metabolism and mitochondrial homeostasis which may play a role in the maturation and antigen presenting function of DCs.
DENDRITIC CELL RESPONSE DURING CHLAMYDIA INFECTION: A ROLE FOR ALPHA ENOLASE

A DISSERTATION
SUBMITTED TO THE FACULTY OF CLARK ATLANTA UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

BY
KHAMIA RYANS

DEPARTMENT OF BIOLOGICAL SCIENCE

ATLANTA, GEORGIA

JULY 2016
I would like to thank God for giving me the strength to continue striving and working hard to achieve my goals. I would like to give special thanks to my advisors, Dr. Nathan Bowen and Dr. Yusuf Omosun, for their guidance and support during my matriculation at Clark Atlanta University. Also special thanks to my lab partner: Danielle McKeithen and my dissertation advisory committee members: Dr. Qing He, Dr. David Logan, and Dr. Marjorie Campbell. I’d also like to thank the faculty, staff, and graduate students at Clark Atlanta University, Morehouse School of Medicine, and Georgia Institute of Technology for providing me the research or administrative assistance that I needed to finish my matriculation at Clark Atlanta University. Lastly, I’d like to thank my parents who encouraged me with their love and support to work hard and be the best that I can be.
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<th>Description</th>
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<tbody>
<tr>
<td>2-DIGE</td>
<td>2-D Difference Gel Electrophoresis</td>
</tr>
<tr>
<td>AKT</td>
<td>Protein Kinase B</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>ANT</td>
<td>Adenine Nucleotide Translocator</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen Presenting Cells</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Tri Phosphate</td>
</tr>
<tr>
<td>BAX</td>
<td>BCL-2 Associated X Protein</td>
</tr>
<tr>
<td>BL</td>
<td>Burkitt Lymphoma</td>
</tr>
<tr>
<td>BMDC</td>
<td>Bone Marrow Dendritic Cell</td>
</tr>
<tr>
<td>CCR7</td>
<td>C-C Motif Chemokine Receptor 7</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
</tr>
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<td>CDC</td>
<td>Center for Disease Control</td>
</tr>
<tr>
<td>cDC</td>
<td>Conventional DCs</td>
</tr>
<tr>
<td>CDP</td>
<td>Committed DC Progenitors</td>
</tr>
<tr>
<td>CLAR</td>
<td>Center for Laboratory Animal Resources</td>
</tr>
<tr>
<td>CLR</td>
<td>C-type Lectin Receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>CO2</td>
<td>Carbon Dioxide</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic Cell</td>
</tr>
<tr>
<td>ECSIT</td>
<td>Evolutionary Conserved Signaling Intermediate in Toll Pathway</td>
</tr>
<tr>
<td>ENO1</td>
<td>Alpha Enolase</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron Transport Chain</td>
</tr>
<tr>
<td>FADH</td>
<td>Hydroflavin Adenine Dinucleotide</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-Macrophage Colony Stimulating Factor</td>
</tr>
<tr>
<td>GPCR</td>
<td>G Protein-Coupled Receptor</td>
</tr>
<tr>
<td>HIF1α</td>
<td>Hypoxia-Inducible factor 1α</td>
</tr>
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<td>Hexokinase</td>
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<td>Interferon</td>
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<td>IKKe</td>
<td>Nuclear Factor-κB Kinase Subunit-ε</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>iNOS</td>
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<td>KD</td>
<td>Knockdown</td>
</tr>
<tr>
<td>KO</td>
<td>Knock Out</td>
</tr>
<tr>
<td>LDHA</td>
<td>Lactate Dehydrogenase A</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LRR</td>
<td>Leucine Rich Repeat</td>
</tr>
<tr>
<td>ME</td>
<td>Mercaptoethanol</td>
</tr>
<tr>
<td>MEM</td>
<td>Modified Eagle's Medium</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>MoPn</td>
<td><em>Chlamydia muridarum</em></td>
</tr>
<tr>
<td>MPC1</td>
<td>Mitochondrial Pyruvate Carrier 1</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian Target of Rapamycin</td>
</tr>
<tr>
<td>NIIC</td>
<td>Notch1 Receptor Intracellular Domain</td>
</tr>
<tr>
<td>NAD+</td>
<td>Nicotinamide Adenine Dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>Hydronicotinamide Adenine Dinucleotide</td>
</tr>
<tr>
<td>NLRP3</td>
<td>Pyrin Domain Containing 3</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>NOD</td>
<td>Nucleotide-Binding Oligomerization Domain Receptors</td>
</tr>
<tr>
<td>OXPHOS</td>
<td>Oxidative Phosphorylation</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral Blood Mononuclear Cell</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-Buffered Saline</td>
</tr>
<tr>
<td>pDC</td>
<td>Plasmacytoid DC</td>
</tr>
<tr>
<td>PD-e1alpha</td>
<td>Pyruvate Dehydrogenase e1alpha</td>
</tr>
<tr>
<td>PGC1α</td>
<td>PPARγ co-activator 1α</td>
</tr>
<tr>
<td>PID</td>
<td>Pelvic Inflammatory Disease</td>
</tr>
<tr>
<td>PKM</td>
<td>Pyruvate Kinase Muscle</td>
</tr>
<tr>
<td>PPARγ</td>
<td>Peroxisome Proliferator-Activated Receptor-γ</td>
</tr>
<tr>
<td>PPR</td>
<td>Pater Recognition Receptors</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene Difluoride</td>
</tr>
<tr>
<td>RBC</td>
<td>Red Blood Cell</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation Assay Buffer</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small Interfering Ribonucleic Acid</td>
</tr>
<tr>
<td>TBK1</td>
<td>TANK-binding Kinase 1</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic Acid Cycle</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy</td>
</tr>
<tr>
<td>Th</td>
<td>T-helper Cell</td>
</tr>
<tr>
<td>TLRs</td>
<td>Toll-Like Receptors</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
</tr>
<tr>
<td>TOM</td>
<td>Translocase Outer Membrane</td>
</tr>
<tr>
<td>TRAF6</td>
<td>TNF Receptor Associated Factor 6</td>
</tr>
<tr>
<td>αKG</td>
<td>α-Ketoglutarate</td>
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</tbody>
</table>
CHAPTER I
INTRODUCTION

*Chlamydia trachomatis* is the most frequently reported bacterial sexually transmitted infection in the United States (69). In 2014, 1.44 million new cases of *Chlamydia* were reported to Center for Disease Control (CDC) from 50 states and the District of Columbia (69). *Chlamydia* can cause severe health problems in women, such as pelvic inflammatory disease (PID), ectopic pregnancy, chronic pelvic pain, and infertility (1,2). Up to 40 percent of females with *Chlamydia* infections develop PID, and 20 percent of those may become infertile (1). It is also important to note that *Chlamydia* infection increases the chance of contracting HIV (1, 2). *Chlamydia* infections also have a major economic impact in the US, with total annual costs of $16 billion in 2010 US dollars (69). Prevention strategies proposed against *Chlamydia* infection includes increased screening and massive treatment (3). There are racial and ethnic disparities in Chlamydial infection, with the disease being five times more prevalent among non-Hispanic blacks compared to non-Hispanic whites (3). It is also estimated that 1 in 15 sexually active females aged 14-19 years has contracted *Chlamydia* at one time or the other (4). However, recent epidemiologic data suggests that rapid treatment of infection prevents the development of some protective immunity against re-infections and that this contributes to tubal factor infertility (5, 6). These findings highlight the need for an
effective vaccine against genital *Chlamydia* infection and indicate that the vaccine strategy is the most reliable and cost effective preventive method, having the greatest potential impact in controlling *Chlamydia* infections and the associated complications in the human population (7).

A major challenge in *Chlamydia* vaccine development is that the experimental vaccines only have a partial and short term protective immunity against *Chlamydia* (6). Our lab has developed the IL-10 (interleukin 10) deficient dendritic cell system with distinct advantages for studies of therapeutic strategies and vaccine development (8). IL-10 deficient bone marrow dendritic cells (BMDCs) stimulated with *Chlamydia* can confer a high level and long-term immunity against reinfection in female mice; and protect against the development of *Chlamydia*-induced tubal pathologies, specifically inflammation and infertility in vivo. IL-10 is a cytokine involved in immunoregulation and inflammation. IL-10 down regulates Th1 (T-helper cell 1) cytokine expression, MHC (major histocompatibility complex) class II antigens, and costimulatory molecules on dendritic cells (8, 9). It also enhances B-cell survival, proliferation, and antibody production (8). This remarkable ability of IL-10 deficient BMDCs indicates that they possess superior immunoregulatory properties required for inducing *Chlamydia* immunity (8, 9).

The IL-10 deficient BMDC system therefore provides a suitable immunotherapeutic strategy to better define the immunologic and biochemical determinants and conditions for inducing an adequate and long-term immunity to protect against *Chlamydia*-induced tubal pathologies. Such immunomodulators could be co-
administered, elicited in vivo, or administered separately to promote the induction of the appropriate and adequate immune response without any associated pathology (4). These immunomodulators could also be used in drug design protocols to shorten the duration of infection with the same beneficial effects. Therefore, studying the various mechanisms involved in host immunomodulation is needed in designing treatment strategies and an efficacious Chlamydia vaccine to prevent reproductive tract complications.

Results from 2-DIGE Proteomics assay showed that alpha enolase (ENO1) was significantly up-regulated in Chlamydia pulsed IL-10 KO bone marrow dendritic cells after 2hr Chlamydia stimulation but not expressed in Chlamydia-pulsed WT BMDCs after 2hr incubation. Therefore, we hypothesized that the high level and early expression of alpha enolase (ENO1) may be involved in antigen uptake, internalization, and DC (dendritic cell) maturation. The central goal of this dissertation is to use our reliable experimental systems and state-of-the-art technological approaches to define the role of ENO1 in regulating the induction of protective immunity against Chlamydia, and determine its effect on DC mechanism of immunomodulation. Therefore, based on this hypothesis, we obtain the following aims:

Aim 1: To confirm the dynamic distribution and expression of ENO1 in dendritic cells.
Aim 2: To investigate the probable role of ENO1 in dendritic cell metabolism post-Chlamydia infection.
Aim 3: To investigate the role of ENO1 in DC maturation and antigen presentation post Chlamydia infection.
CHAPTER II
LITERATURE REVIEW

Dendritic cells (DCs) originate from hematopoietic bone marrow progenitor cells (10). These progenitor cells undergo differentiation into immature dendritic cells (10). Immature dendritic cells continuously search their immediate environment for pathogens like bacteria and viruses (10, 11). Pattern Recognition Receptors (PPRs) like toll-like receptors (TLRs) contribute to detecting such pathogens (11, 12). TLRs distinguish detailed chemical patterns found on subsections of pathogens (10). Once an immature dendritic cell has come into contact with a presentable antigen, the DC becomes activated into a mature phase and commences to migrate through the lymphatic system and into the lymph node (12, 13). Immature dendritic cells phagocytose pathogens and degrade their proteins into fragments and, upon maturation, the DC then presents a portion of the fragment onto its cell surface using the major histocompatibility complex receptors (10, 11). Simultaneously, they upregulate cell surface co-receptors such as cluster of differentiation (CD) CD86, CD80, and CD40 to significantly enhance DCs ability to activate T-cells. C-C motif chemokine receptor 7 (CCR7), a chemotactic receptor, is also upregulated and induces the dendritic cell to travel through the lymphatic system to a lymph node (13). Once in the lymph node DCs function as antigen presenting cells and are able to activate T-cells and B-cells by presenting them with antigens derived from the
pathogen combined with costimulatory signals which are not antigen specific (13).

Dendritic cells can also induce T-cell tolerance (14). Certain C-type lectin receptors (CLRs) on dendritic cell surface, help direct dendritic cells function by instructing it to induce immune tolerance instead of lymphocyte activation (14).

Every helper T-cell is specific to one particular antigen (14). Dendritic cells are able to activate a resting helper T-cell when the matching antigen is presented (15). Dendritic cells can activate both memory and naive T cells, and are the most effective at antigen presentation (15). Mature dendritic cells are able to activate CD8+ T antigen-specific naive cells, the formation of CD8+ memory T cells necessitates the interaction of dendritic cells with CD4+ helper T cells (15). In addition, CD4+ T cells activates the matured dendritic cells and authorizes them to proficiently induce CD8+ memory T cells (15). Monocyte-derived dendritic cells can be generated in vitro from peripheral blood mononuclear cells (PBMCs) or stem cells from the bone marrow (16). Treatment of these cells with interleukin 4 (IL-4) and granulocyte-macrophage colony stimulating factor (GM-CSF) leads to differentiation to immature dendritic cells in about a week (16).

I.I Dendritic Cells in Research

The particular origin and development of the diverse subsets of dendritic cells and their effects on immunity is only slightly understood since dendritic cells are so rare and difficult to isolate (17). Dendritic cells are frequently in communication with neighboring cells within the body (17), either through direct cell–cell contact utilizing the interaction
of cell-surface receptors or via cytokines (17). Stimulating dendritic cells in vivo with an antigen causes it to rapidly begin producing IL-12 (18). IL-12 is a signal that helps induce the naive CD4 T cells to differentiate into Th1 phenotype (18). The significance of antigen presentation function is preparing and activating the immune system to attack antigens that were presented on the surface of the dendritic cell (18). Various cytokines can be produced from dendritic cells that help for antigen presentation and recruitment of leukocytes (18).

II.I  Dendritic Cell Metabolism

Committed DC progenitors (CDPs) produce conventional DCs (cDCs) and plasmacytoid DCs (pDCs) (19). At present, very little is known about the metabolism of committed DC progenitors. However, the differentiation of human monocytes into DCs in response to granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) is accompanied by increased expression of peroxisome proliferator-activated receptor-γ (PPARγ) (20, 21). This is a key transcription factor regulating lipid metabolism, and PPARγ co-activator 1α (PGC1α) regulates mitochondrial biogenesis (22). Published data has shown that expression of PPARγ and PGC1α are followed by increased mitochondrial biogenesis (22). Furthermore, inhibiting mitochondrial respiration in monocytes by the electron transport chain (ETC) inhibitor rotenone, is able to block DC differentiation (22, 23). These studies prove that there is a close relationship between increased dendritic differentiation and citrate synthase activity (24). Citrate is the precursor to isocitrate and subsequently α-ketoglutarate (αKG) in the citric acid cycle
or tricarboxylic acid cycle (TCA cycle) (24). Citrate is also a precursor of fatty acid synthesis (24). In fatty acid synthesis, citrate is exported from the mitochondria and converted into cytosolic Acetyl-CoA; which is an essential transitional component from glycolysis; and goes in to the TCA cycle and fatty acid synthesis (24).

III.I Catabolic Process of Glucose

Glucose is imported into the glycolytic pathway allowing it to be converted into pyruvate in the cytosol (25). Pyruvate then has two possible pathways to follow. In anaerobic conditions pyruvate is converted into lactate, which produces Nicotinamide adenine dinucleotide (NAD+) that is used for the production of Adenosine Tri Phosphate (ATP) during glycolysis (25). Alternatively, in aerobic conditions, pyruvate can enter the mitochondria using mitochondrial pyruvate carrier 1 (MPC1), where it is converted into acetyl-CoA (25). The importance of this decision point in pyruvate metabolism pathways is indicated by the fact that it is regulated at many levels. Important enzymes involved in these processes are encoded by splice variants of transcripts of the pyruvate kinase muscle (PKM) gene (which encodes pyruvate kinase), and catalyzes the production of pyruvate from its precursor phosphoenolpyruvate (26). Oxidation of pyruvate in the mitochondria is stimulated by PKM1 (26). PKM2 stimulates the expression of hypoxia-inducible factor 1α (HIF1α), which initiates the conversion of pyruvate into lactate by inducing of lactate dehydrogenase A (LDHA) expression (26, 27). In mitochondria, acetyl-CoA generated from pyruvate enters the TCA cycle leading to the production of dihydronicotinamide adenine dinucleotides (NADHs) and hydroflavin adenine.
dinucleotides (FADHs), which are utilized as substrates for the electron transport chain (ETC), and support oxidative phosphorylation (OXPHOS) and the production of ATP (25). Citrate can then be disseminated from the mitochondria to assist in the production of acetyl-CoA in the cytoplasm (28). Acetyl-CoA is needed for fatty acid synthesis, a process which is crucial for Toll-like receptor (TLR)-induced activation of DCs (28).

### IV.I Dendritic Cell Resting Phase

Cells at rest generate relatively deficient anabolic loads; the catabolism of multifaceted molecules has the ability to provide substrates for the TCA cycle within mitochondria (25). Catabolism of proteins and triacylglycerols provides amino acids and fatty acids, and generates ATP production through oxidative phosphorylation (OXPHOS) (25). Resting GM-CSF-induced bone marrow-derived DCs (BMDCs) have been shown to use fatty acid oxidation for OXPHOS (29). It is currently unclear whether resting cDCs or pDCs similarly undergo OXPHOS along with fatty acid oxidation, however resting BMDCs also ingest glucose (29).

Based on previous studies, it is highly probable that dendritic cells utilize glucose to synthesize fatty acids which are then consequently oxidized (30). Previous research has proven that this process of glucose oxidation is crucial for the development of memory CD8+ T cells (30). This process has also been shown to be beneficial to T cells in the quiescent stage, since it maintains the conservation of mitochondrial health.
concurrently with the glycolysis and fatty acid synthesis machinery which allows T cells to react more intensely in subsequent re-stimulation by an antigen (30).

V.I Process of Dendritic Cell Activation

The processing of glucose by glycolysis generates pyruvate needed to eventually produce substrates needed for the cell to undergo ATP synthesis (25). Mitochondrial pyruvate carrier 1 (MPC1) carries pyruvate into the mitochondria where it can be converted into acetyl-CoA (25). Acetyl-CoA then promotes the initiation of the TCA cycle (25). An alternative pathway is converting pyruvate to lactate under anaerobic condition. This process enables cells to harvest ATP when there is a limited supply of oxygen, this process is essential for cells to endure hypoxic environments (25).

This pathway is often used in tumor cells even when there is an abundance of oxygen in the environment. The process of pyruvate being converted into lactate in the presence of oxygen is known as Warburg effect (31). Bone marrow dendritic cells show an increase in their consumption of glucose and an enhanced production of lactate (32). This process often occurs after the cells are stimulated with TLR agonist (32). This recently discovered observation reflects two modes of activation through metabolic changes (28, 31). One form occurs early where there is an increase in flux through the glycolytic pathway in dendritic cells (31). This early phase is followed by an alteration of the cellular metabolism, causing BMDCs to commit to the Warburg effect (28). Developmental techniques for measuring real-time changes in extracellular acidification
and oxygen consumption made it possible for this process to be understood (33). When utilizing these new techniques to further understand DC metabolism, researchers observed that glycolysis is amplified within minutes of DC activation by various TLR agonist (28). Studies have also proven that blocking glycolysis using 2-deoxyglucose (an inhibitor of hexokinase (HK)), the first enzyme in the glycolysis pathway, inhibits DC activation (28). This proves that glucose is an essential substrate needed to enhance the response of DCs to TLR agonists (25, 28).

**VI.I Role of Glycolysis in Dendritic Cell Function**

The prompt upsurge in glycolysis which transpires in activated BMDCs is associated with an enhanced lactate production that does not reflect the Warburg effect since OXPHOS is an adequate source for generating ATP required for the cells (28). The transport of pyruvate by means of MPC1 into the mitochondria is vital for early activation of DCs (28). Therefore, one can imply that the main purpose of increased glycolysis appears to be to amplify pyruvate production in order to initiate the TCA cycle. This enables a transient upsurge in additional respiratory capacity (34, 35), giving rise to cells that are more metabolically proficient than resting BMDCs.

It is probable that the increased respiratory capacity may point toward the fact that the cells have the capacity to use TCA cycle intermediates for other pathways within the cell (33). The main importance of the entrance of carbons derived from glucose into the TCA cycle appears to be high fatty acid production (25, 28). Pharmacologically
inhibiting glycolysis post-transcriptionally effects the early stages of BMDCs activation, demonstrating that this pathway is necessary for the synthesis of proteins made in response to DC activation and not essential for gene expression downstream of TLRs (28).

VII.I Late Dendritic Cell Activation Cause Metabolic Change

TLR agonists that activate BMDCs for over 12 hours generally use the Warburg effect for the cells bioenergetic components. These cells normally contain small amounts of quantifiable mitochondrial oxygen consumption (25, 36). It is now understood that BMDCs’ obligation to the Warburg effect following activation is a response to the inhibition of the ETC by nitric oxide (NO) and the cellular expression of inducible nitric oxide synthase (iNOS) (36). Removal of functional OXPHOS by inhibition of the ETC by NO, causes BMDCs to be activated by TLR agonists (36). These TLR agonists are dependent on glycolysis for the production of ATP (36). Understanding how NO production during inflammation has the capacity to inhibit OXPHOS is important (36). It demonstrates that NO has the ability to impact pathogenic microorganism metabolism when they are reliant upon OXPHOS (36).

When cDCs are activated by TLR agonists in vivo they display reduced mitochondrial activity and a boost in glycolysis for a long period needed for cell survival (37). Nevertheless, this variation is described to be independent of iNOS and compelled
by autocrine type I interferon (IFN) signaling via hypoxia-inducible factor 1α (HIF1α) (37).

**VIII.I Regulation of Dendritic Cell Activation via glycolysis pathway**

Dendritic cells often display increased expression of maturation markers, such as CD40, CD80 and CD86 after DC stimulation (38). Additionally, in mouse BMDCs and in human monocyte-derived DCs, rapamycin selectively inhibits certain aspects of TLR-driven DC activation and the expression of IL-6 and IL-10 (39, 40).

Studies have also acknowledged a positive role for the mammalian target of rapamycin (mTOR) target hypoxia-inducible factor 1-alpha (HIF1α) in the TLR-induced activation of BMDCs (41, 42, 43). HIF1 contains α- and β-subunits, and is typically considered to be activated through hypoxia. HIF1 has also been associated with stimulating glycolysis in several systems by promoting the expression of numerous enzymes associated with the glycolytic pathway (31). Recent studies propose that the initial TLR-driven stimulation of glycolysis which transpires throughout DC activation does not need mTOR or HIF1α-mediated signaling (28, 44), and instead depends on protein kinase B (AKT) (35, 45). TANK-binding kinase 1 (TBK1) and inhibitor of nuclear factor-κB kinase subunit-ε (IKKε) which initiates the activation of AKT downstream of TLRs (46). AKT is essential because it phosphorylates and activates (Hexokinase 2) HK2 to assist with the structure of the mitochondrial surface (45, 47). When TBK1, AKT, or Inhibitor-κB kinase ε (IKKε) are inhibited or when HK2 is
blocked from associating with the mitochondria, it depletes the BMDCs ability to activate and react to TLR agonist (28). This signaling axis is significant for the longstanding obligation of DCs to glycolysis resulting from activation by TLR agonists (29, 33, 48).

IX.I Metabolic Signaling in Dendritic Cells

Recent studies have shown that cellular metabolism is not the only foundation of generating ATP to promote DC differentiation and function, but also that proteins produced by metabolic pathways can act as signaling molecules to activate cellular responses. One illustration of this is provided by a significant recent discovery that in M1 macrophages, HIF1α is activated by an intermediate within the TCA cycle called succinate which is produced from glutamate in cells dedicated to Warburg effect and contributes to IL-1β production (49). Extracellular succinate is also capable of inducing DC activation by binding to the G protein-coupled receptor (GPCR) succinate receptor 1 (50). Cellular concentrations of reactive oxygen species (ROS), a byproduct of ETC, are increased when the ETC is inhibited, which activates the nucleotide-binding oligomerization domain receptors (NOD-), leucine rich repeat (LRR-) and pyrin domain-containing 3 (NLRP3) inflammasome, leading to the activation of caspase 1 and the production of IL-1β and IL-18 (51, 52). TLR1, TLR2 or TLR4 agonist have the ability to induce this pathway promoting the activation of TNF receptor associated factor 6 (TRAF6) and its relocation to the mitochondria (53). While in the mitochondria it then interacts with evolutionary conserved signaling intermediate in Toll pathway (ECSIT) leading to high production of ROS (53). The inflammasome activation is significant for
dendritic cell activation and for the ability of the cells to promote adaptive anti-tumor immunity (54).

X.I Functions of Alpha Enolase

Previous studies indicate that Burkitt lymphoma (BL), a high-grade B-cell malignancy, has a low expression of co-stimulatory molecules CD80 and CD86 on antigen-presenting cells (APCs), therefore inhibiting the activation of CD4+ T cells (55, 56, 57). However, when the enolase-like protein in BL is inhibited, there is enhanced antigen presentation in B cells, macrophages and dendritic cells (58). Proteomics data from our lab showed an increase in ENO1 in IL-10 KO dendritic cells compared to WT cells (Figure 1 A). Previous data published from our lab also proves that IL-10 KO dendritic cells elicit high yield of Th2 (8, 9). Th2 was highly expressed in Chlamydia infected mice which rapidly cleared Chlamydia (8). This leads to the importance of understanding the role that enolase may have on the immune system.

Phosphopyruvate hydratase (Enolase, Alpha Enolase 1 (ENO1)) discovered by Lohman and Mayerhof, is an enzyme highly involved in the glycolytic pathway (59). ENO1 is a multifunctional enzyme that aids in catalyzing the reversible conversion of 2-phosphoglycerate into phosphoenolpyruvate in the ninth step of the glycolysis pathway (59, 60). The ENO1 gene is one of the three enolase isoenzymes in mammals (59, 60). Each isoenzyme is a homodimer composed of 2 alpha, 2 beta and 2 gamma subunits (59, 60). ENO1 is associated with cellular growth, hypoxia tolerance and allergic responses
ENO1 has the ability to function as a receptor and activator of plasminogen on the cell surface of several leukocytes and neurons \((60, 61, 62)\). ENO1 can also activate collagenases (enzymes that break down peptide bonds in collagen) and is expressed in mitogens, (which stimulate cell transformation and induce mitosis), cytokines, phorbol ester and bacterial lipopolysaccharide (LPS) endotoxin found in membrane of bacterial cell recognized by toll like receptor 4 (TLR4) in the innate immune response \((60, 64)\). Alternative splicing of ENO1 results in a short isoform that enables it to bind to c-myc promoter and function as a tumor suppressor \((65, 66, 67)\). ENO1 also interacts with the Notch1 receptor intracellular domain (N1IC) and down-regulates c-myc transcription, which will induce the differentiation and maturation of BMDCs \((66)\).
CHAPTER III
MATERIALS AND METHODS

Culturing Bone Marrow Dendritic Cells (BMDCs):

**Materials:** Complete Media: 500ml 1640 RPMI, MEM non-essential amino acid (100X) 10mM 5ml, MEM sodium Pyruvate solution (100X) 10nM 5ml, FBS 50ml, 2 Mercaptoethanol (ME) .5ml, Gentamycin (50mg/ml) 1ml, L-glutamine (100X) 20mM 5ml, Hepes buffer (1M) 5ml, Fungizone (250 ug/ml) 2ml, GM-CSF (10ng/ml) IL-4 (5ng/ml).

100X200mm plastic dishes, 5cc syringes, 25ga needles, 18-22ga needles, forceps, scissors, foam top, ethanol, package of 50ml tubes, 1%Hepes in 1640 RPMI mixture.

**Method:** Sprayed area with 70% ethanol, euthanized mice using CO₂ and cervical dislocation. Mounted the dead mice on dissection board stomach facing upwards and started cutting downwards from the torso of the mouse. With scissors closed, poked inside (gently) until femur is touched. Opened scissors and cut upwards (not cutting the femur) to remove the muscle. Removed femurs from mice, cleaned muscle, and placed femurs to 50ml tubes containing 1% Hepes in 1640 RPMI. Continued the extraction of the DCs under the tissue culture hood in the lab. Poured out 1% Hepes in 1640 RPMI mixture with bones in empty petri dish. Cleaned bones using forceps or scissors to remove tissue and fat. Cut bones at joints, and then continued to cut both ends of bones.
Put cleaned bones in new petri dish with fresh 1% Hepes in 1640 RPMI. After all bones were cleaned they were placed in another empty petri dish and 10ml syringe with a gage of 25 was filled with 1% Hepes in 1640 RPMI and inserted into one of the femur ends. Femoral plug was removed by forcing media into the lumen of femur, until femur turned white. Cells were transferred into 50ml conical tubes. Plates were washed once to make sure that all cells were transferred. 1% Hepes in 1640 RPMI media was refluxed into tubes after centrifugation, until clumps were no longer visible using the 5ml electric pipette set at fast. Cells were washed 3 times with 1% Hepes in 1640 RPMI in 50ml tubes with 5ml of 1% Hepes in 1640 RPMI at 2500rpm at 4°C Celsius for 20min. After the first cells were transferred into a new 50ml tube. During the wash we would reflux the cells using the 1% Hepes in 1640 RPMI media and a 5ml pipette. We waited until bubbles were at the top of the pipette. Then pipetted the 1% Hepes in 1640 RPMI to the new conical tube slowly the capillary action causes the debris to remain stuck to the pipette.

During the wash we created the complete media. We added a concentration of 5ng/ml of IL-4 and 10ng/ul of GM-CSF to the complete media created. The cells were counted and then transferred to cell culture dishes containing 12ml media at 2.67 x 10^7 cells. Plates are incubated for 3 days at 37°C Celsius and 5% CO2. On the third day plates are rocked back and forth media is discarded containing non-adherent cells. Then 10ml of media with GM-CSF& IL-4 was added and placed back into the incubator. On Day 5 dishes were scraped and non-adherent cells were transferred into new dishes and incubated for 2hr. After 2hrs non-adherent cells were transferred into new dishes and placed back into the incubator for an additional 2hrs. After 2 more hours the cells were collected without
scraping and placed into new dishes. These new dishes were re-labeled and placed back into the incubator for 7-10 days. After 7-10 days cells have differentiated into immature dendritic cells.

Lentiviral Transfection of Dendritic Cells:

**Materials:** ENO1-set Si RNA GFP, scrambled siRNA GFP, 6 well plates, polybrene, complete media, WT DCs

**Methods:** Incubated cells at 37 degrees Celsius with 5% CO2 overnight in a 6 well plate containing complete media without IL-4 and GM-CSF overnight prior to viral infection at a density of 0.3 X 10^6. Plates were spun at 2500 rpm for 20min. The media was removed from the wells by pouring off the supernatant and resuspending the cells with 2-3 ml of polybrene (8ug/ml) media mixture per well. 2ul of 106 IU/ml of virus was added to each well. One well was always left uninfected with virus as a control, and 1/3 of the wells contained GFP scrambled virus as positive control. Cells were incubated for 48hrs at 37°C with 5% CO2. Culture medium is then removed and spun at 2500rpm for 20min then poured off. Cells were suspended with 1ml of complete medium in a 6 well plate and ready for use.

Pulsing Dendritic Cells with *Chlamydia muridarum*:

**Materials:** *Chlamydia muridarum* (MoPn), 6 well plates, 1.5ml centrifuge tubes

**Methods:** Each plate contained specified amount of *Chlamydia* stimulation. The amount of bacteria required per cell line was calculated using the ration 5:1 (MoPn: DC).
The calculated amount of bacteria culture was then added to wells according to specified times (less than 4hrs) starting with the longest time first and spinning plates between times at 2200rpm at 25°C. When incubated for longer than 4hrs, plates were spun for 1 hour and incubated at 37°C 5%CO₂ until ready. Cells were then scraped gently from all wells and transferred into 1.5ml centrifuge tubes and spun at 2500rpm for 20min at 4°C. Supernatant was collected and placed in -85°C for cytokine analysis. Cells were placed on ice and ready for use.

**Flow Cytometry:**

**Materials:** DC markers (conjugated antibodies with FITC, PE or 7AAD): CD3, CD4, CD13, CD29, CD39, CD45, CD54, CD70, CD80, CD83, CD86, CD95, CD102, CD119, CD120b, CD121a (IL-1R), CD217 (IL-17R), CD218a (IL18R), CD223 (MHC class II) CD265, CD298, CD305, TLR2, and TLR4, FACs Buffer, 5ml conical FACS tubes, 1.5ml tubes, foil, PBS and FBS

**Methods:** After cells were stimulated with *Chlamydia*, cells are then resuspend in 500ul cold 2% FBS in PBS (wash buffer) and washed once in wash buffer, then spun at 2200rpm for 5mins at 4°C. Cells were then resuspend in 100ul of wash buffer. Then 2.5ul of Fc blocker per 100ul of sample incubated for 10min at 4°C. Cells were then centrifuged at 2200rpm for 12min at 4 degrees Celsius. Supernatant was discarded and resuspended in 50ul. Surface markers and controls were then added to corresponding 1.5ml Eppendorf tubes for 1hr in the dark at 4°C. Cells were washed twice using 500ul of wash buffer spun at 2200rpm for 12min at 4°C. On last wash supernatant was removed
and 500ul of wash buffer was added. Cells are then filtered into 5ml FACS tubes, covered with foil, and stored at 4°C until ready for reading on Guava EasyCyte or BD Accuri C6.

**Confocal Microscopy:**

**Materials:** Wash Buffer (500ml PBS in 10% BSA), 1.5 ml tubes, cold 1X PBS, primary antibodies and secondary antibodies, Fc Blocker, confocal dishes, cold poly-lysine, glutaraldehyde, formaldehyde.

**Methods:** After cells were pulsed, they were gently scraped from wells and transferred to 50ml or 15ml tubes. Cells were then centrifuged at 2200rpm for 15minutes. Supernatant was collected and placed in -85°C. Cells were then resuspended in wash buffer labeled in 1.5ml tubes. Cells were centrifuged at 2200rpm and washed two times at 12min for 4 degrees Celsius and pour off supernatant. Cells were then Fixed by adding freshly made 4% formaldehyde in 0.01% glutaraldehyde in cold 1X PBS. The volume was maintained at around 200ul but was either increased or decreased depending on pellet size. Cells are then incubated for 5min at room temperature. Washed two times with 800ul of cold 1X PBS. Then cells were resuspended in 100ul adding 2.5ul of Block Fc receptor to each sample. Cells were then incubated for 10min at 4°C in the dark. Primary antibodies are then added and undergo incubation for 1hr at 4°C. After the incubation period cells are then spun at 2200rpm for 12min at 4°C. Cells were then washed 2 times with wash buffer at 2200rpm for 12min at 4°C. During last wash supernatant was discarded and responded in 100ul wash buffer. Secondary antibody and
DRAQ5 were added and incubated for 1hr at 4°C. Cells were then washed 3 times and on last wash 100ul of wash buffer was added and cells were now ready for viewing. Prior to preparing the cells, confocal dishes were labeled accordingly and coated with 1mL of cold poly-lysine and incubated overnight in the culture hood.

**Adoptive Transfer:**

**Materials:** Need 106 cells per mouse, BD 1ml TB Syringe with Intradermal Bevel Needle 26G, Depo-Provera, 15ml tubes

**Methods:** We used siRNA transfected DCs and WT DCs pulsed for only 2hrs with *Chlamydia*. Cells were scraped from 6well plates and placed into 15ml tubes then spun at 2500rpm for 20min at 4°C. Supernatant was collected and placed in -85°C. Complete media without IL-4 & GM-CSF was added to tubes to resuspend cells and count. There were 106 cells per 100ul of complete media. Cells were then placed on ice until ready. Depo-Provera was prepared at 25mg/ml using 100ul per mouse. In Center for Laboratory Animal Resources (CLAR) facility biohazard room inject mice with Depo-Provera subcutaneously between ears. Intraperitoneal injection of cells at 106cells/100ul complete media from siRNA transfected groups pulsed with *Chlamydia ENO1* knockdown DCs and Scramble control DCs were added into mice according to respective cages. Likewise, WT DCs also pulsed with *Chlamydia* was added intraperitoneally to each mouse in respective cages at same concentration. After 1 week mice were infected vaginally with MoPn and then swabbed vaginally every 3 days. Swabs were placed in -85 degree C until needed for infectivity assay.
T-Cell Co-culture:

**Materials:** Syringe 40um filter MACS Buffer 50mL Tubes, 1X PBS Complete Media (without cytokines), RBC Lysis Buffer (10X) CAT#: 420301, 96 – well round bottom plate, MACS Separator, MACS LS Columns γ-irradiator, TC – 10 Cell Counter, Pan T Cell Isolation Kit II (MACS 130 – 095 – 130), XTT Cell Viability Kit (Cell Signaling #9095), 1.5 ml tubes, 1640 RPMI and 1% HEPES

**Methods:** Cells were collected after DC cell culture and centrifuged at 2500rpm 20min at 4°C then resuspended in 5mL of Complete Media. Cells were then counted ratio DC:TC = 1:5 CT:TC = 5:1 of DCs to T-cells were calculated for plating cells into round bottom 96 well plate. Cells were plated at 50μl of DCs at concentration of 1 x 105, 50μl of C. trachomatis (CT) at 25 x 105, and 50μl T – cells at concentration of 5 x 105. DCs were separated into 1.5ml tubes per CT infection. Non – UV DC’s were then separated spun at 2200rpm for 1hr at 25°C, then incubated at 1hr at 37°C. Infected media is then removed and resuspended in 200μl of complete media and put on ice. Now γ-irradiate all DCs with 2000rad for about 3min 10sec. Tubes were kept on ice until after T – cell harvest.

For T-cell harvest: Spleen was removed from mice and transported into 1640 RPMI with 1% HEPES. A 40μm filter is placed on top of an empty 50mL conical tube and with syringe plunger. The syringe plunger is then used to mash spleen into the filter, allowing the fluid to collect in the 50mL conical. The filter is then rinsed with 1X PBS as often as needed. Cells are then spun at 1200rpm 10min 4°C and supernatant is removed. RBC lysis buffer is then created (500ul of 10X RBC Lysis Buffer to 4.5mL of
1X PBS) and resuspended in pellet in 5mL of 1X RBC Lysis Buffer. Cells were then incubated at room temp for 4-5 min. Next we added 20ml of 1X PBS to the solution and centrifuged at 1200rpm 10 min 4°C and remove supernatant then washed cells twice with 1X PBS at 1200rpm for 10 min 4°C. After last wash, cells were then resuspended in 35mL 1X PBS and T – cells were counted.

UV DC stimulation with T-cell: T-cells were then spun at 22000rpm for 1 hr at 25°C, and incubated for 1 hr at 37°C. Cells were then resuspended in 40μl of MACS buffer per 107 total cells and added to 10μl of PAN T – Cell Biotin – Antibody Cocktail per 107 total cells. Cells were mixed well and incubated in the dark for 5 min at 4°C. 30ul of MACS buffer was added per 107 total cells and 20μl of Anti-Biotin Micro Beads per 107 total cells. Cells were mixed well and incubated in the dark for 10 min at 4°C. The MACS LS Column was rinsed with 3ml of MACS Buffer and flow through was discarded. T-cell suspension was then added to column and flow through was collected. This flow through represented the enriched T – cells. Column was then washed one time with 3 mL of MACS buffer and combined with the previous collected flow through. Cells were spun at 1200rpm for 10 min at 4°C and resuspended in 10mL of complete media then cells were counted. Cells were kept on ice until ready to use. After 2 hrs of incubation at 37°C, supernatant was then removed and stored at -85°C, then resuspended in 200μl of complete media. T – cells are now added to the corresponding wells with UV-DCs and bringing the volume to 250ul with complete media. Cells are incubated 5 days.

On Day 5 150μl of the supernatant is removed and stored at -85°C. Cells were then ready to proceed with XTT Cell Viability Kit. Reagents were thawed and created at 1:50
volume ratio of XTT detection solution (Electron coupling solution: XTT Reagent). 50 μl of XTT detection solution was added per well and incubate at 37°C for 3hrs a spectrophotometer was then used to read at 450nm.

**Infectivity Assay:**

**Materials:** Swabs, Transport Media (SPG media w/ T-Med), dry ice, 15ml tubes, beads

**Methods:** Prepared Transport Media under hood by adding 2 beads per 15ml tube and 1.5ml of transport media per tube. Transport Media and a box with dry ice was brought into the CLAR facility Biohazard room. Mice were swabbed vaginally Swab mice vaginally every 3 days until day 15. After day 15 mice were swabbed once every week. After each swab was placed in 15ml tube of Transport Media, the tube was then immediately placed on dry ice. Samples were transported back into the lab transporting and placed in 85°C until analyzing for infectivity rate.

**Fertility Assay:**

**Materials:** Male mice, scissors, needles 18-22 Ga, foam tops

**Method:** With permission from CLAR facility place in corresponding cages, 1 male mouse per 2 female mice. After 31 days (or when instructed by CLAR faculty) mice were sacrificed and number of pups were counted.
Western Blotting Analysis:

**Materials**: Pierce BCA Protein Assay Kit, RIPA buffer, heat block, running buffer, transfer buffer, electrophoresis unit, TGX gels Bio-Rad, nitrocellulose paper, protein marker ladder, 5ml pipet, transfer apparatus, blocking buffer, TBST, shaker, primary antibodies, secondary antibodies, LAS – 4000 machine, Solution A and Solution B from Luminex (to detect bands), foil, PBS, forceps, box to hold membrane, Cy3 and Cy5.

**Method**: Dendritic cell lysates were denatured, and then ran on TGX gels (Bio-Rad) for 1 hour. Proteins were then transferred on to nitrocellulose membrane (Bio-Rad) using wet transfer method. After 1 hour, blots were washed, blocked with 5% milk then incubated with primary antibody for ENO1 (from Abcam) overnight at 4°C. Subsequently, blots were washed three times in TBS/Tween followed by incubation of the HRP conjugated secondary Abs (R&D systems) and Amersham WB Cy3 secondary (GE Healthcare) for 1 hr. Blots were washed and developed using Western ECL substrate and viewed in a Bio-Rad Gel Doc XR+ System. The images were saved as Tiff files.

**Modified Western Method**: We followed the Cy5 Total Protein Normalization procedure as shown by McWhirter and colleagues (74). DC cell lysates were stained with Cy5 Amersham WB (GE Healthcare) for 30 min at room temperature and ran on TGX gels (Bio-Rad) for 1 hour. Proteins were then transferred onto PVDF membrane (Bio-Rad) using The Trans-Blot Turbo Transfer System (Bio-Rad) for 7 min. Blots were washed, blocked with 5% milk then incubated with primary antibody overnight at 4°C. Subsequently, blots were washed five times in TBS/Tween followed by incubation of
HRP conjugated secondary Abs (R&D systems). Blots were washed and developed using Western ECL substrate in Bio-Rad Gel Doc XR+System. Viewed using fluorescence for protein detection. Cy5 total protein normalization in Western blot analysis.

**Statistical analysis:**

The data derived from different experiments was analyzed and compared by performing a 1- or 2-tailed t-test. Data presented as the mean ± SE of at least three independent experiments. The relationship between diverse experimental groups was evaluated by analysis of variance (ANOVA) (Microsoft Excel 2015 Redmond, WA). Statistical significance was judged at P < 0.05.
CHAPTER IV
RESULTS

Dynamic Distribution of ENO1

The results in Figure 1A indicate ENO1 protein levels were increased after IL-10 KO DCs were stimulated with *Chlamydia*. Western blot and confocal microscopy analysis was performed to confirm the proteomics results (Figures 1B and 1C). The confocal images confirmed the dynamic distribution of ENO1 in IL-10 KO DCs after *Chlamydia* stimulation. After confirming that ENO1 levels were maintained at high levels in IL-10 KO DCs after *Chlamydia* stimulation we proceeded by using lentiviral technology to successfully knockdown ENO1 completely in WT DCs after 48 hours (Figure 2A).
Figure 1A & B: ENO1 Expression in IL-10 KO DCs.

A). Dendritic Cell lysates from WT DCs and IL-10 KO DCs stimulated with and without *Chlamydia*. The Cy5 represents red dye and Cy3 represents green dye. The red color indicates high expression of protein in IL-10 KO DCs while the green color indicates high expression of protein in WT DCs. The yellow color indicates that the protein expression is the same for both WT and IL-10 KO DCs. Table 1 in the figure above depicts spot numbers corresponding to differentially expressed proteins in proteomics image. Alpha enolase is shown in red font and is upregulated (>3.0 fold) in IL-10 KO DCs after 2 hours of *Chlamydia* stimulation. B). Western Blot analysis showed that after 2 hours stimulation with Chlamydia ENO1 expression was lower in WT DCs compared to IL-10KO DCs.
Figure 1C: Distribution of ENO1 in Dendritic Cells.
Confocal microscopy analysis of ENO1 expression in WT and IL-10 KO DCs stimulated with *Chlamydia*. The graph is a representation of the confocal images that express ENO1. There is a significant difference (p<0.05) in ENO1 expression between IL-10KO DCs and WT DCs regardless of *Chlamydia* stimulation. The confocal confirms that ENO1 is expressed most abundantly in the cytoplasm of the dendritic cell. This experiment was repeated 3 times. This confirms that ENO1 is expressed most abundantly in the cytoplasm of the dendritic cell. This experiment was repeated 3 times.
Figure 2A: ENO1 knockdown Lentiviral Infection.
ENO1 knockdown was used to knockdown ENO1 in WT DCs at 0 hours, 24 hours and 48 hours. Scramble siRNA was used as a control for ENO1 knockdown confirmation. Western blot analysis confirms expression of ENO1 in DCs transfected with ENO1 knockdown. ENO1 expression is present in siRNA Scramble control and ENO1 knockdown 0 hour and 24 hour transfection. ENO1 expression is completely knocked down after 48hrs of transfection. Therefore, WT cells transfected with ENO1 knockdown at 48 hours will be used continually to further analyze ENO1 effects on Dendritic Cell function. This experiment was repeated 3 times.

The Effect of ENO1 on DC Immunoregulation

In order to further understand the role of ENO1 in the metabolic regulation of DCs we measured the concentration of Pyruvate (Figure 3A). Pyruvate concentration was lower in ENO1 knockdown DCs compared to WT DCs. Due to lower concentration of pyruvate one can predict that ENO1 may regulate the metabolic pathways which have an effect on DC function since pyruvate is a crucial step eventually leading to the citric acid cycle and the electron transport chain which is responsible for high yield of ATP production needed for DC activation and maturation. Figure 3B indicates the modified protein analysis Figure 3C show proteins associated with mitochondrial function. ANT 1/2/3/4 is responsible for mitochondrial export of ATP. ANT 1/2/3/4 show low expression in uninfected ENO1 knockdown DCs, but when stimulated with Chlamydia...
this expression increases. While in WT DCs the expression of ANT 1/2/3/4 remains consistent with or without *Chlamydia* stimulation. Translocase outer membrane (TOM) 20 expression is low in ENO1 knockdown DCs stimulated with *Chlamydia*. While in WT DCs, TOM20 expression remains constant regardless of *Chlamydia* stimulation. BAX (BCL-2 associated X protein) is highly expressed in unstimulated ENO1 knockdown DCs, yet is not expressed in *Chlamydia* stimulated ENO1 knockdown DCs. BAX expression is low in WT DCs with or without *Chlamydia* stimulation. BCL-2 expression is low in ENO1 knockdown DCs unstimulated and is not expressed in ENO1 knockdown DCs. BCL-2 is not expressed in WT DCs unstimulated or stimulated with *Chlamydia*. Cytochrome C is highly expressed in ENO1 knockdown DCs unstimulated with *Chlamydia* and is not expressed in ENO1 knockdown DCs stimulated with *Chlamydia*. While in WT DCs Cytochrome C is expressed in unstimulated DCs and not expressed in *Chlamydia* stimulated DCs. This may indicate that the mitochondrial structure is much less intact when ENO1 knockdown in the cells. In order to observe the cells undergoing apoptosis we use flow cytometry to observe apoptotic events (Figure 3D). It was evident that apoptosis was decreased after DCs were stimulated with *Chlamydia* but there was a significant increase in ENO1 knockdown DCs verses WT DCs.

This means that ENO1 knockdown DCs may have a less efficient antigen presenting function. We then proceeded to using transmission electron microscopy (TEM) to view the mitochondrial structure (Figure 3E). We discovered that the mitochondrial membrane structure in ENO1 knockdown DCs was highly permeable and the cristae of the mitochondria was completely dissolved in comparison to infected WT
DCs. Mitochondrial structural changes have been known to affect the production of ATP which is crucial for producing the energy needed for DC maturation.

**Figure 3A: Pyruvate Concentration in *Chlamydia* stimulated Dendritic Cells.**
Pyruvate concentration was obtained in cell lysate and supernatant. Pyruvate concentration was measured using spectrophotometer. Concentration of pyruvate is shown post-*Chlamydia* stimulation at 0min, 30min, 60min, 120min, and 240min. There is a significant difference (p<0.05) of high concentration of Pyruvate at 30min, 120min and 240min. This graph shows that ENO1 knockdown DCs stimulated with *Chlamydia* maintain a low concentration of pyruvate in comparison to WT DCs stimulated with *Chlamydia*. 
Figure 3B: Pyruvate Dehydrogenase Expression in DCs.
Proteins which were normalized using ImageQuant Reader (GE Healthcare). The total protein was detected and used for normalization using Cy5 dye and proteins of interest were probed using Cy3 secondary from the Amersham System Protocol (GE Healthcare). The ratio of Cy3/Cy5 indicates the differentially expressed proteins. The expression of pyruvate dehydrogenase was not different in ENO1 knockdown DCs in comparison to WT DCs.
Figure 3C: Mitochondrial Structure.
Transition electron microscopy (TEM) technology indicates the structural difference of the mitochondria using. The depiction shows WT and ENO1 knockdown DCs unstimulated and stimulated with \textit{Chlamydia} for 13hrs. After stimulation the red arrows indicate a major morphology change of mitochondria in ENO1 knockdown DCs. The mitochondria cristae have completely disappeared and the outer membrane structure is enlarged.
Figure 3D: Expression of Mitochondrial Associated Proteins.
Proteins which were normalized using ImageQuant Reader (GE Healthcare). The total protein was detected and used for normalization using Cy5 dye and proteins of interest were probed using Cy3 secondary from the Amersham System Protocol (GE Healthcare). The ratio of Cy3/Cy5 indicates the differentially expressed proteins. Cytochrome C, BAX, TOM 20 all had lowered expression in chlamydia stimulated DCs with their ENO1 knocked down. Bcl-2 expression was low and similar in all sample sets.
Figure 3E: Apoptosis Analysis.

Analysis of apoptotic events using Flow Cytometry analysis with the FITC Annexin V Apoptosis Detection Kit with 7-AAD (Biolegend). The Green-B Fluorescence is FITC detection and Red-B Fluorescence is 7-AAD detection. DCs were stained with CD11c Pey-CY7 stain and gated for further analysis. Quadrant 1 (top left) Quadrant 2 (top right), Quadrant 3 (lower left), Quadrant 4 (lower right). Cells in Quadrant 1 indicate necrosis pathway while cells in Quadrant 3 are live cells. Cells in Quadrant 4 indicate cells undergoing apoptosis. Chlamydia stimulation for ENO1 knockdown DCs and WT DCs undergo less apoptotic events. But Chlamydia stimulated ENO1 knockdown DCs undergo significantly high expression (p<0.05) of apoptosis events verses Chlamydia stimulated WT DCs. Experiment was repeated 3 times.

ENO1 knockdown DCs regulates DC activation and maturation

It is clear that ENO1 may have an effect on DC function therefore we observed DC surface markers involved in maturation and activation. (Figure 4A) indicates that the expression of TLR4 is significantly lower in infected WT DCs compared to Chlamydia stimulated ENO1 knockdown DCs. MHC II antigen presenting receptor is a crucial DC receptor for antigen presentation during Chlamydia infection. MHC II and CD80 (a co-stimulatory receptor) were less expressed in infected ENO1 knockdown DCs vs. infected
WT DCs, although CD40 expression in infected ENO1 knockdown DCs was higher than in infected WT DCs. This indicates that ENO1 has an effect on DC maturation. Lowered DC maturation results in lower DC activation to Th1 cells associated with rapid clearance of Chlamydia infection. It is clear that ENO1 may have an effect on DC function therefore we observed DC surface markers involved in maturation and activation. Figure 3a indicates that the expression of TLR4 is significantly lower in infected WT DCs compared to infected ENO1 knockdown DCs. Figure 4B demonstrates that during Chlamydia infection ENO1 knockdown DCs expression of Th1 cytokines IL-12, MIP-1beta, MIP-1alpha, and RANTES were significantly lowered in comparison Chlamydia infected WT DCs while simultaneously showing increase in Th2 cytokines IL-10 and IL-14. DC – T cell co-culture is demonstrated in Figure 4C and 4D. Figure 4C shows that Th1 cytokines RANTES, IL-1beta, IL-1alpha, and IL-9 were significantly lowered while Th2 cytokines IL-13, IL-17A, IL-10, and IL-5 were highly expressed infected ENO1 knockdown DCs. Figure 4D also indicates lower total t-cell proliferation in ENO1 knockdown DCs co-cultured with T-cells from spleen of Chlamydia infected mice.
Figure 4A: Dendritic Cell Maturation Markers.
Figure 4 is a flow cytometry analysis using BD Accuri C6 analyzer showing Toll Like Receptor 4 (TLR4) expression. DCs were stained with CD11c Pey-CY7 stain and gated for further analyzation. Cells were stained with FITC conjugated TLR4. Samples were repeated 3 times. TLR4 expression was significantly lowered in ENO1 knockdown DCs stimulated with *Chlamydia* in comparison to WT DCs stimulated with *Chlamydia*. TLR4 expression is significantly lower in unstimulated ENO1 knockdown DCs verses unstimulated WT DCs. Flow Cytometry analysis using Guava EasyCyte flow cytometer showing CD80, CD40, MHC II, CD86 expression in DCs stained with CD11c Pey-CY7 gated for further analyzation. Cells were stained with FITC conjugated MHC II, FITC conjugated CD80, FITC Conjugated CD40 and PE Conjugated CD86. Samples in double stained with PE and FITC were compensated. Green-B-Fluorescence indicates FITC staining and Yellow-B Fluorescence indicates PE staining. Samples were repeated 3 times. The graph depicts the flow cytometry dot plot data showed significant differences (p<0.05) between WT DCs and ENO1 knockdown DCs. The expression of CD86, CD80 and MHC II were lower in ENO1 knockdown DCs after *Chlamydia* stimulation vs. WT DCs. While CD40 expression increased expression in ENO1 knockdown DCs stimulated with *Chlamydia*. TLR4 expression is significantly lower in unstimulated ENO1 knockdown DCs verses unstimulated WT DCs.
Figure 4B: Dendritic Cell Cytokine Expression.

Bio-Plex Pro Mouse Cytokine 23-Plex multiplex array was used for cytokine analysis according to manufacturer’s guidelines (Bio-Rad). Cytokines IL-4, IL-17A, IL-10, IL-1 alpha, IL-1 beta, IFN-gamma, TNF alpha, MCP-1 IL-12(p70), MIP-1alpha, MIP-1beta, RANTES which induce T-helper cell 1 response. At 1hr Chlamydia stimulation there is a significant (p<0.05) increase of IL-12(p70), MIP-1alpha, MIP-1beta, and RANTES in WT DCs verses ENO1 knockdown DCs. There is a significant (p<0.05) increase of IL-10 and IL-4 in ENO1 knockdown DCs verses WT KD DCs.
Figure 4C & D: DC Antigen Presenting Function and T-cell Proliferation

C). There is a low expression of RANTES, IL-1beta, IL-1alpha and IL-9 cytokines in ENO1 knockdown DCs co-cultured with T-cells verses WT DCs co-cultured with T-cells. These cytokines aid in the induction of T helper cell 1 (Th1) response. IL-13, IL-10 and IL-15, IL-17A show high expression in ENO1 knockdown DCs co-cultured with T-cells in comparison to WT DCs co-cultured with T-cells. D). The T-cells were collected from spleen of Chlamydia infected WT mice and co-cultured with WT DCs and ENO1 knockdown DCs. ENO1 knockdown DCs showed less T-cell proliferation in comparison to WT DCs.

The Effect of ENO1 on DC function in vivo

We performed adoptive transfer of ENO1 knockdown DCs into C57/BL6 mice via tail intravenous injection. This adoptive transfer was repeated multiple times. However, due to the inability to completely restrain the mice, we believe that the intravenous injection of the ENO1 knockdown DCs may not have been performed optimally or maybe the amount of cells used was not sufficient. Therefore, only 3 dates gathered from the infectivity analysis were statistically significant. Although Figure 5A indicates there does seem to be a trend, ENO1 knockdown transfected mice maintaining
Chlamydia infection throughout term. While Chlamydia stimulated WT DC mice cleared Chlamydia at a faster rate.

**Figure 5A: ENO1 knockdown DCs Infectivity assay.**
Graph shows WT mice with Chlamydia stimulated ENO1 knockdown DCs (ENO1 knockdown DCs) and WT mice with Chlamydia stimulated WT DCs, and WT mice with no DCs adoptively transferred. This data shows significant decrease (p<0.05) in Chlamydia infection at 15, 21, and 28 days post-Chlamydia stimulation in comparison to mice with ENO1 knockdown DCs.
CHAPTER IV

CONCLUSION/DISCUSSION

Understanding the importance of ENO1 as it relates to the dendritic cell function and activation is still ongoing. It is important to note that the proteomics data shows several molecules which may also contribute to the increase in antigen presenting function of IL-10 KO DCs. ENO1 is one of the three isotypes of the enolase family and has been shown to play a role in inflammation (72). It is understood that plasminogen is a ligand for ENO1 membrane bond receptors (73) and confocal analysis has proven that there is a dynamic distribution of ENO1 on the surface of dendritic cells. Therefore, the inflammatory responses in Chlamydia may also be triggered by the effect of plasminogen on these receptors. We observed that TLR4 and co stimulatory cytokines CD80 and CD86 decreased in expression after ENO1 knockdown DCs were stimulated with Chlamydia. This implicates that when dendritic cells are stimulated with Chlamydia and ENO1, a key glycolytic enzyme is not present, the cells ability to produce high yields of ATP in the TCA cycle is decreased. This would affect the rate of maturation thereby decreasing DC activation and antigen presenting function. Interestingly, the inhibition of ENO1 contributes to increase in cytokines which are associated with activation Th1, which proves that ENO1 has some effect on DCs ability to activate Th2. Th2 is known to enhance rapid clearance of Chlamydia infection. Lowering the yield of Th2 decreases
dendritic cell ability to activate Th2 cells needed in clearing the infection. Even when DCs are co-stimulated with total T-cells collected from spleen of *Chlamydia* infected mice cytokines associated with Th2 activation were still lowered when ENO1 was knocked down in these dendritic cells. Because ENO1 is a glycolytic enzyme it was important to understand the role that ENO1 has on mitochondria which is the location of TCA cycle and plays important role in apoptosis of the cell. When dendritic cells are deficient in ENO1 it is clear that the function of the mitochondria is disrupted. Therefore, pyruvate which is produced through the process of glycolysis, is inhibited leading to decrease expression of enzymes needed to undergo the TCA cycle as well as enzymes needed to maintain the structure of the mitochondria. Disrupting these mechanisms may in fact contribute to the decrease in antigen presenting function and maturation, since it was previously stated that ATP is essential in contributing to the proteins needed for these processes. ENO1 has several functions as described that may also be contributing to the change in DC function after stimulation. ENO1 may also serve as a target for therapy in lowering inflammation that are dependent on the expression of Th2. Alpha Enolase is highly expressed throughout the cell and has numerous functions, therefore it is important to further elucidate the role that Alpha Enolase plays in inflammatory responses.
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