Interplay Between Nitric Oxide-Mediated Metabolic Rewiring And Immune Functions In Dendritic Cells

INTERPLAY BETWEEN NITRIC OXIDE-MEDIATED METABOLIC REWIRING AND IMMUNE FUNCTIONS IN DENDRITIC CELLS

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ABSTRACT

Recent years have highlighted the importance of metabolic processes in controlling immune responses at the cellular level. Studies have shown that metabolic changes during immune cell activation support energy needs and influence inflammation and cell function in both innate and adaptive immunity, strongly suggesting potential methods to regulate inflammation. Arginine metabolism is particularly crucial in the immune responses of myeloid cells, such as macrophages and dendritic cells (DCs). These cells produce two main arginine catabolizing enzymes, inducible nitric oxide synthase (iNOS) and arginases (ARG). iNOS utilizes L-arginine and oxygen to produce nitric oxide (NO), a vital component in fighting infections, but also harmful at high levels. Arginases convert L-arginine into L-ornithine and urea; ornithine builds polyamines for tissue repair and collagen, and can promote the expression of anti-inflammatory genes.

DCs perform numerous functions, including phagocytosis, the production of chemokines and cytokines, the clearance of infections, and the activation of T cells to bridge innate and adaptive immunity. Our previous research demonstrated that: 1) DCs increase iNOS and NO production upon activation, shifting their metabolism toward glycolysis due to NO-mediated respiration inhibition; 2) a threshold of NO production exists — a concentration of NO that leads to a decline in mitochondrial respiratory function. Studies have observed ARG expression in DCs exhibiting tolerogenic features, suggesting that ARG may play a role in regulating iNOS via substrate competition. However, given the heterogeneity of DC subtypes, it remains unclear whether the known iNOS-producing DCs exhibit this ARG expression pattern, and whether competitive substrate usage by ARG affects iNOS activity, particularly in relation to the NO-respiration threshold.

In this dissertation, using murine bone marrow-derived DCs (BMDCs) as a model, we examined whether arginase activity can limit iNOS function by decreasing arginine availability, thereby altering NO-driven immune functions in DCs. Our results showed that when arginase activity is blocked, NO levels increase, lowering the NO-respiration threshold. This shift in the threshold influences how long DCs survive after activation and how effectively they control the replication of pathogens, *Listeria monocytogenes*. Overall, our findings support the hypothesis that iNOS activity is partly regulated by arginases through substrate competition in DCs. By limiting NO production, arginase helps prevent mitochondrial damage and extends cell survival, although it may also reduce the cell's ability to fight pathogens. This balance is crucial for proper immune function, underscoring the importance of arginine metabolism in regulating iNOS activity to mediate inflammation and facilitate effective immune responses.

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DEDICATION

This work is dedicated to my advisor, Dr. Eyal Amiel, whose expertise, insightful feedback, and unwavering support were instrumental in the completion of this dissertation. I am deeply grateful for his mentorship in both science and life. It has truly been an honor to serve as his apprentice.

To my inner child, for her strength and courage to be healed, willingness to continually learn to be authentic and vulnerable,
and her determination and trust in me.

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LIST OF ABBREVIATIONS

Abbrebiations	Definition
AA	Arachidonic Acid
ACOD1	aconitate decarboxylase 1
ADC	arginine decarboxylase
AGAT	L-arginine:glycine amidinotransferase
APC	antigen-presenting cells
ARG	Arginase
BEC	*S*-(2-boronoethyl)-L-cysteine *; Arginase inhibitor*
BH4	tetrahydrobiopterin
BMDC	Bone-Marrow derived dendritic Cells
CaM	calmodulin
cAMP	cyclic AMP
CAT	cationic amino acid transporter
CCL	Chemokine Receptor ligand
CCR	CC-chemokine receptor 2
CCR	Chemokine Receptor
cDCs	conventional-DCs
CLPs	common lymphoid progenitors
CLRs	C-type lectin receptors
CMPs	common myeloid progenitors
CO	Carbon monoxide
COX-2	cyclooxygenase-2
CREB	cyclic AMP-responsive element-binding protein

DAMP	damage-associated molecular patterns
DC	Dendritic Cell
eIF2α	eukaryotic initiation factor alpha
eNOS	endothelial NOS
EPRs	E-type prostanoid receptors
ETC	electron transport chain
FAD	flavin adenine dinucleotide
FAD	flavin adenine dinucleotide
FLT3	FMS-like tyrosine kinase 3 ligand
FMN	flavin mononucleotide
GM-CSF	granulocyte-macrophage colony-stimulating factor
GMPs	granulocyte-macrophage progenitors
GSH	glutathione
HIF1-alpha	hypoxia-inducible factor alpha
HO	heme oxygenase
HRE	hypoxia response element
HSCs	Hematopoietic Stem Cells
IDO	Indoleamine 2,3-dioxygenase
IFNs	Interferons
IKK	IκB kinase
IKK-epsilon	*IkB kinase-epsilon*
IL	interleukin
IRFs	*interferon regulatory factors*
KEAP1	Kelch-like ECH-associated protein 1
LDs	Lipid droplets

LPS	lipopolysaccharide
LXR	liver X receptor
M1	Pro-inflammatory macrophages
M2	Anti-inflammatory macrophages
M-CSF	macrophage-colony stimulating factor
MDPs	monocyte-dendritic cell progenitors
MHC	Major Histocompatibility Complex
moDCs	Monocyte-derived DCs
MPPs	multipotent progenitors
mTOR	mammalian target of rapamycin
MyD88	Myeloid Differentiation Primary Response Protein 88
NADP	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NFkB	Nuclear factor kappa B
nNOS	neuronal NOS
NO	Nitric Oxide
NOHA	Nω-hydroxy-L-arginine
NOR-NOHA	nor-Nω-hydroxy-L-arginine
NRF2	erythroid 2-related factor 2
OCR	Oxygen consumption rate
ONOO-	peroxynitrite
OXPHOS	oxidative phosphorylation
PAMP	Pathogen-associated molecular patterns
pDCs	plasmacytoid DCs
PG	prostaglandin

PI3K / PKB	phosphoinositide 3-kinases/protein kinase B
PPP	pentose phosphate pathway
PRDX	peroxiredoxin
PRRs	pattern recognition receptors
PTMs	post translational modifications
RNS	reactive nitrogen species
ROS	Reactive Oxygen Species
S1P	Sphingosine-1-phosphate
S1PRs	Sphingosine-1-receptors
SEITU	*S*-ethylisothiourea *; iNOS inhibitor*
SOD	superoxide dismutase
STAT	signal transducer and activator of transcription 3
TCA	Tricarboxylic Acid Cycle
TGF-beta	transforming growth factor beta
Th-1	type-1 immune response
Th-2	type-2 immune response
Tip-DCs	TNF-α/iNOS-producing DCs
TIRF-beta	TIR domain-containing adaptor-inducing interferon-β
TLRs	Toll-like receptors
TNB-1	*TANK-binding kinase 1*
TNF-alpha	tumor necrosis factor alpha
Tregs	regulatory T cells
UDPG	uridine diphosphate glucose

CHAPTER 1: COMPREHENSIVE LITERATURE REVIEW

1.1 THE IMMUNE SYSTEM

1.1.1 Properties and Overview of Immune Responses

Although invisible to the naked eye, the human body is home to approximately 30–40 trillion microbial cells, comprising a diverse array of bacteria, viruses, and fungi, which account for the same or more than the average number of human cells in an adult body.(Sender et al., 2016) Some of these microbes live harmoniously and symbiotically with us, while others invade for their own survival when the conditions favor them.(Casadevall & Pirofski, 2014) The microbes that pose a challenge to our well-being and induce harmful symptoms are called pathogens. In addition to pathogens, the human body encounters threats from non-biological entities (foreign substances) such as microplastics, pollutant gases, smoke, various chemicals, and high-energy radiation like X-rays and UV rays.(Vivier & Malissen, 2005) Both biological and non-biological intrusions can result in severe damage, cause cellular mutations leading to cancer or other diseases, and, in extreme cases, may result in death. This type of harmful foreign substance is known as an immunogen. The immune function, or immunity, refers to our body's defensive state to protect us from such intrusions.(Iwasaki & Medzhitov, 2015; Vivier & Malissen, 2005) The immune system can achieve immunity through a collaborative network of defense mechanisms (systems) involving organs, cells, proteins, and protective molecules. The immune system can be divided into two systems: the innate immune system and the adaptive immune system. (Chaplin, 2010; Iwasaki & Medzhitov, 2015)

1.1.2 Innate Immunity: The Early Defense

The body's first line of defense in the immune system is known as innate immunity. Often referred to as natural or innate immunity, this form of immunity is present at birth and does not require prior exposure to a pathogen to perform its function. Therefore, innate immunity acts as a primary and rapid-response mechanism against pathogenic threats(Chaplin, 2010; Iwasaki & Medzhitov, 2015; Vivier & Malissen, 2005). It involves a range of conserved, nonspecific barriers and cellular defenders that respond immediately upon encountering pathogens. Key components of this defense include physical barriers, such as skin and mucous membranes, chemical mediators like lysozyme and interferons (IFNs), and cellular defenders, including phagocytes like macrophages and dendritic cells (DCs).(Eisenbarth, 2019) These innate defenders also recruit additional phagocytes to the infection site, controlling or inhibiting pathogen replication while awaiting the arrival of the specific and secondary defense mechanisms.(Chaplin, 2010; Eisenbarth, 2019; Iwasaki & Medzhitov, 2015; Vivier & Malissen, 2005)

1.1.3 The Innate Immune System: Myeloid Cells

Cellular defenders in both the innate and adaptive immune systems stem from hematopoietic stem cells (HSCs) located in the bone marrow, which also serves as the primary lymphoid organ and has an exceptional capacity for self-renewal. (Anderson et al., 2021) Based on the expression of specific transcription factors and engagement of specific signaling pathways, HSCs develop into multipotent progenitors (MPPs), serving as precursors for common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs). (Figure 1) Depending on their transcriptional programming, CLPs and CMPs can mature in specific locations such as the thymus, and in peripheral or secondary lymphoid organs like lymph nodes and the spleen. This maturation leads CLPs to differentiate into lymphocytes (T cells, B cells, and natural killer cells) and CMPs into innate immune cells.(Bosteels & Janssens, 2025; Eisenbarth, 2019) (Figure 1.1) Following the divergence between lymphoid and myeloid lineages, CMPs can be further divided into two main groups: monocyte-dendritic cell progenitors (MDPs) and granulocyte-macrophage progenitors (GMPs). As these progenitors mature, they gradually lose their ability for self-renewal while acquiring functional specialization, resulting in mature myeloid cells like granulocytes, monocytes, macrophages, and dendritic cells (DCs). (Figure 1.1) Importantly, all these differentiation steps are tightly regulated, with no significant indications of trans-differentiation between hematopoietic lineages under normal circumstances. (Y.-J. Liu et al., 2001)

The Connection between Innate and Adaptive Immunity

The innate immune system functions by identifying conserved molecular patterns through germline-encoded pattern recognition receptors (PRRs), which include Toll-like receptors (TLRs), C-type lectin receptors (CLRs), and NOD-like receptors (NLRs).(Carroll et al., 2024; Jang et al., 2015) PRRs help detect common patterns found in groups of microbes (Pathogen-associated molecular patterns; PAMPs) or those arising from damaged cells (damage-associated molecular patterns; DAMPs). This detection triggers pro-inflammatory responses and begins the process of clearing pathogens through the processes of phagocytosis, the release of antimicrobial peptides, and the production of cytokines to activate additional immune defenses.(Underhill & Goodridge, 2012) During phagocytosis, the phagocytosed pathogen vesicle fuses with lysosomes, which are membrane-bound organelles containing digestive enzymes, leading to the breakdown of pathogens into molecular fragments. Some phagocytic cells also take on the role of antigen presentation, loading the degraded molecular fragments (antigens) onto membrane-bound proteins called Major Histocompatibility Complex (MHC).(Neefjes et al., 2011; Pishesha et al., 2022) DCs are prominent examples of professional antigen-presenting cells (APCs). Once activated, DCs can exit the infection site and migrate to draining lymph nodes, where adaptive immune cells like B and T cells circulate, waiting for activation by APCs.(Eisenbarth, 2019; Y.-J. Liu et al., 2001) Understanding DC biology and its immune regulatory mechanisms is crucial in immunology, both due to this intermediary role between innate and adaptive immunity and because DCs initiate the majority of antigen-specific immune responses.

Overview of DCs

1.1.3.1.1 Cellular Characteristics and Heterogeneity of DCs

All DCs have migratory ability, produce chemokines and cytokines, and phagocytose and present antigens to T-cells; however, DCs can be classified as either conventional or non-conventional types, based on their development (ontogeny), genetic signatures, expressed surface markers, and primary functional roles.(Anderson et al., 2021; Belz & Nutt, 2012) Initially, based on specific progenitors in the bone marrow and the necessary transcription factors for differentiation, monocyte/DC progenitors evolve into common DC progenitors (CDPs) that express the transcription factor encoded by the Zbtb46 gene.(Satpathy et al., 2012) (Figure 1.1) CDPs can mature into conventional-DCs (cDCs), known for their high phagocytic ability with their professional T cell priming role. Additionally, CDP can develop into plasmacytoid DCs (pDCs), which can produce large quantities of cytokines and chemokines. The development of CDPs is dependent on the cytokine FMS-like tyrosine kinase 3 ligand (FLT3L).(Satpathy et al., 2012)

In contrast, granulocyte/macrophage progenitors (GMP) can differentiate into both monocytes and granulocytes, which consist of basophils, eosinophils, and neutrophils. (Figure 1.1) They depend on the cytokine macrophage-colony stimulating factor (M-CSF) to evolve into monocyte-derived macrophages, while granulocyte-macrophage colony-stimulating factor (GM-CSF) is necessary for differentiation into DCs.(Anderson et al., 2021; Eisenbarth, 2019) Additionally, they can differentiate into Langerhans cells, a type of skin-resident DC, influenced by transforming growth factor beta (TGF-β). Unlike conventional DCs (cDCs), myeloid cells derived from GMP do not express the transcription factor ZBTB46(Satpathy et al., 2012); they instead depend on the chemokine receptor CC-chemokine receptor 2 (CCR2) for recruitment to sites of infection.(Anderson et al., 2021) (Figure 1.1) Monocyte-derived DCs (moDCs) perform various functions, such as clearing pathogens and preparing the inflammatory microenvironment by producing and releasing pro-inflammatory cytokines and molecules.(Eisenbarth, 2019; Sallusto & Lanzavecchia, 2010) However, defining the subtypes of DCs remains difficult due to plasticity in their characteristics and functions in response to environmental cues. This adaptability complicates efforts to categorize them using fixed markers or functions. Additionally, overlapping markers and functions among certain subtypes further complicate DC subset classification efforts.(Merad et al., 2013)

1.1.3.1.2 Inflammatory DCs (Monocyte-derived DCs) and their activation

Inflammation serves as the body's protective reaction to harmful stimuli, such as injury, infection, and tissue damage. This response may present as pain, redness, swelling, and heat in the affected area, aiming to eliminate the harmful agent and initiate the healing process.(Furman et al., 2019; Min et al., 2018) For example, in the case of a bacterial infection, monocytes circulating in the blood are recruited to the site of infection, where they differentiate into moDCs.(Eisenbarth, 2019) These cells are noted for producing the pro-inflammatory cytokine tumor necrosis factor alpha (TNF-α) and inducible nitric oxide synthase (iNOS), which is why they are also referred to as TNF-α/iNOS-producing DCs (TipDCs; also known as inflammatory DCs; iDCs)(Serbina et al., 2003). As in other classified DC subsets, TipDCs can recognize a type of bacterial structural molecule called lipopolysaccharide (LPS) through their pattern recognition receptor (PRR), toll-like receptor 4 (TLR4), and its associated extracellular protein, MD-2. The binding of the LPS-MD2 complex to TLR4 results in the dimerization of TLR4, which further facilitates the recruitment of adaptor proteins, such as Myeloid Differentiation Primary Response Protein 88 (MyD88) and TIR domain-containing adaptor-inducing interferon-β (TRIF).(Laird et al., 2009; Sakai et al., 2017) The role of MyD88 is especially crucial in DCs, as it leads to the activation of downstream signaling molecules, including the key transcription factor Nuclear factor kappa B (NF-κB)(Sakai et al., 2017), which regulates the production of inflammatory cytokines and the DC maturation process. (Laird et al., 2009; Y.-H. Li et al., 2002)

1.2 Inflammatory Response

1.2.1 Canonical Signaling pathway (NFkB) in inflammatory DCs

Nuclear factor-κB (NF-κB) is a family of inducible transcription factors that control a wide range of genes related to immune and inflammatory responses. This family comprises five members: NF-κB1 (also called p50), NF-κB2 (p52), RelA (p65), RelB, and c-Rel.(T. Liu et al., 2017) (Figure 2) Normally, NF-κB proteins are kept in the cytoplasm by inhibitory proteins, such as members of the IκB family. NF-κB activation occurs primarily through two pathways—the canonical and noncanonical (or alternative)—both of which are vital for regulating the immune and inflammatory responses, although their signaling mechanisms differ.(Hayden & Ghosh, 2011) Ligand binding to PRRs triggers the canonical pathway, where activation starts with IκBα phosphorylation by the IκB kinase (IKK) complex, leading to ubiquitin-dependent degradation of IκBα via the proteasome. (Figure 1.2) Once free, NF-κB moves into the nucleus to function as a transcription factor, enhancing the expression of genes encoding for iNOS, TNF-α, interleukin (IL)-1β, IL-6, IL-12p40, cyclooxygenase-2 (COX-2), which are involved in inflammation, cell proliferation, chemokine, and adhesion molecule production. (Hayden & Ghosh, 2011; T. Liu et al., 2017; Sakai et al., 2017) (Figure 1.2)

1.2.2 TRIF-dependent signaling pathway in inflammatory DCs

Besides the key adaptor protein MyD88, TLR signaling also recruits TRIF, which can independently activate NF-κB and promote the expression of inflammatory genes, particularly type 1 interferons (IFNs).(Hayden & Ghosh, 2011; Laird et al., 2009) (Figure 1.3) TRIF stimulates kinases like TANK-binding kinase 1 (TBK1) and IkB kinase-epsilon (IKKε), leading to phosphorylation of interferon regulatory factors (IRFs) and their movement into the nucleus.(Israël, 2010; Shen et al., 2008) (Figure 1.3) These activated IRFs not only drive inflammation but can also support NF-κB activation, creating a synergistic boost to the activation response.(Newton & Dixit, 2012) Supporting this, LPS-stimulated murine DCs exhibit increased phosphorylation of TBK1, IKKε, and Akt, facilitating metabolic adaptation in DCs characterized by enhanced glycolysis and subsequent expression of iNOS, a crucial enzyme responsible for nitric oxide (NO) production.(Everts et al., 2014a, 2014b; Shen et al., 2008) In conclusion, the MyD88- and TRIF-dependent pathways are crucial for coordinating the innate immune response and have important roles in various diseases and cellular functions.

1.3 The Interplay between Metabolism and Immune Functions

1.3.1 Immune Regulation by L-Arginine Metabolism

A growing number of studies have demonstrated that the metabolic programming, or reprogramming, of immune cells is associated with various disease states, including autoimmunity, cancer, allergy, infectious diseases, atherosclerosis, neurodegenerative disorders, and metabolic diseases.(J. Chen et al., 2021; Corrado & Pearce, 2022; Grzywa et al., 2020; Murray et al., 2015) Arginine plays a fundamental and critical role in maintaining the homeostasis and overall function of the immune system, serving as a significant modulator of immune system activation.(Bronte & Zanovello, 2005) This importance is a central focus of the field of immunometabolism, which recognizes the broad impact of metabolites on immune function. Arginine availability, its synthesis, and its catabolism are highly interconnected aspects that can determine divergent pro-inflammatory or anti-inflammatory immune outcomes.(Bansal & Ochoa, 2003; Das et al., 2010) As a semi-essential amino acid, arginine can play a key role in situations where the body's metabolic requirements exceed its production, such as during pregnancy or in pathological conditions like malignant diseases.(Bansal & Ochoa, 2003; Martí I Líndez & Reith, 2021) Beyond its role as a building block for protein synthesis, arginine serves as a substrate for distinct metabolic pathways that profoundly affect the biology of various immune cells, including macrophages, DCs, and T cells.(Martí I Líndez & Reith, 2021)

1.3.2 The recognition of L-arginine in Inflammation

L-arginine is one of the dibasic proteinogenic amino acids, first isolated and identified in the late 18th century. The 19th century saw many discoveries related to L-arginine biology. One major finding was that L-arginine promoted growth in chicks and young rats but not in fully grown chickens and rats, leading to the initial belief that L-arginine is essential for young but not adult mammals.(Starikova et al., 2023) These discoveries regarding the L-arginine metabolism pathway contributed to the understanding of the importance of dietary L-arginine intake. There are three ways mammals obtain L-arginine: (1) through in-vivo dietary intake and absorption, (2) via endogenous de novo synthesis from L-citrulline (the precursor amino acid for L-arginine), and (3) through recycling from protein degradation or breakdown.(Bansal & Ochoa, 2003; Brosnan & Brosnan, 2004; Mistry et al., 2002) Studies have shown that the small intestine is the main source of L-arginine in growing mammals, whereas in healthy adult mammals, this organ shifts to become a key producer of circulating L-citrulline for subsequent L-arginine synthesis in the kidney, also known as the intestine-renal L-arginine axis.(Brosnan & Brosnan, 2004;

Mistry et al., 2002) This observation reinforced the initial idea of L-arginine being a semi-essential amino acid in mammals. Although the differing L-arginine requirements between young and adult individuals were established, these observations did not fully capture the complex role of L-arginine metabolism in the context of disease or pathogenesis until the late 19th century, when the utilization of L-arginine by macrophages via iNOS was first reported.(MacMicking et al., 1997; Nathan & Xie, 1994)

Studies have demonstrated a connection between low plasma L-arginine levels and poor outcomes in sepsis or delayed recovery from trauma.(Martí i Líndez & Reith, 2021; Wijnands et al., 2015) As a result, arginine supplementation has been investigated as a means to increase plasma L-arginine; however, several factors remain undefined, including the ideal timing, dosage, route of administration, and the amount of arginine utilized by the body. In mammals, four main enzymes break down arginine: arginine decarboxylase (ADC), L-arginine:glycine amidinotransferase (AGAT), arginase (ARG), and NOS. ADC is highly active in the brain, where it converts arginine into agmatine, supporting brain function. AGAT helps produce creatine from arginine, supplying quick energy to muscles. Conversely, ARG and iNOS are mainly found in immune cells, especially myeloid-lineage cells like macrophages and DCs.(Wu et al., 2009)

1.3.3 Nitric Oxide Synthase (NOS)

<u>Overview of NOS Catalysis and Domain Architecture</u>

NOS are crucial enzymes responsible for producing NO, a lipophilic gaseous molecule that shows antimicrobial function in the immune system. As a cytosolic oxidoreductase, a type of enzyme that catalyzes the transfer of electrons from one molecule (the reductant) to another (the oxidant), NOS uses electrons from NADPH and oxygen to convert L-arginine into L-citrulline and NO.(Nathan & Xie, 1994) Successful catalytic activity of NOS depends on two key requirements: (1) availability of cofactors, and (2) dimerization to form a homodimer. The cofactors involved include flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), heme, tetrahydrobiopterin (BH4), and calmodulin (CaM). Each monomer has two distinct domains separated by a CaM binding region: the C-terminal reductase domain and the N-terminal oxygenase domain. CaM has an EF-hand domain that binds calcium ions (Ca2+). This calcium binding induces a conformational change in CaM, exposing a hydrophobic region that facilitates its binding to the oxygenase domain of NOS. When CaM binds, NOS undergoes structural transformations, resulting in the formation of a functional NOS homodimer.(Campbell et al., 2014; Gachhui et al., 1997; Xia et al., 2009) (Figure 1.4) Dimerization (also referred to as 'coupled' NOS) promotes the oxygenase domains of each monomer of NOS to interact with each other and creates a head-to-head alignment. Additionally, dimerization-mediated oxygenase interface provides structural flexibility for NOS' substrate (L-arginine) and cofactors (heme and BH4) to bind to NOS. In line with this, studies report that the presence of H4B and L-arginine, which bind within the oxygenase domain, further stabilizes the dimerization process of NOS monomers.(Smith et al., 2013) (Figure 1.4)

Upon the formation of a functioning homodimer, electrons are obtained from NADPH and begin to be transferred to FAD, then to FMN, within the reductase domain. What makes the NOS reaction interesting is that it demonstrates trans-domain electron transfer, meaning that electrons are transferred from FMN to the heme in the oxygenase domain of the opposite monomeric domain. (Figure 1.4) This reduced heme plays a crucial role in NOS activity, as it facilitates the binding and activation of molecular oxygen within the oxygenase domain of the dimer, subsequently helping to hydroxylate L-arginine to form the intermediate product Nω-hydroxy-L-arginine (NOHA). NOHA then reacts with the activated oxygen species at the heme site, resulting in its oxidation and the production of L-citrulline and NO.(Beckman & Koppenol, 1996; Doman et al., 2022) (Figure 1.5)

Mammals possess three isoforms of nitric oxide synthases (NOS): neuronal NOS (NOS1;nNOS), inducible NOS (NOS2; iNOS), and endothelial NOS (NOS3; eNOS). The nomenclature of each isoform corresponds to the cell type in which it was initially identified.(Daff, 2010; MacMicking et al., 1997) NOS enzymes can be classified into two groups based on their expression levels and calcium dependency for catalytic activity. As a constitutively and minimally expressed enzyme, nNOS and eNOS require a stable calcium concentration for efficient Ca2+-Calmodulin-mediated dimerization.(Lin et al., 2000; McMurry et al., 2011) Conversely, inducible NOS (identified initially in macrophages) exhibits a higher affinity for CaM, rendering its activity unaffected by low intracellular Ca2+ levels once CaM is bound.(Jordan et al., 1995; S.-J. Lee et al., 2001) Furthermore, iNOS remains active post-expression as long as co-factors and substrate availability are maintained, due to its independence from calcium.(S.-J. Lee et al., 2001) Additionally, iNOS can produce nitric oxide at levels up to 1000 times greater than e/nNOS.(Daff, 2010; Nagpal et al., 2013)

NO-mediated cellular modifications

NO is an excellent example of a pleiotropic gas, which means that this gaseous molecule can simultaneously influence several different biological functions or traits within an organism. For example, NO can induce toxic effects or help manage cellular oxidative damage, depending on its concentration and its interaction with other molecules. To explain, NO alone is less reactive compared to other highly reactive oxygen species, but the oxidized form of NO can become reactive nitrogen species (RNS), such as peroxynitrite, causing PTMs on various macromolecules(Beckman & Koppenol, 1996). (Figure 1.6) The two major NO-driven PTMs are nitrosylation and nitration. One of the NO species, the nitrosonium ion (NO+) not only binds to metal groups such as ferrious ion, cupper, zinc (M-nitrosylation), but also it rapidly binds to thiol groups of proteins (S-NO; cysteine-nitrosylation) (Kim, 2011). (Figure 1.6) Nitrosylated protein cysteine thiols can form sulfenic, sulfinic, or sulfonic acid, protein intra- or intermolecular disulfide bridges(Parí et al., 2013). Also, nitrosylated proteins can transfer the nitrosonium group to other proteins (trans-nitrosylation). Among the known S-nitrosylation target proteins, a glycolytic enzyme Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) can trans-nitrosylate other proteins such as sirtuin-1 (nuclear type lll histone deacetylase), histone deacetylase (HDAC2), and DNA-activated protein kinase (DNA-PK)(Kornberg et al., 2010). A mass spectrometric study in various mouse tissues revealed that many metabolic enzymes are S-nitrosylated by NO(Doulias et al., 2013). The nitrosylation protein modifications have important regulatory functions in a number of cellular processes, because the addition of NO+ group alters the protein activities. However, it remains unclear the specific cellular conditions under which metabolic enzymes become nitrosylated and whether the nitrosylated enzymes contribute to metabolic switch in DCs (Doulias et al., 2013; Koshiishi et al., 2007).

Besides S-nitrosylation cellular modification, NO can also react with superoxide anion (O2•-) to produce a potent reactive oxygen species, peroxynitrite (ONOO-). ONOO- interacts with several amino acids such as tyrosine (Y), tryptophan (W), cysteine (C), and methionine (M) and adds a nitro group (-NO2) to them. This introduction of a nitro group to a protein is called nitration(Brown, 2001). (Figure 1.6) Nitrotyrosine has been widely studied, and the nitrotyrosine on a protein is found to elicit either gain or loss of protein function. The production of superoxide is regulated by antioxidant enzymes, such as superoxide dismutase (SOD), under normal conditions; however, the level of superoxide often exceeds the removal capacity of SOD. Furthermore, SOD loose its activity when nitrosotyrosine is formed, leading to increased production of ONOO- (Yamakura & Kawasaki, 2010). Mitochondrial enzymes, aconitase, ATPase, and cytochrome c are also the target of nitrotyrosine modification. Unlike reversible S-nitrosylation, nitrotyrosine modification is long-lasting and protein degradation is a way to remove it. Therefore, a high amount of NO build-up in DCs proportionally enhances the chance of mitochondrial respiration disruption, consequently resulting in apoptosis (Cassina et al., 2000; Rafael Radi et al., 2002).

Studies have reported the regulation of iNOS via NO through either a negative or a positive feedback loop.(Graham et al., 2018; M. Lee & Choy, 2013) The NO's affinity towards metal ions makes the NOS enzyme itself a target of NO-mediated modification, as the cofactor heme sits within the oxygenase domain of NOS. NO-bound heme can inhibit the proper oxidation and electron transfer in NOS activity, leading to uncoupling of the dimer and promoting the generation of ROS such as superoxide anion.(Koshiishi et al., 2007) Other groups have found that the accumulation of NO can cause S-nitrosylation of the key transcription factor for iNOS expression, NF-κB, thereby reducing its capacity for DNA binding and leading to downregulation of the Nos2 transcript.(Koshiishi et al., 2007; Marshall et al., 2004) Some also argue that S-nitrosylation can promote the expression of iNOS by stimulating other signaling cascades, such as the Ras pathway.(M. Lee & Choy, 2013)

NO-mediated antimicrobial effects

Thanks to the cellular modifications mediated by NO mentioned earlier, NO plays a beneficial role for the host by demonstrating broad antimicrobial effects against various pathogens, including bacteria, fungi, viruses, and parasites.(J. Chen et al., 2021; Formaglio et al., 2021; Lisi et al., 2021; Okda et al., 2025) NO-driven reactive nitrogen species (RNS) can directly damage pathogen DNA, cause PTMs on essential proteins that disrupt cellular functions, or induce lipid peroxidation, compromising the integrity of pathogen cell membranes.(Beckman & Koppenol, 1996; Roberts et al., 2024a) Studies also indicate that in respiring bacteria, NO can interfere with the assembly of the FtsZ ring, a key structure for cytokinesis, by inactivating enzymes involved in purine nucleotide biosynthesis and the ETC.(Jones-Carson et al., 2020; Roberts et al., 2024a) Additionally, NO can disrupt bacterial quorum sensing (QS), a communication system that controls virulence and biofilm formation by either directly targeting QS regulator AgrA or by penetrating the biofilm matrix.(Roberts et al., 2024b; Urbano et al., 2018) As a result, there have been efforts to develop NO-releasing compounds as therapies, particularly to target antibiotic-resistant bacteria and other pathogens.(Kaplish et al., n.d.; Poh & Rice, 2022)

NO-producing DCs

As mentioned above, monocyte-derived DCs, also known as Tip-DCs, markedly increase iNOS expression during inflammatory responses,(Schmid et al., 2012; Serbina et al., 2003) while the other two isoforms remain minimally expressed by immune cells in these conditions. NO can convert into reactive nitrogen species (RNS), which can modify or inhibit various enzyme activities and functions and potentially cause mutations in genes.(Espey et al., 2002; Hess et al., 2005) Although NO's high reactivity might increase host-cell damage, it also acts as a powerful molecule for pathogen clearance. In mice infected with influenza viruses, increased pathology correlates with greater recruitment of DCs to the lungs. Additionally, the clearance of Listeria monocytogens (L. m.) is hindered when moDC/iDC recruitment to the spleen is blocked in mice.(Mitchell et al., 2011; Neuenhahn et al., 2006) These in vivo studies emphasize the crucial protective role of NO in host immune defense and demonstrate the importance of NO-producing DCs during infection. Bone marrow-derived dendritic cells (BMDCs), differentiated using granulocyte-macrophage colony-stimulating factor (GM-CSF), are a valuable in vitro model for representing monocyte-derived dendritic cells (moDCs) in inflammatory conditions conditions.(Jones et al., 2017) Furthermore, growing evidence suggests a close link between NO and the metabolic reprogramming of DCs during inflammation.

1.4 Hallmark of inflammatory DC activation

1.4.1 Acute metabolic programming

Immune cells increase their metabolism upon activation to support and perform their immune functions, such as chemokine and cytokine production, clonal expansion, and expression of cell membrane migratory molecules.(Chapman & Chi, 2022; Rehill et al., 2024; Viola et al., 2019) DCs are no exception to this increased metabolic feature of activated immune cells, as they exhibit increased glycolysis upon stimulation, characterized by an initial surge known as the "glycolytic burst."(Thwe et al., 2017) (Figure 1.7 left) This burst occurs within 4-6 hours after activation. LPS-triggered TLR activation initiates the TANK-binding kinase 1/Inhibitor-κB kinase ε (TBK1/IKKϵ) signaling pathway, which upregulates pro-inflammatory and glycolytic genes, including Aconitate decarboxylase (a TCA cycle enzyme converting cis-aconitate to itaconate) and the mitochondrial citrate carrier (solute carrier family 25 member 1; Slc25a1). In the cytosol, newly imported glucose becomes phosphorylated to glucose-6-phosphate (G6P), which has multiple fates.(Everts et al., 2014a; Kierans & Taylor, 2024; Kornberg et al., 2010)

Initially, G6P is metabolized through a series of glycolytic steps to produce adenosine triphosphate (ATP), the primary energy currency of the cell. Pyruvate, resulting from this process, either converts into lactate within the cytosol or translocates into the mitochondria to form acetyl-CoA, which then enters the tricarboxylic acid (TCA) cycle.(Kierans & Taylor, 2024; Pearce & Everts, 2015) (Figure 1.7 left) During the TCA cycle, the carbons derived from acetyl-CoA contribute to the generation of reducing equivalents, namely nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH2), which subsequently donate electrons to the electron transport chain (ETC).(Choi et al., 2021) The ETC comprises a series of protein complexes situated in the inner mitochondrial membrane, responsible for establishing an electrochemical proton gradient by actively pumping protons across the membrane. The proper flow of electrons through the ETC depends critically on the presence of oxygen at its terminus, serving as the ultimate electron acceptor and being reduced to water. The electrochemical proton gradient thus generated provides the driving force for ATP synthase to catalyze the phosphorylation of adenosine diphosphate (ADP) into ATP. In conclusion, this cellular respiration process, which involves the reduction of oxygen concurrent with ADP phosphorylation, is known as oxidative phosphorylation (OXPHOS).(Choi et al., 2021) Moreover, studies reported that this enhanced glycolysis and elevated influx of pyruvate into the TCA cycle appear to serve more purposes than simply generating additional ATP via OXPHOS.(Martínez-Reyes & Chandel, 2020) One of the TCA cycle metabolites, citrate, can be exported into the cytosol via mitochondrial citrate shuttle protein, slc25a1, and is utilized for the fatty acid synthesis and fatty acid-derived signaling molecule generation (such as prostaglandins) process.(Everts et al., 2014b; O'Neill, 2011) (Figure 1.7 middle) It is speculated that the fatty acid synthesis is required during DC activation to enhance the capacity of both endoplasmic reticulum (ER) and Golgi, as DCs may undergo ER stress and unfolded protein response (UPR) as a result of increased protein synthesis, including chemokines/cytokines and co-stimulatory receptors. (Everts et al., 2014b; Williams & O'Neill, 2018)

G6P can also be rerouted within the cytosol into the pentose phosphate pathway (PPP), leading to the production of the reducing agent nicotinamide adenine dinucleotide phosphate (NADPH) and pentose sugars.(Pearce & Everts, 2015; Xu et al., 2022) (Figure 1.7 middle) NADPH plays a crucial role not only in iNOS activity and NADPH oxidases that generate reactive nitrogen species (RNS) and reactive oxygen species (ROS), but also in fatty acid synthesis, nucleotide production, and cellular antioxidant defenses.(Kong et al., 2010; Segal et al., 2012) Additionally, the pentose sugars formed serve as the backbone for de novo nucleotide synthesis, aiding in the increased gene transcription in DCs.(Mullen & Singh, 2023)

1.4.2 Sustained glycolysis and NO-mediated metabolic programming

Unlike the initial phase, the later phase of increased glycolysis, which transpires more than six hours after activation, is entirely dependent on the production of nitric oxide (NO) in these cells.(Everts et al., 2012) The protein iNOS expression can be detected in LPS-stimulated DCs around 4 – 5 hours post-activation, and it catalyzes the conversion of two molecules of O2 and L-arginine into L-citrulline and nitric oxide (NO). NO readily reacts with free iron (Fe2+), iron in iron–sulfur ([Fe-S]) centers, or iron within haemproteins.(Crack et al., 2013; Crack & Le Brun, 2019; Fitzpatrick & Kim, 2015) These [Fe-S] centers are found in essential enzymes, such as the TCA cycle enzyme Aconitase and the ETC Complexes I and II.(Palmieri et al., 2020; Read et al., 2021) NO also reversibly inhibits ETC Complex IV (Cytochrome c oxidase) by blocking its oxygen binding site due to its structural similarity to O2.(Alderton et al., 2001) As NO levels increase, the inhibition becomes irreversible, and NO transforms into a more reactive nitrogen species (RNS) (Figure 1.7 right). This ETC blockade causes the cell to reroute carbon flux, preventing acetyl-CoA from entering the TCA cycle, thereby shifting energy production to glycolysis even in the presence of oxygen—a process known as aerobic glycolysis.(Everts et al., 2012) In addition to ETC complexes, enzymes of the TCA cycle are also targets of NO and RNS. These metabolic enzymes undergo NO-driven post-translational modifications (PTMs) following chemical reactions with RNS, as detailed below.(Bailey et al., 2019; Doulias et al., 2013) It is hypothesized that these PTMs induce changes in TCA cycle flux within activated DCs. Collectively, the accumulation of NO triggered by Toll-like receptor (TLR) activation results in the inhibition of ETC and the disruption of mitochondrial respiration (oxidative phosphorylation, OXPHOS). As a result, DCs adapt by sustaining a high rate of aerobic glycolysis to meet their energy (ATP) production and biosynthetic requirements. One of the outcomes of sustained increased glycolysis is a low cellular pH due to the build-up of lactic acid, and a reduction in the cellular ATP/ADP ratio, which can alert the cell to its nutrient-requiring state.(Kozlov et al., 2020; Saedeleer et al., 2012) (Figure 1.7 right)

The NO-mediated stalling of ETC and low ATP levels activate the phosphoinositide 3-kinases/protein kinase B (PI3K/Akt) signaling axis, which includes the mammalian target of rapamycin (mTOR) and hypoxia-inducible factor alpha (HIF1α) signaling pathway.(Perrin-Cocon et al., 2018) To clarify, increased cellular energy demand and limited nutrient availability stimulate the protein kinase mTOR, which plays a vital role in regulating cell growth and metabolism.(Snyder & Amiel, 2019) This activation leads to the increased expression and activity of the transcription factor hypoxia-inducible factor 1-alpha (HIF1α). Upon activation, HIF1α translocates into the nucleus and binds to the hypoxia response element (HRE). This interaction subsequently initiates the transcription of genes that enable cells to adapt to nutrient deficiency and/or a hypoxic cellular environment. (Corcoran & O'Neill, n.d.) (Figure 1.7 right)

1.5 Inducible-NOS regulation in DCs

Divergent Genetic Regulation of iNOS

Animal models are essential tools in biomedical research, with mice being the most common. The C57BL/6 (B6) strain is one of the earliest established inbred laboratory strains, created through sibling mating over at least 20 generations in a sterile environment. This process confers advantages such as easy breeding and genetic consistency, making B6 ideal for in vivo studies in areas like Alzheimer's disease, atherosclerosis, infectious diseases, inflammation, and metabolic disorders. Although many human diseases and drugs have been developed using these inbred mice, models incorporating greater genetic diversity can offer more relevance to human conditions. Wild-derived mice are crucial for exploring varied genetic backgrounds in a controlled way. In this context, researchers attempted to develop a "wildling" B6 model by transferring B6 embryos into wild mice, aiming to create a model that better mimics human genetics while retaining the benefits of the B6 strain.(Rosshart et al., 2019) Thus, we conducted a study comparing immune responses in different mouse strains to better understand how genetic variation influences NO production in DCs.

The commonly used B6 strain exhibits high NO levels and rapid NO production, possibly due to the artificial selection and identical genetic background, which may not accurately represent human immunity.(Anderson et al., 2021) The wild-derived PWD strain, with greater genetic diversity and closer similarities to human NOS2, produces significantly less NO upon stimulation, correlating with lower Nos2 gene and iNOS protein expression. Experiments with the consomic model—including a chromosome 11 segment from PWD in B6 genetic background—reveal that NO production and mitochondrial activity differ notably between strains, affecting cellular metabolism and antimicrobial effectiveness.(Snyder et al., 2022) To briefly explain, B6 iNOS produced higher levels of NO than 11.2's at a low dose of LPS (~10 ng/mL). This difference in NO production caused B6 DCs' mitochondrial respiration to shut down, while 11.2 DCs' respiration remained intact. The altered respiratory status was associated with reduced DC survival and lifespan; however, higher NO levels in 11.2 DC showed better control of the intracellular pathogen *Listeria monocytogenes* replication.(Snyder et al., 2022) Overall, the findings demonstrate that natural genetic diversity has a significant impact on NO regulation, mitochondrial function, cell survival, and pathogen defense. Our results from 11.2 strongly suggest a concept of NO-threshold mitochondrial respiration inhibition. The PWD strain emerges as a more physiologically relevant model for human immune responses, emphasizing the importance of considering genetic variation in immunology research to better translate findings from mice to humans.(Snyder et al., 2022)

Regulation of iNOS via metabolite UDP-glucose

After confirming the genetic regulation of iNOS activity with corresponding NO levels and concentrations—parameters linked to host protection and cytotoxic responses—we further investigated the threshold for NO-mediated inhibition of mitochondrial respiration. We aimed to examine the regulation of iNOS activity and mitochondrial respiration in BMDCs, using uridine diphosphate glucose (UDPG), a known iNOS-regulating metabolite involved in glycogen synthesis and a ligand for the purinergic receptor P2Y14.(Ma et al., 2020) Although UDPG does not significantly alter inflammatory gene expression at high LPS doses, it exhibits a minor activating effect, increasing certain cytokines and T cell activation markers without affecting NO production or mitochondrial respiration under these conditions.(Vagher et al., 2025) However, at lower LPS doses, UDPG enhances NO production and iNOS-linked OCR, especially near the threshold where NO begins to inhibit mitochondrial function. Blocking P2Y14 with a specific inhibitor reduces pro-inflammatory cytokine expression and restores mitochondrial function, indicating that UDPG/P2Y14 signaling actively promotes inflammation and metabolic reprogramming in response to low-dose LPS.(Vagher et al., 2025) Long-term P2Y14 inhibition decreases NO levels and improves cell survival, without significantly affecting DC activation markers such as CD40 and CD86. Overall, the findings reveal a novel role for UDPG/P2Y14 signaling in modulating the mitochondrial respiration threshold and inflammatory responses, especially under conditions where NO begins to impact cellular metabolism. This highlights the significance of endogenous metabolic signaling pathways in regulating immune cells, suggesting potential therapeutic targets for controlling inflammation.(Vagher et al., 2025)

1.6 Arginases

1.6.1 Arginase mechanism of action and its roles

Alongside UDPG, a growing number of studies have revealed the influence of metabolites on the immune system.(Kelly & Pearce, 2020; Q. Li & Hoppe, 2023) In mammals, arginine metabolism by Arginase (ARG), in addition to iNOS, is strongly associated with chronic inflammation and immune dysregulation. This linkage includes conditions such as cancer, autoimmunity, allergic lung asthma, and infectious diseases.(Das et al., 2010; Grzywa et al., 2020) ARG is an enzyme that hydrolyzes L-arginine into L-ornithine and urea. It functions as a homotrimer, composed of three identical subunits, each containing an active site where L-arginine binds and the catalytic reaction occurs. Each active site features a binuclear manganese cluster (two Mn2+ ions) as a cofactor. These manganese ions coordinate with water molecules, positioning and stabilizing water to serve as a nucleophile, allowing it to attack L-arginine and hydrolyze it. (Bansal & Ochoa, 2003)

ARG has two main roles: (1) Ammonia detoxification, where it catalyzes the final step of the urea cycle, removing toxic ammonia produced during protein breakdown; and (2) ornithine regeneration.(Canè et al., 2025) Although ornithine is not incorporated into proteins, it is vital for many metabolic processes. In the urea cycle, ornithine produced by ARG enters mitochondria, binds with carbamoyl-phosphate, and helps produce L-citrulline. The L-citrulline then translocates to the cytosol and can be recycled back into L-arginine, completing the arginine-ornithine-citrulline cycle. Additionally, ornithine is a precursor for synthesizing polyamines, including putrescine, spermidine, and spermine, which are crucial metabolites for cell growth, differentiation, and immune function. (Luiking et al., 2009; Wijnands et al., 2015)

1.6.2 Isoforms and expression of Arginase

There are two types of ARG isoforms: cytosolic ARG1 and mitochondrial ARG2. Although both isoforms exhibit identical catalytic activity, they differ in their enzymatic functions and physiological roles, which depend on the cell types and conditions in which they are expressed.(Canè et al., 2025; Z. Li et al., 2022) ARG1 is predominantly expressed in hepatocytes owing to its integral association with the urea cycle, given that the liver serves as the primary organ responsible for detoxification and urea synthesis. Nonetheless, ARG1 expression has also been documented in myeloid cells, including polymorphonuclear neutrophils (PMNs), macrophages, and myeloid-derived suppressor cells (MDSCs).(Canè et al., 2025; Das et al., 2010) Additionally, in murine myeloid studies, Arg1 induction is observed in response to type-2 immune response (Th2)- related cytokines, such as IL-4 and IL-13. These cells are present within the tumor microenvironment and are implicated in suppressing the immune response. Conversely, mitochondrial ARG2 is highly expressed in the kidney and other extrahepatic cell types, including macrophages, endothelial cells, tubular epithelial cells, and specific T cell subsets.(Caldwell et al., 2018, p. 2; Munder et al., 1999)

1.6.3 Role of Arginase in Tolerogenic Immunity

Arginase plays a key role in shaping adaptive immune responses, primarily by reducing L-arginine availability, an essential nutrient for T-cell activity.(Dunand-Sauthier et al., 2014; Rodriguez et al., 2017) When myeloid cells deplete arginine via arginase, T cell responses are significantly suppressed, as this limits T cell proliferation and cytokine production. This process contributes to immunosuppression in chronic inflammation and tumor growth. Removing Arg2 in CD8+ T cells has been shown to boost anti-tumor immunity and slow tumor progression.(Líndez et al., 2020) Additionally, ARG2 expression in human regulatory T cells (Tregs) supports the idea that ARG enhances immunosuppressive functions and helps maintain metabolic resilience, further reinforcing its role in immune regulation.(Lowe et al., n.d.) Besides T-cells, studies have also reported that increasing Arg1 in DCs correlates with a tolerogenic phenotype. (Domogalla et al., 2017; Simioni et al., 2017)

Tolerogenic dendritic cells are a specialized subset of DCs that encourage antigen-specific tolerance rather than causing inflammation.(Domogalla et al., 2017) They mainly work by suppressing effector T cell responses and aiding the development of T-reg cells. This makes tolerogenic DCs vital for maintaining immune system balance and preventing autoimmune diseases. Unlike immature DCs, which rely on weak co-stimulatory signals to induce tolerance, tolerogenic DCs have active mechanisms, such as expressing surface inhibitory molecules and releasing anti-inflammatory cytokines, allowing for more effective immunosuppression.(Khoshnejad et al., 2020; van Wigcheren et al., 2023) ARG1 is a key enzyme expressed by tolerogenic DCs, playing a role in their suppressive function. For instance, all-trans retinoic acid (ATRA) significantly increases Arg1 in BMDCs, leading to decreased T cell stimulation and direct inhibition of T cell expansion. The suppressive effect of ATRA-treated DCs is partly reversed by Arg1 inhibitors, highlighting Arg1's importance.(Bhatt et al., 2014) Similarly, lactoferrin (LF) treatment reduces T cell proliferation by BMDCs (LF-BMDCs) and promotes Foxp3+ Treg differentiation, with increased Arg1 levels observed in these cells.(Park et al., 2020) The suppressive activity of LF-BMDCs can also be partially restored by Arg1 inhibitors, supporting its role in their tolerogenic effects.(Park et al., 2020) Additionally, ectopic expression of interleukin-35 (IL-35) in mouse splenic DCs induces Arg1(Panfili et al., 2019), giving them an immunosuppressive phenotype in vivo driven by Arg1 activity. Collectively, these findings demonstrate that Arg1 expression and activity are hallmarks of tolerogenic DCs, essential for their ability to modulate immune responses. Although the connection between ARG and immune tolerance has been established, further research is needed to clarify the mechanisms and define this particular tolerogenic subtype of DCs. Research on macrophages, which share the same myeloid lineage as DCs, has helped shed light on ARG's regulatory role in inflammation.(Munder et al.,

1999) These cells can express both iNOS and ARG, exhibiting more distinctive inflammatory responses, either anti-inflammatory or pro-inflammatory, depending on the stimulus.(Y. Liu et al., 2021)

1.6.4 Regulation of iNOS by ARG in macrophages

Monocyte-derived macrophages (M0) can be activated and differentiate into either pro-inflammatory M1 or anti-inflammatory M2 (alternatively activated) types. Although their expression levels vary, both M1 and M2 use L-arginine via two main enzymes: iNOS and ARG. These enzymes compete for the same L-arginine substrate, influencing the macrophage's functional state and role in immune responses.(Boucher et al., 1999; S. Chen et al., 2023) The balance between iNOS and arginase activity is crucial in determining whether macrophages adopt a pro-inflammatory (M1) or anti-inflammatory/wound-healing (M2) phenotype. Specifically, Type-1 (Th1) cytokines like interferon-gamma or LPS stimulation primarily induce iNOS expression, while Th2 cytokines such as IL-4 and IL-10 strongly upregulate ARG1 and ARG2. The co-regulation of iNOS and arginase involves separate transcriptional pathways, and their crosstalk has been extensively studied.(S. Chen et al., 2023; Munder et al., 1999) In LPS-activated RAW 264.7 cells, Arg2 activity increases proportionally with iNOS.(Dowling et al., 2021) In rats, high iNOS expression and activity, coupled with low Arg1 activity in peritoneal macrophages, help resist Toxoplasma gondii by producing NO and maintaining low polyamine levels.(Z. Li et al., 2012) Conversely, mouse macrophages, which are more prone to T. gondii, show low iNOS and high Arg1 levels.(El Kasmi et al., 2008) Inhibiting arginase in mouse macrophages enhances NO production, reducing T. gondii infection and growth.(El Kasmi et al., 2008) This highlights the importance of substrate competition between iNOS and arginase in controlling T. gondii proliferation via NO levels. Owing to the shared myeloid lineage and similar innate immune functions between monocyte-derived macrophages and DCs(Anderson et al., 2021), our group hypothesized that iNOS is partially regulated by Arginase via substrate competition in DCs.

DISSERTATION OVERVIEW AND CONCLUDING REMARKS

This dissertation investigates the intricate mechanisms that regulate immune responses, emphasizing the pivotal role of DCs and the significant impact of L-arginine metabolism on their function and cellular reprogramming during inflammation.(Rodriguez et al., 2017) Inflammatory DCs, known as Tip-DCs, are early responders and regulators of inflammation and critically control certain bacterial infections. LPS stimulation activates TLR4 signaling pathways, leading to NF-κB activation, a key transcription factor that induces inflammatory gene expression, including iNOS.(Shen et al., 2008) Since NO has two counteracting roles— self-cytotoxic and antimicrobial/protective, the regulation and balance of its production is a critical determination point in immune cellular fate. Our group and others have demonstrated that NO, when produced at certain levels, can inhibit mitochondrial respiration in DCs in BMDCs, a compelling in vitro model for Tip-DCs.(Bailey et al., 2019; Serbina et al., 2003) Having previously identified that there is a delicately regulated NO threshold that controls NO-mediated respiration inhibition, the work of this dissertation expands on this framework by introducing a regulatory checkpoint of this axis via arginine substrate competition.(Snyder et al., 2022; Vagher et al., 2025) The next chapter will examine how arginase, another crucial enzyme that breaks down L-arginine and competes with iNOS for the same substrate, regulates NO production and influences the NO-respiration threshold in BMDCs. Understanding the interplay between L-arginine metabolism and the competitive dynamics involving iNOS provides deeper insights into DC activation, function, and survival in inflammatory settings.

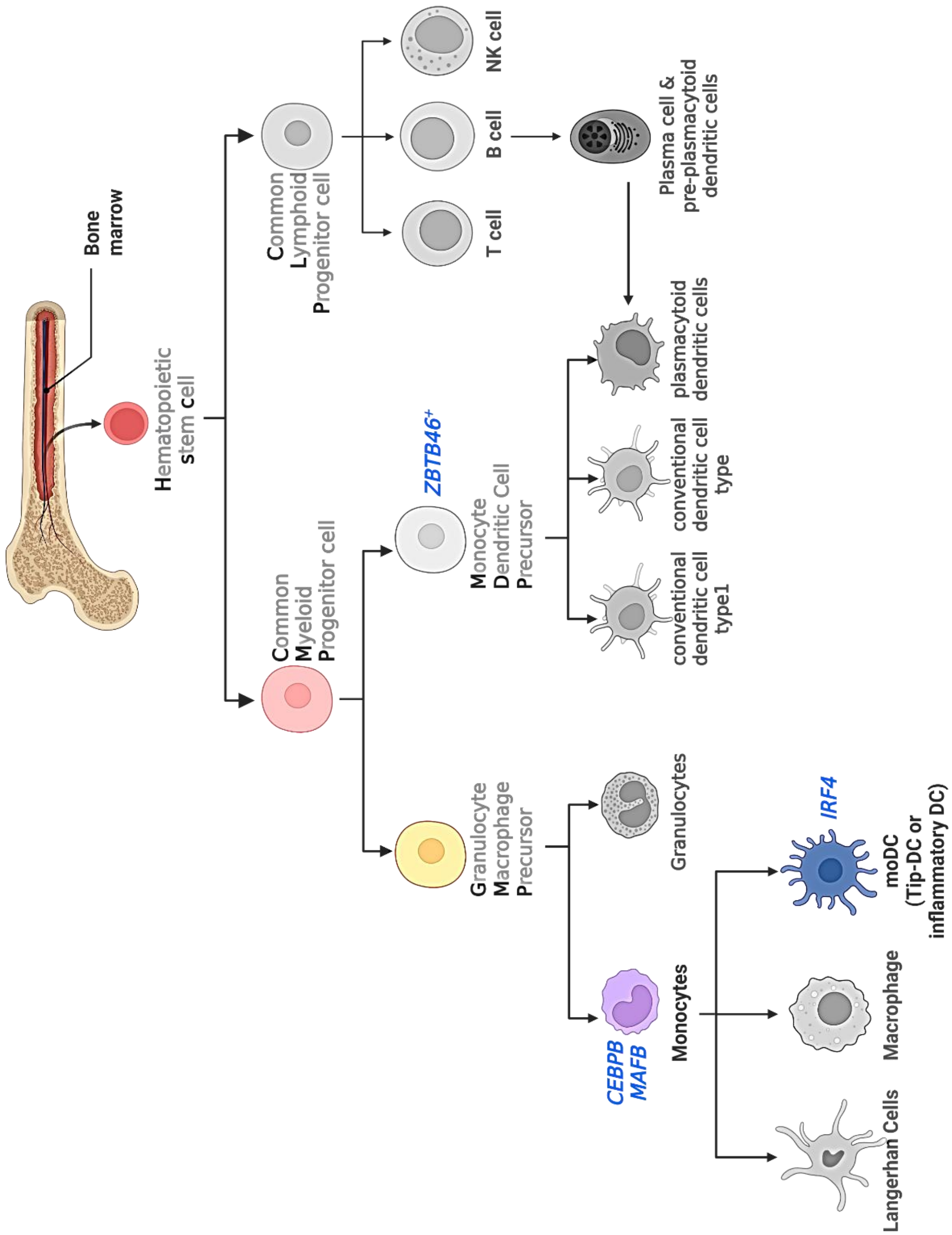

Figure 1. 1 Immune Cell Lineage Chart.

Monocyte-derived dendritic cell (moDC) maturation (color). Blue indicates known transcription gene markers. Created with BioRender.com & Adapted from Anderson et al. 2021 Nature Reviews Immunology & Satpathy et al. 2012 Nature Immunology

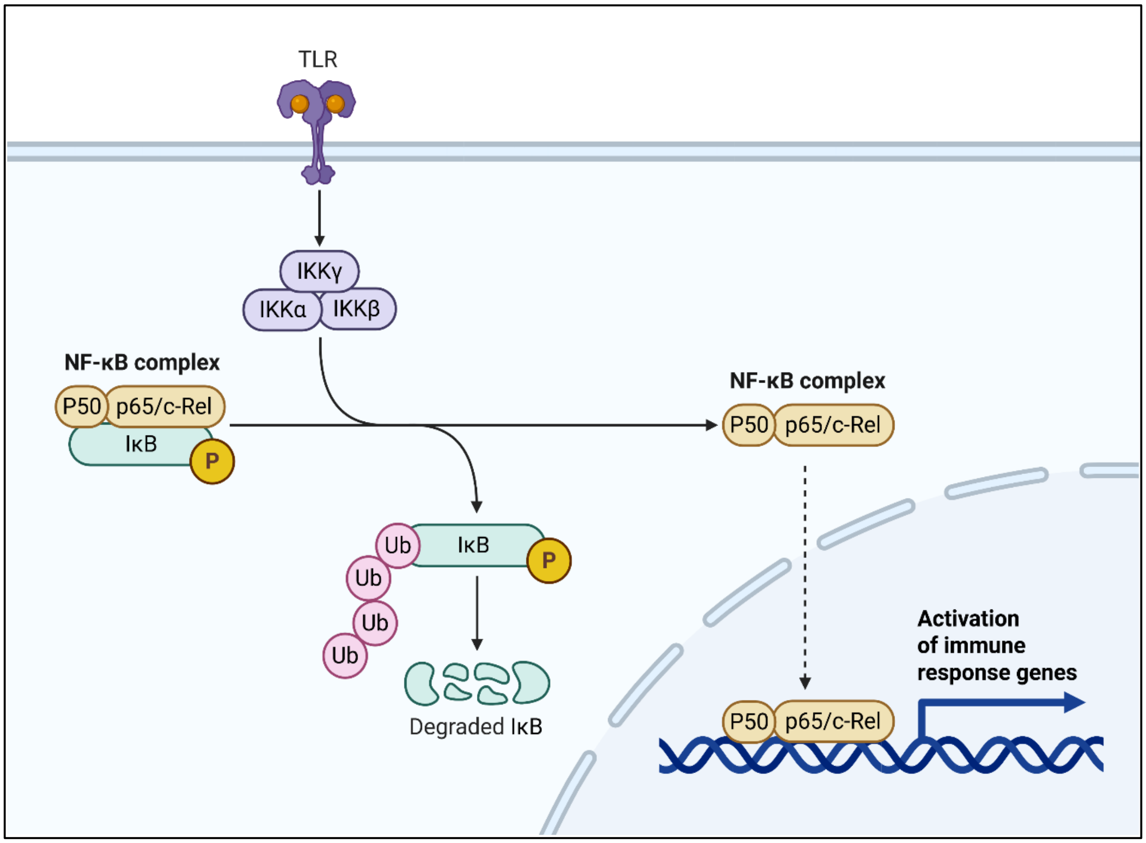

Figure 1. 2 TLR stimulation-mediated canonical signaling cascade.

TLR4 (Toll-like receptor 4) signaling activates the NF-κB (nuclear factor kappa B) pathway, a crucial process in the innate immune response and inflammation. This pathway leads to the production of pro-inflammatory cytokines, which help the body fight infection, but can also contribute to various diseases if dysregulated. The figure was created using a template from BioRender.com

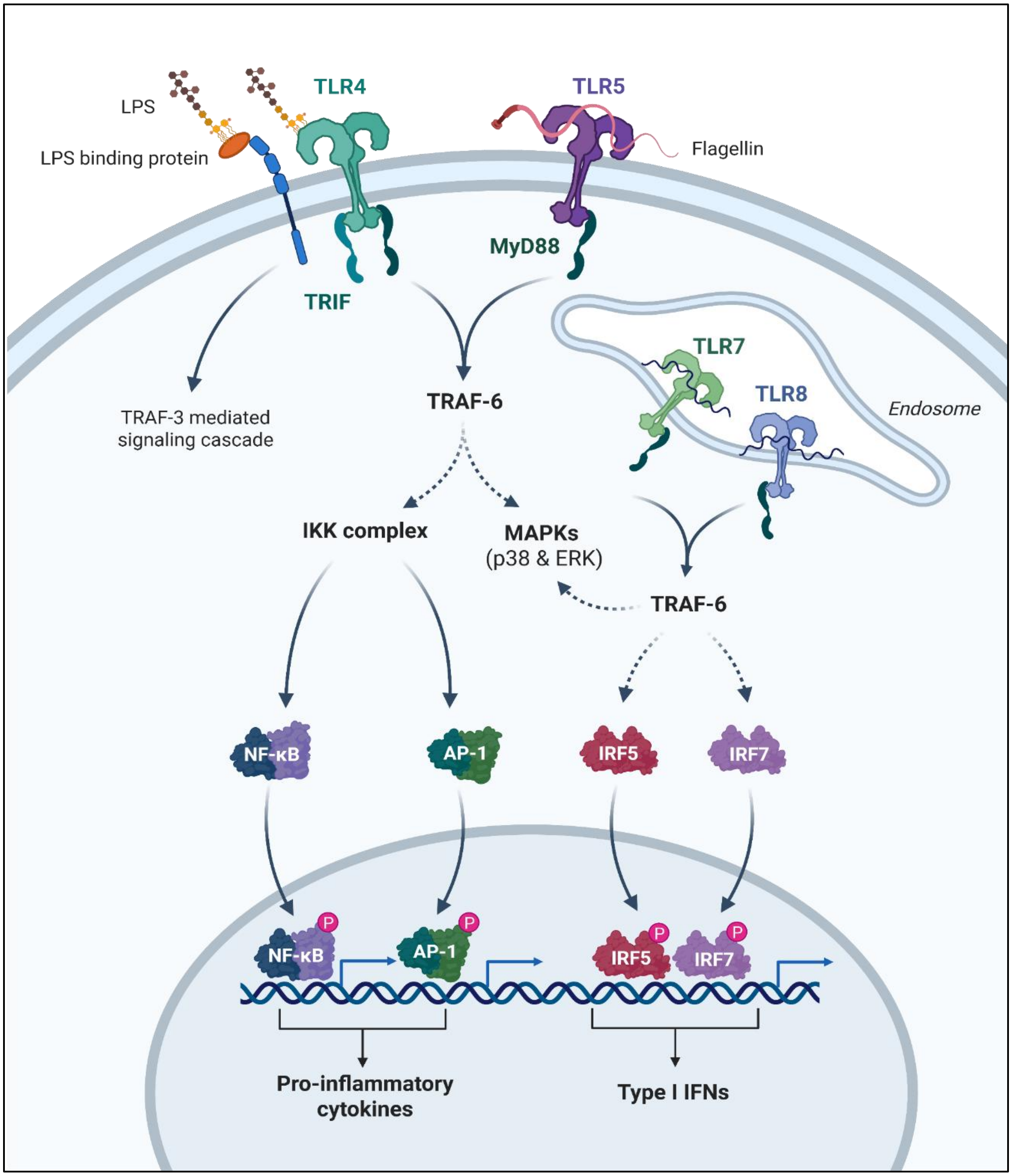

Figure 1. 3 Synergistic effects of TRIF and MyD88-driven signaling pathways.

MyD88 and TRIF, key signaling molecules in the Toll-like receptor (TLR) pathway, can exhibit synergistic effects when activated together, leading to enhanced immune responses, particularly in cytokine production. This synergy is not simply additive; it involves complex intracellular interactions and is influenced by the timing and dosage of TLR agonist stimulation. The figure was created using a template from BioRender.com

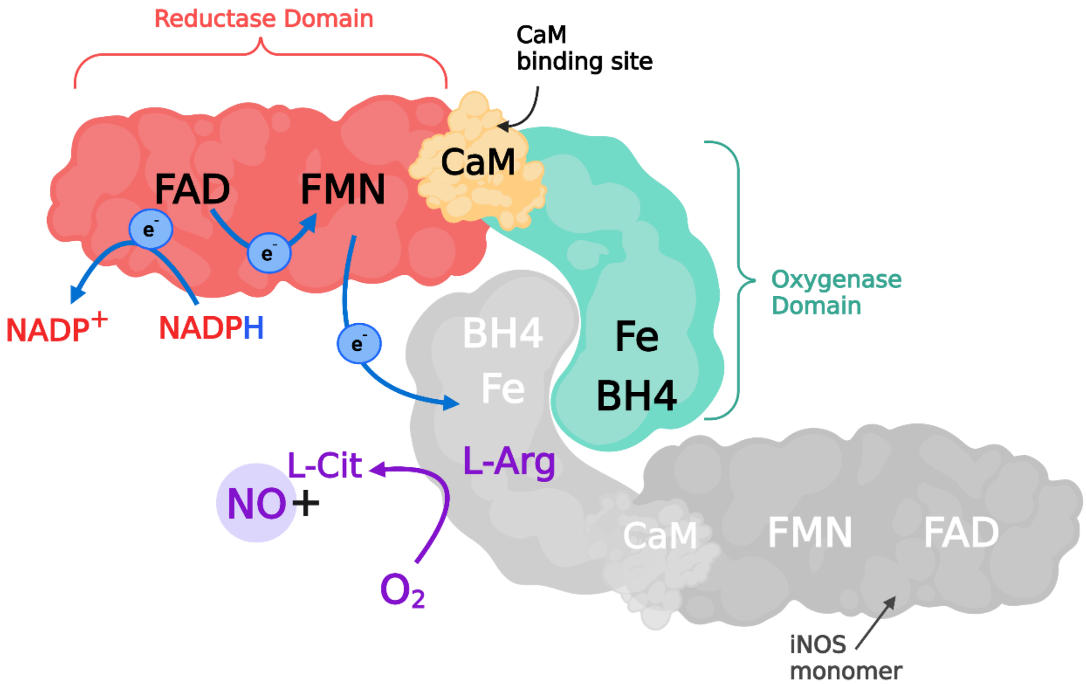

Figure 1. 4 Structures and Mechanisms of Nitric Oxide Synthase (iNOS)

Illustrating the dimerized form of inducible Nitric Oxide Synthase structure. Each monomer contains a reductase domain, a calmodulin (CaM) binding site, and an oxygenase domain. Cofactors are included nicotinamide adenine dinucleotide phosphate (NADPH; electron donor), flavin adenine dinucleotide (FAD), Flavin mononucleotide (FMN), Heme (containing Fe), and tetrahydrobiopterin (BH4). Created with BioRender.com & the figure was adapted from Doman et al. 2022 Bioorganic & Medicinal Chemistry

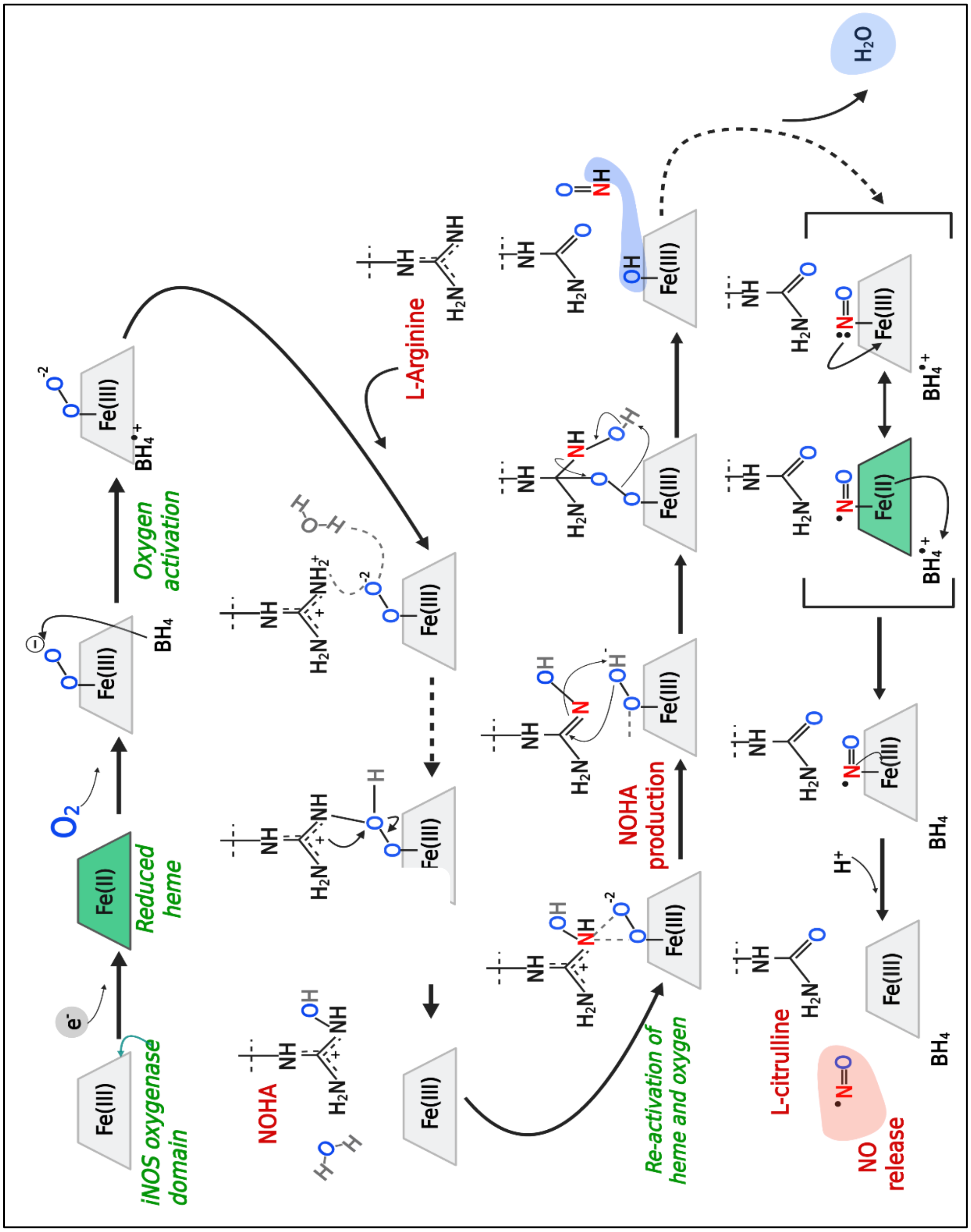

Figure 1. 5 iNOS oxygenase domain: Mechanism of Action (detail)

The overall reaction involves a series of electron transfers from NADPH through the flavins (FAD and FMN) to the heme group, which then activates molecular oxygen. The heme group, in the presence of BH4, then facilitates the two-step oxidation of L-arginine, generating the intermediate Nω-hydroxy-L-arginine (L-NOHA) and then ultimately, L-citrulline and NO. Figure was generated with BioRender.com

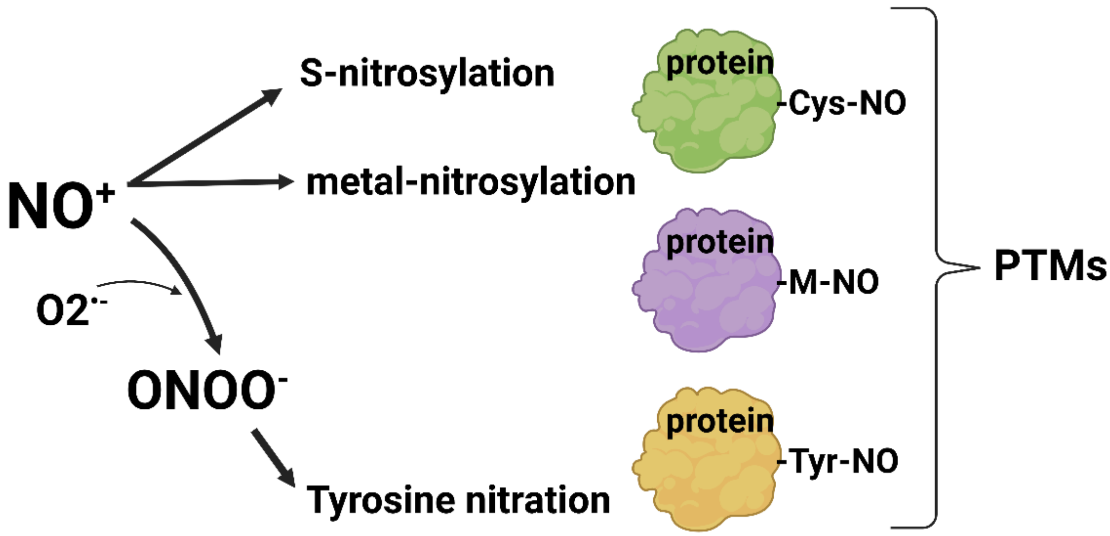

Figure 1. 6 Nitric Oxide-mediated Post-Translational Modifications.

Nitrosylation and nitration are two distinct post-translational modifications (PTMs) of proteins, both involving NO, but differing in the specific chemical groups added. Nitrosylation primarily involves the addition of a nitrosyl group (NO) to a molecule, often a thiol group (-SH) of cysteine residues, forming S-nitrosothiols (SNOs). Nitration, on the other hand, involves the addition of a nitro group (-NO2) to molecules, commonly tyrosine or tryptophan residues in proteins. Figure was created using BioRender.com

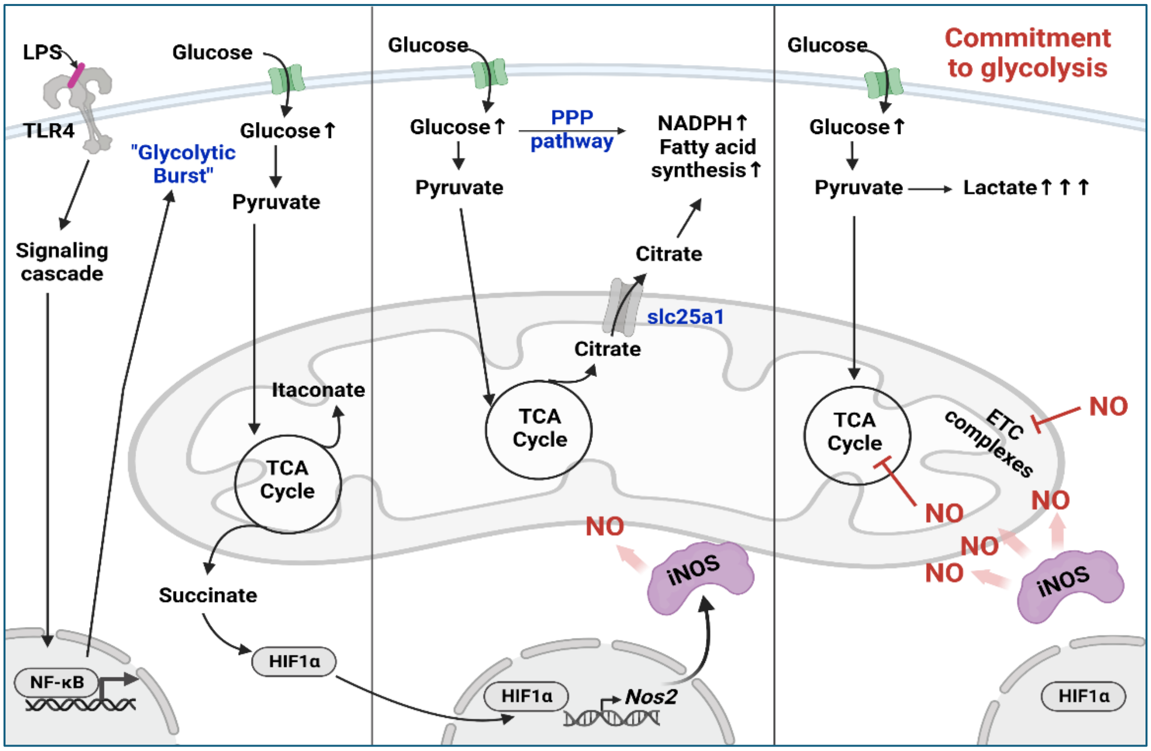

Figure 1. 7 Initial, Intermediary, and Late-phase of NO-mediated metabolic remodeling.

<u>Left panel (Initial phase)</u>: Upon TLR4 activation, DCs show increased glycolysis (this phenomena is referred to as "glycolytic burst"), while upregulating pro-inflammatory genes, including *Nos2*, via canonical signaling pathways, such as NFkB-mediated transcription.
<u>Middle panel (Intermediary phase)</u>: As elevated glycolytic rate reaches the maximal capacity, one of the TCA cycle metabolite citrate can be transported to cytosol, subsequently aiding in fatty acid synthesis process. In addition, newly imported glucose is shuted to penpose phosphate pathway (PPP) to produce NADPH and nucleotide building blocks.
<u>Right panel (Late phase)</u>: As iNOS-produced NO builds up in the system, it elicits mitochondrial respiration inhibition, leading DCs to commit to aerobic glycolysis. Metabolic rewiring in DCs refers the phenomena when DCs utilize metabolic pathways (carbon flux), as cellular respiratory is blocked by NO.

Figure was created using BioRender.com

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CHAPTER 2: L-ARGININE SUBSTRATE COMPETITION BETWEEN INDUCIBLE NITRIC OXIDE SYNTHASE AND ARGINASE REGULATES NO-MEDIATED INHIBITION OF MITOCHONDRIAL RESPIRATION IN DENDRITIC CELLS.

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ABSTRACT

L-Arginine metabolism plays a crucial role in regulating the metabolic state and function of immune cells, significantly influencing the inflammatory responses within tissue microenvironments. The competition for substrates between two primary L-arginine catabolizing enzymes, inducible nitric oxide synthase (iNOS) and arginase (Arg), represents a key metabolic regulatory axis in myeloid cells. Although the interplay between NOS and Arg during the inflammatory polarization of mouse macrophages is well-researched, there exists a crucial knowledge gap regarding how this regulatory axis affects NO-mediated respiratory suppression. Our study explores the hypothesis that inflammatory dendritic cells (DCs) influence iNOS-dependent nitric oxide (NO) production through substrate competition with Arg. By utilizing murine bone marrow-derived DCs (BMDCs) as a model for iNOS-producing inflammatory DCs, we highlight the impact of arginine competition on the expression of vital metabolic enzymes and on metrics of cellular respiration, host cell viability, and the phagocytic control of the intracellular pathogen Listeria monocytogenes replication. We demonstrate that the cytosolic isoform of arginase (Arg1) is co-expressed with iNOS, and pharmacological inhibition of Arg1 activity results in increased iNOS expression and enhanced NO production, which in turn increases NO-mediated suppression of mitochondrial respiration under low LPS-stimulating conditions. Additionally, inhibiting ARG1/iNOS competition accelerates NO-mediated cell death and enhances the NO-mediated control of Listeria monocytogenes infection and replication. These results reveal the role of innate arginine substrate competition as a regulatory mechanism of DC metabolism and function. This research offers new mechanistic insights into the delicate regulatory pathways that uphold cellular homeostasis during the innate immune response to microbial challenges.

INTRODUCTION

In recent years, there has been heightened awareness of the critical regulatory role that metabolic processes play in cellular immune responses. Research into how the immune system interacts with metabolic processes has shown that metabolic changes linked to activation in immune cells not only support cellular energy production but also influence the inflammatory state and functionality of cells in both the innate and adaptive immune branches.(Canè et al., 2025; Chen et al., 2021) Grasping the complex interplay between metabolism and cellular immune functions offers the potential for new insights into the intricate regulation of inflammation at the cellular level.(Chen et al., 2021; Corrado & Pearce, 2022; Møller et al., 2022) Among various metabolic processes with regulatory significance, arginine metabolism has historically attracted attention for its vital role in the immune responses of the myeloid lineage.(Bronte & Zanovello, 2005; Geiger et al., 2016; S. Clemente et al., 2020) For instance, as extensively documented, classically activated macrophages (M1) utilize arginine to generate pro-inflammatory molecules and cytokines, such as nitric oxide (NO), which helps to suppress tumor growth. Conversely, alternatively activated macrophages (M2) follow a different arginine metabolic pathway, leading to the production of ornithine, an amino acid linked to anti-inflammatory effects.(Kelly & O'Neill, 2015) In addition, the reduction of arginine levels has been linked to suppression of T cell activation by downregulating the CD3ζ chain, a key segment of the T cell receptor.(Geiger et al., 2016; Rodriguez et al., 2002)

Many innate immune cells, such as neutrophils, macrophages, and dendritic cells (DCs), express two major mammalian arginine-metabolizing enzymes: inducible nitric oxide synthase (iNOS) and arginases (ARG).(Munder et al., 1999; Salimuddin et al., 1999) iNOS oxidizes L-arginine in the presence of equimolar oxygen to generate nitric oxide (NO) and L-citrulline. This simple diatomic gaseous molecule, NO, is important in providing antimicrobial host protection and elicits auto-cytotoxic effects at high concentrations.(Snyder et al., 2022) NO acts as a potent antimicrobial agent, potentially forming reactive nitrogen species (RNS), disrupting various essential enzyme activities, and leading to genetic mutations.(Daff, 2010; Doulias et al., 2013; Koshiishi et al., 2007) Both isoforms of ARG (cytosolic ARG1 and mitochondrial ARG2) carry the same catalytic function, hydrolyzing L-arginine to L-ornithine and urea. Not only does L-ornithine serve as a precursor for polyamines, essential key molecules for fiber and collagen generation, but it can also function as a gene expression modulator by promoting the upregulation of anti-inflammatory genes.(Das et al., 2010) Because of these distinct characteristics of iNOS and ARG, high iNOS-expressing M1 and high ARG-expressing M2 are often viewed as pro-inflammatory and anti-inflammatory/immune-tolerogenic, respectively, underscoring the importance of the regulation of arginine metabolism as a key player in inflammatory outcomes in these cells.(Munder et al., 1999)

DCs are critical modulators of infection that execute many immune effector functions, including antigen sampling, phagocytosis of pathogens, pathogen clearance, and linking between innate and adaptive immunity through their regulation of T cell activation. Under inflammatory conditions, blood-circulating monocytes can differentiate into inflammatory DCs, also known as Tumor necrosis factor (TNF)—α/iNOS-producing DCs (TipDCs).(Serbina et al., 2003) Previously, our group has reported that: 1) DCs upregulate iNOS and NO upon activation in an

LPS-dose-dependent manner, which prompts a long-term metabolic commitment to glycolysis due to NO-mediated inhibition of mitochondrial respiration(Snyder et al., 2022; Thwe et al., 2017); 2) There is an observed NO-mediated "respiratory inhibition threshold" associated with discrete LPS stimulatory doses, referring to the stimulatory dose that impedes cellular mitochondrial respiration.(Snyder et al., 2022; Vagher et al., 2025) In other words, at certain levels of inflammatory stimulation, LPS-activated DCs can produce NO at levels that impair mitochondrial respiration and reduce DC lifespan, demonstrating that these doses of LPS induce auto-cytotoxic effects at the cellular level. In the macrophage literature, numerous studies have shown that an intrinsic substrate competition between ARG and iNOS enzymes regulates NO production.(Boucher et al., 1999; Canè et al., 2025; Ckless et al., 2008; Santhanam et al., 2007) This leads us to hypothesize that this substrate competition may regulate the NO-mediated respiration threshold in iNOS-expressing DC subsets. In this work, we test the hypothesis that the functional balance between these enzymes may be crucial for determining whether NO-mediated innate immune responses are protective or pathological, depending on the nuanced regulation of NO production.

RESULTS

LPS stimulation co-induces iNOS and Arg1 expression

In these studies, we investigate the regulation of iNOS/NO via substrate competition by ARG in DCs, murine bone marrow-derived DCs (BMDCs), which model key characteristics of inflammatory monocyte-derived DCs (also known as Tip-DCs), were chosen as an in *vitro* study model to test the role of arginase competition using pharmacological inhibitors for both iNOS and ARG enzymes, *S*-ethylisothiourea (SEITU – iNOS inhibitor) and *S*-(2-boronoethyl)-L-cysteine (BEC – ARG inhibitor), respectively. To assess the role of arginine substrate competition in our model, we first sought to establish the expression regulation of arginine-utilizing enzymes, iNOS and ARG, at doses we have previously determined to be below the NO-mediated respiratory inhibition threshold (LPS 5 ng/mL) and above (LPS 100 ng/mL). The relative gene expression analysis via RT-qPCR between unstimulated vs stimulated DCs indicated that DCs co-induce the expressions of *Nos2* and *Arg1*, encoding for iNOS and Arg1 proteins respectively, upon both doses of LPS stimulation (**Figure 2.1A**). However, the expression level of *Arg2*, the gene responsible for Arg2, was not regulated by LPS stimulation (**Figure 2.1A**). This co-expression pattern of protein expression by Western blot for iNOS and Arg enzymes reflected the gene expression data, with upregulation of iNOS and Arg1 and constitutive expression of Arg2 under the high dose-LPS (100 ng/mL) (**Figure 2.1B)**.

After confirming the coexpression of iNOS and both isoforms of arginases, we further tested each enzyme's functionality by measuring levels of enzyme products. As direct measurement of NO is challenging, the Griess nitrite assay, which oxidizes NO to the more detectable nitrite (NO_2^-), was utilized to assay iNOS activity; L-ornithine was quantified for Arg1/2 activity (**Figure 2.1C and 2.1D**). The Griess assay indicates a strong correlation between NO production and stimulating LPS-dose (**Figure 2.1C**). The increase of NO production plateaus at higher LPS doses (LPS 50-100 ng/mL), implying iNOS enzyme-substrate complex saturation (**Figure 2.1C**). Though no significant change in the level of L-ornithine was detected across the LPS doses (0 – 25 ng/mL), a relatively steady level of L-ornithine suggests the presence of functioning arginases in DC (**Figure 2.1D**), and a lack of changes may indicate the sensitivity limitations of the assay used. Collectively, these data indicate that LPS stimulation induces both iNOS and Arg1 in a dose-dependent manner, while the mitochondrial Arg2 is constitutively expressed.

To test the dependence of extracellular L-arginine on iNOS activity, we assessed NO production at either low (10 ng/mL) or high (100 ng/mL) LPS doses at a variety of L-arginine concentrations (**Figure 2.1E**). Arginine-depleted cell culture media supplemented with dialyzed FBS was used to mitigate arginine contributions from media serum supplementation and to control the amount of extracellular arginine. The unstimulated control had no detectable NO regardless of various supplemented arginine concentrations, reflecting the absence of iNOS protein detection in unstimulated DCs (**Figure 2.1B and 2.1E-F**). Nitrite increases were observed for both doses of LPS in a manner that was proportional to L-arginine media supplementation (**Figure 2.1E**). The negligible nitrite levels detected at zero L-arginine versus 2000 μM L-arginine supplementation under the same LPS-dose suggest that iNOS activity is more dependent on extracellular substrate concentration than intracellular L-arginine levels (**Figure 2.1E**). To further control for the extracellular substrate dependence of iNOS, D-arginine, the enantiomer of L-arginine, was supplemented (**Figure 2.1E – 2.1H**) to generate the control condition where only an intracellular substrate pool was available to the cells. D-Arginine-supplemented groups exhibited similar levels of nitrite accumulated as observed under no extracellular substrate conditions (0 μM L-arginine), confirming the dependence on extracellular arginine for the iNOS reaction in this context (**Figure 2.1F-2.1H**).

Collectively, these data support that DCs co-express the cytosolic arginine metabolizing enzymes, iNOS and Arg1, in an LPS-dose-dependent manner. Also, the results from the two stereoisomers of arginine addition indicate that the extracellular substrate amount is crucial for optimal iNOS activity. Based on our findings that the mitochondrial isoform Arg2 level was minimal and maintained its constitutive expression upon LPS stimulation, we focused our subsequent experiments on the cytosolic arginine metabolizing enzymes, iNOS and Arg1.

Nitrite accumulation corresponds with mitochondrial respiratory shutdown and decreased cell survival rate in DCs.

Free radical NO has wide-reaching biochemical effects, such as inhibiting mitochondrial electron transport chain (ETC) complexes and inducing protein post-translational modification (PTM) of cysteine.(Koshiishi et al., 2007; Palmieri et al., 2020) Thus, next, we examined the effect of NO production on mitochondrial respiration. We used the Seahorse XF flux analyzer to measure real-time oxygen consumption rate (OCR) data (**Figure 2.2A**).(Pelletier et al., 2014) Specific OCR measurements can be obtained by calculating rate changes between various points of the mitochondrial stress test curve; for example, the ATP production-linked OCR can be measured from the rate change after oligomycin injection, an ATP synthase inhibitor. Making use of the fact that DCs upregulate high levels of iNOS expression and iNOS uses oxygen in its reaction, we added a competitive iNOS inhibitor (*S*-ethylisothiourea; SEITU) as the final injection during the measurement (**Figure**

2.2A). DCs show a flattening trend of the mitochondrial OCR with increasing LPS dose and NO accumulation (**Figure 2.2B**). This OCR reduction was inversely correlated with iNOS-linked OCR levels (**Figures 2.2B and 2.2C**). Co-stimulation of LPS and iNOS-inhibitor, SEITU, rescues this NO-mediated OCR inhibition as previously published (data not shown)(Snyder et al., 2022; Vagher et al., 2025). Repeatedly, we observe that more than 80% of the mitochondrial OCR drops when the detected nitrite level reaches around 15-20 μM (**Figure 2.2D**). We will refer to this discrete level of nitrite accumulation, which correlates with the state where DCs have lost most of their mitochondrial OCR, as the 'NO-respiratory inhibition threshold.'

To demonstrate the effect of NO-respiration inhibition on DC biology, LPS-stimulated DCs were cultured for 96 hours (4 days) post-activation. DCs were analyzed by flow cytometric analysis to determine their survival. As previously reported(Snyder et al., 2022; Vagher et al., 2025), cell survival is inversely correlated with nitrite levels, demonstrating that the percent of dying cells increases due to NO-mediated loss of cellular respiration (**Figure 2.2E**). iNOS inhibition rescues the NO-mediated cell death, further validating that NO accumulation reduces cellular respiration and survival (**Figure 2.2F**). Taken together, our data show that the NO-driven reductions in respiration and cell survival become more prominent as the detected nitrite level exceeds the NO-respiratory threshold.

Arginase inhibition by BEC leads to increased iNOS and Arg1 expression and enhanced NO production

After confirming the co-induction of iNOS and Arg1 proteins in DCs in response to LPS stimulation, we evaluated the impact of competitive arginase inhibitors to further verify the concurrent enzymatic activities of iNOS and Arg1. We assessed several amino acid-derived arginase inhibitors, including S-(2-boronoethyl)-L-cysteine (BEC), which resembles the guanidinium group of arginine due to its boronic acid structure and demonstrates greater inhibitory effectiveness against Arg1 compared to other inhibitors. Another inhibitor, Nω-Hydroxy-nor-L-arginine (nor-NOHA), acts as a structural analog of the arginase intermediate, Nω-hydroxy-L-arginine (NOHA).(Doman et al., 2022) While both inhibitors (BEC and nor-NOHA) resulted in heightened NO production and reduced arginase activity, we identified a nitrite signal not driven by iNOS with nor-NOHA. This effect may stem from the unintended reaction of the compound, leading to the release of biologically active NO-like molecules.(Momma & Ottaviani, 2020) Consequently, we selected BEC as our pharmacological Arg1 inhibitor for these studies, along with the iNOS inhibitor SEITU. Given our results, research utilizing nor-NOHA should consider the spontaneous production of NO-like compounds.

To examine the influence of inhibitors on iNOS and Arg1 expression levels, DCs were stimulated with various LPS doses with either or both iNOS (SEITU) and Arg (BEC) inhibitors (**Figure 2.3**). When DCs remained unstimulated (LPS 0 ng/mL), no changes were observed in *Nos2* and *Arg1* expressions regardless of the presence of inhibitors (**Figure 2.3A and B, left).** Under the low-dose stimulating condition (LPS 10 ng/mL), BEC treatment significantly heightened both *Nos2* and *Arg1* expressions compared to the condition of no drug, though only when iNOS was active (**Figure 2.3A and B, middle**). This finding suggests the possibility that the expression of these genes may be under the same transcriptional regulation.

The augmentation of *Nos2* with BEC was not observed for the high LPS dose condition (LPS 100 ng/mL), suggesting a plateau effect of stimulation at that dose (**Figure 2.3A, right**). Moreover, notable reduction in *Nos2* and *Arg1* expressions under the iNOS inhibiting conditions suggests that the expression of these genes is influenced by NO levels (**Figures 2.3A and B**). To determine whether the corresponding protein expression recapitulates this BEC effect on gene expression, Western blotting for iNOS and Arg1 was conducted (**Figures 2.3C-D**). A positive correlation between LPS-dose and iNOS and Arg1 expressions was observed under all treatment conditions (**Figure 2.3C**). As was observed for the gene expression, Arg-inhibition resulted in increased protein expression of iNOS and Arg1, while iNOS inhibition reduced levels of both enzymes. These findings support the model of a NO-mediated positive feedback loop controlling gene/protein expression (**Figure 2.3C**) as has been previously reported.(Bogdan, 2001; M. Lee & Choy, 2013) Notably, the augmentation of iNOS and Arg1 expression by BEC treatment was most evident at the lower end of the low dose (LPS 5 ng/mL), presumably the conditions where substrate competition may be most impactful (**Figure 2.3C**).

To further characterize the BEC-mediated protein expression regulation at this lower dose of LPS (5 ng/mL), duplicate biological samples were analyzed separately and compared to the unstimulated control (**Figure 2.3D**). As shown in the aforementioned results, no iNOS expression is detected in unstimulated control, and a low level of iNOS expression is observed upon LPS stimulation (**Figure 2.3D**). The

BEC-driven iNOS expression boost was strongly noted at this LPS dose, and a reduction was observed when the combination of BEC and SEITU was used (**Figure 2.3D**). This pattern of augmentation by BEC and reduction by SEITU plus BEC was comparable in Arg1 expression as well. However, a slight increase in Arg1 expression in unstimulated control was observed (**Figure 2.3D**). As a reported BEC-ARG crystal structure indicates, this slight increase of Arg1 under no LPS condition may be mediated by BEC binding to the active sites of Arg1 trimer and enzyme trimer stabilization.(Di Costanzo et al., 2005)

To test the impact of altered expression patterns on metabolic enzyme activity, functional assays were performed to further validate BEC's efficacy as the arginase inhibitor and the presence of enzymatic/catalytic competition between iNOS and Arg1 (**Figures 2.3E and 2.3F**). Griess nitrite assay results corroborate that BEC-mediated iNOS upregulation promotes significantly higher NO production which is then diminished in the presence of SEITU (**Figure 2.3E**). No statistically significant reduction in ornithine level was recorded when comparing the no-drug vs BEC-treated groups. Moreover, a pronounced increase in ornithine levels in the presence of SEITU provides additional evidence of enzymatic competition between iNOS and Arg1 (**Figures 2.3E and 2.3F**).

BEC-mediated Arginase inhibition increases the extracellular substrate bioavailability for iNOS

To monitor the changes in the activities of iNOS and Arg1 in the presence of inhibitors, unstimulated or LPS-stimulated (1 ng/mL) DCs were treated with BEC or SEITU. Cells were collected every three hours after a six-hour post-stimulation time point, when iNOS can be detected, then analyzed by mass spectrometry (MS)-based metabolomics (**Figures 2.4A–F**). MS results verified that BEC treatment significantly increases the arginine pool (**Figure 2.4A and D**) and efficiently inhibits Arg1 activity, as indicated by the ablated arginase product ornithine (**Figure 2.4B and E**). The positive slope of citrulline began to decline around 16 hours post-stimulation but was rescued by BEC treatment, strongly suggesting the initiation of substrate competition between iNOS and Arg1 (**Figure 2.4C and F**). Taken together, these data indicate that Arg1 is co-induced with iNOS upon LPS stimulation, facilitating a substrate-competitive subcellular environment that partially regulates the activity of iNOS.

After confirming BEC-mediated iNOS activity enhancement (**Figure 2.4C and F**), we aimed to determine whether this effect was due to the metabolism of pre-existing intracellular arginine pools or an increase in the metabolism of the extracellular substrate pool. To evaluate this, DCs were stimulated with or without the inhibitors and supplemented with various concentrations of arginine. D-Arginine supplementation served as a negative control to assess the level of intracellular arginine utilization by iNOS, particularly in the presence of inhibitors (**Figures 2.4G and 2.4H**). With the same LPS stimulation dose (LPS 10 ng/mL) and without extracellular arginine supplementation, BEC treatment did not result in a nitrite increase (**Figure 2.4G**). In contrast, the BEC-mediated NO increase was evident under the same LPS dose (10 ng/mL) when 200 μM of L-arginine was supplemented (**Figure 2.4H**). The absence of a BEC-mediated NO production effect in the D-arginine condition supports the conclusion that competition between iNOS and Arg1 predominantly occurs over extracellular substrate sources (**Figure 2.4H**).

The coinduction of iNOS and Arg1 with BEC and co-reduction with SEITU in LPS-stimulated DCs (**Figure 2.3**), as well as matching NO production levels, hinted that the expressions of iNOS and Arg1 may be under the same transcriptional control. LPS stimulation is reported to trigger signaling cascades that lead to the activation of a crucial transcription factor, Nuclear Factor kappa B (NFκB), via phosphorylation. The inactive form of NFkB resides in the cytosol, bound by its regulatory protein; however, once phosphorylated, it moves to the nucleus to perform its role as a transcription factor. While the phosphorylation status alone does not confirm the functional activity of NFkB, the ratio of phosphorylated/total NFkB provides indirect insight into how inhibiting either iNOS or Arg1 might affect NFkB status. Consequently, we aimed to assess NFkB levels at various LPS doses with and without inhibitors, all under the same stimulation hour (**Figure 2.4I**). Total NFkB upregulation was observed even at minimal LPS stimulation (5 ng/mL), compared to the unstimulated control (**Figure 2.4I, left**). After observing the reduction in phosphorylated NFκB with SEITU treatment, as well as the genetic and protein analysis results, alongside a significant increase in NO production from BEC treatment, we anticipated more substantial NFκB phosphorylation with BEC treatment than in the LPS-alone groups. Surprisingly, although the groups treated with BEC did not exhibit a distinct phosphorylated/total NFκB ratio compared to the unstimulated control, the decrease in the ratio seen with the BEC and SEITU combination reaffirmed that the levels of endogenous NO positively influence NFκB activation (data not shown).

Inhibition of the substrate competition between iNOS and Arg1 expedites NO-mediated respiration shutdown in DCs

So far, our results have indicated the existence of competition for the substrate L-arginine between iNOS and Arg 1 in LPS-stimulated DCs. The inhibition of arginases by BEC significantly enhanced NO production. Therefore, we set out to investigate whether this BEC-mediated NO boost impacts DC metabolism, particularly cellular respiration, due to its paramount significance for cellular energy production (**Figure 2.5**). To monitor the changes in respiration, OCR was measured using the Seahorse Flux analyzer in the presence of the inhibitors (**Figure 2.5A**). The unstimulated DCs showed no change in OCR regardless of the inhibitors (**Figure 2.5A, left**). However, the overall OCR rate flatlined as the LPS dose increased (**Figure 2.5A**), indicating that less oxygen is being utilized by the cells as more NO is produced. BEC-mediated NO boost was sufficient to induce an NO-driven shutdown of respiration in DCs even at low-dose LPS (5 ng/mL) (**Figure 2.5A, middle**). This NO-respiration inhibition was rescued when iNOS activity was blocked by SEITU, further confirming that the reduction in OCR under BEC was NO-driven (**Figure 2.5A**). To discern the influence of iNOS regulation through substrate competition with Arg1 on different cellular oxygen-consuming activities, a range of LPS doses (0 - 100 ng/mL) was used with the inhibitors (**Figure 2.5B – 2.5D**). The specific activity-linked OCR was determined by calculating the changes in OCR in response to the drug. For instance, the change in OCR after SEITU injection indicates the rate of oxygen usage by the iNOS enzyme (**Figure 2.5B**). In alignment with the aforementioned results, the OCR measurements also support BEC' s significant augmentation of iNOS activity (**Figure 2.5B and 2.5 E**). The fascinating result is that this iNOS-linked OCR was inversely correlated with mitochondrial- and

ATP-production-linked OCRs (**Figure 2.5 B-D**), indicating that DCs lose their capacity for cellular respiration even at LPS doses where they typically maintain intact respiratory capacity (**Figure 2.5E and 2.5G**). Cells lacking mitochondrial respiration must rely on other metabolic pathways, such as glycolysis and/or fatty acid oxidation, as their primary energy sources, and this metabolic rewiring can alter the outcomes of cellular fates.(Everts et al., 2012; Møller et al., 2022; Palmieri et al., 2020) In other words, this finding strongly suggests that iNOS regulation by Arg 1 via substrate competition is impactful enough to drive cellular metabolic alterations.

The regulation of iNOS by Arg1 regulates cellular survival and host-protective cellular immune function

To further investigate the role of iNOS regulation by Arg1 in DC biology, LPS-stimulated DCs were treated with inhibitors for about 96 hours (approximately 4 days) to test the effects of NO accumulation (**Figure 2.6A**). Flow cytometric analysis to assess cellular survival showed no significant cell death observed at 24 hours post stimulation regardless of the inhibitors used or the LPS dosage (low or high) (**Figure 2.6B-C**). On Day 4, however, a notable increase in cell death was seen with BEC treatment, particularly evident at the lower dose of 5 ng/mL (**Figure 2.6B-C, right**). The substantial reduction in cell death under iNOS-inhibitory conditions confirms that the heightened cell death associated with BEC treatment was indeed caused by NO (**Figure 2.6A-C**). In conclusion, increased NO production due to Arg1 inhibition reduces the NO threshold that inhibits respiration and decreases long-term cell survival rates.

By exploring the intriguing dual effects of NO—its cytotoxic and protective roles—we investigated how iNOS regulation by Arg1 affects DC antimicrobial function using *Listeria monocytogenes*. Because *L. monocytogenes* is an intracellular pathogen, it exploits phagocytes to replicate within them while avoiding other immune defenses. To assess NO's role in controlling bacterial replication, we co-cultured DCs with *L. monocytogenes*, ensuring the bacteria were taken up through phagocytosis. Remaining unphagocytosed bacteria were subsequently eliminated using the antibiotic gentamicin. Afterward, DCs containing internalized bacteria were incubated for 18-20 hours in a medium supplemented with BEC or SEITU to examine the effect of BEC-induced NO enhancement on bacterial clearance (**Figure 2.6D-F**). We first confirmed successful DC activation with whole bacteria by measuring NO production via the Griess nitrite assay. We observed a positive correlation between NO production and the infection levels, indicated by the multiplicity of infection (MOI) (**Figure 2.6D**). While both the no-drug and BEC conditions showed dose-dependent increases in nitrite accumulation, BEC condition groups showed an overall elevated NO production compared to control (**Figure 2.6D**). After the incubation period, DCs were lysed and cultured to quantitate the relative density of replicated bacteria (**Figure 2.6E-F**). Notably, discernible bacterial colonies were found between groups with active iNOS (no drug and BEC groups) and those with inhibited iNOS (SEITU and SEITU+BEC groups) (**Figure 2.6E-F**). The increased NO production due to Arg1 inhibition markedly hindered bacterial growth, an effect that was reversed when iNOS activity was blocked (**Figure 2.6E-F**). These findings demonstrate that Arg1-mediated iNOS regulation through substrate competition significantly impacts DC biology and influences the outcomes of inflammatory immune responses.

DISCUSSIONS

These studies demonstrate the phenomenon that iNOS activity in LPS-activated DCs is tonically regulated by substrate competition for L-arginine with Arginase. Our findings show that ARG partially regulates NO production via substrate competition, which impacts the NO-mediated respiration inhibition threshold and influences metabolic reprogramming, cell death, and antimicrobial activity in stimulated DCs. We found that activated DCs express both iNOS and arginases (Arg1 and Arg2). Notably, the mitochondrial isoform Arg2 remains constitutively low, even in the presence of LPS activation, while the cytosolic isoform Arg1 is upregulated in response to LPS stimulation. We report that iNOS activity is more influenced by the extracellular substrate concentration compared to existing intracellular L-arginine levels, as activated DCs produced limited NO when D-arginine was added to the medium. However, as the levels of exogenous arginine increase, the impact of BEC on nitrite production diminishes (data not shown). These findings support the recently proposed model of interaction between ARG and NOS, indicating that the regulation of NOS activity by ARG requires an environment that reduces (or restricts) arginine availability.(Momma & Ottaviani, 2022)

Given the positively charged side chain of arginine, the import of L-arginine into the cell necessitates a stereospecific cationic amino acid transporter (CAT) system. The CAT family in immune cells comprises a constitutive type (CAT1) and an inducible type (CAT2). The inducible CAT2 transporter has been reported to be highly associated with innate immunity and iNOS activity, as the CAT2$^{-/-}$ mice showed suppressed immune response with reduced NO production against *Helicobacter pylori* infection.(Barry et al., 2011) In support of this, our q-RT PCR results revealed that LPS-stimulated DCs upregulated CAT2-B by over 200-fold compared to unstimulated control, while CAT1 expression remained unchanged compared to the unstimulated control (data not shown). Together, these results substantiate the co-expression of iNOS and arginases, implying a substrate-competitive microenvironment, likely for the extracellular L-arginine pool or newly imported L-arginine pool.

Our analysis of genetic and protein expression revealed a significant increase in iNOS and Arg1 levels following BEC treatment, which was inhibited by SEITU. Correspondingly, the Griess nitrite assay showed a marked increase in NO production with BEC treatment (**Figure 3**), while mass spectrometry metabolite tracing also validated the effective inhibition of Arginase activity by BEC (**Figure 4**). These findings suggest that one of the possible mechanisms of the effect of Arg inhibition is a positive feedback loop driven by nitric oxide (NO) itself. Although we did not observe an increase in NF-κB phosphorylation with BEC treatment, the decreased phosphorylated/total NF-κB ratio under iNOS-inhibiting conditions, compared to LPS alone, supports the idea that NO can enhance iNOS expression at the transcriptional level.

The role of NO in regulating iNOS expression remains debated, and whether it has a positive or negative influence on iNOS expression appears to be contingent upon the cell type and/or context.(Ckless et al., 2008; Graham et al., 2018; M. Lee & Choy, 2013; Momma & Ottaviani, 2022) One the one hand, it has been reported that NO can inhibit iNOS expression via *S*-nitrosylation of NFkB, leading to reduced DNA binding.(Ckless et al., 2008; Hernansanz-Agustín et al., 2013; Marshall et al., 2004) Furthermore, the NO-mediated increase in reactive nitrogenous and oxidant species (RNS/ROS) can bolster anti-inflammatory responses, thereby suppressing iNOS expression.(Graham et al., 2018) On the other hand, the opposite effects have also been reported, whereby NO stimulates iNOS expression by *S*-nitrosylation of a signaling protein, Ras, and through the NO-mediated activation of soluble guanylate cyclase (sGC), initiating G-protein receptor-associated signaling cascades.(M. Lee & Choy, 2013; Marshall et al., 2004)

Another possible explanation for the positive feedback of NO on iNOS expression is the activation of hypoxia-inducible factor-1 (HIF-1), a transcription factor that regulates cellular responses to low oxygen levels or acidic environments.(Perrin-Cocon et al., 2018; Tannahill et al., 2013) Studies indicate that NO can influence HIF-1 activity, leading to increased iNOS expression.(Melillo et al., 1995) The accumulation of NO generates hypoxic conditions, as the iNOS catalytic reaction requires oxygen. Additionally, NO-induced mitochondrial inhibition shifts cellular metabolism towards glycolysis, accumulating lactate and decreasing intracellular pH.(Jantsch et al., 2008; Melillo et al., 1995) This commitment to glycolysis, driven by NO, produces a subcellular environment that activates HIF-1, thereby establishing a NO-*Nos2* feedback loop. Accordingly, this NO-driven cellular commitment to glycolysis supports fatty acid synthesis,(Castoldi et al., 2020; Doulias et al., 2013; O'Neill, 2011) leading to the generation of various lipid-based molecules, including prostaglandin E2 (PGE2), mevalonate (precursor molecule for cholesterol), and low-density lipoproteins (LDLs). These lipid molecules, in collaboration with inflammatory cytokines, can enhance Arg1 transcription via signal transducer and activator of transcription 3 (STAT3) and liver X receptor (LXR)-dependent mechanisms.(Canè et al., 2025; Kim et al., 2016; Pourcet et al., 2011) *Nos2* expression can be regulated both at the transcriptional and translational levels. The low availability of L-arginine has been shown to affect the translation of iNOS mRNA in astrocytes by facilitating the phosphorylation of eukaryotic initiation factor (eIF2α), which subsequently inactivates eIF2α.(J. Lee et al., 2003)

The negative relationship between iNOS-specific OCR and the cellular respiration associated with mitochondrial/ATP production suggests a delicate equilibrium between the cytotoxic and protective roles of NO in DCs. In support of this model, Arg1 inhibition results in cellular exhaustion, which decreases long-term survival rates, while increasing iNOS-dependent antimicrobial activity through the regulation of bacterial replication. Many studies have aimed to adjust the balance of nitric oxide's opposing effects by modifying iNOS activity. For example, dietary arginine supplementation has been employed to enhance immune function in patients with sepsis, trauma, or cancer.(Bansal & Ochoa, 2003; Das et al., 2010) Nevertheless, the results of these studies have varied. Possible explanations for the inconsistent results include: 1) differing cellular regulation of mitochondria,(Faas & De Vos, 2020; Li & Hoppe, 2023) 2) cellular reactions to high NO activity,(Kelly & O'Neill, 2015) 3) recycling of substrates from other metabolites,(Lange et al., 2019; Qualls et al., 2012) and 4) arginine consumption by other microorganisms.(Xiong et al., 2016)

The NO-mediated cellular and biological alterations generally arise from NO-derived reactive nitrogenous or oxidative species (RNS/ROS), which are under tight cellular homeostatic control.(Espey et al., 2002; Koshiishi et al., 2007) The accumulation of NO increases the likelihood of *S*-nitrosylation on the regulatory protein Kelch-like ECH-associated protein 1 (KEAP1). *S*-Nitrosylation of KEAP1 results in the detachment of KEAP1 from the transcription factor erythroid 2-related factor 2 (Nrf2) in the cytosol, leading Nrf2 to translocate to nucleus where it upregulates genes that protect against oxidative cellular stress.(McNeill et al., 2015; Wakabayashi et al., 2010; Wei et al., 2018) Nrf2 activation gives rise to the expression of antioxidant proteins,(Han et al., 2001) including peroxiredoxins (PRDXs)(Graham et al., 2018; Rhee et al., 2012) and heme oxygenase (HO).(Chung et al., 2008)

HO-1 is an effective enzyme that breaks down heme, yielding free iron, biliverdin, and carbon monoxide (CO). Its activity provides various protections against cellular stress, including NO-mediated post-translational modifications (PTMs). Extensive research shows a connection between NO and CO, as CO acts as a biphasic regulator of NO (low CO levels enhance NO production, while high levels decrease it). The two gases have comparable molecular weights, solubilities, and production rates, and can bind ferrous heme.(Chung et al., 2008; Hartsfield, 2002) Moreover, instances of CO-bound iNOS heme and reduced iNOS translation due to CO have been noted. Consequently, HO-1 helps lessen the generation of reactive nitrogen species (RNS) and reactive oxygen species (ROS) by inhibiting both iNOS synthesis and activity, HO-1 indirectly influences iNOS by competing with its electron donor, NADPH. In essence, the reduction in NADPH availability for iNOS, caused by HO-1 can suppress NO production, even when L-Arg levels are adequate. (Di Pietro et al., 2020; D. W. Lee et al., 2017; Sawle et al., 2005)

Unpublished preliminary data from our lab indicate that the expression of *Hmox1*, the gene responsible for HO-1 protein, is significantly increased by BEC treatment, which enhances NO production and further elevates *Hmox1* levels in DCs compared to unstimulated controls (data not shown). While the cellular protection provided by HO-1 and the interaction between NO and CO are well-documented, a significant gap remains in our understanding of the impact of HO-1 regulation on iNOS regarding NO-mediated respiration inhibition. We hypothesize that the upregulation of HO-1 raises the NO-respiration threshold in LPS-stimulated DCs, implying that DCs would maintain their respiration at high doses of LPS, even when DCs lose cellular respiration. This knowledge gap presents a critical area for research, as it could shed light on how to manipulate DC immune effector functions for therapeutic applications.

One intriguing area of research in amino acid metabolism and immune regulation is Indoleamine 2,3-dioxygenase (IDO).(Kelly & Pearce, 2020; Mellor & Munn, 2004) This enzyme breaks down the amino acid tryptophan into kynurenine. A significant amount of research has demonstrated the link between IDO's immunosuppressive role (tolerogenic function) and T cell regulation through kynurenine-mediated gene expression and tryptophan depletion from the microenvironment. Furthermore, IDO and Arg1 are co-expressed in DCs under the influence of anti-inflammatory cytokines, such as transforming growth factor-β (TGF-β).(Mellor & Munn, 2004; Mondanelli et al., 2017) Based on our findings of iNOS-Arg1 co-induction and the discovery by the Mondanelli *et al.* group that polyamines, a downstream product of Arg1 (L-ornithine), can enhance IDO1 expression and bolster immunosuppressive effects in DCs,(Mondanelli et al., 2017) we hypothesize that inhibiting IDO1 in DCs will reduce the NO-respiration threshold to a lesser extent than inhibiting Arg1 in LPS-stimulated DCs.

In conclusion, this study examines the hypothesis that arginase activity in activated DCs restricts iNOS activity by reducing arginine availability. We demonstrate that iNOS activity is regulated by arginase activity due to competition for the common substrate, L-arginine. The Arginase-inhibition mediated NO enhancement lowers the NO-mitochondrial respiration inhibitory threshold, causing a shutdown of cellular respiration at low LPS stimulatory doses where respiration is typically unaffected. The shifting of the NO-respiratory inhibition threshold further influences DC cellular and immune functions, including effects on post-activation survival rate and the replication control of the intracellular pathogen, *Listeria monocytogenes*. These findings support our hypothesis that iNOS-expressing DC cellular function is tonically regulated by arginine substrate competition with ARG, with important implications for the field of immunology and inflammation. Our findings indicate that arginase activity curbs the loss of NO-mediated mitochondrial respiration in DCs, resulting in extended post-activation lifespan, but also limiting antimicrobial capabilities. From a broader perspective, the data from these studies underscore the critical importance of tight regulation of iNOS activity in NO-producing immune cells, with significant implications for cellular respiration, cell survival, and immune function.

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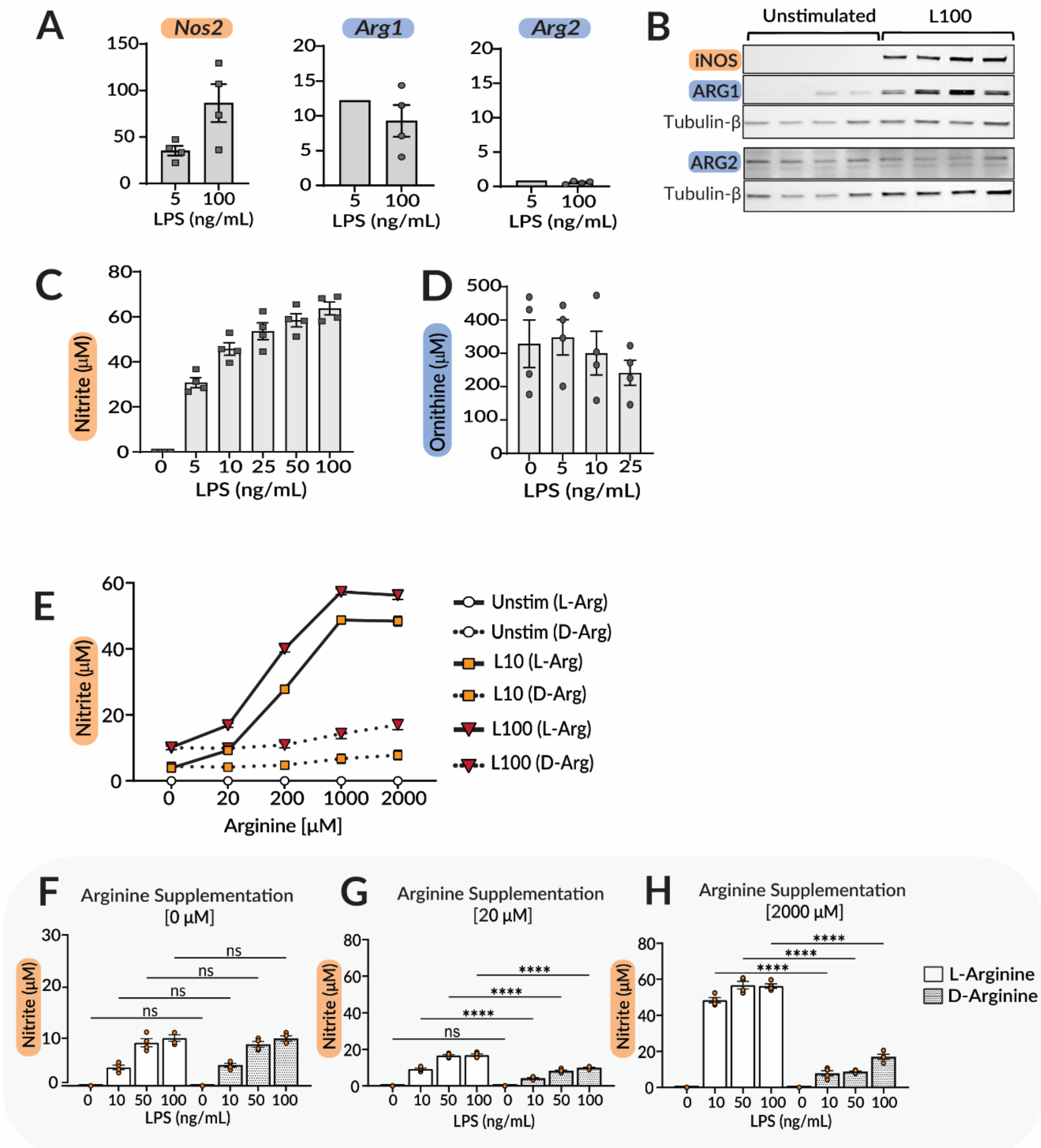

Figure 2. 1 LPS stimulation co-induces iNOS and Arg1 expression

(A) *Nos2, Arg1*, and *Arg2* gene transcription assessed by RT-qPCR from BMDCs were left unstimulated (U) or stimulated for 24 h with LPS at 5 or 100 ng/mL. Normalized to 18s rRNA unstimulated condition via $2^{-(\Delta\Delta Ct)}$ method. (B) Western blot for iNOS, Arg1, and Arg2 from protein lysates isolated from BMDCs stimulated for 24 hours with 0 (unstimulated) and 100 ng/mL LPS. Tubulin-β serves as a loading control. (C) Griess nitrite assay and (D) Ornithine assay on media supernatant from BMDCs stimulated with a range of LPS doses (0 – 100 ng/mL) for 24 hours. (E) Griess nitrite assay on media supernatant from BMDCs stimulated with LPS 0, 10, and 100 ng/mL under a range of exogenous arginine supplementation for 24 hours. (F) – (H) bar graph results from Griess Nitrite results (E) under 0, 20, and 2000 µM arginine supplementation. (A)-(H), n=4. Analyzed by one-way ANOVA, n=4. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. All p-values and tests are indicated on the figures. If a p-value is not stated, the data are considered non-significant ($p > 0.05$).

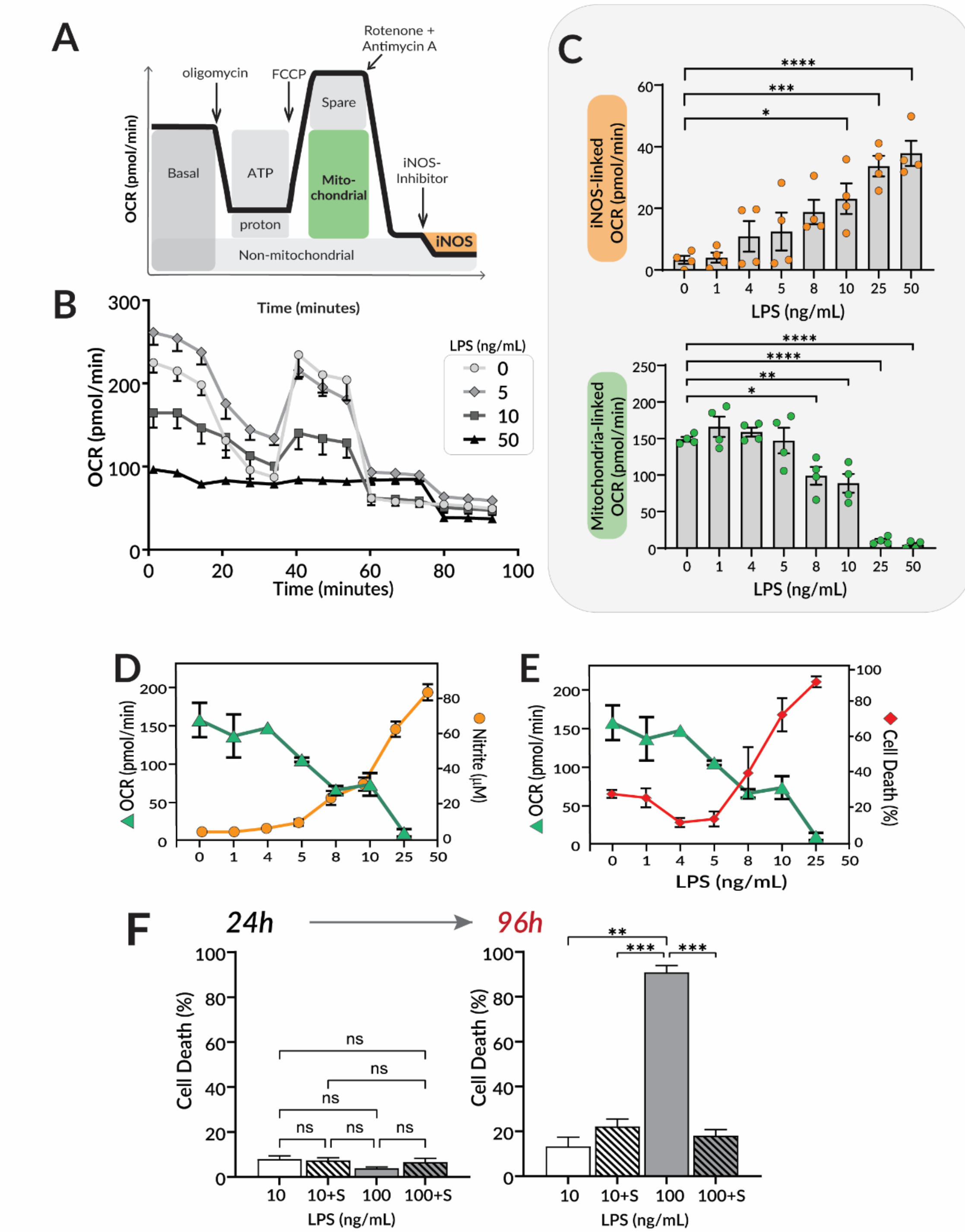

Figure 2. 2 Nitrite accumulation corresponds with mitochondrial respiratory shutdown and decreased cell survival rate in DCs

(A) depicts an example of an OCR kinetic trace for the mitochondrial stress test of extracellular flux analysis. The vertical arrow indicates the drug injection. Specific-linked OCRs are indicated with a text box. (B) Mitochondrial stress test kinetic trace of extracellular flux analysis on BMDCs stimulated for 24 hours with a range of LPS doses (

0 – 50 ng/mL). Analyzed by one-way ANOVA, n=4 (C-top) iNOS-linked OCR calculated from kinetic traces in (B) by subtracting the average OCR of post-iNOS inhibitor from the average OCR of post-rotenone/Antimycin A. (C-bottom) Mitochondrial-linked OCR calculated from kinetic traces in (B) by subtracting the average OCR of post-rotenone/Antimycin A from the average OCR of baseline before oligomycin injection. (D)The overlapping line graphs of mitochondrial-OCR calculated from kinetic traces (B) and Griess nitrite assay done on the identical sample supernatant. (E)The overlapping line graphs of mitochondrial-OCR calculated from kinetic traces (B) and flow cytometric cell death data from LPS-stimulated BMDCs for 96 hours under the same LPS doses as (B). (F) Percentage of dead cells assessed by flow cytometry using a fixable Live/Dead stain on BMDCs treated with 10 or 100 ng/mL LPS +/- iNOS inhibitor, SEITU (S), for 24 - 96 hours. Analyzed by one-way ANOVA, n=4. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. All p-values and tests are indicated on the figures. If a p-value is not stated, the data are considered non-significant ($p > 0.05$).

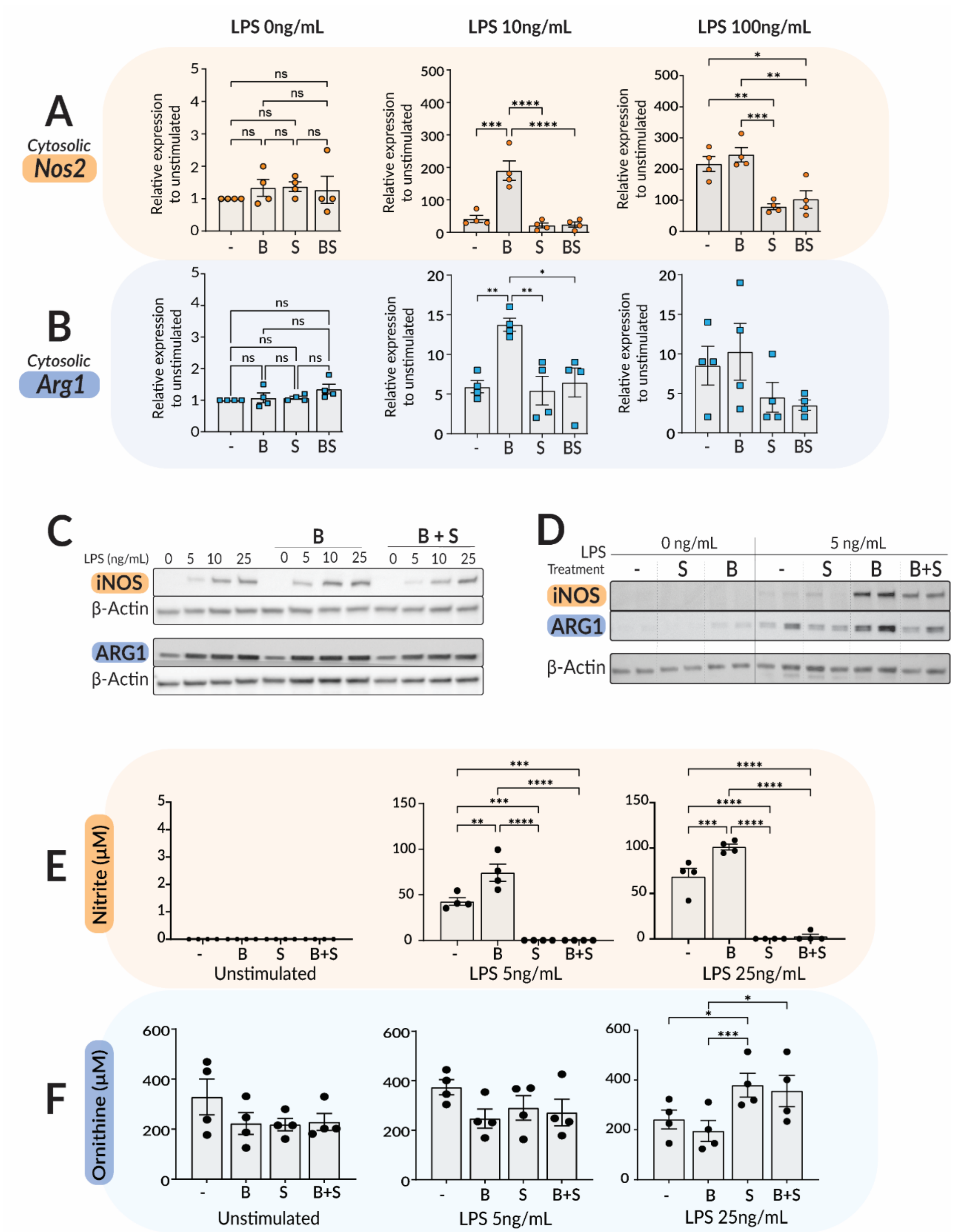

Figure 2. 3 Arginase inhibition by BEC leads to increased iNOS and Arg1 expression and enhanced NO production.

All figures drug indication: Single dash line- no drug/ B-BEC(Arg inhibitor) / S-SEITU(iNOS inhibitor)/ BS-BEC+SEITU. (A) - (B) *Nos2* and *Arg1* gene transcription assessed by RT-qPCR from BMDCs were left untreated (-) or drug-treated for 24 h with

LPS at 0, 10, and 100ng/mL. Normalized to 18s rRNA unstimulated condition via $2^{-(\Delta\Delta Ct)}$ method. (C) - (D) Western blot for iNOS and Arg1 from protein lysates isolated from BMDCs stimulated for 24 hours with various LPS doses at 0, 5, 10, and 25 ng/mL. β-Actin serves as a loading control. (E) Griess nitrite assay and (F) Ornithine assay on media supernatant from BMDCs stimulated with a range of LPS doses (0, 5, and 25 ng/mL) for 24 hours. (A)-(F), n=4. Analyzed by one-way ANOVA, n=4. * p <0.05, ** p 0.01, *** p <0.001, **** p <0.0001. All p-values and tests are indicated on the figures. If a p-value is not stated, the data are considered non-significant (p > 0.05).

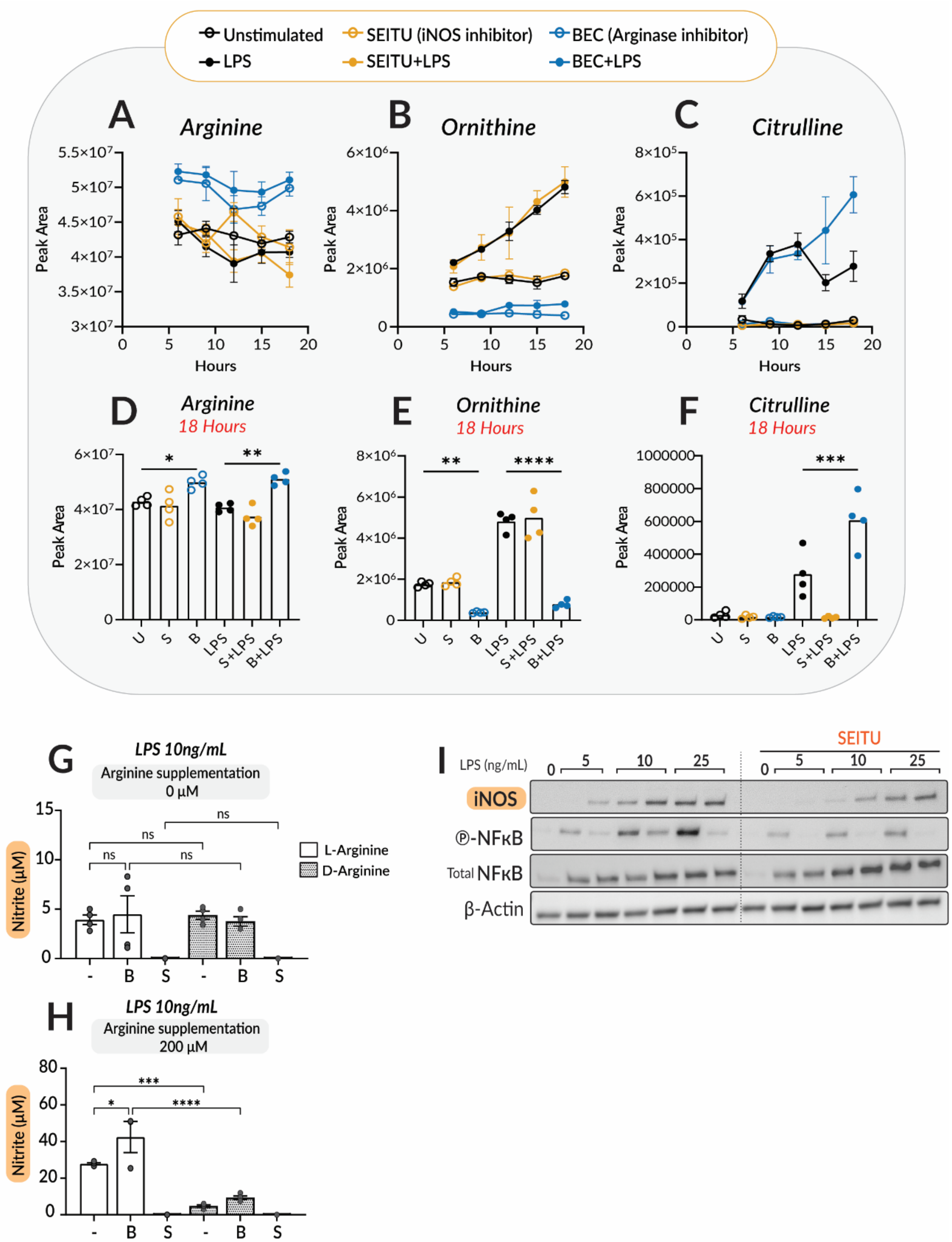

Figure 2. 4 BEC-mediated Arginase inhibition increases the substrate availability for iNOS and boosts NO production

All sub-panels: U is unstimulated, S is the iNOS inhibitor *S*-ethylisothiourea (SEITU) at 500 µM, B is the arginase inhibitor S-(2-boronoethyl)-L-cysteine (BEC) at 250 µM (A) –

(F). Accumulation of metabolites arginine, ornithine, and citrulline was assessed by mass spectrometry at LPS 1 ng/ml. Kinetic graphs display metabolite levels in DCs stimulated for up to 20 hours, with measurements taken every 3 hours post-6-hour stimulation (A through C). Bar graphs display metabolite levels in DCs stimulated for 18 hours (D through F). (G) – (H) Griess nitrite assay on media supernatant from BMDCs stimulated with LPS 10 ng/mL under exogenous arginine supplementation either 0 (G)or 200 µM (H) for 24 hours. (I) Western blot for iNOS, Phosphorylated-NFkB, and total NFkB from protein lysates isolated from BMDCs stimulated for 24 hours with various LPS doses at 0, 5, 10, and 25 ng/mL. β-Actin serves as a loading control. The two representative mouse results are shown. (A through H) N = 4 mice; each point represents an individual mouse. Analyzed by one-way ANOVA and Tukey test, significant adjusted p values for U versus B and LPS versus B+LPS are reported. Adjusted p values less than 0.05 (*), 0.01 (**), 0.001 (**), and 0.0001 (****) are denoted on graphs.

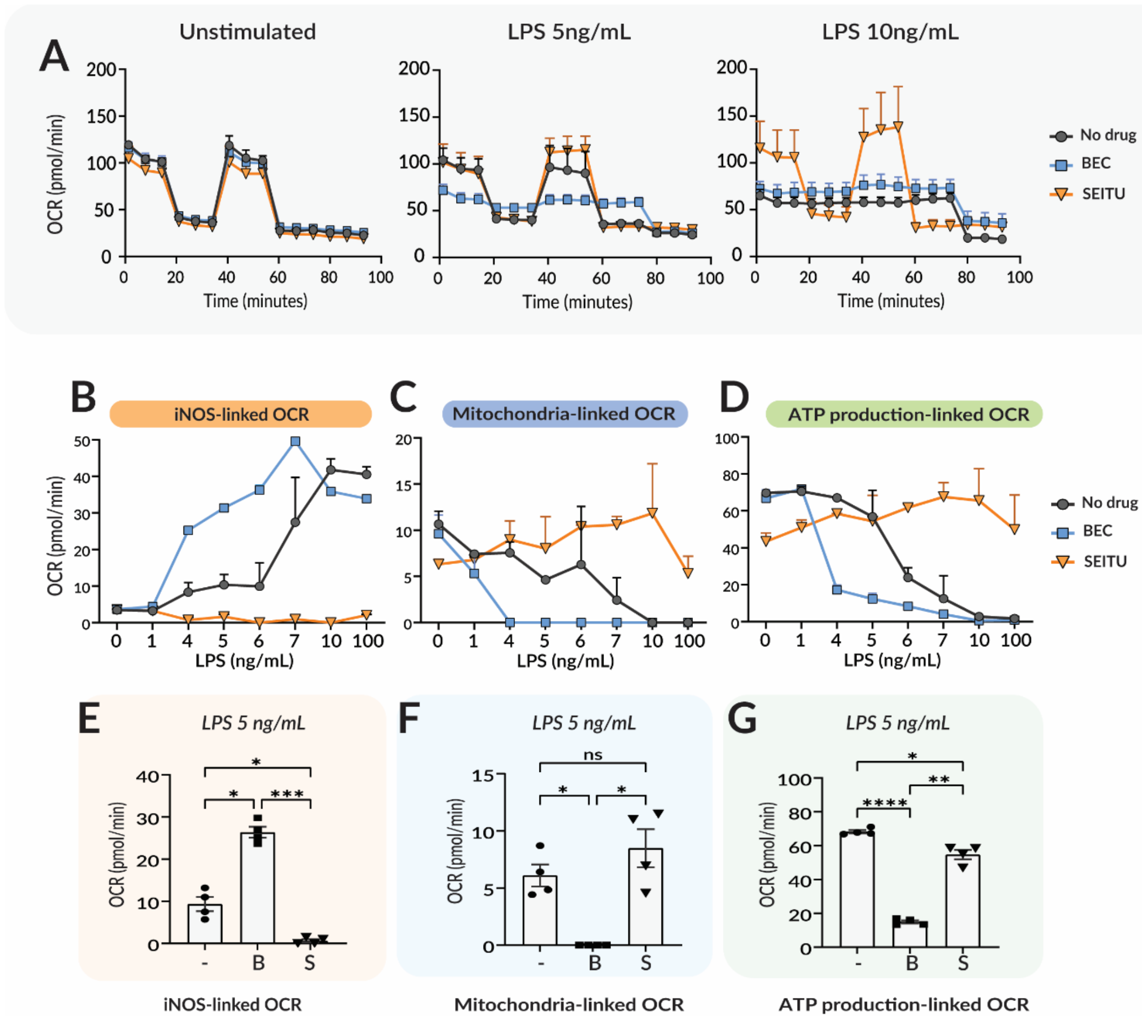

Figure 2. 5 Inhibition of the substrate competition between iNOS and Arg1 expedites NO-mediated respiration shutdown in DCs

(A) Mitochondrial stress test kinetic trace of extracellular flux analysis on BMDCs stimulated for 24 hours with a range of LPS doses (0, 5, and 10 ng/mL). (B) iNOS-linked OCR calculated from kinetic traces in (A) by subtracting the average OCR of post-iNOS inhibitor from the average OCR of post-rotenone/Antimycin A. (C) Mitochondrial-linked OCR calculated from kinetic traces in (A) by subtracting the average OCR of post-rotenone/Antimycin A from the average OCR of baseline before oligomycin injection. (D)The overlapping line graphs of mitochondrial-OCR calculated from kinetic traces (A). (E through G) Bar graph data showing each specific-linked OCR at LPS 5ng/mL from graphs (B through D). n=4. Analyzed by one-way ANOVA, n=4. * p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001. All p-values and tests are indicated on the figures. If a p-value is not stated, the data are considered non-significant (p > 0.05).

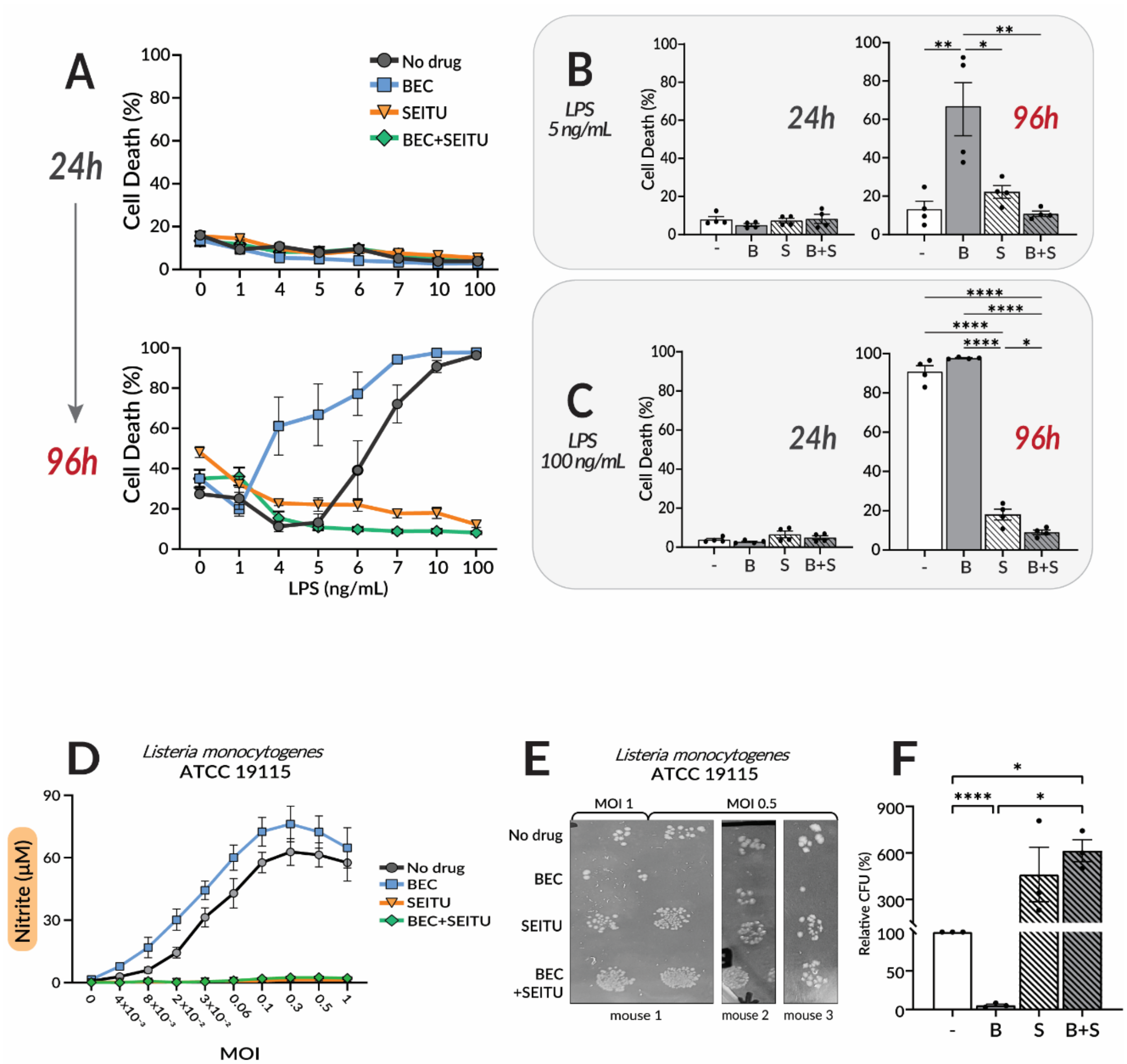

Figure 2. 6 The regulation of iNOS by Arg1 regulates cellular survival and host-protective cellular immune function

All sub-panels: U is unstimulated, S is the iNOS inhibitor *S*-ethylisothiourea (SEITU) at 500 μM, B is the arginase inhibitor *S*-(2-boronoethyl)-L-cysteine (BEC) at 250 μM. (A) Percentage of dead cells assessed by flow cytometry using a fixable Live/Dead stain on BMDCs treated with a range of LPS (0-100ng/mL) +/- inhibitors for 24 - 96 hours. (B) Bar graph showing at LPS 5ng/mL for 24 and 96h from graph (A). (C) same as (B), except at LPS 100ng/mL. (D) Griess nitrite assay on media supernatant from BMDCs co-cultured with *Listeria monocytogenes* under a range of multiplicity of infection range for 18 hours. (E) Gentamicin protection assay result showing *L. cytogeneses* growth after 18 hours of replication after phagocytosed by DCs. (F) Normalized bar graph from bacterial colony forming unit (CFU) shown in (E). Analyzed by one-way ANOVA, (A through C) n=4. (D through F) n=3. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. All p-values and tests are indicated on the figures. If a p-value is not stated, the data are considered non-significant ($p > 0.05$).

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CHAPTER 3 COMPREHENSIVE LITERATURE REVIEW

AVENUES OF FUTURE INTEREST IN THE FIELD OF IMMUNOMETABOLISM

The overarching context of this study pertains to our group's (The Amiel Laboratory) extensive, decades-long investigation into the regulation of the balance between the antimicrobial and auto-cytotoxic effects of inflammation-related NO production at the cellular level.(Snyder et al., 2022; Thwe et al., 2017; Vagher et al., 2025) This dissertation offers a comprehensive analysis of how metabolic competition for a single metabolite, arginine, can significantly impact cellular respiration, viability, and immune function. In this regard, it is pertinent to emphasize other critical metabolites and metabolic pathways that can influence iNOS activity and NO synthesis, thereby governing this delicate equilibrium. In the discussion section, various mechanisms of metabolite-mediated iNOS regulation will be examined, including the roles of lipids, TCA cycle metabolites, and the interactions between iNOS and antioxidant enzymes.

3.1 Metabolite-mediated Regulation of iNOS

3.1.1 Via Lipid-mediated molecules

Innate immune cells like macrophages and DCs that produce nitric oxide show increased citrate levels, a metabolite of the TCA cycle, as their metabolism accelerates to fulfill energy needs during immune activation.(Pearce & Pearce, 2013) The mitochondrial citrate is transported to the cytosol via the mitochondrial carrier protein Slc25a1, where it facilitates lipid synthesis and the formation of lipid-derived molecules (**Figure 3.1**) (Everts et al., 2014; O'Neill, 2011, 2014) This is supported by studies that demonstrate blocking Slc25a1 or fatty acid synthase reduces PGE2 production.(Infantino et al., 2011) Moreover, NO-mediated suppression of OXPHOS decreases β-oxidation, leading to an enlargement of lipid droplets (LDs). LDs store neutral lipids such as diacylglycerols, triacylglycerols, arachidonic acids, and prostaglandins. (**Figure 3.2)** Lipidomic analyses using mass spectrometry confirm that NO-induced mitochondrial inhibition results in the accumulation of neutral lipids.(Castoldi et al., 2020; Qiu & Simon, 2016) Additionally, prostaglandin synthesis is not solely about lipid production; prostaglandins also regulate the immune response by influencing the expression of iNOS. Therefore, in subsection 3.1.1, we will examine what these lipid-mediated molecules are and review the known mechanisms of iNOS regulation, followed by a hypothesis regarding how these metabolites may influence NO's biological role in DCs.

Prostaglandins

PGs are small molecules derived from arachidonic acid (AA) in cell membranes, produced by cyclooxygenases (COXs). There are two isoforms: COX-1 (prostaglandin H synthase-1) and COX-2 (prostaglandin H synthase-2). COX-1 is constitutively expressed in most tissues, supporting functions like homeostasis and gastric protection, while COX-2 is primarily found in myeloid and stromal cells and is associated with pathological processes. Non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin and ibuprofen work by inhibiting COX activity to control inflammation. (Biringer, 2021) PGE2, the most abundant type of PGs, is released into extracellular space after synthesis. (**Figure 3.2**) It binds to four PGE receptors, known as E-type prostanoid receptors 1-4 (EP1–EP4), each of which is linked to a distinct G protein, resulting in diverse signaling effects.(**Figure 3.3**) Notably, EP2R and EP4R are coupled to Gs proteins, which increase intracellular cyclic AMP (cAMP). Elevated cAMP activates transcription factors, such as cyclic AMP-responsive element-binding protein (CREB) and NF-kB. (Harizi et al., 2003; Kalinski, 2012) Depending on its concentration and the receptor activated, PGE2 can have pro- or anti-inflammatory effects, such as suppressing NK cell activity, reducing alveolar macrophage phagocytosis, and regulating DC differentiation and migration.(**Figure 3.3**)(Harizi, 2013; Scandella et al., 2002)

The interaction between NO and PGs was first observed in experiments with LPS-stimulated macrophage cell lines, RAW264.7. In these studies, both iNOS and COX-2 expressions were detected, and PG production decreased upon treatment with an iNOS inhibitor.(Salimuddin et al., 1999) In support of this, many infectious stimuli are known to induce COX-2 expression through TLR/NF-κB signaling pathways. Additionally, studies examining an exogenous NO donor have shown that a high level of NO enhances the expression of COX-1 and COX-2 mRNA levels.(Martínez-Colón & Moore, 2018; Salvemini et al., 1993) Several mechanisms have been suggested to explain this NO's influence on COXs: (1) NO may S-nitrosylate COX enzymes to boost their activity, based on the findings that iNOS can bind and activate COX-2 specifically via S-nitrosylation of a cysteine residue near the enzyme's catalytic domain.(S. F. Kim et al., 2005) or (2) NO-derived peroxynitrite (ONOO-) may nitrate tyrosine-385 on COX, causing enzyme inactivation. (Deeb et al., 2006) Furthermore, cytosolic phospholipase A2α, which produces AA and plays a rate-limiting role in PGE2 synthesis, can also be the target of S-nitrosylation, a protein modification, leading to increased activity. This NO-promoted PGE2 synthesis could be a key immune regulatory point due to its association with DC migration. (Diao et al., 2021)

As a crucial component connecting innate and adaptive immunity, DCs need to migrate to lymph nodes to activate antigen-specific T cells. PGE2 facilitates DC migration by increasing the expression of the chemokine receptor CCR7, which detects signals produced by stromal cells and high endothelial venule (HEV) cells within the lymph node, specifically CCL19 and CCL21.(Muthuswamy et al., 2010) As a feedback mechanism, CCL19 can induce CCR7 internalization, thereby reducing the attraction of naïve T cells to the lymph node. Studies have found that PGE2 plays a suppressive role in CCL19 expression, leading to an increase in CCR7 levels in DCs.(Muthuswamy et al., 2010; Scandella et al., 2002) Moreover, PGE2 can enhance the production of IL-1β, an inflammatory cytokine.(Molina-Holgado et al., 2000) These data indicate that PGE2 promotes DC migration and helps set the inflammatory tone, while lowering T cell attraction. Therefore, considering the link between PGE2, NO, and DC migration, we hypothesize that altering PGE2 levels could influence NO production and subsequently regulate mitochondrial respiration. This regulation may be particularly important in situations requiring tight control over bacterial or viral amplification, especially those that target adaptive immune cells, such as *Mycobacterium tuberculosis* and Epstein-Barr virus.(Liu & Kelly, 2008; Sander et al., 2017; Sheppe et al., 2018)

Sphingosine

Sphingosine is an amino alcohol that serves as the core component of key cell membrane lipids, including sphingolipids (such as sphingomyelin and glycosphingolipids).(Spiegel & Milstien, 2011) In addition to providing structural support, sphingosine contributes to immune regulation during inflammation by influencing iNOS activity.(Machida et al., 2008a) Research indicates that its phosphorylated form, sphingosine-1-phosphate (S1P), acts as a bioactive lipid capable of triggering either anti-inflammatory or pro-inflammatory responses.(Chi, 2011; Spiegel & Milstien, 2011) Intracellular S1P indirectly promotes the transcription factor NF-κB via interactions with E3 ubiquitin ligase RTAF2 (a key inhibitory component of the NFkB pathway), leading to its activation and the expression of inflammatory genes, such as *NOS2*.(Alvarez et al., 2010) However, extracellular S1P can influence macrophage polarization towards the M1 (pro-inflammatory) state through signaling via S1P2 and S1P3 receptors, resulting in increased levels of iNOS and pro-inflammatory cytokines, such as TNF-α.(Heo & Im, 2019) Conversely, S1P binding to the S1P1 receptor generally triggers anti-inflammatory effects by blocking the NF-κB pathway.(Machida et al., 2008b) Although these mechanisms are complex and cell- and context-dependent, evidence suggests a link between S1P's regulation of NO in DCs, based on NO-related metabolic changes and the development and/or enlargement of the lipid droplets. Specifically, five S1P receptor types (S1PR1 through S1PR5) are expressed in mammals.(Yuan et al., 2021) While more research is needed to clarify their roles, S1PR1 and S1PR3 have been linked to increased DC migration.(Maeda et al., 2007; Spiegel & Milstien, 2011) When an S1P analog was used to inhibit S1PRs, DC migration from the lamina propria to mesenteric lymph nodes decreased.(Zou et al., 2023) Thus, we propose that elevated S1P levels may attract dendritic cells, particularly migratory types such as Langerhans cells, to infection sites. This could boost antimicrobial responses alongside NO-producing DCs. However, if this large number of phagocytes depletes the extracellular L-arginine pool, it could weaken the NO-antimicrobial effect, as substrate concentration directly influences iNOS functionality.

Oxysterols and Nuclear Receptors

DCs alter their metabolic processes, transitioning from primarily relying on beta-oxidation of fatty acids to a more glycolytic pathway to facilitate their activation and function.(Møller et al., 2022; O'Neill, 2014) As NO accumulates and its concentration reaches the NO-respiratory threshold, DCs reconfigure their carbon flux, committing to aerobic glycolysis.(Snyder et al., 2022; Vagher et al., 2025) Glycolysis indirectly supports cholesterol synthesis by supplying essential precursors and energy. To explain, glycolysis converts glucose into pyruvate, which can subsequently be transformed into acetyl-CoA within the mitochondria. This mitochondrial acetyl-CoA may follow one of two pathways: (1) it enters the tricarboxylic acid (TCA) cycle to contribute to ATP synthesis via oxidative phosphorylation (OXPHOS), or (2) it is exported to the cytosol as citrate. Cytosolic citrate is then reconverted to acetyl-CoA and utilized in cholesterol biosynthesis.(Hu et al., 2023; Jin et al., 2023) Notably, mevalonate is a key intermediate in the cholesterol synthesis pathway and has been associated with the induction of "trained immunity" in innate immune cells.(Bekkering et al., 2018) The cholesterol produced can also undergo oxidation to form oxysterols. Research indicates that certain oxysterols, such as 25-hydroxycholesterol (25-HC) and 24,25-epoxycholesterol, can bind to and activate the liver X receptor (LXR).(Töröcsik et al., 2009) Activation of LXR in macrophages has been shown to suppress TLR-mediated innate immune responses, leading to a reduction in the production of inflammatory mediators, including IL-6, IL-1β, COX-2, and iNOS. (Hong et al., 2011; Joseph et al., 2004; Pourcet et al., 2011) Furthermore, it has also been reported that LXR activation enhances arginase expression, suggesting its role in promoting an anti-inflammatory phenotype.(Pourcet et al., 2011) Consequently, oxysterols that activate LXR may indirectly inhibit iNOS expression by attenuating the overall inflammatory response. Therefore, we hypothesize that modulating oxysterol production or activating LXR will reduce iNOS activity through substrate competition caused by increased Arginase expression induced by LXR. In essence, we suggest that heightened LXR activity may have a similar effect to arginase overexpression, lowering the NO-respiratory threshold in DCs.

3.1.2 Via anti-inflammatory Metabolite: Itaconate

Earlier in this dissertation, we showed how activated DCs (alongside M1 macrophage examples) reprogram their metabolism by increasing glycolysis and reducing mitochondrial respiration through NO.(Palmieri, Gonzalez-Cotto, et al., 2020; Snyder et al., 2022; Vagher et al., 2025) In subsection 3.1.1 above, we discussed how this shift to aerobic glycolysis in DCs also enhances lipid production, leading to the creation of immune-regulatory bio-lipid molecules. Besides lipid-mediated molecules, other metabolites have been found to contribute to setting the inflammatory phenotype.

Upon TLR stimulation, the downstream signaling cascades via NFkB induce the expression of metabolic enzymes, including aconitate decarboxylase 1 (ACOD1; also known as CAD and IRG1). ACOD1 utilizes one of the TCA cycle byproducts/metabolites, cis-aconitate, to produce itaconate (**Figure 3.4**) (Choi et al., 2021; O'Neill, 2014)Under normal conditions, cis-aconitate is primarily involved in the TCA cycle and is converted to isocitrate by the enzyme Aconitase. However, similar to many of the metabolic enzymes that catalyze redox chemical reactions, such as the ETC complexes, Aconitase also contains an Fe-S cluster, which is a common target of NO binding and reaction.(Bailey et al., 2019; Palmieri, McGinity, et al., 2020) Aconitase catalyzes two conversion reactions: 1) citrate to cis-aconitate and 2) cis-aconitate to isocitrate. (**Figure 3.4**) Inhibition of Aconitase by NO is reversible at low concentrations, but at high NO levels, RNS/ROS cause iron to be released from the Fe-S cluster, making the inhibition irreversible. This block of aconitase activity by NO creates a pause in the TCA cycle, promoting the accumulation of cis-aconitate (the substrate metabolite for Aconitase).(Palmieri, Gonzalez-Cotto, et al., 2020) Subsequently, this backup of cis-aconitate becomes destined to undergo ACOD1's catalytic activity, producing a large amount of itaconate.(O'Neill & Artyomov, 2019; Ryan et al., 2020) Recent studies have reported that this metabolic byproduct of the TCA cycle, itaconate, can influence immune response towards anti-inflammation via several mechanisms(Song et al., 2023) :(1) Itaconate can reduce the phosphorylation of p65, a key component of the NF-κB complex, thus preventing its translocation to the nucleus and hindering the transcription of iNOS.(Bourner et al., 2024) (2) Itaconate can also induce activating transcription factor 3 (ATF3) expression, which negatively regulates IκBζ, a member of the IκB family of proteins and is known to be a regulator of the NF-κB signaling pathway.(Peace & O'Neill, n.d.) By driving the indirect inhibition on IκBζ, it reduces the production of pro-inflammatory genes, including Nos2. (3) some studies also argue that a derivative form of itaconate, 4-octyl itaconate (4-OI), can activate the nuclear factor erythroid 2-related factor 2 (NRF2) pathway, the main regulating pathway for antioxidant and anti-inflammatory responses. (Marrocco et al., 2021; Yang et al., 2023)

What makes itaconate an intriguing metabolite is that it can elicit host-anti-inflammatory effects while simultaneously exerting pathogen-controlling actions.(Luan & Medzhitov, 2016) Specifically, itaconate acts as a direct antimicrobial agent by disrupting bacterial cell membrane stability or interfering with the formation of extracellular polysaccharides, which are essential for biofilm development.(Peace & O'Neill, n.d.) Correspondingly, certain bacteria, such as *Yersinia pestis* and *Pseudomonas aeruginosa*, have evolved enzymes capable of degrading itaconate as a means of evading the host immune response. (Sasikaran et al., 2014) In summary, we hypothesize that the presence of extracellular itaconate may mitigate nitric oxide (NO)-mediated inhibition of cellular respiration by preventing NF-κB activation, while concurrently maintaining antimicrobial activity through direct interaction with the pathogen's structural components.

3.2 The impact of Cellular Anti-oxidant Responses on iNOS

In our thorough review of the mechanisms and biological roles of NO in DCs, as well as the opposing effects of immune regulation by NO, some may question whether NO production is truly beneficial in inflammatory contexts, considering the potential risk of NO-mediated cellular modifications and host-cytotoxic effects on both immune and surrounding cells.(Belenichev et al., 2024; P. K. M. Kim et al., 2001; Shreshtha et al., 2018) Unlike pathogens vulnerable to NO-driven cellular changes, mammals possess protective mechanisms such as antioxidant systems that mitigate the cytotoxic effects of NO while still enabling its antimicrobial function.(Begara-Morales et al., 2016) The accumulation of NO increases the likelihood of S-nitrosylation on the regulatory protein Kelch-like ECH-associated protein 1 (KEAP1). S-Nitrosylation of KEAP1 results in the detachment of KEAP1 from the transcription factor Nrf2 in the cytosol, leading Nrf2 to translocate to the nucleus where it upregulates genes that protect against oxidative cellular stress. (Wakabayashi et al., 2010) Nrf2 activation gives rise to the expression of antioxidant proteins, including peroxiredoxins (PRDXs) and heme oxygenase (HO).(Begara-Morales et al., 2016; Luo et al., 2018) Hence, in this subsection 3.2, we will explore the association of these Nrf2-driven antioxidant proteins with iNOS expression, function, and NO effects in DCs. **(Figure 3.5)**

3.2.1 Via Heme Iron homeostasis: Heme oxygenase-1 and carbon monoxide

Studies have found that the inflammatory responses can drive the coexpression of heme oxygenase (HO) with iNOS. Although inflammatory stimuli can induce both HO and iNOS, they are known to regulate each other, as the HO product, carbon monoxide (CO), and NO undergo a dynamic interplay in biological systems.(Chung et al., 2008) There are two isoforms of HO, one is constitutively expressed at low levels (HO-2), and the other's expression is inducible (HO-1) and observed in myeloid immune cells like DCs.(Park et al., 2010) HO-1 is an efficient enzyme that breaks down heme, releasing free iron, Biliverdin, and CO. Notably, HO-1 offers multiple protective mechanisms against cellular stress, including NO-mediated post-translational modifications (PTMs): 1) iNOS shares many features with cytochrome P450 enzymes (CYP), with heme being an essential cofactor in the active site. HO-1 can decrease iNOS translation by reducing intracellular free heme levels.(Cinelli et al., 2020; Datta et al., 1999; Khatsenko, 1998) 2) Biliverdin, the second product of HO-1, is easily reduced to Bilirubin, a lipophilic antioxidant. Given NO's lipophilicity, Bilirubin is more effective than cytosolic antioxidants like Glutathione or Superoxide dismutase.(Idelman et al., 2015) 3) Extensive research highlights the crosstalk between NO and CO, as CO acts as a biphasic regulator of NO—small amounts promote NO production, while higher levels inhibit it. Both gases have similar molecular weights, solubilities, basal production rates, and can bind ferrous heme.(Hartsfield, 2002) Observations include CO binding to iNOS heme and CO-mediated inhibition of iNOS translation.(H. S. Kim et al., 2008) 4) HO-1 also indirectly influences iNOS by competing with its electron donor, NADPH.(Gozzelino et al., 2010; Ting et al., 2023) **(Figure 3.5)**

Although cellular protection mediated by HO-1 and the crosstalk between NO and CO are extensively documented, a notable gap persists in our comprehension of how HO-1 regulation influences iNOS in relation to NO-induced inhibition of respiration. Addressing this knowledge gap is of critical importance, as it may yield valuable insights into the modulation of DC immune effector functions for therapeutic purposes. According to data presented in this dissertation, LPS-stimulated DCs produce NO at levels that impair mitochondrial respiration and reduce the lifespan of these cells.(Snyder et al., 2022; Vagher et al., 2025) Consequently, we infer that the concentration of stimulatory LPS is crucial in mitigating these cytotoxic effects by regulating iNOS expression levels or by influencing the substrate bioavailability for iNOS through Arginase inhibition.(Boucher et al., 1999; Ckless et al., 2008) Additionally, preliminary unpublished data from our laboratory indicate that LPS activation leads to a dose-dependent upregulation of HO-1 expression, which correlates with the NO production curve in DCs. Additionally, pilot experiments using CO-releasing molecules (CORM) treatment revealed a decrease in nitrite detection, a derivative of NO. These findings support the notion that distinct NO thresholds define different cellular respiration states and immune responses, with activity potentially influencing these thresholds. Considering the interaction between NO and CO, along with NO's dual roles in dendritic cells, we propose that HO-1 reduces NO-driven host cell damage by suppressing iNOS activity and expression. Furthermore, inhibiting HO-1 could decrease the LPS-induced NO-respiration threshold. **(Figure 3.6)**

3.2.2 Via Cellular Redox Homeostasis: Peroxiredoxin and Glutathione

Along with HO-1, several other antioxidant enzymes are upregulated by Nrf2, including peroxiredoxin (PRDX) and glutathione (GSH).(Graham et al., 2018; Ma, 2013) By lowering ROS levels, PRDX can help prevent oxidative stress and damage, ensuring proper immune cell function. They can serve as both positive and negative regulators of immune signaling, influencing the strength and duration of immune responses.(Rhee, 2016) There are six PRDX isoforms (PRDX1 through PRDX6), based on their subcellular presence, in mitochondria, peroxisomes, the cytosol, and the nucleus. Though all six isoforms have been identified in mammals, specific PRDX isoforms have been linked to myeloid cell function, such as macrophages and DCs.(Graham et al., 2018; Riddell et al., 2010; Rivollier et al., 2006) PRDXs are thiol peroxidases that reduce various peroxides, including hydrogen peroxide (H2O2). The reaction of NO with superoxide (O2.-) leads to the formation of peroxynitrite (ONOO-). By detoxifying H2O2 via PRDXs activity, the degree of ONOO- generation can be modulated. Additionally, not only do PRDXs modify the redox environment, but they can also diminish signals that activate iNOS, thus indirectly regulating iNOS. (Bast et al., 2010; Ingram et al., 2019; S.-U. Kim et al., 2013)

Glutathione (GSH), a key cellular antioxidant, plays a vital role in regulating iNOS activity. The interplay between GSH and iNOS is complex and involves multiple mechanisms. Research shows that high levels of GSH or its oxidized form, GSSG, can decrease iNOS expression by reducing the stability of Nos2 mRNA.(Chen et al., 2000) Additionally, since both iNOS and GSH function as oxidoreductases, they depend on NADPH as a cofactor for their catalytic actions. Therefore, in situations of limited NADPH, these enzymes may compete with each other for the shared cofactor, NADPH.(Baldelli et al., 2019) This competition affects the cell's redox balance and NO synthesis, which can influence cellular functions and may contribute to inflammatory responses, particularly in the context of NO-mediated respiration inhibition. Additionally, macrophage studies during Mycobacterium tuberculosis infection points out that GSH can help boost NO's antimicrobial effect, as GSH plays a role as the NO carrier (nitroso-glutathione; GSNO), leading to the nitrosylation of the bacteria.(Venketaraman et al., 2005) To advocate the regulatory associations of PRDX and GSH on iNOS, several therapeutic lifestyle interventions have been developed to promote the expression of these anti-oxidant enzymes. (Subedi et al., 2019) Several studies report that digesting herbal compounds, such as sulforaphane (a type of cruciferous vegetables like broccoli) and curcumin (a chemical produced by turmeric, a member of the ginger family), may help alleviate inflammatory symptoms (that may be driven by high iNOS activity in combination with high levels of pro-inflammatory cytokines) by activating Nrf2 pathway. (Shahcheraghi et al., 2021; Subedi et al., 2019) These herbal supplements are reported to upregulate Nrf2-related antioxidant proteins, including HO-1, PRDX, and GSH.(Saha et al., 2020) Considering all these findings collectively, we hypothesize that supplementation with the aforementioned chemicals will attenuate iNOS expression and the NO-mediated cytotoxic role in DCs by augmenting the expression of antioxidant enzymes that regulate iNOS. Furthermore, we propose that inhibiting PRDX and GSH will intensify NO-driven cytotoxic effects, leading to a reduction in the NO-respiratory threshold in DCs.

CONCLUDING REMARKS

This dissertation offers a comprehensive analysis of how metabolic competition for amino acid arginine affects cellular respiration, viability, and immune function in inflammatory DCs (also known as Tip-DCs). These cells are highly phagocytic and serve as early responders, regulating inflammation and playing a crucial role in fighting certain bacterial infections. Our study demonstrates that activating DCs with LPS triggers TLR4 signaling pathways, resulting in NF-κB activation and increased iNOS expression. We also examine the importance of the balance between NO's dual functions—cytotoxicity and antimicrobial/protective effects—in DCs. NO plays a key role in regulating DC immune cell fate by affecting cellular respiration, survival, and bacterial control. This work introduces a regulatory checkpoint based on arginine substrate competition by arginase. This enzyme reduces L-arginine levels and competes with iNOS for the same substrate, thereby influencing NO production and the threshold of NO-respiration in DCs. By controlling substrate availability for iNOS, arginase modulates the intensity of NO production, shifting the balance toward host-protective effects. Additionally, reviewing various metabolites and cell-derived molecules that regulate iNOS—either directly or indirectly—reveals numerous potential iNOS/NO regulatory pathways involving complex mechanisms and interactions. Understanding how metabolites and metabolic status influence iNOS regulation in DCs will enhance our knowledge of DC biology and help explain how DCs set the inflammatory tone in response to other molecular signals and specific metabolic settings.

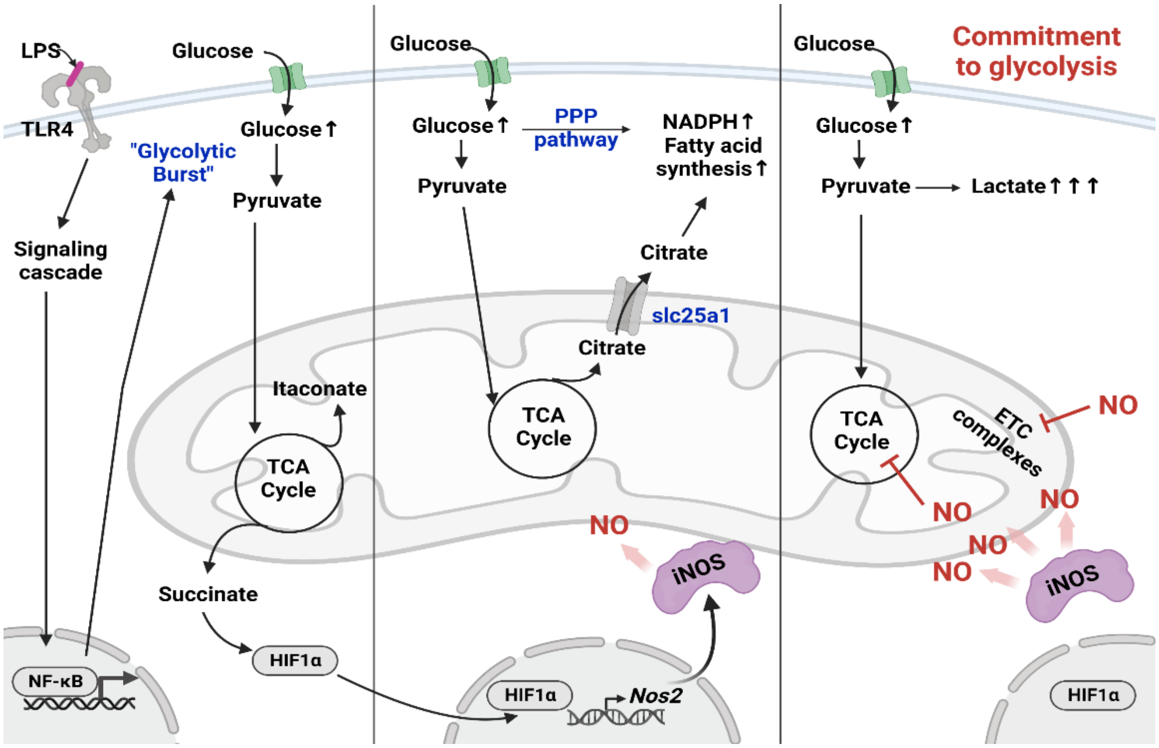

Figure 3. 1 Initial, Intermediary, and Late-phase of NO-mediated metabolic remodeling.

Left panel (Initial phase): Upon TLR4 activation, DCs show increased glycolysis (this phenomena is referred to as "glycolytic burst"), while upregulating pro-inflammatory genes, including *Nos2*, via canonical signaling pathways, such as NFkB-mediated transcription. **Middle panel (Intermediary phase)**: As elevated glycolytic rate reaches the maximal capacity, one of the TCA cycle metabolite citrate can be transported to cytosol, subsequently aiding in fatty acid synthesis process. In addition, newly imported glucose is shuted to penpose phosphate pathway (PPP) to produce NADPH and nucleotide building blocks. **Right panel (Late phase)**: As iNOS-produced NO builds up in the system, it elicits mitochondrial respiration inhibition, leading DCs to commit to aerobic glycolysis. Metabolic rewiring in DCs refers the phenomena when DCs utilize metabolic pathways (carbon flux), as cellular respiratory is blocked by NO.

Figure was created using BioRender.com

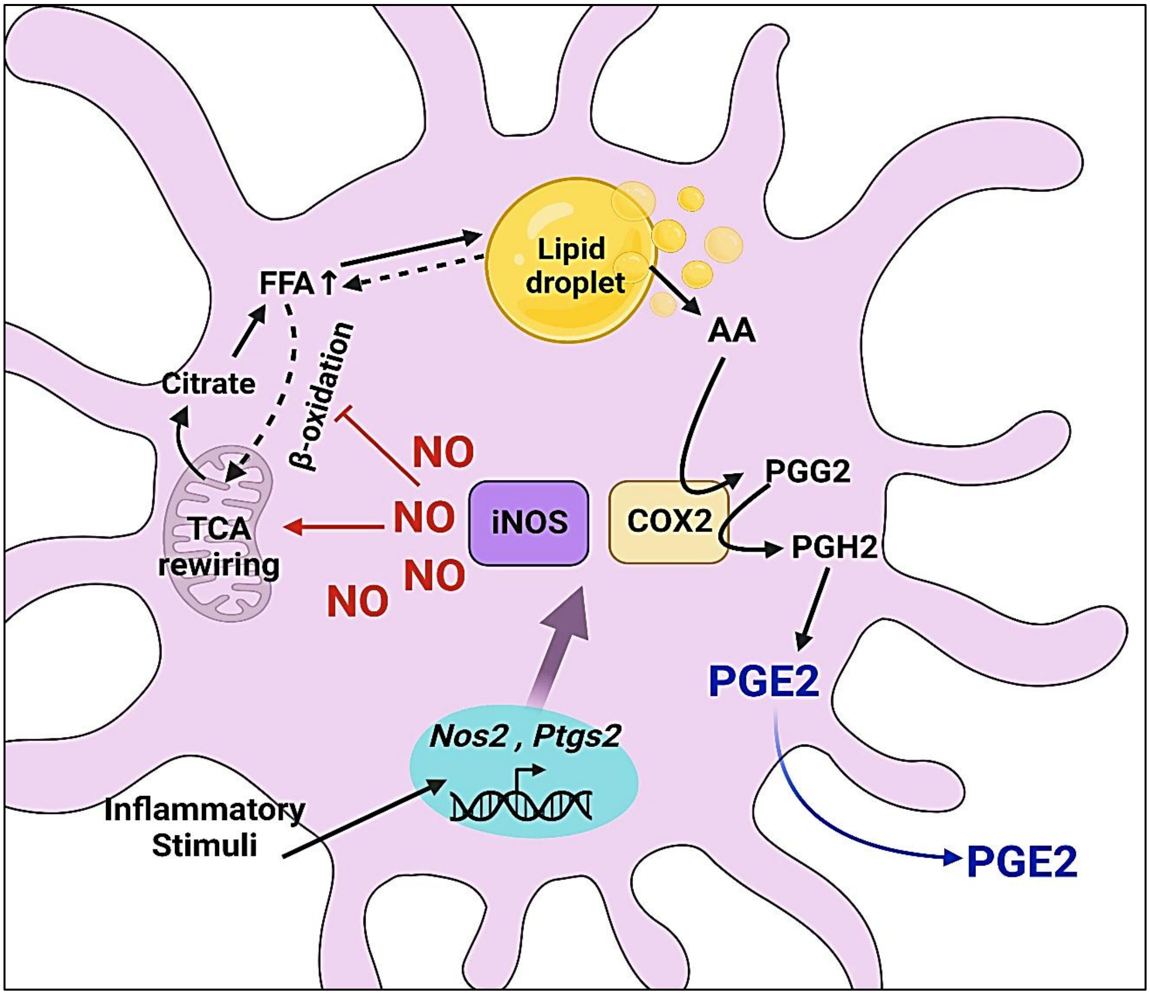

Figure 3. 2 Fatty acids and lipid droplet generation in macrophages and Dendritic Cells.

Inflammatory stimuli, such as the bacterial structural component lipopolysaccharide (LPS), can co-induce Nos2 and Ptgs2, which encode for inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), respectively. Upon NO-mediated cellular respiration inhibition, one of the TCA cycle citrate is exported out to the cytosol, incorporating the synthesis of fatty acids. The accumulation of fatty acids can lead to the development or enlargement of lipid droplets (LDs). LDs are intracellular organelles involved in lipid storage and metabolism. Cyclooxygenase-2 (COX-2), a key enzyme in prostaglandin E2 (PGE2) synthesis, can be recruited to lipid droplets, leading to increased PGE2 production within these organelles. PGE2, a pro-inflammatory molecule, plays a role in various cellular processes, including inflammation and tumor growth.

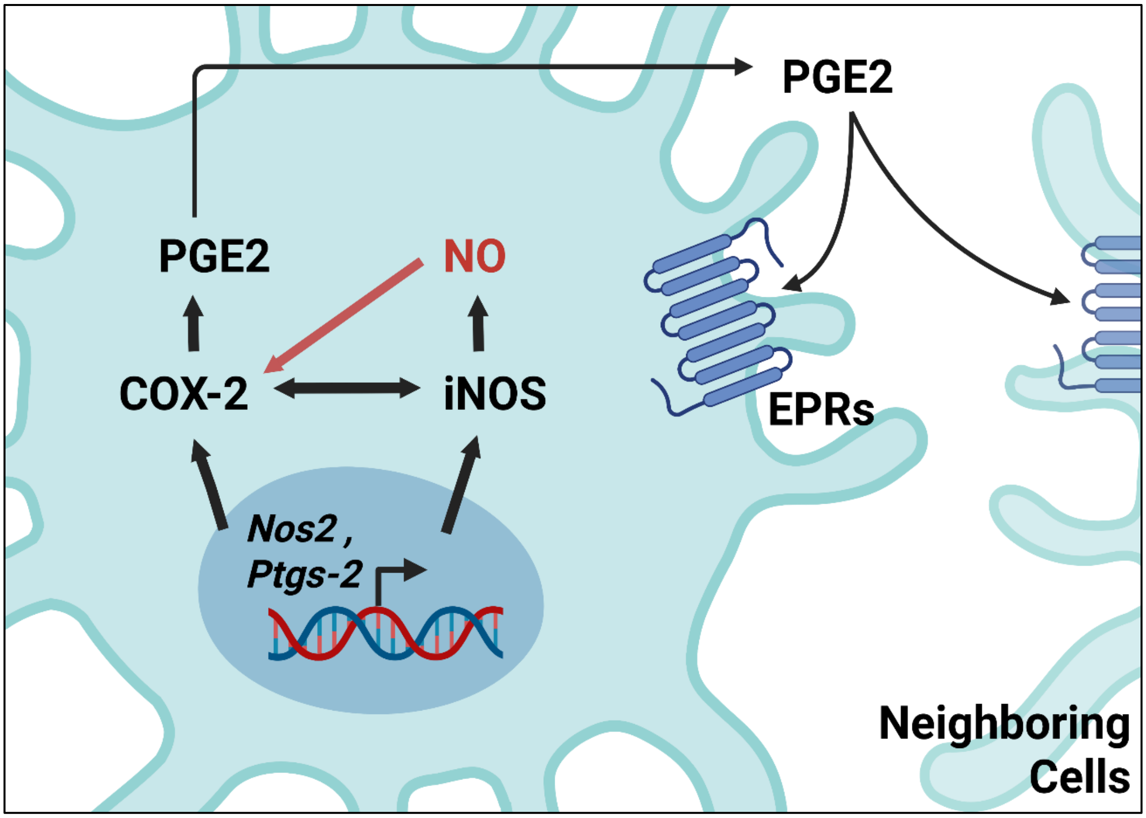

Figure 3. 3 Prostaglandin E2 generation and recognition via E-type prostanoid (EP) receptors in DCs.

Prostaglandin E2 (PGE2) plays a crucial and complex role in various bodily functions, including inflammation, pain management, blood flow regulation, and temperature regulation. It exerts its effects through binding to specific cell surface receptors, a family of G protein-coupled receptors (GPCRs) known as E-type prostanoid (EP) receptors. There are four distinct EP receptors: EP1, EP2, EP3, and EP4. Moreover, iNOS-produced NO can either promote or inhibit COX-2 enzyme, mediating PGE2-related immune regulation.

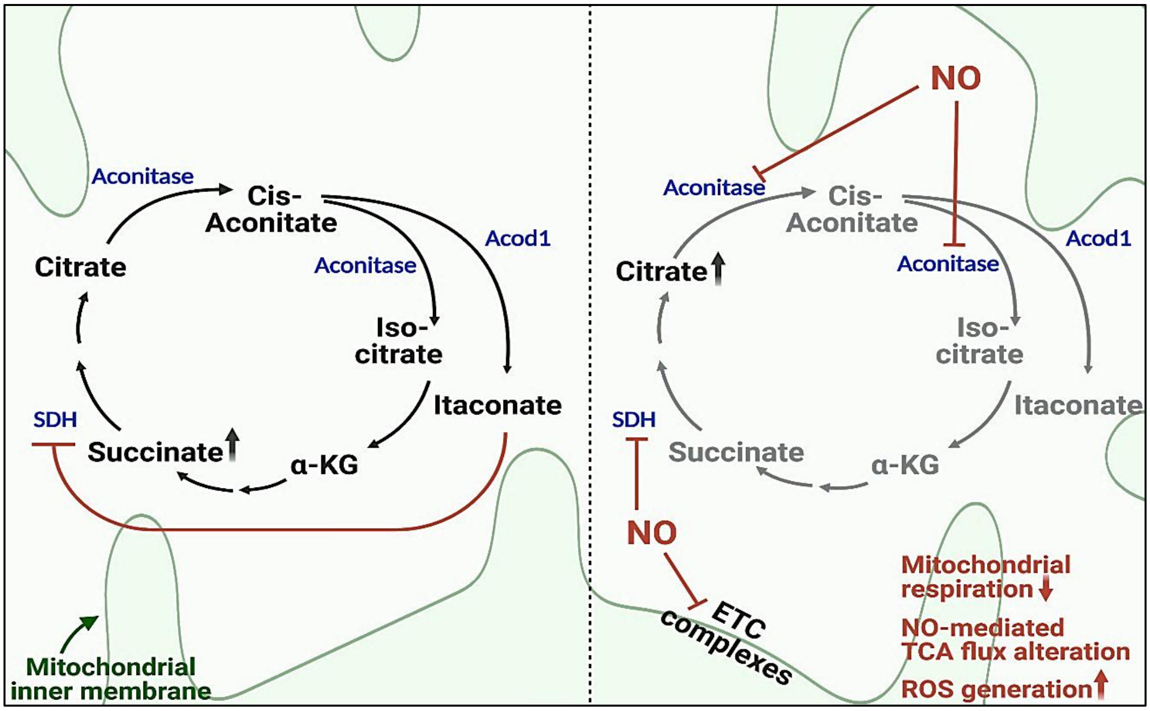

Figure 3. 4 NO-mediated Aconitase inhibition drives Itaconate production via ACOD1.

Aconitate decarboxylase 1 (ACOD1), also known as immunoresponsive gene 1 (IRG1), is a mitochondrial enzyme that utilizes *cis*-aconitate as a substrate. ACOD1 catalyzes the decarboxylation of *cis*-aconitate to produce itaconate. This diversion of *cis*-aconitate from the TCA cycle to itaconate production represents a shift in cellular metabolism. Therefore, the inhibition of aconitase by NO leads to an accumulation of its substrate, *cis*-aconitate, promoting the production of itaconate by ACOD1.

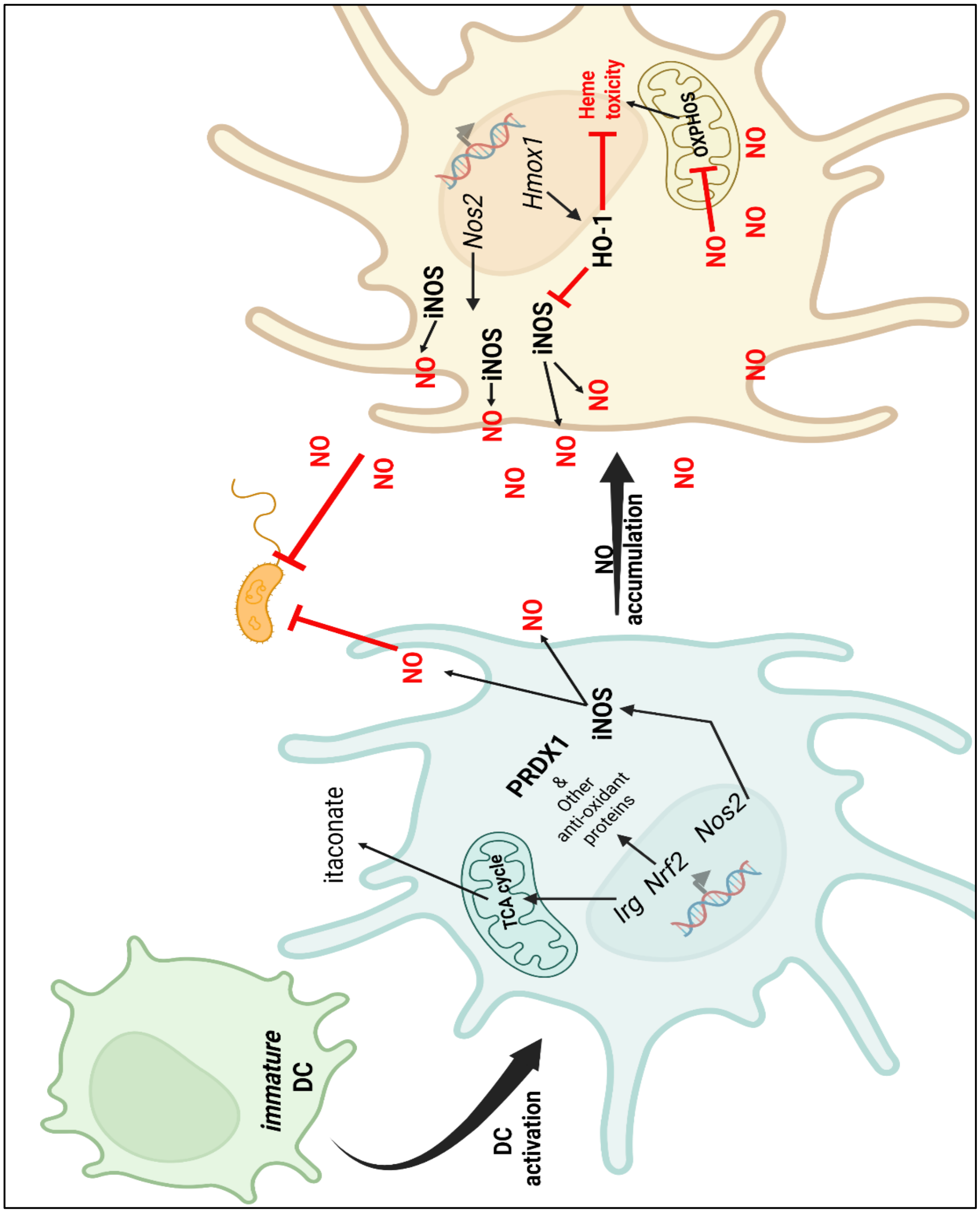

Figure 3. 5 Antioxidant system-mediated iNOS regulation in DCs.

NO can induce the Nrf2 pathway and HO-1 expression primarily by modulating the interaction between Nrf2 and Keap1, facilitating Nrf2 nuclear translocation and stability, which leads to induction of various antioxidant enzymes such as heme-oxygenase (HO-1) or Peroxiredoxin (PRDX). These antioxidant enzymes play critical roles in cellular defense against oxidative stress and inflammation by modulating iNOS activity and NO-related modifications either directly or indirectly, depending on the concentrations and cell types.

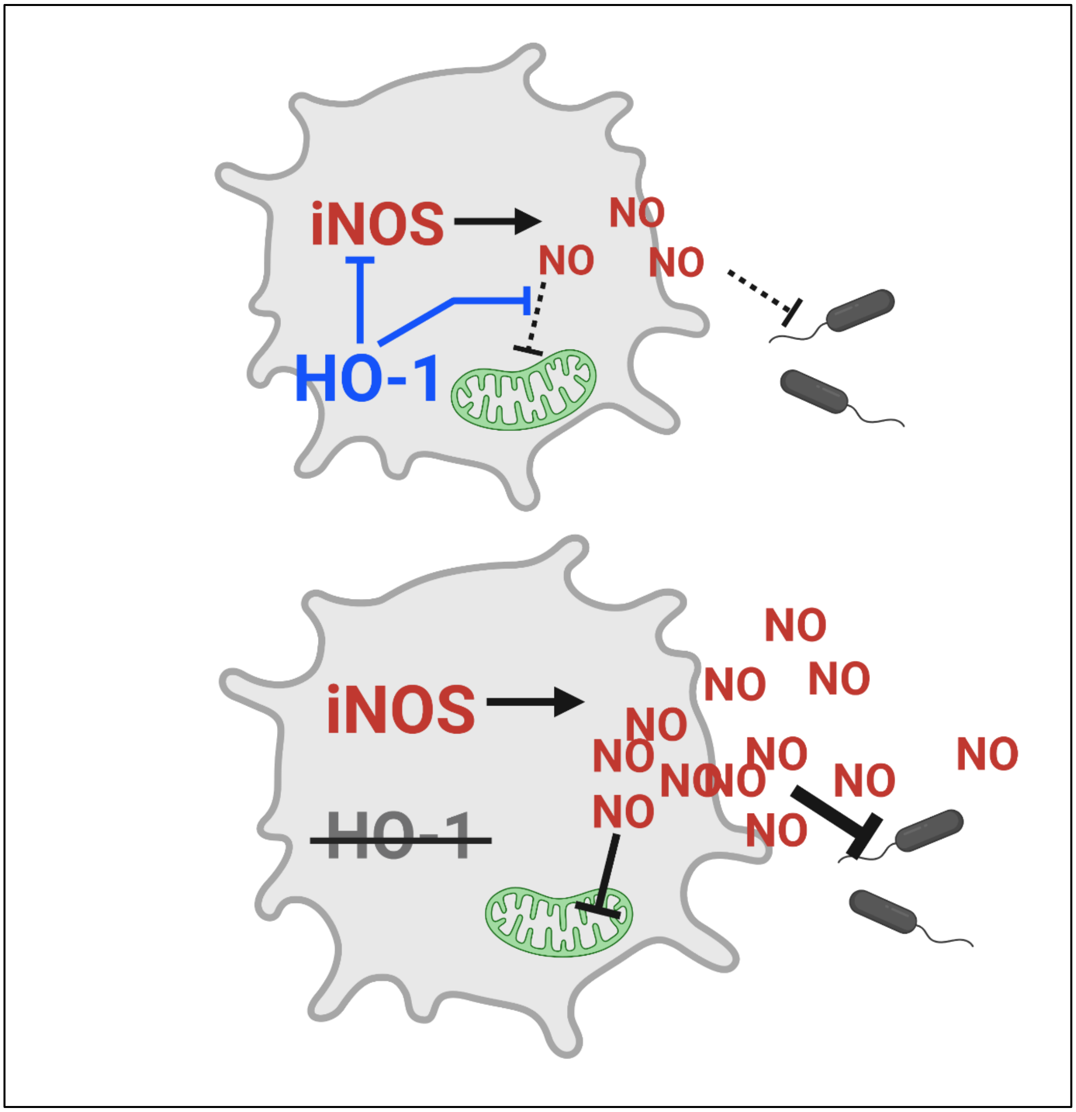

Figure 3. 6 Descriptive illustration showing the regulatory roles of HO-1 on iNOS expression and function.

The inducible form of heme oxygenase (HO-1) can limit the availability of free heme, a substrate for iNOS, thereby decreasing iNOS protein levels and subsequent NO production. Moreover, Carbon monoxide (CO), a byproduct of HO-1 activity, can directly inhibit iNOS activity. This HO-1-mediated iNOS inhibition can dampen and/or prevent NO-mediated cellular respiration inhibition, though this may lead to reduced NO's antimicrobial potency.

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CHAPTER 4: COMPREHENSIVE METHODS

Mice

C57BL/6J mice (Jackson Laboratory Stock #000664) were all purchased from The Jackson Laboratory and bred at the vivarium at the University of Vermont (Protocol Number: PROTO201900021). Mice were maintained under specific pathogen-free conditions under protocols approved by the Institutional Animal Care and Use Committees. Mice were sacrificed between 6 and 10 weeks of age using CO_2 and cervical dislocation.

Mouse bone marrow-derived DC (BMDC) culture and activation

Bone marrow-derived DCs were generated as previously described(Snyder et al., 2022). Briefly, cells obtained from murine femur flush were seeded at 8 x 10^6 cells/10 cm petri dish in 10 mL of complete DC medium (RPMI 1640 containing 10% FBS, 100 U/mL penicillin/ streptomycin, and 2 mM L-glutamine, and 55 mM beta-mercaptoethanol, all from Life Technologies). To promote DC differentiation, a type of growth factor, Granulocyte-macrophage colony-stimulating factor (GM-CSF), was added to seeded cells at 20 ng/mL (GM-CSF #200-15, Fujifilm/Irvine Scientific) and incubated for 6-7 days in a humidified incubator set at 37°C with 5% CO_2. On Days 3 and 5, 15 mL of media was added and swapped, respectively. On day 7, cultures were checked under the microscope, and then the collected DCs were resuspended in a fresh complete DC media. For 96 plate tests, 20,000 cells were seeded per well to ensure an even coverage of the culture surface without overcrowding. The TLR4 ligand LPS from *Escherichia coli* 0111:B4 was added at several concentrations indicated in the figures (#tlrl-3pelps, InvivoGen). The iNOS inhibitor *S*-ethylisothiourea (SEITU) was used at 500 µM (#81275, Cayman Chemical), and arginase inhibitor *S*-(2-boronoethyl)-L-cysteine (BEC) was used at 250 µM (#10170, Cayman Chemical). DMSO was used as solvent for the various inhibitors used. Stimulated DCs with/without inhibitors were incubated for 18-24 hours as indicated in each figure.

Nitrite quantification (Griess Nitrite Assay)

After the indicated stimulation duration, the media supernatant was harvested, and Griess nitrite assays were conducted immediately. Nitrite content was measured using the Griess nitrite assay according to manufacturer instructions (#G7921, Molecular Probes). Indirect quantification of NO via measurement of the stable intermediate nitrite is a validated and established method.(Tsikas, 2007)

L-Ornithine quantification

After the indicated stimulation duration, the media supernatant was harvested, and ornithine assays were conducted immediately according to the manufacturer's instructions (Abcam ab252903: Fluorometric Ornithine Assay Kit). The cell-free complete DC media was used as a negative control (or as a background ornithine level). Then the media's ornithine level was subtracted from all calculated ornithine concentrations of testing DCs to determine DC-mediated ornithine production.

Mass spectrometry-based metabolomics

Metabolites from frozen cell pellets were extracted at 2e6 cells/mL in ice cold lysis/extraction buffer (methanol:acetonitrile:water 5:3:2). Samples were agitated at 4 °C for 30 min followed by centrifugation at 10,000 g for 10 min at 4 °C. Protein and lipid pellets were discarded, and supernatants were analyzed (10 µL per injection) on a Vanquish UHPLC system (Thermo, San Jose, CA, USA) and run on a Kinetex C18 column (150 x 2.1 mm, 1.7 µm – Phenomenex, Torrance, CA, USA) with a 5 min reverse phase gradient exactly as previously described.(Nemkov et al., 2019) The UHPLC system was coupled via electrospray ionization (ESI) to a Q Exactive mass spectrometer (Thermo, Bremen, Germany), scanning in Full MS mode (2 µscans) at 70,000 resolution in the 65-950 m/z range in negative and positive ion mode (separate runs). Metabolite assignments were determined using Maven (Princeton, NJ, USA), following conversion of .raw files into .mzXML by RawConverter. Chromatographic and MS technical stability were assessed by determining CVs for in a technical mixture of extract run every 10 injections.

Seahorse Real-time extracellular flux analysis: Mitochondrial stress test with an iNOS inhibitor

The Seahorse mitochondrial stress test with an iNOS inhibitor was performed as previously described(Snyder et al., 2022; Thwe et al., 2017). Seahorse plates were seeded with 2 x 10^5 cells per well and stimulated with the indicated LPS doses in complete DC medium. At indicated time points, DCs were switched to and analyzed in XF running buffer (unbuffered RPMI 1640, 5 mM glucose, 10% FBS) per the manufacturer's instructions to obtain real-time changes in extracellular acidification rates (ECAR) and oxygen consumption rates (OCR). Where indicated, ECAR and/or OCR were analyzed in response to 1 µM oligomycin (Oligo, #J61898MA, Thermo Scientific), 1.5 µM fluoro-carbonyl cyanide phenylhydrazone (FCCP, #15218, Cayman Chemical), and 100 nM rotenone (#150154, MP Biomedicals) plus 1 µM antimycin A (Rot/Ant, #A8674, Sigma Aldrich), 500 µM SEITU. All drug dilutions were done in plain XF unbuffered RPMI. All Seahorse assays were run on a Seahorse XFe96 machine (Agilent Cell Analysis, Agilent Seahorse XF Analysis, Santa Clara, CA, RRID:SCR_014526).

Reverse-Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

Twenty-four-well plates were seeded with 1x10^6 cells per well and stimulated for 24 hours. CellStripper (Corning #25058) was used to harvest cells, which were then centrifuged at 400 g for 5 minutes to pellet. Pellets were stored at -80^{o}C prior to RNA extraction. RNA was extracted according to the manufacturer's instructions for the Qiagen RNeasy Mini Kit (Qiagen #74104) and RNase-Free

DNase Set (Qiagen #79254). Extracted RNA concentration and purity were determined by nanodrop (ThermoScientific NanoDrop 2000 Spectrophotometer) and 200 ng of RNA was used in the cDNA synthesis reaction according to the manufacturer's instructions for the qScript cDNA Super MIX kit (QuantaBio #95047). The qPCR reaction was performed according to the manufacturer's instructions for the DyNAmoColorFlash SYBR Green (ThermoFisher Scientific #F416S) kit, using the specified *Nos2, Arg1, Arg2, 18s rRNA* (for housekeeping gene) or *beta-Actin* (for housekeeping gene (See below for detailed primer information) and 1 µl of product from the cDNA synthesis reaction, on a Quant Studio 3 Real-Time PCR System, 96-well, 0.2 ml (ThermoFisher Scientific #A28137, RRID:SCR_018712).). We calculated the ΔCt between the housekeeping gene 18s r*RNA* and the target genes (*Nos2, Arg1, and Arg2),* and then calculated ΔCt of testing group was compared to the ΔCt of the unstimulated control, to determine the relative gene expression levels (ΔΔCt).

Primer sequences were adopted from (Cloots et al., 2013; Vagher et al., 2025) and primers were purchased through ThermoFisher Scientific.

Gene	**Direction**	**Sequence 5' to 3'**
Nos2	Forward	AGTTCGTCCCCTTCTCCTGT
Nos2	Reverse	CCTTGTTCAGCTACGCCTTC
Arg1	Forward	GAACCCAACTCTTGGGAAGAC
Arg1	Reverse	GGAGAAGGCGTTTGCTTAGTT
Arg2	Forward	CACCTCTCACCACTGTATCTGG

Arg2	Reverse	CCAGGAAAATCCTGGCAGTTGTG
Lat1	Forward	GAAGGACCCCGACCGG
Lat1	Reverse	AACAGCCACCAGGAAGATGG
Cat1	Forward	CTGGTGGACCTCATGTCCATT
Cat1	Reverse	GCTCATTCTGATCTACTCGATCTAGCT
Cat2b	Forward	GATCCATTTTCCCAATGCCTC
Cat2b	Reverse	TGGAATTGATTTGAGCTAGACATTTG
18s ribosomal RNA	Forward	AGTTAGCATGCCAGAGTCTCG
18s ribosomal RNA	Reverse	TGCATGGCCGTTCTTAGTTG
β-actin	Forward	AGTGTGACGTTGACATCCGTA
β-actin	Reverse	GCCAGAGCAGTAATCTCCTTCT

<u>Flow cytometry</u>

96-well plates were seeded with 2 x 10^5 cells per well and stimulated for the indicated duration of time. Cells were centrifuged at 400 g for 5 minutes. Cell media supernatant was aspirated and saved. Serum-free PBS was added to the wells and cells were put on ice for 10 min prior to transferring to round bottom 96-well plates for staining. Cells were washed with serum-free PBS and resuspended 1:10,000 in fixable Far Red viability stain in PBS for 30 min (#L34973; Molecular Probes/Invitrogen). Cells were washed with Cell Staining Buffer (#420301, BioLegend, hereafter called CSB) at 400 g for 5 min and then fixed in PBS containing 1% formaldehyde for 15 min (#30525-89-4; Alfa Aesar). For extracellular staining, CSB was used for all wash steps and Ab incubations.

Prior to all Ab incubations, cells were incubated for 10 min with TruStain FcX PLUS (anti-mouse CD16/32) Ab (#156604; BioLegend, RRID:AB_2783137) at 1:200 to block Fc receptors for 10 min. All following Ab incubations were 30 min at room temperature as follows: CD11c-PE/Cy7 (1:416.5; #117317; BioLegend, RRID:AB_493568), CD11b-allophycocyanin/Cy7 (1:3000 #101225; BioLegend, RRID:AB_830641), CD40-FITC (1:400; #124607; BioLegend, RRID:AB_1134090), and CD86-PacBlue (1:400; #105021; BioLegend, RRID:AB_313168). Single-color controls were prepared in parallel with samples and underwent all incubation and wash steps. For the viability dye single-color control, cells were heat killed at 55°C for 10 min and then pooled in an equal ratio with live cells prior to staining and fixation. Samples were run on an Aurora (Cytek Biosciences, RRID:SCR_019826). All flow analysis was performed using FlowJo Software version 10.10 (BD Biosciences, RRID:SCR_008520).

Western blot

Twelve-well cell culture plates were seeded with 2 x 10^6 cells per well and stimulated for 24 hours. Cells were harvested using cell lifters and then centrifuged at 400 g for 5 minutes to pellet. Supernatants were saved to be used in matching Griess and ornithine analyses. Pellets were stored at -80°C before use. Once ready to use, pellets were resuspended in Cell Lysis Buffer (#9803; Cell Signaling Technology) containing Pierce Protease and Phosphatase Inhibitors (#A32959; Thermo Fisher Scientific). Samples were kept on ice for 30 min with periodic mixing to achieve adequate cellular lysis. Cells were spun at 9600 g for

10 min, and the cellular debris (pellet) was removed by transferring the supernatant into a new tube. Protein concentration was determined using a Pierce BCA Assay Kit according to manufacturer's instructions (#A32959; Thermo Fisher Scientific), and all samples were normalized to 1 mg/ml in sample buffer containing 2-ME. Samples were heated at 95°C for 5 min prior to loading a 4-15% Mini-PROTEAN TGX Precast Protein Gel (#4561086; Bio-Rad Laboratories) with a molecular weight ladder (#161-0375; Bio-Rad Laboratories). Samples were run at 200V for 30 min, then transferred onto nitrocellulose membranes, according to the manufacturer's instructions (#1704270; Bio-Rad Laboratories) using a Trans-Blot Turbo Transfer System (#1704150; Bio-Rad Laboratories). Five percent BSA in Tris buffer saline with Tween-20 (TBST) was used for blocking and all antibody incubations. Overnight incubations at 4°C on a rocker in primary antibodies were followed by washing with TBST and then 1-h incubations in secondary antibodies (see below for the antibody information). SuperSignal West Pico PLUS Chemiluminescent Substrate (#4579; Thermo Fisher Scientific) was used for chemiluminescence signal detection, and blots were imaged on a Syngene PXi using GeneSys (RRID:SCR_015770).

Antibodies	**Company**	**Catalog #**
iNOS	Cell Signaling Technology	2982
Arg1	Cell Signaling Technology	93668
Arg2	Cell Signaling Technology	55003
Phospho-Nfkb p65	Cell Signaling Technology	3033
Total Nfkb p65	Cell Signaling Technology	8242

Tubulin-β	Cell Signaling Technology	2128
β-Actin	BioLegend	643802
Anti-Rabbit IgG, HRP-conjugated	Cell Signaling Technology	7074
Anti-mouse IgG, HRP-conjugated	Cell Signaling Technology	7076

L- and D-Arginine supplementation

Arginine-free DC complete media was prepared by mixing RPMI 1640 SILAC (Thermo Fisher #88365), L-lysine (Sigma-Aldrich #L8662 / Target concentration ~0.218 mM), 10% v/v Dialyzed FBS (Thermo Fisher #26400044), 100 U/mL penicillin/ streptomycin, 2 mM L-glutamine, and 55 mM beta-mercaptoethanol. A titrating concentration of exogenous L-arginine (Sigma-Aldrich #A8094) and D-arginine (ThermoFisher #A16222.06) was added to the Arg-free DC media, according to the experimental plan. In order to cover the full range of arginine concentrations, 0 – 2 mM arginine was used since the normal DC culture media contains ~1.137 mM arginine (ThermoFisher #11875-085).

GM-CSF differentiated DCs were washed in Arg-free DC media twice to remove residual extracellular L-arginine. Then DCs were incubated in Arg-free media for ~1 hour in the cell culture incubator (humidified 37°C /5% CO_2) to promote a depletion of intracellular L-arginine pool. DCs were washed, stimulated with LPS (with/without inhibitors) in arginine-supplemented media, then incubated for ~24hours. Supernatants were collected for Griess nitrite detection.

Bacteria / Gentamicin Protection Assay

The following protocol/procedures were adapted from bio-protocols (Sharma & Puhar, 2019; Subashchandrabose & Mobley, 2014).

Listeria monocytogenes strain ATCC 19115 was used for all bacterial experiments. Cultures were grown from frozen stocks in BHI broth (Remel #R45272) at 37°C on an orbital shaker at 200 rpm overnight to achieve a linear growth phase. To remove accumulated listeriolysin O, a toxin produced by *L. monocytogenes,* the bacterial culture was washed with fresh antibiotic-free media, then spun at 7,500 g for 10 minutes at 4°C. This culture washing step was repeated 3 times. Then, the resuspended bacteria concentration was determined by OD_{600} readings (Spectrophotometer: Eppendorf BioPhotometer). Then the bacteria were diluted according to a pre-determined multiplicity of Infection (MOI). Bacteria and DCs were co-incubated for 1 hour at 37°C with 5% CO_2 to allow bacterial internalization. Cells were washed and then incubated in 50 µg/mL gentamycin (Gibco #15750-060) for 30 minutes to kill the unphagocytosed bacteria. Duplicates of the bacteria-DC coculture plate were set, one for the initial bacteria count (0h post-coculture) and another for the NO-impacted bacteria count (18h post-coculture). The cells in the initial count plate underwent an osmotic lysis process with cell culture grade sterile water, and then the lysate was subsequently plated on BHI agar plates. After 18h of incubation in the presence of either SEITU or BEC, the supernatant was collected, and the cells were lysed with water. In order to achieve a better side-by-side comparison, a dot-inoculation method was used. Briefly, a drop of 2 – 5 µL lysate was loaded on the agar plate, then incubated at 37°C to allow bacterial colonies to grow. The colony-forming units (CFU) of each testing condition were calculated.

Quantification and Statistical Analysis

Throughout the manuscript, “N” refers to independent cell cultures from individual mice, except for a few replicate experiments in which individual femurs from one mouse were cultured and differentiated separately as biological replicates. Those will be indicated in the figure legends. All figures are representative of at least three independent experiments.

All statistical tests were performed in GraphPad Prism using t tests or one-way ANOVA and pairwise comparisons on data where appropriate to determine statistical significance. All p-values reported are adjusted p values. All p-values and tests are indicated on the figures. If a p-value is not stated, the data was determined to be non-significant (greater than $p=0.05$). For all statistical analyses, $P > 0.05$ not significant (ns), $P \leq 0.05$ *, $P \leq 0.01$ **, $P \leq 0.001$ ***, $P \leq 0.0001$ ****.

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