OVERCOMING SELF-REACTIVITY THROUGH IMMUNOLOGICAL AND METABOLIC REDOX MODULATION

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OVERCOMING SELF-REACTIVITY THROUGH IMMUNOLOGICAL AND METABOLIC REDOX MODULATION

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The importance of reactive oxygen species (ROS) is illustrated by their crucial roles in immunology and disease pathologies. ROS can activate redox-dependent transcription factors, promoting a host of proinflammatory immune responses that are exacerbated during oxidative stress. The aim of this thesis is to determine how redox modulation impairs self-reactivity and aberrant inflammation in diabetes. Prevention of CD4+ T_H1 T cell activation is critical for restricting autoreactive immune responses and maintaining pancreatic β cell integrity in type 1 diabetes. Moreover, decreasing the inflammatory milieu and subsequent complications is necessary for restoring insulin sensitivity in type 2 diabetes. Although current immunosuppressive therapies are invaluable for transplantations, small molecule inhibitors with low toxicity are necessary for stopping autoreactivity and treating inflammatory-driven metabolic diseases. We utilized a catalytic antioxidant (CA) in diabetogenic models based on previous work demonstrating that redox modulation promotes T cell hyporesponsivness and impairs innate cell cytokine secretion by blocking NF-κB activation. Additionally, CA sustains health of isolated islets, delays islet allograft rejection, and inhibits transfer of diabetes into young NOD.*scid* mice.

First, the mechanisms behind CA-mediated CD4+ T_H1 T cell hyporesponsivness were investigated *in vitro* and *in vivo* using diabetogenic murine experiments with a focus on the redox-dependent sheddase TACE and one of its substrate, LAG-3, a negative regulator of T cell

activation. Ability to track type 1 diabetes progression through a serum biomarker, soluble LAG-3, was also assessed from both murine and human samples. Next, CA-mediated alteration(s) of immune cell metabolism was characterized. Effects on glycolysis and oxidative phosphorylation were assessed to determine additional mechanisms of regulation and where treatment efficiency wanes. Lastly, redox modulation was evaluated in treatment of high-fat diet-induced type 2 diabetes. Markers of inflammation and diabetic complications were measured to ascertain the severity of insulin resistance.

Collectively, this work is a distinct contribution to the knowledge of CA treatment and its ability to 1) inhibit diabetogenic T_H1 responses through regulation of a redox-dependent metalloprotease and subsequent cleavage of a negative T cell surface marker; 2) prevent self-reactivity through metabolic regulation; and 3) reduce inflammation and complications in high-fat diet-induced type 2 diabetes.

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LIST OF ABBREVIATIONS

ALB = albumin

ALR = alloxan-resistant

ALT = alanine aminotransferase

AMPK = AMP-activated protein kinase

APC = antigen presenting cells

ARE = antioxidant response element

ATP = adenosine triphosphate

BAX = Bcl-2 associated X protein

BCG = bacillus-calmette-guerin

Bcl = B cell lymphoma

BCR = B cell receptor

BDC = Barbara Davis Center

Ca = calcium

CA = catalytic antioxidant

CD = cluster of differentiation

ChgA = chromogranin A

CHO = Chinese hamster ovary

CO_2 = carbon dioxide

CP = connecting peptide

CTL = cytotoxic T lymphocyte

Cu/Zn SOD = copper/zinc SOD

CVB4 = coxsackie virus B4

DC = dendritic cells

DMEM = Dulbecco's Modified Eagle Medium

DN = double negative

DNA = deoxyribonucleic acid

EAE = experimental autoimmune encephalomyelitis

ELISA = enzyme-linked immunosorbent assay

ER = endoplasmic reticulum

FCCP = carbonylcyanide-p-trifluoromethoxyphenylhydrazone

FFA = free fatty acid

GLOB = immunoglobulin

GLP-1 = glucagon-like peptide 1

Glut1 = glucose transporter 1

GPX = glutathione peroxidase

H&E = hematoxylin and eosin

H_2O_2 = hydrogen peroxide

HBSS = Hank's Balanced Salt Solution

HDL = high density lipoprotein

HepG2 = liver hepatocellular carcinoma

HFD = high-fat diet

HIF-1 = hypoxia-inducible factor 1

HLA = human leukocyte antigen

HOCL = hypochlorous acid

IDO = indoleamine 2,3-dioxygenase

IFN = interferon

IFNγR = interferon-gamma receptor

IGF1 = insulin growth factor 1

IL = interleukin

iNOS = inducible nitric oxide synthase

IPGTT = intraperitoneal glucose tolerance test

IRS = insulin receptor substrate

JAK = Janus kinase

JNK = c-Jun amino-terminal kinases

LAG-3 = lymphocyte activation gene 3

LN = lymph node

LPS = lipopolysaccharide

M = BDC-2.5 mimotope

MCP = monocyte chemoattractant protein

MHC = major histocompatibility complex

MMF = mycophenolate mofetil

MnSOD = manganese SOD

mRNA = messenger ribonucleic acid

mTOR = mammalian target of rapamycin

Na = sodium

NAD = nicotinamide adenine dinucleotide

NFAT = nuclear factor of activated T cells

NF-κB = nuclear factor kappa B

NK = natural killer

NO = nitric oxide

NO_2^- = nitrite

NOD = nonobese diabetic

NOX = NADPH oxidase

Nrf = nuclear respiratory factor

O_2^- = superoxide

Oligo = oligomycin

$ONOO^-$ = peryoxynitrite

PDX1 = pancreatic duodenal homeobox 1

PGC-1 = PPARγ coactivator 1

qRT-PCR = quantitative real-time polymerase chain reaction

ROS = reactive oxygen species

Rot = rotenone

SAPK = stress-activated protein kinases

SEM = standard error of the mean

sLAG-3 = soluble LAG-3

SOD = superoxide dismutase

STAT = signal transducer and activator of transcription

STZ = streptozotocin

TACE = tumor necrosis factor alpha converting enzyme

TCA = tricarboxylic acid

TCR = T cell receptor

TFAM = transcription factor A mitochondrial

Tg = transgenic

TGFβ = transforming growth factor beta

T_H = T helper

TLR = toll-like receptor

TNFR1 = tumor necrosis factor receptor 1

TNFα = tumor necrosis factor alpha

TP = total serum protein

Treg = regulatory T cell

UCP = uncoupling protein

UPR = unfolded protein response

VEGF = vascular endothelial growth factor

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1.0 INTRODUCTION

1.1 TYPE 1 DIABETES

Type 1 diabetes or insulin-dependent diabetes mellitus is an autoimmune disorder involving immune-mediated recognition of islet beta cells by autoreactive T cells. The self-reactive T cell response leads to the liberation of ROS and proinflammatory cytokines, resulting in the destruction of pancreatic beta cells in the islets of Langerhans and loss of insulin secretion. Insulin deficiency causes increased blood glucose levels and hyperglycemic side effects. Patients with type 1 diabetes must constantly prevent hyperglycemia by administering exogenous insulin or in the situation of severe hyperglycemic unawareness, by undergoing islet transplantation. Although the majority of the cases of diabetes affecting children are of the type 1 subset, adults are also susceptible. In particular, genetic predisposition based on the human leukocyte antigen (HLA) genotype is one of the main risk factors for type 1 diabetes (1-3). Individuals at the highest risk for type 1 diabetes development have the DR3/4-DQ8 HLA haplotype, which is often screened for in first-degree relatives of patients with diabetes (4; 5). In addition, a host of putative environmental triggers such as viral infection, infant diet, and cold weather have been hypothesized to contribute to the activation of autoreactive T cells and disease vulnerability. Particularly, coxsackie B4 virus (CVB4) has been implicated as one of the viral infections that can trigger onset of disease (6). CVB4 is capable of infecting the pancreatic beta cells, which

results in local inflammation, damage, and release of islet antigens that could potentially stimulate autoreactive T cells (6). Despite the cause, type 1 diabetes is ultimately due to a loss of beta cells mediated by autoreactive T cell attack (2).

Type 1 diabetes affects people in all geographical locations, but has a high incidence rate in people living in North America and Europe (7). The worldwide frequency of type 1 diabetes is expected to double within the next 15 years (8). Childhood incidence is of special interest, since the majority of type 1 diabetes patients are juveniles. In a study conducted in 2000, Finland, Sardinia, Portalegre, Puerto Rico, and Canterbury were found to have the highest numbers of type 1 diabetes cases, particularly in the 10-14 age range (7), and the global distribution patterns had not changed significantly since the 1970s. These results illustrate that certain locations have a higher susceptibility of developing type 1 diabetes, as further supported by a map arranged by the International Diabetes Federation (**Figure 1**). Together, type 1 and type 2 diabetes, which is characterized by a loss of insulin sensitivity mainly occurring in obese individuals (8) (discussed in Chapter 4), afflicts more than 194 million people all over the world, with at least 10% of the U.S. cases being type 1 (9). With epidemiological advances enabling better disease monitoring, the prevalence of cases around the globe justifies type 1 diabetes as a true chronic epidemic.

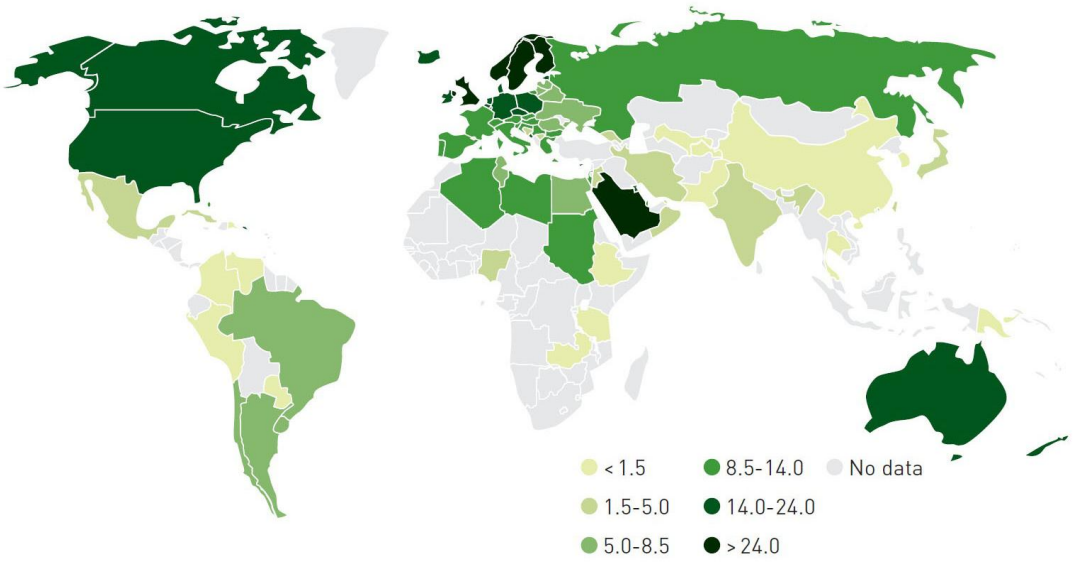


Figure 1: New cases of type 1 diabetes (0-14 years per 100,000 children per year), 2011. *Used with permission from the International Diabetes Federation* (10).

1.2 OXIDATIVE STRESS

Oxidation-reduction or redox reactions are pivotal to maintaining life through respiration, metabolism, and energy supply. Mitochondria, which are known to be the powerhouses of the cell, possess the ability to utilize nutrients to generate energy (redox potential) via the electron transport chain, which donates electrons to oxygen to yield adenosine triphosphate (ATP) and H_2O (11; 12). Consequently, oxygen free radicals, beginning with superoxide (O_2^-), are non-enzymatically leaked from the mitochondria and react with other molecules to create other reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), peryoxynitrite (ONOO-), and hydroxyl radical (OH-), all of which can alter DNA, proteins, carbohydrates, and nucleic acids (13-15) and may eventually lead to irreversible damage. The inability of a cell's antioxidant defenses to prevent oxidative injury and accretion of severe ROS-mediated damage over time will eventually lead to cell death (15-17). In order to maintain a reducing environment, several

cellular antioxidant defenses are in place, including glutathione, glutathione peroxidase (GPX), catalase, and three different superoxide dismutase (18) enzymes: SOD1, 2, and 3, located in different sub-cellular and extracellular locations. A basal level of "accidental" superoxide is accumulated in healthy individuals (11; 19), which has been widely hypothesized to be responsible for aging and the associated pathologies (20-22). Redox reactions are imperative to preserving cellular metabolism yet must be strictly regulated. Imbalances between ROS and antioxidants can initiate oxidative stress, which without proper resolve, can manifest into disease. During chronic oxidative stress caused by environmental factors (i.e. UV light, ionizing radiation, toxic substances), infections, or lack of dietary antioxidants, an inequity of cellular reducing equivalents capable of detoxifying the increased burden of ROS has marked effects on normal cellular processes. However, in times of oxidative stress, normal cellular respiration is also still functioning, resulting in dsyregulated mitochondrial free radical production and disparity between ROS generation and antioxidant defenses (16; 23). The combination of stress-induced and conventional mitochondrial dysfunction can manifest into disease states, including cancer (24-26), rheumatoid arthritis (27; 28), neurological disorders (29-32), pulmonary diseases (33), and type 1 diabetes (34-37).

Once was thought to be solely derived from the mitochondria, reactive oxygen species have now been shown to be produced by an important family of enzymes that play a role in the immune system (38-40). The NADPH oxidase (NOX) enzymes are designed to combine NADPH and oxygen to actively generate superoxide. Activated phagocytes, such as macrophages, monocytes, and dendritic cells (DCs), as well as neutrophils, form ROS within the phagosomal membrane for efficient killing of a wide array of invading pathogens (41). The protection afforded by the phagocytes is crucial, but not without side effects. Production of

highly permeable reactive oxygen species (i.e. H_2O_2) causes leakage of these molecules from phagocytes and therefore, unwanted effects on bystander cells (42; 43). In an environment high in oxidative stress, these bystander reactions drive increased activation of the immune system, cell damage, and progression to disease. For example, NOX-derived ROS have been shown to stimulate mitogenic signaling and proliferation (44; 45), which can have potentially deleterious consequences on the promotion of tumorigenesis (46; 47) and in the context of autoimmunity, can lead to T cell expansion (48). Additionally, H_2O_2 can augment monocyte chemokine receptor surface expression critical for migration to sites of infection and inducing inflammation (49), and can promote VEGF signaling to trigger angiogenesis, with implications in cancer metastasis and tumor progression (50). Furthermore, ROS generated from both mitochondria and NADPH oxidase complexes can act intra-cellularly as well as inter-cellularly as signal transduction molecules. Hydrogen peroxide has been shown to inactivate protein phosphatases (51), as well as to activate protein tyrosine kinases (52; 53) and metalloproteases, through the oxidation of critical cysteine residues (54; 55). Phosphatases such as SHP-1 serve to decrease inflammation by inhibiting tyrosine kinase activity, yet this type of regulation is lost upon cysteine oxidation (56-59). Similarly, latent metalloproteases require oxidation for activation and, in the presence of hypochlorous acid (HOCL) and H_2O_2, secretion of chemotactic mediators (L-selectin and proinflammatory TNFα) is highly increased (60), thus enhancing inflammation. In addition, H_2O_2 has been demonstrated to freely cross the plasma membrane and activate NF-κB, a redox-dependent transcription factor (61; 62). NF-κB plays a major role in immunity by promoting proinflammatory cytokine production, cell proliferation, and inflammation. In general, receptor-ligand interactions are known to generate ROS (63; 64). LPS can facilitate the binding of toll-like receptor 4 (TLR4) to NADPH oxidase 4 (Nox4), causing a subsequent release of ROS (65),

resulting in the activation of NF-κB and generation of proinflammatory cytokines IL-1β and TNFα (64). In a highly oxidized environment, the binding of pathogens to innate cell receptors can lead to hyperresponsiveness (66), suggesting that inflammation is secondary to oxidative stress (36; 67). Not only are phagocytic cells critical for early pathogen recognition through receptor-ligand interaction, they are also necessary for activation of the adaptive immune response. Following antigen recognition by phagocytic antigen-presenting cells (APC), an adaptive immune response develops in secondary lymphoid organs, through synapse formation of APCs with lymphocytes, as well as from critical innate-derived ROS and third signal proinflammatory cytokines (TNFα, IL-1β) enhancing T cell activation, proliferation, and effector function (48; 68). APC engagement with T cells first occurs between MHC-peptide and the TCR, known as the first signal. Second signal is a costimulatory signal, including T cell CD28 interaction with CD80/CD86 molecules on the APC surface. The soluble 'third' signal is made up of cytokines and ROS, critical for the differentiation of T cells into specific lineages and for promoting effector functions (69; 70). Within this interaction, the H_2O_2 made by the phagocytes is able to traverse the synapse and act upon the T cells, at concentrations ranging from 10-100 μM (71; 72), resulting in a feed-forward mechanism stimulating T cell-specific NF-κB activity and subsequent proinflammatory cytokine production. Similar effects of ROS are also seen on B cells (73). Moreover, antigen stimulation of the T cell receptor (74) also drives endogenous production of H_2O_2 through the T cell's own NOX enzyme (39; 75). Intracellular H_2O_2, depending on the abundance and the context, can then signal and lead to T cell proliferation (75; 76), T cell effector function (48; 63; 77) or at high doses, cause apoptosis (75; 76). Therefore, in the presence of oxidative stress, an inability to balance oxidation with antioxidant enzymes can

drive chronic inflammation from both the innate and adaptive arms of the immune response (78), manifesting into many clinically-relevant diseases, such as type 1 diabetes.

1.3 OXIDATIVE STRESS IN TYPE 1 DIABETES

Despite a multitude of efforts in trying to specify the exact etiology, the cause of type 1 diabetes is still not fully elucidated. The combinatorial effects of genetic susceptibility, environmental factors, and dietary deficiencies are known to contribute to disease origin; however, the impact of oxidative stress in a genetically susceptible individual is of particular interest. Oxidative stress, as stated above, occurs when the generation of ROS overcomes the scavenging abilities of antioxidants. Such instances may be mediated by genetic lack of antioxidant enzymes as well as environmental triggers like viral infections. Overall, oxidative stress has been linked to beta cell cytotoxicity (79-81) and has been suggested to play a role in type 1 diabetes pathology (82-85). Several studies show that the total serum antioxidant status, as measured by urate, Vitamin C, and total plasma antioxidant levels, of prediabetic and type 1 diabetes patients is lower in comparison to age-matched controls (86; 87), which inevitably leads to greater oxidative modification of proteins and lipids (88). Other literature illustrates a connection between viruses, ROS production, and type 1 diabetes onset. ROS are made following viral infection from activated phagocytes (89; 90), as mentioned previously, and work to not only cause cellular injury but also can activate inflammatory, redox-dependent transcription factors, such as NF-κB, perpetuating inflammation. Viral-mediated ROS production or a reduction in antioxidants can have severe consequences, as beta cells are more prone to oxidative damage than most other tissues. The beta cell mitochondria have exceptionally low levels of the phase II enzymes

glutathione peroxidase, superoxide dismutase, and catalase (35; 91-93). Because of this low antioxidant defense, beta cells can be clearly disrupted by oxidative stress, and in genetically predisposed individuals, result in easy targets for a subsequent cytokine-mediated autoimmune attack. Mitochondrial and NOX-derived ROS both have implications in beta cell destruction and type 1 diabetes. Increased glucose causes rapid induction of the tricarboxylic acid (TCA) cycle within the beta cell mitochondria, which not only causes greater energy production but also leads to augmented ROS generation (94). The superoxide leaked from mitochondria can then form H_2O_2 and work to uncouple glucose metabolism from insulin secretion (95). Ultimately, high levels of mitochondrial ROS can cause beta cell death (96; 97). Intriguingly, models of type 1 diabetes induce disease by generating toxic amounts of ROS within the islets (i.e. streptozotocin and alloxan) (98). Alloxan is easily taken up by beta cells (99), where it is reduced into dialuric acid and subsequently reoxidized to establish a redox cycle (100). ROS generated by alloxan treatment have been shown to promote islet beta cell DNA fragmentation, culminating in cell death (101). In contrast, an alloxan-resistant strain of mice, the ALR mouse, shows increased ROS dissipation and resistance to islet destruction (34; 102; 103), further implicating the importance of oxidative stress in type 1 diabetes. Streptozotocin (STZ), on the other hand, causes beta cell DNA alkylation and eventually drains the cellular nicotinamide adenine dinucleotide (NAD+) and ATP source in an effort to repair the DNA (104). Xanthine oxidase is then able to utilize dephosphorylated ATP as a substrate for superoxide production (105). Additionally, STZ metabolism increases the levels of islet cell nitric oxide (NO) (106), which together with superoxide can generate ONOO-. Detection of ONOO- in prediabetic nonobese diabetic (NOD) mouse islets (107) suggests the importance of this source of ROS in beta cell death (85). Similarly, NOX enzymes have been detected within the

pancreatic beta cells (108; 109). Hyperglycemia can increase the assembly of NOX enzymes through its p47phox subunit, and therefore, enhance superoxide generation (110) and facilitate beta cell death.

1.3.1 General immunology of type 1 diabetes

Autoimmune diseases manifest from a complex immune response involving players of both innate and adaptive immunity, leading to loss of self tolerance. In type 1 diabetes, the exact mechanism(s) by which each arm of the response initiates and furthers the disease is still not entirely clear. Genetic predisposition is a susceptibility factor in type 1 diabetes, yet the reactivity towards an autoantigen is the critical immunological trigger that eventually elicits disease (1; 3).

Autoantigens are self-molecules present in the host that under the right circumstances have the ability to stimulate an immune response. Innate cells process and present self antigens to members of the adaptive immune system. Autoantibodies and autoreactive T cells interact with autoantigens to drive type 1 diabetes pathogenesis. In type 1 diabetes, the presence of autoantibodies was discovered by detecting the reaction of serum with sections of human pancreas (111). The exact molecules recognized by the antibodies are islet cell surface and cytoplasmic autoantigens. Such antigens associated with type 1 diabetes include glutamic acid decarboxylase 65 (GAD65), protein tyrosine phosphatase (IA2), and insulin (IAA) (111; 112). GAD65 is an enzyme present in human beta, alpha, and delta cells that mediates GABA synthesis and in conjunction with another islet expressed protein, IA2, is accountable for the strongest autoantibody recognition of pancreatic islets (111). Insulin, along with its precursor proinsulin, are the only known autoantigens specifically to the beta cells (113). Other molecules

demonstrated as potential autoantigens include: ICA12, osteopontin, nephrin, CD38, heat shock protein 65, rat-specific ICA69, chromogranin-A and the zinc solute carrier, ZnT8 (111; 114; 115).

1.3.2 Contribution of the innate immune system in type 1 diabetes pathology

The innate immune system is responsible for providing the initial defense and regulation when the host encounters a foreign antigen (116). Its function, in turn, is to eventually stimulate the adaptive response for a more specified and powerful protection. However, if the innate system is unable to properly engage and activate the adaptive cells, it may be the first breach in a cascade of normally appropriate events that will lead to subsequent failures and autoimmunity.

NOD mice spontaneously develop type 1 diabetes, characterized by an early infiltration of dendritic cells and macrophages into the islets, prior to lymphocytic infiltrate. This infiltrate of accessory cells is the first sign of autoimmunity (117; 118) Since dendritic cells are thought to be the only APC able to activate naïve T cells, their function in autoimmunity is as important as their adaptive immune counterparts (116; 119). Like typical T cell activation, autoreactive T cells are also primed by DCs that migrate to the thymus after antigen uptake (120). The specific antigen recognized by and mechanism of activation of DCs that mediate type 1 diabetes are still only speculative. DCs may be activated by environmental factors, the recognition of islet-associated proteins, or through interactions with already activated resident macrophages (6; 116). As mentioned previously, CVB4 infection has been linked to type 1 diabetes onset (6). One such scenario could involve viral infection of the beta cells where the damage that occurs influences the production of proinflammatory cytokines and a local immune response. Usually, the cytokines promote a response against the virus and in non-predisposed individuals, clearance of

infection without exacerbated incident. However, in genetically susceptible individuals, this cytokine burst can be the trigger point that initiates an autoimmune response. The cytokines produced can cause the mobilization of endogenous antigen, referred to as cryptic epitopes, which can be taken up by resident DCs to activate autoreactive T cells via a bystander approach, or to elicit an initial strong response that eventually subsides and reveals other epitope targets, a process known as epitope spreading (6; 121-123). Following viral recognition by DCs in susceptible individuals, there is an upregulation of costimulatory molecules that can actually lower the threshold for the activation of autoreactive T cells (116). Primed T cells can then home to the pancreas and cause greater damage of the beta cells. In addition to viral-mediated autoreactive mechanisms, pancreatic DCs have also been shown to recognize CD154 (CD40 ligand) molecules transgenically expressed on beta cells followed by migration to the lymph nodes to stimulate autoreactive T cells and cause insulitis (120). Hsp60, which is a heat shock protein expressed on the surface of beta cells during islet inflammation, is another molecule that can be seen as a danger signal for the DCs (116; 124). These data demonstrate that the presence of certain molecules on the surface of pancreatic cells, and not just those that are liberated after inflammatory damage, can also activate the DCs. Besides direct recognition of islet-associated molecules, DCs can be induced to better present self-antigens as well (116; 119). Despite their role in tolerance and the stimulation of Tregs in protecting against type 1 diabetes (125), plasmacytoid DCs that phagocytose apoptotic cells have been shown to release IFN-α (126), which can enhance the ability of conventional DCs to present self-antigens (119).

Although resident macrophages are present in the pancreas at all times, acquisition of antigen is required for macrophage activation and the production of cytokines. As described above, genetic and environmental factors can lead to cell destruction, releasing beta cell-specific

antigens as well as ROS (127). The ROS created by the initial insult to the islets are able to stimulate the activation of redox-dependent NF-κB and other transcription factors within the macrophages (128). Activated macrophages secrete a mixture of proinflammatory cytokines such as TNFα, IL-6, IL-1β, and more ROS, which can initiate damage of the pancreatic beta cells (129-131). IL-1β can cause extensive cytolysis of beta cells (132) through the upregulation of iNOS and subsequent generation of NO (133; 134). By contrast, TNFα enhances IL-1β-mediated islet destruction through activation of APCs and T cells (135-137), but does not cause direct beta cell apoptosis *in vivo* (137). Like DCs, macrophages will also phagocytose dying beta cells and present antigen in the context of their MHC molecules. Subsequent to antigen presentation, macrophages will release cytokines and ROS that can help to direct the innate response in activating other cells such as DCs, αβ T cells, natural killer (NK), natural killer T (NKT), and γδ T cells (116). In addition, the combination of NO, IL-1β, and TNF-α produced by recruited macrophages can have detrimental effects by mediating islet cytotoxicity and beta-cell death (138). Macrophages from NOD mice have also been described as being deficient in phagocytosis, especially in the uptake of apoptotic cells (116). Phagocytosis, in this regard, helps to limit inflammation caused by the presence of recently killed cells. If macrophages are unable to engulf the anti-inflammatory apoptotic cells, these cells could progress to a necrotic state and elicit greater inflammation near the islets, leading to even more beta cell death (139).

NK and NKT cells are also implicated in type 1 diabetes. NK cells are one of the first cells, along with DCs and macrophages, to migrate into the pancreas prior to disease onset (119). These cells themselves do not cause disease, but can interact with autoreactive conventional T cells to stimulate their proliferation and activation (119). However, NK cells can also carry out regulatory functions in the pancreatic infiltrate. For instance, NK cells can directly lyse immature

DCs (116), since DCs present at the pancreas before antigen recognition have low levels of MHC class I expression. Oxidative stress has been linked to reduced NK cell numbers and expression of their activation receptor (140; 141). Similarly, NKT cells, which bridge the gap between innate and adaptive immune cells, also have important regulatory functions in autoimmunity. NKT cells are reactive to CD1d (142), a rare antigen-presenting molecule. A decrease in CD1d-restricted NKT cells correlates with an increase in diabetes expression in both NOD mice and humans (116; 119). CD1d-NKT cells play regulatory roles in immunity, based on their ability to produce large amounts of IL-4, which skews T cells to a T_H2 subset (116). Cytokines released from T_H2 cells inhibit the development of the T_H1 subset, which is the major population of CD4+ cells involved in type 1 diabetes, as discussed below (143-146). After CD1d-NKT cell transfer, diabetes risk is partially reduced in NOD mice (147). Notably, NOD mice have deficiencies in both NK and NKT cells (148; 149)

Overall, DCs, macrophages, NK, and NKT cells have all been identified as having some abnormalities in patients with type 1 diabetes (116; 119). Therefore, if people are genetically susceptible to disease, modulation of the innate immune system could be valuable in prevention and treatment of disease. Although necessary for an initial defense, the innate immune system does not elicit as potent a response as the adaptive immune system. ROS and cytokines released by APCs not only promote beta cell damage, but also help to generate an adaptive immune response, which in type 1 diabetes, is the crucial step in autoimmune destruction. It is well established that chronic exposure of antigens to innate immune cells in a highly oxidized environment will lead to MHC-peptide presentation, perpetuating an adaptive immune response (150; 151) (**Figure 2**) The adaptive B cell-produced antibodies in type 1 diabetes are responsible

for disease prediction and can act in combination with innate molecules to cause even more damage that leads to beta cell loss (116; 144-147; 152; 153).

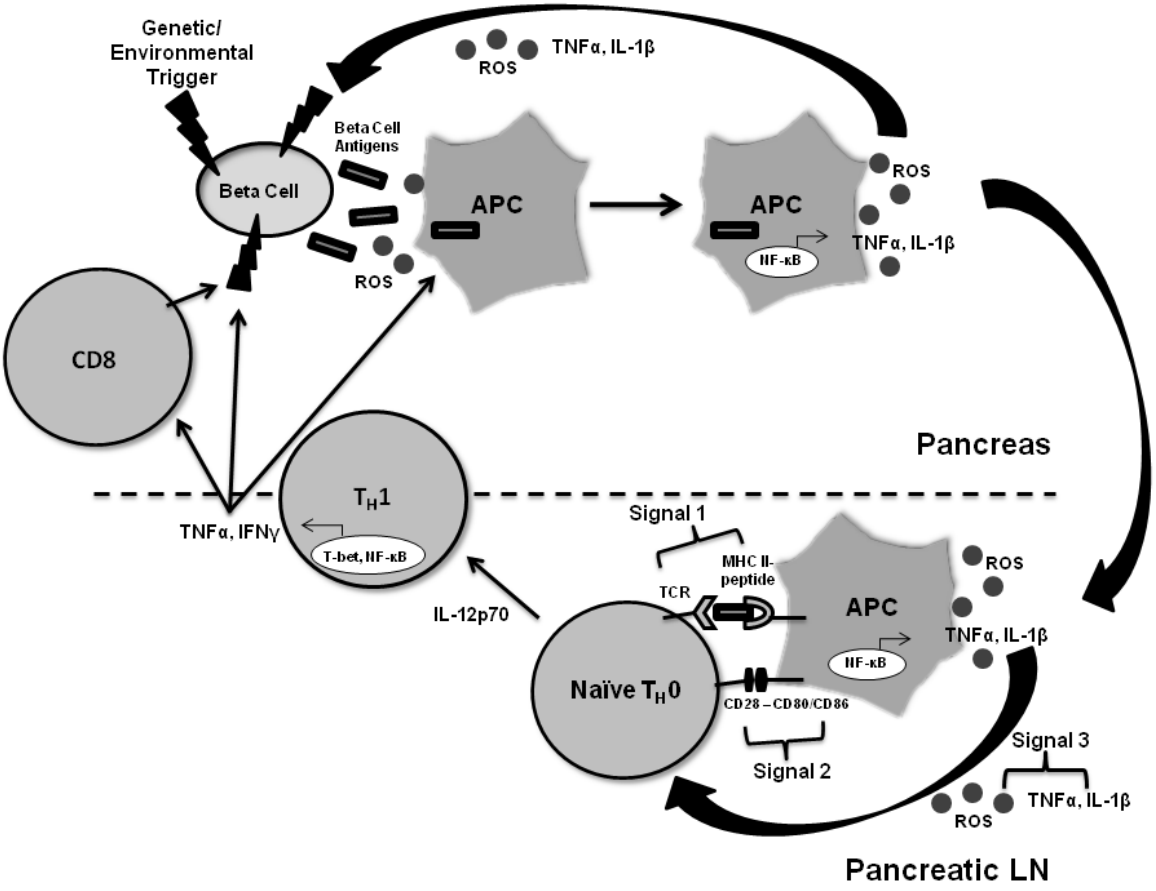


Figure 2: Role of redox in the immunopathology of type 1 diabetes. An initial genetic predisposition or environmental insult to the beta cell triggers the release of beta cell antigens as well as the production of ROS. Beta cell antigens are phagocytosed, and ROS are able to stimulate redox-dependent transcription factors such as NF-κB, which leads to APC activation and cytokine secretion. ROS and proinflammatory cytokines secreted by APCs act as the third signal within the T cell-APC immunological synapse, which occurs in the pancreatic lymph node. ROS play a critical role in the progression of naïve T_H0 cells to cytokine-secreting T_H1 cells. Release of IFNγ by T_H1 cells then works directly on the beta cells as well as activates more APCs and CD8+ cells, all of which can impart deleterious effects on the islets (154).

1.3.3 Role of the adaptive immune system in type 1 diabetes pathology

The adaptive response is primarily made up of B and T cells. Each of these cells produces a surface and/or soluble receptor that are specific for the antigen presented by the innate immune cells. B cells first produce a B cell receptor (BCR) and then later differentiate into antibody-secreting plasma cells, whereas T cells only generate a membrane-bound TCR (74). In type 1 diabetes, B cells have relatively unclear mechanisms for contributing to disease. Prior to diabetes onset, autoantibodies formed against islet autoantigens can be detected at high levels in the serum (111; 155). Antibodies against more than one autoantigen, along with HLA genotyping, can reliably predict diabetes susceptibility (111). Despite their formation, however, if mouse antibodies are transferred into a naïve murine host, they do not cause disease (111; 155). Therefore, the autoantibodies themselves are not sufficient to trigger disease onset, which is a paradox to passive immunity. However, more in depth studies have linked the autoantibodies to other possible pathogenic mechanisms. Soluble autoantibodies can activate the innate immune response through either Fc receptors or complement receptors and further drive the activation of NKT cells, mast cells, macrophages, or γδ T cells that drive the inflammatory response (156; 157). The membrane-bound forms of antibodies, or the BCR itself, also have mechanisms attributing to disease. Autoantibodies that remain attached to B cell surfaces can help to improve antigen capture and presentation through MHC class II, characterizing B cells as APCs for CD4+ T cell activation (158). Along with DCs and macrophages, B cells have also been detected in the infiltrate of the pancreatic islets preceding insulitis (155). Depleting B cells through the use of an anti-CD20 monoclonal antibody, rituximab, decreases diabetes incidence in NOD mice and most likely acts to generate regulatory B cell populations that help control disease, especially in an adoptive transfer model (155). Rituximab clinical trials in recent onset patients show a reduction

in the amount of insulin needed after one year of treatments; however, differences between control and treated groups are absent after two years, making the therapy only temporarily efficacious (159). Although the exact mechanisms by which B cells contribute to type 1 diabetes are unclear, antibody formation and the ability of B cells to present antigens are helpful in predicting diabetes and priming the autoreactive T cells, respectively.

Autoreactive T cells are the main effectors of the beta cell damage and apoptosis that eventually lead to the onset of type 1 diabetes. Unlike B cells, T cells can directly promote apoptosis through cell lysis and the production of proinflammatory cytokines that induce stress upon the highly sensitive beta cells (116; 144-147; 152; 153). In the context of continuous beta cell ablation in the beginning stages of type 1 diabetes, APCs phagocytose dying cells and migrate to the pancreatic lymph node to interact with naïve T cells via the immunological synapse. It is this interaction that enables T cell proliferation and effector function to occur. In the presence of all three necessary signals: 1) MHC-peptide, 2) costimulation, and 3) soluble third signal (in this case consisting of ROS, IL-1β and TNFα), T cells become activated via NFAT and NF-κB (160-164). Furthermore, IL-12 released from APCs can differentiate CD4+ T cells into the T_H1 lineage via signaling through STAT4 (165-168). The specific cytokines produced depend on the subset of CD4+ helper T cells involved in the pathogenesis. T helper cells can be divided into several populations: T_H1, T_H2, T_H17, and T follicular helper (TFh) cells. T_H1 cells are involved in cellular immunity, which increases the killing abilities of both macrophages and cytotoxic T lymphocytes (CTL), and produce cytokines such as IFN-γ, TNF-α, IL-1β and IL-2 (145; 146). T_H2 cells help to increase B cell proliferation, antibody production, and produce cytokines such as IL-4, IL-5, IL-6, IL-10, and IL-13 (146). T_H17 cells produce IL-21, IL-22, and IL-17, and TFh cells generate IL-2, IL-10, and IL-21 (169). An imbalance of

T_H1/T_H2 is known to be involved in the pathogenesis of type 1 diabetes (146). Several instances have been discovered in which promoting T_H2 subsets through overexpression of IL-4 (170), blocking IL-12-induced T_H1 differentiation (146), using an anti-IFN-γ antibody (171) or negatively vaccinating (172) in an effort to skew the immune response can actually reduce the incidence of disease. T_H17 cells are thought to play harmful roles in mouse models of multiple sclerosis and collagen-induced arthritis (173). Despite early controversial studies (174-177), T_H17 cells have also demonstrated pathogenic roles in type 1 diabetes, with increased levels in pancreatic islets (178) and the ability to enhance proinflammatory cytokine-induced apoptosis of human islets (179). A relatively new T helper cell subset, TFh cells, is important for helping B cells to produce antibodies within the germinal centers (180). In the context of type 1 diabetes, excessive TFh activity caused a breakdown of tolerance, the development of anti-islet antibodies and fast progression to diabetes (181). Additionally, IL-21, which is produced from TFh, is critical for pancreatic infiltration (182), making TFh a relevant population of T cells in type 1 diabetes development.

In classic type 1 diabetes pathology, CD4+ T_H1 cells home to the site of antigen production, the beta cells, and recruit other T cells and more APCs through the secretion of IFNγ. IFNγ has some indirect effects on beta cells, including potentiating the maturation of pancreatic APCs, which can then elicit an even greater T cell response (183). Additionally, neutralization of IFNγ in NOD mice has been shown to reduce both diabetes and insulitis (171), whereas a null mutation in the IFNγR gene in NOD mice leads to delayed insulitis and inhibition of spontaneous diabetes (184). Proinflammatory cytokines TNFα, IL-1β, and IFNγ all play a role in beta cell death primarily through activation of redox-regulated transcription factors NF-κB and STAT1 (145; 185; 186). Combinations of TNFα with IFNγ or IL-1β are necessary for primary

murine beta cell death (187), and TNFα/IFNγ act synergistically to activate the stress-activated pro-apoptotic JNK/SAPK pathway, which promotes beta cell apoptosis via p53 and intracellular ROS (188). The activation of NF-κB can also increase iNOS and Fas expression, potential inducers of cell death, while downregulating the anti-apoptotic protein Bcl-2 (189). Apoptosis of beta cells is also mediated partially by T cell expression of Fas ligand, TNFα, and perforin/granzyme (137; 189). Specifically, CD4+ T cells are thought to be sufficient for type 1 diabetes onset (144; 190), whereas CD8+ T cells seem to play in later stages of diabetes, being responsible for direct beta cell killing (191). CD4+ cells help to coordinate beta cell antigen-specific damage indirectly through enhancing effector function of cytotoxic T lymphocytes (CTLs) (152; 153). It is known that synergy between both diabetogenic CD4+ and CD8+ T cells results in absolute transfer of diabetes in rodent models (192; 193). Although specific to the model of autoimmune diabetes, TNFα secretion from CD4+ T cells can bind to TNFR1 on beta cells and cause apoptosis (194), while CD8+ T cells can kill NOD beta cells by a Fas-dependent mechanism (195) or by perforin release (196). CD8+ T cells can elicit an antigen-specific killing response via lysis of beta cells (152; 153) in much the same fashion as the lysis of virally infected cells. Ultimately, T cell exacerbation of beta cell death comes from endogenous generation of ROS and cytokines following APC activation (197) that can perpetuate islet destruction through a feed forward mechanism.

Regulatory T cells (Treg) are another adaptive cell subset important in type 1 diabetes resolution of pathogenesis. Tregs are specialized cells that suppress activation of effector T cells and preserve tolerance to self-antigens (198), making them highly important in controlling autoimmunity. Regulatory T cells are predominately CD4+Foxp3+ and curb activation and function of self-reactive T cells in the periphery. In type 1 diabetes, CD4+CD25+Foxp3+ Tregs

have been shown to play a role in disease inhibition (198), including the prevention of diabetes development in NOD mice (199-203). The suppression itself can be due to a number of different surface cell molecules present on Tregs (CTLA-4, LAG-3, and PD-1) as well as immunosuppressive cytokine release (IL-10, TGF-β) (204). CD8+ Tregs are also important in regulating autoimmune responses. CD8+CD25+Foxp3+ Tregs measured in a collagen-induced murine model of arthritis can reduce disease severity and suppress T cell effector function (205). Furthermore, CD8+CD122+ Tregs can localize to the central nervous system, decreasing inflammation in the experimental autoimmune encephalomyelitis murine model of multiple sclerosis (206). In the NOD mouse, CD8+ Tregs are important for GAD-induced transferred tolerance (207) and for reversal of autoimmunity (208). Another cell capable of immune suppression of type 1 diabetes is the αβ-$TCR^{+}CD3^{+}CD4^{-}CD8^{-}$ double negative (DN) Treg cell. DN T cells mediate the inhibition of self-peptide reactive CD8+ T cells (209). DN T cells are considered unconventional regulatory cells because they are activated in an antigen-specific manner to directly kill effector T cells. DN Tregs first acquire peptide-MHC from APCs and then interact with effectors via Fas/FasL, which causes apoptosis of the autoreactive T cells. Human studies have identified a clear deficiency in Treg functionality in type 1 diabetes patients (210). The mechanism(s) behind this inadequacy has not been fully elucidated; however, no current evidence suggests an overabundance of ROS to be the culprit. In the context of an oxidative stress environment, Treg abilities are actually preserved. Instead, it is likely that the significant ROS-mediated T_H1 inflammation plays a role in preventing sustained Treg suppression against self-antigen (211-213), resulting in hyperactivation and autoreactive T cell attack of the beta cells.

Despite the differences between the innate and the adaptive immune system, interplay between the two makes autoimmune diabetes an extremely complicated and contentious disease. Overall, ROS are crucial in not only activating the initial infiltrating macrophages and DCs (214) via the common denominator NF-κB, but also for subsequently driving an adaptive T_H1 immune response that is necessary for total ablation of beta cells and progression to type 1 diabetes (189; 190) **(Figure 3**). Therefore, therapies would be most beneficial if there was not only protection of the beta cells from ROS, but also inhibition of the ROS-mediated autoimmune attack, possibly by preventing NF-κB activation, the ensuing inflammation, and the initiation of the adaptive immune response.

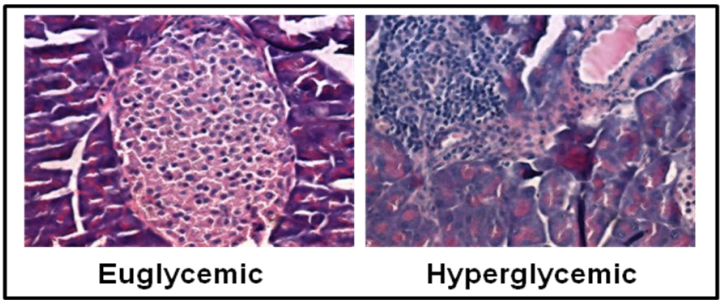


Figure 3: Islet infiltration in type 1 diabetes. Pancreata from NOD mice were taken at hyperglycemia (18 wks) or during euglycemia as a control. Sections stained with hematoxylin and eosin.

1.4 TYPE 1 DIABETES THERAPY AND EFFORTS FOR PREVENTION

Currently, the only mainstream treatment for type 1 diabetes is daily insulin injection. Because of the loss of insulin and impairment in blood glucose tolerance, several severe side effects may occur in type 1 diabetes patients if insulin levels are not exogenously and consistently maintained. Acute illnesses include ketoacidosis and hypoglycemia, whereas long term complications may include heart attack, stroke, poor wound healing, retinopathy, and

nephropathy (9), all of which are a burden on countries with limited healthcare systems. In the United States, people diagnosed with diabetes spend 2.3 times more on medical costs than those individuals without diabetes (215). With the costs of chronic disease treatment increasing annually (8), insulin, needles, and other diabetes testing supplies will only become more expensive. In addition, dysregulated glycemic control occurs in patients failing to manage their daily insulin levels (216). New studies are being conducted in order to eradicate costs and compliance issues of patients. One such study proposed the effectiveness of inhaled insulin (216); but upon establishment by Pfizer, the inhaler did not allow for precise insulin dosing delivery and was discontinued (217). Beta cell and pancreas transplantations have also been implemented over the years; although like any transplant, immunosuppressive drugs must be taken long-term in order for the host not to reject the tissue (9) and several islet donors are typically needed per transplant (218). Unfortunately, only 44% of islet transplantation recipients remain insulin independent at 3 years post-transplant (219). Another highly promising area of research is the use of exogenous stem cell sources for reconstitution of insulin-producing beta cells (9; 220). Autologous inducible pluripotent stem cells may be a potential source of new beta cells, with studies in NOD mice proving successful for reversing diabetes (221; 222). However, the underlying issue with stem cells is effectively stopping the autoimmunity so as not to provide another source of antigen to already endogenously activated autoreactive T cells. Other means of treatment, preferably before onset, are thus a necessary consideration.

With improvements in detecting susceptibility, type 1 diabetes is becoming a predictable disease. In addition, better immunological understanding of diabetes can help to identify autoantigens and clarify the mechanisms behind beta cell destruction. The window of time for preventative therapy, however, dwindles as prediction accuracy increases and beta cell mass

decreases (113) (**Figure 4**). Therefore, prophylactic treatments or diabetes vaccination are challenging but not entirely inconceivable. Different pharmacological agents or antibody administrations, such as mycophenolate mofetil (MMF) and anti-CD3, respectively, have the ability to translate into preventative treatments in humans and animal models (223; 224). Both of these therapeutics help in suppressing the autoimmune response. MMF has been shown to block activated T and B cell proliferation and antibody formation with enhanced specificity (223). Anti-CD3 treatment acts as a partial activating signal, inducing tolerance in T cells and skewing the response to more of a T_H2 phenotype, increasing anti-inflammatory cytokines and inducing CD8+ regulatory T cell production (224; 225). A clinical trial was conducted in 2006 utilizing MMF alone or in combination with Daclizumab, which is a monoclonal antibody specific for IL-2 receptor alpha (CD25), to patients recently diagnosed with type 1 diabetes. Both drugs should theoretically inhibit the activation of autoreactive T cells, thus possibly preserving any remaining beta cells. Although the hypothesis had support from previous animal studies (226; 227), the trial failed to limit the destruction of residual beta cells and was concluded in 2008 (228). Similarly, Teplizumab, an anti-CD3 antibody, showed promise in preclinical studies in recent onset diabetics (229), yet also failed to reach efficacy endpoints in a clinical trial, where no differences existed in insulin dependency between Teplizumab and placebo-treated patients (230). TrialNet, the international network of clinical centers, is currently recruiting high-risk individuals for a Teplizumab trial as well as other first-degree relatives and recent onset patients for various other therapeutic procedures (231). However, the high prevalence of failures in the clinic has sparked the necessity for alternative approaches as well as combinatorial treatments, as reviewed by Phillips et al (232).

Vaccination against type 1 diabetes might seem quite paradoxical in the typical sense of an immunization. For protection against autoimmunity, a vaccine would have to promote tolerance to an antigen (a 'negative' vaccination) or drive the system towards a different immunological subset. In a multivalent vaccine strategy described by our group, immunization of NOD mice with islet lysate drove a more T_H2-type response, skewing the autoreactive T cell pool away from autoantigen recognition, effectively preventing and reversing type 1 diabetes (172). Conversely, a clinical trial of autoantigen immunization in recent onset patients showed no effect in preserving insulin production (233). A recent and more hopeful clinical trial utilizing the Bacillus-Calmette-Guerin (BCG) vaccine, currently administered for tuberculosis, showed reversibility of disease in long-term type 1 diabetes patients following immunization (234). Reversal of disease was attributed to the killing of insulin-specific autoreactive T cells, induction of regulatory T cells, and the regeneration of new beta cells. Despite the indirect procedure with the BCG vaccine, such findings are clearly worth more in-depth trials, especially in recent onset patients. In light of all of these trials, we are testing a different kind of treatment targeting an important aspect of type 1 diabetes pathology – oxidative stress. While investigations are ongoing regarding catalytic antioxidant usage as a stand-alone therapy or in combination with other drugs, prophylactic treatments targeting redox modulation may be particularly promising, as will be discussed in detail below and in the upcoming chapters.

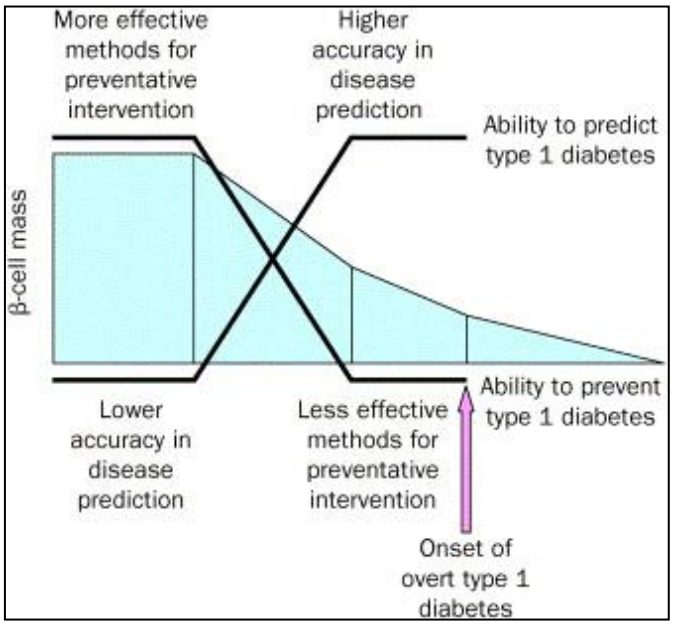


Figure 4: The ability to predict type 1 diabetes increases as beta cell mass decreases, resulting in less effective methods for preventative strategies. *Used with permission from The Lancet* (113).

1.5 REDOX MODULATION

GPX, SOD, and catalase are categorized as the most crucial antioxidant enzymes; however, islets inherently contain only a fraction of these enzymes in comparison to liver, which possesses the highest percentage of antioxidant gene expression (235). Because of the low antioxidant defenses present in pancreatic islets, therapeutic strategies to enhance antioxidants are potentially of greater therapeutic benefit. Overexpression of GPX1, SOD1 (Cu/Zn SOD), SOD2 (MnSOD), or SOD mimetic, an enzyme mimicking superoxide dismutase activity, administration in insulinoma cell lines afforded protection from ROS and reactive nitrogen species (145) *in vitro* (36; 236; 237). Usage of SOD mimetics in other inflammatory models has also demonstrated decreases in proinflammatory cytokines (238; 239). Furthermore, stable transfection of insulin-producing RINm5F cells with GPX, catalase, and Cu/Zn SOD resulted in protection from

cytokine toxicity induced by the combination of IL-1β, TNFα, and IFNγ (240). Antioxidant overexpression has been linked to not only protection against ROS and cytokines, but also to enhanced cell proliferation and decreased death. PDX1, a transcription factor necessary for beta cell differentiation, survival and insulin gene transcription (241), is also very responsive to ROS (242). Oxidative stress causes cytoplasmic relocation of PDX1, increased degradation of the protein, and subsequent dysfunction of beta cells (243; 244). Upon GPX1 overexpression within the islets, PDX1 protein exhibits stability and enhanced function in type 2 diabetes models , which can also have implications in type 1 diabetes for stabilizing beta cell survival. Other experiments have utilized transgene or adenoviral technology to overexpress antioxidant genes within the beta cells to specifically show islet-intrinsic, as opposed to autoimmune protection from type 1 diabetes. These studies have yielded conflicting results. For example, overexpression of metallothionein and catalase in beta cells was unable to delay or inhibit spontaneous diabetes onset in NOD mice and reduced activation of the PDX1 survival pathway (245). Metallothionein proteins are intracellular, cysteine-rich molecules with potent redox capabilities (246). Similarly, transgenic expression of extracellular SOD under control of the insulin promoter in beta cells does not confer any difference in type 1 diabetes incidence in comparison to control NOD mice (247). These results suggest that basal levels of ROS production are necessary for beta cell function, possibly by triggering appropriate insulin signaling and regulating cell survival (248). In contrast, overexpression of thioredoxin, a redox-regulated protein which helps repair ROS-damaged proteins and DNA, affords protection of beta cells from autoimmune and STZ-induced diabetes (249). Beta cell-specific transgenic expression of catalase and metallothionein is also able to protect isolated islets from hydrogen peroxide, which induces beta cell mitochondrial damage (95) and cytotoxicity (250), and reduce the effects of STZ treatment (250-252).

Transgenic expression of heme oxygenase-1, which has crucial cytoprotective functions against oxidative stress and inflammation, can reduce insulitis severity and delay spontaneous diabetes in NOD mice (253), and alloxan-induced diabetes is also decreased following overexpression of Cu/Zn SOD in beta cells (254). Moreover, precedence for the importance of enhancing islet-associated antioxidant levels has been demonstrated at the genetic level, in which mice resistant to alloxan treatment (ALR mice) exhibit protection from diabetes (103; 255). This finding provides further justification for the need for necessary experiments to determine "druggable" targets based upon modulation of antioxidant function.

Because of global reductions in ROS, systemic administration of antioxidants, in comparison to overexpression studies, shows more consistency in ameliorating type 1 diabetes. Administration of 16 mg/kg/day of a potent antioxidant, Lazaroid, which inhibits lipid peroxidation, to young NOD mice resulted in a reduction of diabetes incidence from 89% in controls to 44% in the treated animals (256). Furthermore, after multiple low-dose administration of STZ, addition of zinc sulphate to the drinking water was able to increase islet metallothionein levels in mice, inhibiting the onset of type 1 diabetes (257). Also, intraperitoneal injection of butylated hydroxyanisole antioxidant was able to attenuate the production of proinflammatory cytokines by islets and macrophages (258), thereby lowering insulitis and hyperglycemia (259). Such uniformity in these results, versus the transgenic expression of multiple antioxidants, as discussed above, may relate to the ability of systemic therapies to not only protect the beta cells but to also inhibit immune system activation and inflammation. Adenoviral delivery of systemic heme oxygenase to NOD mice decreased insulitis and type 1 diabetes incidence; however, this alleviation was associated with a decrease in mature DCs and T_H1 effector function (260). Additionally, ALR mice resistant to alloxan-induced diabetes contain specific genetic

modifications conferring systemic elevation of antioxidants, resulting in neutrophils with reduced superoxide bursts (261). In an *in vitro* system using the antioxidant probucol, which can delay alloxan-induced (262) and spontaneous diabetes in rats (263), macrophages exhibit decreased H_2O_2 production, thus maintaining islet viability (264).

1.6 CATALYTIC ANTIOXIDANT

Further reports on the effects of systemic antioxidants on innate immunity include studies from our lab utilizing manganese metalloporphyrin-based catalytic antioxidants (CA) with bone marrow-derived macrophages. In particular, manganese metalloporphyrins are a class of superoxide dismutase (18) mimics, constructed with a manganese center, 4 quaternized cationic pyridyl nitrogens, and varying side chain alkyl groups imparting lipophilicity differences (265) (**Figure 5**). CA display extreme stability, efficacious bioavailability, localizing to the nucleus, cytosol, and mitochondria, and low toxicity (TD_{50}=91.5 mg/kg), maximizing their therapeutic potential (266-268). The metal center within the CA catalyzes superoxide dismutation, mimicking SOD activity (269; 270), and affords scavenging capabilities of a broad range of

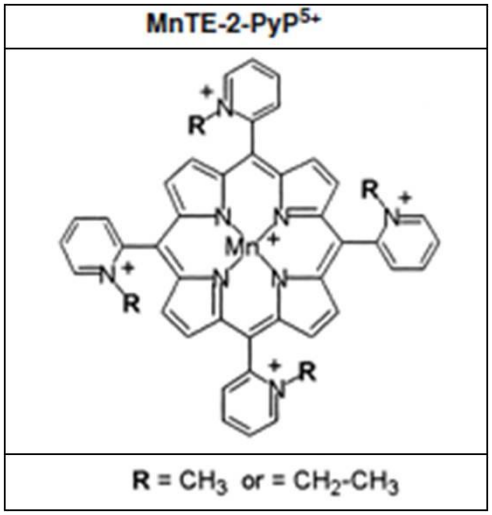


Figure 5: MnTE-2-PyP5+. Structure of the catalytic antioxidant used in our studies. *Used with permission from JBC* (271).

Following treatment with CA, LPS-induced production of nitrite (NO_2-), O_2- TNFα, and IL-1β by macrophages was significantly reduced, in comparison to control (36; 64) (**Figure 6**). This effect was mediated in part by the ability of CA to oxidize the p50 subunit of NF-κB within the nucleus, inhibiting its binding to DNA and subsequent transcription of proinflammatory cytokines (64). CA, therefore, acts as an oxidioreductase, with pro-oxidative capabilities within the negatively-charged nucleus and antioxidant abilities throughout the rest of the cell (268; 272). Redox modulation of transcription factor DNA binding has previously been demonstrated for NF-κB, as well as for other eukaryotic transcription factors such as AP-1 and AP-2 (273; 274). Inhibition of NF-κB has been well established as an effective method of thwarting the immune response and resolving inflammation to maintain beta cell integrity (275; 276); however, we are the first to illustrate a link between metalloporphyrin catalytic antioxidants, blockade of NF-κB activation, and delayed autoimmune diabetes, as described below.

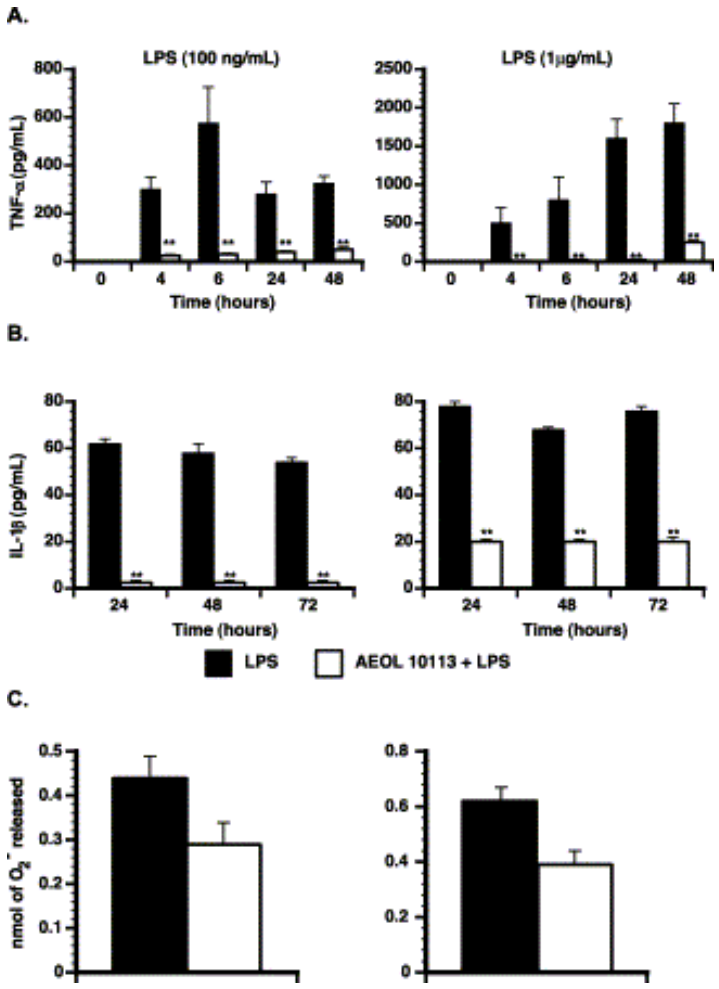


Figure 6: Catalytic antioxidants decrease proinflammatory cytokine synthesis in LPS-stimulated NOD bone marrow-derived macrophages. Culture supernatants from LPS-stimulated macrophages were harvested at 24-72h and analyzed for (A) TNF-α and (B) IL-1β by ELISA. (C) Superoxide levels were measured by cytochrome *c* reduction. Results are representative of the means (±SEM) of at least four independent experiments, each performed in triplicate. ***p < .001* versus LPS-challenged macrophages. *Used with permission from FRBM* (64).

1.6.1 Prevention of type 1 diabetes

The activation of macrophages and T cells relies on oxidative stress, which ultimately leads to the progression of type 1 diabetes. Based upon this fact, CA was also investigated in the context

of CD4+ and CD8+ T cells. The BDC-2.5 TCR-Tg T_H1 cell clone, specific for the protein chromogranin A (ChgA), a member of the granin family of neuroendocrine secretory proteins (115), causes rapid onset of diabetes upon transfer into NOD.*scid* recipients (277). Pretreatment of NOD.*scid* mice with CA prior to adoptive transfer of the BDC-2.5 clone inhibits infiltration of T cells into the pancreas, significantly delaying type 1 diabetes onset. To further delineate the mechanism of diminished T cell effector function, *in vivo* treatment of NOD and BDC-2.5 TCR-Tg mice with CA was able to decrease innate-derived third signal synthesis, primarily consisting of TNFα, resulting in antigen-specific T cell hyporesponsiveness (48). Similar results were found upon CA treatment in the context of CD8+ T cells, reducing proliferation, cytokine production, and cytolytic effector molecules (278). Interestingly, by inhibiting NADPH oxidase in NOD animals (NOD.*Ncf1*^{m1J}) in an effort to genetically mimic systemic CA administration, NOX-derived superoxide production is not only eliminated, but T cells also show reduced T_H1 responses, with protection from type 1 diabetes onset (163). Earlier studies by Chaudhri et al. supported our findings by demonstrating attenuation of T cell proliferation and IL-2R expression following antioxidant treatment (279; 280). These results point to the possible importance of redox modulation in not only regulating the innate immune cells, but also impacting the T cells, which formulate an adaptive immune response crucial for the autoimmune attack in type 1 diabetes (**Figure 7**).

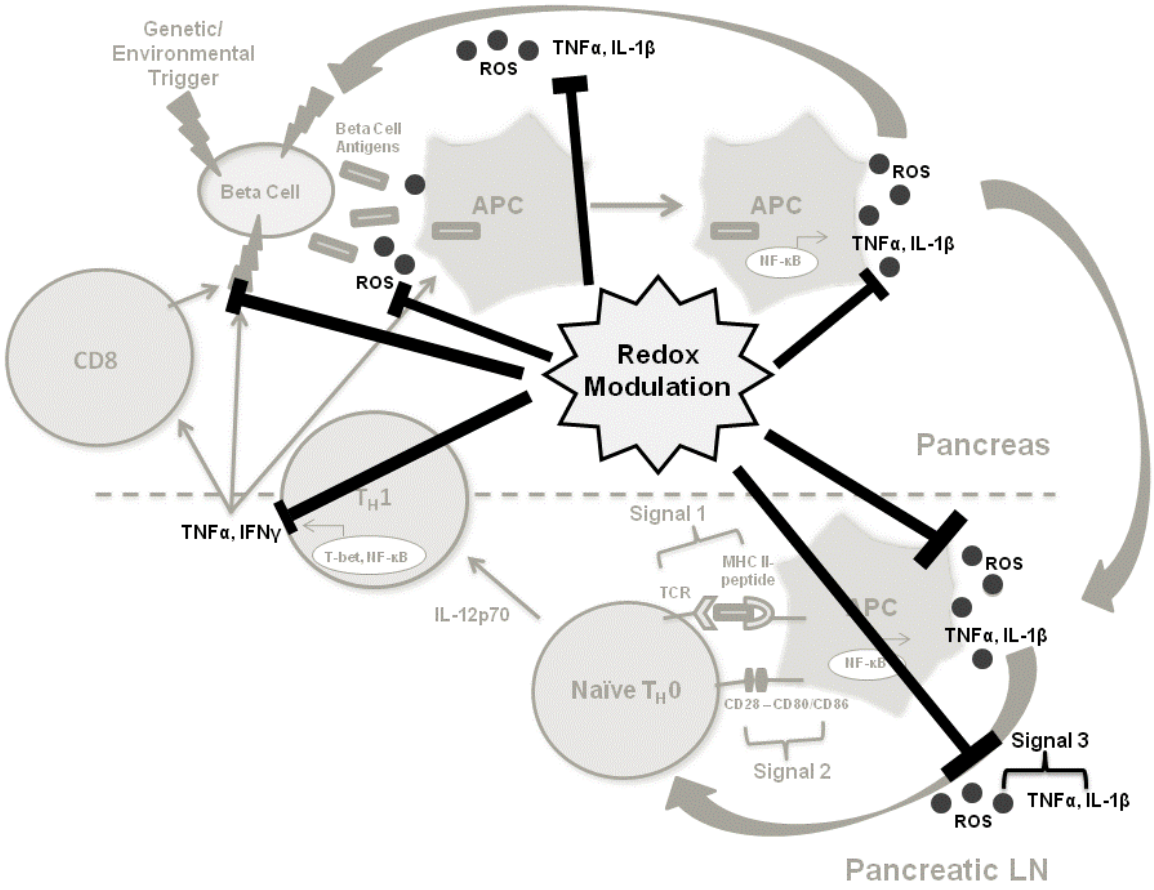


Figure 7: Role of redox modulation in controlling ROS-mediated beta cell destruction. Redox modulation has shown promise in blocking the production of ROS and its ability to activate APCs, resulting in diminished T_H1 cell activation and effector function, which ultimately may help regulate beta cell destruction (154).

1.6.2 Potential for alleviation of ER stress

In addition to decreasing oxidative stress imposed on the islets, which can directly damage beta cells or indirectly stimulate the autoreactive immune response to become activated, redox modulation may also be useful for decreasing deleterious ER stress within the beta cells. Because the beta cells are constantly making insulin and insulin must be folded properly for secretion, the importance of balancing a high protein-folding load with survival of the cells increases

substantially in comparison to other non-secretory cells (281). An overload of misfolded proteins may eventually result in cell death, if not properly resolved. An early study by Lo et al. highlighted the susceptibility of beta cells to ER stress by overexpressing MHC class II proteins in islets, essentially overwhelming the protein folding machinery and leading to apoptosis (282). Other more recent studies show biochemical connections between ER stress-induced apoptosis and beta cell death, through both calcium-dependent and independent molecules (283-285). To reconcile protein misfolding within the ER, the unfolded protein response, or UPR is consequently triggered (286; 287). The UPR acts as a backup mechanism to protect cells from accumulating unfolded proteins and to restore the balance between the protein folding machinery and the secretory pathway (288). However, an accumulation of unfolded proteins during severe ER stress is sometimes unable to be resolved by the UPR, as characterized in the Akita mouse which contains a mutation in the proinsulin 2 gene. This mutation disrupts insulin folding, retains it within the ER, activates UPR, yet still eventually leads to beta cell death (289; 290). Moreover, ROS have been suggested to support the UPR towards a more proapoptotic than proadaptive level (291) further illustrating the importance of regulating oxidative stress to maintain beta cell survival. Although the UPR paradoxically utilizes an oxidative environment within the ER to correctly fold proteins (i.e. disulfide bond formation), sustained oxidative stress can drive the UPR to a level that promotes apoptosis (291; 292). Additionally, the abundance of ROS present during continued unabated ER stress can trigger apoptosis in neighboring cells as well. This is especially critical in islet beta cells, where the ability to handle oxidative stress is already reduced because of low levels of antioxidants (35; 91-93). More pertinent is when unresolved ER stress leads to dying beta cells containing the misfolded proteins. These cells can be taken up by resident pancreatic APCs and presented to autoreactive T cells within the pancreatic lymph

nodes. This type of event may stimulate the reactivity of T cells to formerly tolerated 'neo-autoantigens', which can ultimately promote more beta cell destruction and eventual development of autoimmune diabetes (293; 294). A study conducted by Malhotra, et. al shows that antioxidant treatment of CHO cells results in not only decreased oxidative stress, but also decreased misfolded proteins, reduced activation of the UPR, and enhanced secretion of proteins (281). Thus, it appears that a temporal or redox balance is essential for optimal beta cell function. In situations where the beta cell may experience environmental stressors that lead to disruption of the ER-machinery, the results may set in motion both ER-stress-induced UPR and the expression of misfolded proteins in an oxidative environment, further providing an optimal milieu for driving autoreactive T cells to become activated. Therefore, redox modulation may serve yet another purpose: to help reduce ER stress and subsequently maintain beta cell viability.

1.6.3 Promise of CA in transplantation

The ability to predict susceptibility to type 1 diabetes is becoming increasingly accurate (295), and therefore, prophylactic treatment of patients with antioxidant therapeutics is not out of the realm of possibilities. However, a more feasible option for individuals with chronic hyperglycemia is islet transplantation. Islets, like any other transplantable organ, are in short supply; however, maintaining function and viability of transplanted islets is the major drawback of the procedure (251). Not only are islets susceptible to immune rejection, but hypoxia during isolation and transplantation is the primary cause of beta cell death (296). Because of their low resistance to ROS (35; 91-93), beta cells are especially vulnerable to oxidative damage and ischemia-reperfusion injury (297; 298). In order to combat this weakness, the application of antioxidants seems a suitable alternative, as they have shown promise in liver and kidney

transplantations (258; 299). Longer allograft survival times have been demonstrated with mouse islets soaked with hydroxyl-radical inhibitors prior to transplantation (300) and with multiple *in vivo* administrations of SOD and catalase prior to and after islet transplantation (301). Likewise, transduction of islets with heme oxygenase-1 or SOD2 genes improved viability and insulin secretion *in vitro* (302), with greater functionality upon transplantation, in comparison to controls (303), respectively. Furthermore, we have also demonstrated benefit using the catalytic antioxidant approach, whereby adding CA during and after human islet isolation enhanced cell survival and function, allowing for normalization of STZ-induced diabetic NOD.*scid* mice (304). Additionally, CA is not only able to protect human islets from STZ cell damage, but can also protect murine islets from both antigen-independent, innate-mediated inflammation, and antigen-dependent T cell-mediated allograft rejection (297). Overall, unlike common anti-rejection drugs, which are outstanding at suppressing the adaptive immune response but fail to shield islets from ROS/inflammation (305; 306), our CA treatment is non-toxic to islets and can alleviate both the alloimmune (297) and autoimmune response (36; 48; 64; 278).

1.6.4 Other potential avenues for CA usage

Although redox has been extensively studied in the context of both type 1 diabetes and type 2 diabetes (94; 307), the plethora of literature discussed above shows the implications of ROS in all stages of autoimmune type 1 diabetes, including the primary "trigger", the initiation of insulitis by the innate immune system, and the acquisition of T cell-mediated autoreactivity. These studies open the door to novel ideas of redox modulation, such as targeting ROS-dependent immunological metalloproteases (54; 55; 60) or disrupting the autoreactive T cell pool, as described (48; 163; 278). Moreover, a study evaluating self-antigen-primed T cells

demonstrates how NO is able to reduce FOXP3 expression and subsequently decrease Tregs in autoimmune disorders (308), illustrating how intricate and vast the role of redox is on the immune response and where future studies may focus. In addition to effects on the target organ(s) and the immune system, autoimmunity also gives rise to systemic problems, and in the context of diabetes, ROS have been characterized as crucial elements promoting hyperglycemia-induced diabetic complications, especially those involving the vasculature (16; 309). One important study conducted by Ling, et. al provided evidence of oxidative stress-mediated vascular complications in prediabetic NOD mice (310), which exemplifies the importance of ROS in not only exacerbation of disease, but also on initiation of type 1 diabetes and non-hyperglycemic associated pathologies. Furthermore, antioxidants, such as Vitamin E, can not only dampen vascular activation (311), but can also grant protection from the loss of secondary target organ function, such as the kidneys (312). Therefore, oxidative stress affects every aspect of type 1 diabetes and the benefit of redox modulation may be more significant than once thought.

1.7 SUMMARY

Although optimal treatments must take into consideration the limitations associated with current antioxidant therapies, including bioavailability, immunogenicity-limited cellular accessibility, and cost of production, the advent of newer non-peptidyl small compounds may allow for better alleviation of oxidative stress. Antioxidant therapy should restore balance between oxidation and reduction, leading to resolution of inflammation, thus decreasing the autoimmune destruction of the islet beta cells. The following chapters will discuss how CA treatment, specifically, (1) promotes autoreactive T cell hyporesponsiveness; (2) reduces immune cell metabolism, in mouse

models of type 1 diabetes; and (3) modulates inflammation and diabetic complications in a high-fat diet model of type 2 diabetes.

2.0 MODULATION OF REDOX BALANCE LEAVES MURINE DIABETOGENIC TH1 T CELLS "LAG-3-ING" BEHIND

2.1 ABSTRACT

Preventing activation of diabetogenic T cells is critical for delaying type 1 diabetes onset. The inhibitory molecule LAG-3 and metalloprotease TACE work together to regulate T_H1 responses. The aim of this study was to determine if regulating redox using a catalytic antioxidant (CA) could modulate TACE-mediated LAG-3 shedding as a way to impede diabetogenic T cell activation and progression to disease. A combination of *in vitro* experiments as well as *in vivo* analyses using NOD mouse strains was conducted to test the effect of redox modulation on LAG-3 shedding, TACE enzymatic function, and disease onset. Systemic treatment of NOD mice significantly delayed type 1 diabetes onset. Disease prevention correlated with decreased activation, proliferation, and effector function of diabetogenic T cells, reduced insulin-specific T cell frequency, and enhanced numbers of LAG-3+ cells. Redox modulation also affected TACE activation, diminishing LAG-3 cleavage. Furthermore, disease progression was monitored by measuring serum soluble LAG-3, which was decreased in CA-treated mice. Therefore, affecting redox balance by CA treatment reduces the activation of diabetogenic T cells and impedes type 1 diabetes onset via decreasing T cell effector function

and LAG-3 cleavage. Moreover, soluble LAG-3 can serve as an early T cell-specific biomarker for type 1 diabetes onset and immunomodulation.

2.2 INTRODUCTION

In addition to direct cell-mediated killing of beta cells in type 1 diabetes, soluble inflammatory mediators, including cytokines and ROS, often precede the later stages of fulminant beta cell destruction. Regulation of local and systemic redox affects activation and proliferation of a variety of immune cells and protects tissues/cells from innate and cell-mediated damage (154). Based on previous studies showing the importance of ROS in chronic inflammation, our lab has utilized a CA to modulate both innate and adaptive immunity in type 1 diabetes. CA is a manganese metalloporphyrin (Mn(III) mesotetrakis (N-alkylpyiridinium-2-yl) porphyrin; MnTE-2-PyP^{5+}) that catalyzes superoxide dismutation, mimicking superoxide dismutase activity (269). CA also scavenges a broad range of ROS, including superoxide, hydrogen peroxide, peroxynitrite, and lipid peroxyl radicals (64; 269; 313). The importance of blocking ROS to hinder autoreactive immune responses and type 1 diabetes has been demonstrated within the NOD.*Ncf1* mouse model, where the p47phox mutation in NADPH oxidase leads to protection from spontaneous diabetes onset in 70% of the females (163). CA treatment mimics this mutation, yet serves as a physiologically relevant possibility for disease intervention and clinical use. CA activity regulates proinflammatory immune processes by decreasing TNFα, IL-1β, and ROS synthesis from activated antigen-presenting cells (48), likely by inhibition of NF-κB-dependent gene transcription and efficient innate immune activation (64). CA can translocate to the nucleus and inhibit the binding of the p50 NF-κB subunit to the DNA

via oxidation of the critical cysteine at position 62, resulting in significantly reduced proinflammatory cytokine production (64; 314). Additionally, CA induces CD4+ T cell antigen-specific hyporesponsiveness (48) and decreases the cytolytic activity of CD8+ T cells (278), delaying islet allograft rejection (297). In the context of type 1 diabetes, diabetogenic BDC-2.5 T cell clones exhibit impaired diabetes transfer in CA-treated NOD.*scid* recipient mice (36).

Although the effects of modulating the redox balance are profound, the mechanism of CD4+ antigen-specific hyporesponsiveness and decrease in proinflammatory cytokine production after CA treatment is still poorly understood. Our previously published work showed that TNFα secretion is reduced in CA-treated macrophages (48). A Disintegrin and Metalloproteinase-17 (ADAM17), or Tumor Necrosis Alpha Converting Enzyme (TACE), is a metalloprotease responsible for cleaving pro-TNFα from the cell surface. Many metalloproteases, such as TACE, are redox-dependent enzymes, initially formed as latent zymogens that become active upon oxidation of specific cysteine residues (Cys522 and Cys600) in their disintegrin/cysteine-rich region, releasing the prodomain from their catalytic subunit (**Figure 8**) (54; 55; 315; 316). TACE is synthesized in the rough ER, matured in the Golgi, and is then expressed as a transmembrane protein on the cell surface (317). We hypothesize that CA treatment may not only scavenge ROS, decrease proinflammatory cytokine production, and inhibit NF-κB activation, but may also inhibit TACE, altering the cleavage kinetics of T cell surface proteins. Support for this hypothesis derives from studies demonstrating that TACE is responsible for the shedding of key transmembrane proteins, such as TNFα, Notch, epidermal growth factor receptor ligands, L-selectin (CD62L), and CD223 (LAG-3), making it an essential enzyme in normal immune function (318-323). The inhibition of TACE activation via specific TACE inhibitors, such as TAPI-1, has recently gained popularity in the clinic, with vast

increases in the production of these agents (323; 324). Diseases that are targeted by this type of therapy include multiple sclerosis, systemic lupus erythematosus, rheumatoid arthritis, Sjögrens syndrome, and atopic dermatitis (323; 324). The most promising TACE inhibitors, however, failed phase II trials as a result of mechanism-based hepatotoxicity and/or lack of efficacy (325). Therefore, small molecules that can afford the same type of protection with a better toxicity profile would be beneficial.

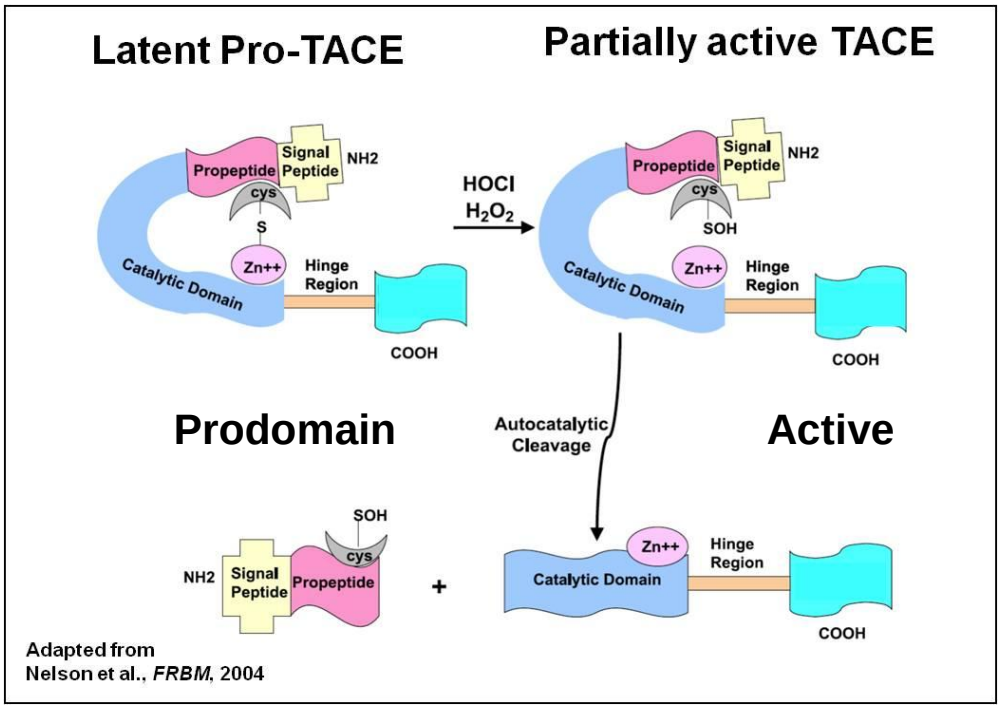


Figure 8: The TACE metalloprotease is redox-dependent. TACE is formed initially as a latent zymogen, with a thiol interaction between a cysteine and zinc residue keeping the enzyme inactive. Upon oxidation of the critical cysteine switch, sulfonic acid is formed, breaking the thiol bond with zinc. An autocatalytic cleavage event then occurs to release the active TACE enzyme from its prodomain. *Used with permission from FRBM (54).*

Lymphocyte Activation Gene 3 (LAG-3) is a negative regulator of immune cell activation expressed on activated CD4+ and CD8+ T cells and plasmacytoid dendritic cells (326; 327). Upon TCR binding with MHC class II, LAG-3 levels increase on the surface of T cells, resulting in attenuated TCR-dependent T cell activation and eventual clonal exhaustion (**Figure**

9) (328), possibly by physical competition with CD4 for MHC interaction (329). LAG-3 tempers the immune response, prevents any aberrant activation, and controls the expansion of the T cell pool (328). IL-12 is thought to upregulate human LAG-3, possibly indicating overlapping regulatory elements with CD4 (326; 330); however, the transcription factor(s) responsible for LAG-3 expression have yet to be identified. To allow CD4 to bind efficiently to class II, LAG-3 is cleaved by TACE (315; 321). LAG-3 follows cyclical kinetics, in which its upregulation and cleavage is eventually followed by a re-upregulation to promote immune response contraction and homeostasis (**Figure 10**).

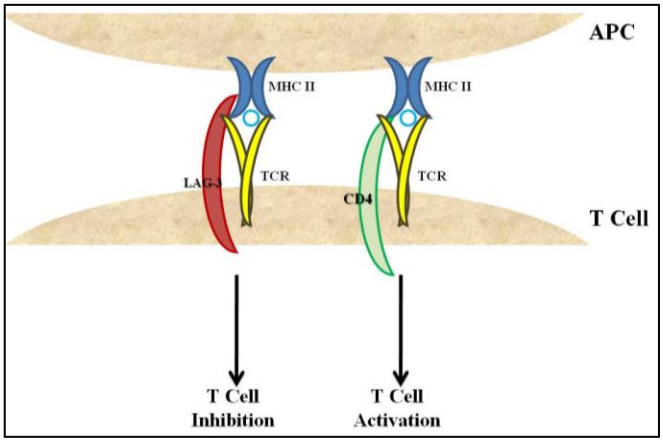


Figure 9: LAG-3 is a negative T cell regulator. Following TCR interaction with MHC-peptide, LAG-3 is upregulated and binds to class II MHC to prevent T cell activation. Upon TACE-mediated cleavage of LAG-3, CD4 is then able to bind to class II MHC and promote T cell activation and effector function.

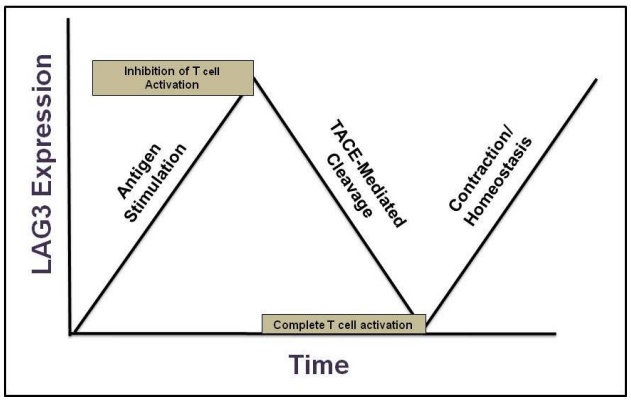


Figure 10: **LAG-3 protein expression displays cyclical kinetics.** LAG-3 is initially upregulated upon antigen stimulation in order to inhibit aberrant T cell activation. Once LAG-3 is shed by TACE, CD4 binds efficiently to MHC class II and progresses the immune response to complete T cell activation. After the cells have effectively cleared the pathogen, LAG-3 is re-upregulated to promote contraction and immune response homeostasis.

LAG-3 knockout mice demonstrate increased T cell proliferation and IFN-γ cytokine production (328), and antibody-mediated LAG-3 blockade results in enhanced CD69 expression and T cell differentiation (331). Additionally, recent studies report that LAG-3(-/-) NOD mice demonstrate accelerated spontaneous diabetes (332; 333), further indicating a potential immunoregulatory function of LAG-3. Soluble LAG-3 (sLAG-3) is a surrogate measure of TACE activity (315; 321) and an additional marker of T cell activation (334; 335). Indeed, serum levels of sLAG-3 are considered a biomarker of T cell activation in breast cancer (334). Therefore, in the context of type 1 diabetes, sLAG-3 could serve as a surrogate marker of autoreactive T cell activation, a predictive biomarker of diabetes progression from preclinical to clinical disease, or a measure of immuno-intervention efficacy.

In this study, we demonstrate the effects of CA treatment on the TACE redox state, coupled with LAG-3 expression and T cell activation, to promote autoreactive T cell hyporesponsiveness and reduce type 1 diabetes onset.

2.3 RESEARCH DESIGN AND METHODS

2.3.1 Materials

NOD.BDC-2.5.TCR.Tg, NOD, NOD.*scid*, and 6.9TCR/NOD.C6 (C6.6.9) mice were bred and housed under specific pathogen-free conditions in the Animal Facility of Rangos Research Center at Children's Hospital of Pittsburgh of UPMC (Pittsburgh, PA). Female mice at 4-10 wks of age were used in all experiments. All animal experiments were approved by the Institutional Animal Care and Use Committee of the Children's Hospital of Pittsburgh and were in compliance with the laws of the United States of America. LAG-3-PE (C9B7W), CD40L-APC, PD-1-FITC (eBioscience, San Diego, CA), CD4-APC, CD69-PE, CD40-PE, CD44-FITC, CTLA-4-PE, FasL-PE (BD Biosciences, San Diego, CA), goat anti-mLAG-3 (R&D Systems, Minneapolis, MN), anti-mTbet (4B10) (Santa Cruz, CA), and rabbit anti-mTACE (Abcam, Cambridge, MA) were used for flow cytometry and Western blots. Antibody pairs for IFN-γ ELISAs were purchased from BD Biosciences and mouse DuoSet TNFα and hLAG-3 ELISAs were purchased from R&D Systems. MnTE-2 catalytic antioxidant (CA) was a generous gift from James Crapo, MD at National Jewish Health. CA was prepared as previously described (48) and used at 68 μM in all in vitro experiments.

2.3.2 CA pellet implantation and spontaneous type 1 diabetes assessment

NOD female mice were implanted with a 14-day sustain release CA pellet (2.1 mg/kg/day) subcutaneously at the nape of the neck. Control animals were left untreated. Animals were reimplanted with CA pellets every 2 weeks until 29 weeks of age. Spontaneous type 1 diabetes

incidence was monitored by blood glucose starting at 12 weeks of age. Overt diabetes was defined as two consecutive readings >300 mg/dl.

2.3.3 In vitro T cell assay

BDC-2.5.TCR.Tg, NOD, or C6.6.9 splenocytes from 6-8 wk old mice were seeded in 96-well round-bottom plates or 12-well plates with 0.5-1 µM of BDC-2.5 mimotope (M) (EKAHRPIWARMDAKK) or 2.5 µg/ml concanavalin A (ConA) (336) in supplemented DMEM (48) (Invitrogen Life Technologies). TAPI-1 (Calbiochem, Darmstadt, Germany) was supplemented daily at 4 µM as indicated. At 24-96h post-stimulation, cells were collected for flow cytometry or for preparation of whole cell lysates. Supernatants were harvested for ELISA.

2.3.4 Surface staining and flow cytometric analysis

Cells were stained as previously described (278). Fluorescence was measured on a FACSAria (BD Biosciences). Flow cytometric analysis was done using FlowJo Software v6.4 (Tree Star, Ashland, OR). All samples were gated on CD4+ cells. Fold change was calculated as (Control/No antigen)/(CA/No antigen).

2.3.5 Cytokine measurements by ELISA

sLAG-3 ELISAs were performed as described (321). IFN-γ and TNFα ELISAs were performed according to manufacturer's instructions. All ELISAs were read on a SpectraMax M2 microplate

reader (Molecular Devices, Sunnyvale, CA), and data was analyzed using SoftMax Pro v5.4.2 (Molecular Devices).

2.3.6 Immunization for LAG-3 detection

One day prior to immunization, 28 NOD mice were treated intraperitoneally (i.p.) with CA (10 mg/kg) or HBSS. Mice were injected with 50 µg of insulin emulsified in Complete Freund's Adjuvant (CFA) s.c. at the base of the tail and treated i.p. for 7 days. On days 0.5, 1, 2, 3, 4, 6, and 8, 2 mice/group were sacrificed, and inguinal lymph nodes (LNs) were harvested for flow cytometry of LAG-3. Non-immunized mice served as negative controls. Similar to insulin immunizations, NOD mice (n=6/group/experiment) were pretreated with CA or HBSS, immunized with hen egg lysozyme (HEL) (100 ug) or NOD.*scid* islet cells (5000) and treated with CA or HBSS for 7 days. On day 8, LNs were harvested and stimulated for an *in vitro* recall assay. Supernatants were collected at 2-3 days post-stimulation and utilized for IFN-γ ELISA.

2.3.7 Intracellular cytokine staining and ELISPOT assay

NOD insulin immunization +/- CA was conducted as above. Primary intracellular IFN-γ was detected in inguinal LN cells isolated 6 days after insulin immunization. After surface staining for CD4, LN cells were prepared as described (278), stained with APC-labeled mouse anti-IFN-γ (BD Biosciences) or isotype controls, and analyzed by flow cytometry. Antigen recall ELISPOT assays were also conducted 6 days after immunization using LN cells ($2.5x10^5$ in triplicate) seeded in IFN-γ-precoated strips from Mabtech (Sweden) with 25 µg insulin. After 2 days of

incubation at 37°C in a 5% CO_2 humid air chamber, plates were developed following the manufacturer's instructions. Frequency = $2.5x10^5$/avg# spots/treatment.

2.3.8 Preparation of cell lysates and western blotting

BDC-2.5.TCR.Tg splenocytes were stimulated with M +/- CA. Whole cell lysates from splenocytes or from CD4+ T cells, isolated after stimulation via MACS separation using the CD4+ T cell Isolation Kit (Miltenyi Biotec, Auburn, CA), were prepared as described (337). Membrane lysates were obtained by lysis in 50 mM Tris, pH 7.4, 150 mM NaCl buffer supplemented with inhibitor cocktails, centrifugation at 72,000 x g for 30 min, removal of supernatants, and sonication with Tris buffer+1% NP-40. Protein concentration of all lysates was determined by BCA protein assay (Thermo Fisher, Rockford, IL). Protein lysates were separated on 8% or 4-20% (TACE) SDS–PAGE gels. For fluorescein-5-maleimide (F5M) detection (Thermo Scientific, Rockford, IL), 25 µg lysates were labeled with 1.5 µM F5M for 10 min on ice in the dark. After labeling, the lysate was filtered over 3K centricon filters to remove any remaining label, samples were ran on SDS-PAGE gels, and fluorescence was detected. Western blots were performed as described (64) with antibodies to LAG-3 (1:1300), Tbet (1:1000), TACE (1:2000), and β-actin (1:10,000) in 5% BSA in TBST. Secondary antibodies were from Jackson ImmunoResearch, West Grove, PA. Chemiluminescence was detected using ECL Plus reagent (Amersham Pharmacia Biotech, Buckinghamshire, UK). Blots were analyzed using Fujifilm LAS-4000 imager and Multi Gauge software (Fujifilm Life Science, Tokyo, Japan).

2.3.9 LAG-3 mRNA quantification

BDC-2.5.TCR.Tg splenocytes were isolated and stimulated with 1 µM M +/- CA for 72-96h. Cells were collected, pelleted, and stored at -80°C before RNA isolation. RNA was isolated using the RNAeasy Kit (Quiagen, Valencia, CA), followed by cDNA synthesis using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). The qRT-PCR was performed using the following primer pair: LAG-3 (forward) 5'-AGTGACTCCCAAATCCTTCGGGTT-3', (reverse) 5'-GGGACGCCACACAAATCTTTCCTT-3'; GAPDH (forward) 5'-TGCATCCTGCACCACCAACT-3', (reverse) 5'-CTGGCATGGCCTTCCGTGTT-3'. Quantitative RT PCR was performed using a Light Cycler 2.0 (Roche, Indianapolis, IN). The reaction mixtures containing SYBR Green were generated following the manufacturer's protocol. The cycling program was: initial denaturation at 95°C for 10 min, 40 cycles of amplification with a denaturation step at 95°C for 5 sec, an annealing temperature of 60°C for 15 sec, and an extension step at 72°C for 20 sec. All samples were normalized to GAPDH, and CA-treated samples were compared to control samples arbitrarily set to 1.

2.3.10 Soluble LAG-3 (sLAG-3) immunoprecipitation

Supernatants from BDC-2.5.TCR.Tg splenocyte stimulations were concentrated in 30K Amicon Ultra-15 centrifugal filter units (Millipore). Samples >30K were immunoprecipitated using 1 µg of LAG-3 antibody as described (337). Western blot was performed as above.

2.3.11 In vitro TACE fluorogenic assay

BDC-2.5.TCR.Tg splenocytes were stimulated for 24h with M +/- CA +/- 200 μM TAPI-1 in 96-well black fluorescence plates. TACE specific fluorogenic substrate (Mca-P-L-A-Q-A-V-Dpa-R-S-S-S-R-NH_2, Fluorogenic Peptide Substrate III, R&D Systems) diluted to 10 μM in 50 mM Tris buffer, pH 9.0 was added for 6h at 37°C. Fluorescence was read at an excitation of 320 nM, emission of 405 nM. The average fold change in activity = Stimulated Cells/Unstimulated vs. Stim+CA/Unstim vs. Stim+TAPI/Unstim.

2.3.12 Adoptive transfer of diabetes

One day prior to adoptive transfer, 6 NOD.*scid* mice were treated i.p. with CA (10 mg/kg) or left untreated. BDC-2.5.TCR.Tg splenocytes were adoptively transferred (10^7/mouse) intravenously (i.v.) into NOD.*scid* recipients on day 0. Mice were treated daily with CA. Serum was collected every 4 days post-transfer for sLAG-3 ELISA. Mice positive for glucosuria after daily urinalysis were monitored by blood glucose levels, and overt diabetes was determined as above. Mice were monitored for disease onset up to 28 days post-transfer, when splenocytes were isolated for *in vitro* analysis.

2.3.13 sLAG-3 levels from NOD and human samples

NOD females and males (n=4/group) were bled for serum every other week starting at 6 weeks of age until 14 weeks of age. sLAG-3 was detected by ELISA according to the manufacturer's instructions. Mice positive for glucosuria after daily urinalysis were monitored by blood glucose

levels, and overt diabetes was determined as above. Human serum samples (type 1 diabetes, healthy controls, first-degree relatives) were graciously provided by: Dorothy Becker (Children's Hospital of Pittsburgh), Clayton Matthews (University of Florida), and Ranjeny Thomas (University of Queensland). hLAG-3 ELISAs were blocked and samples/standards were diluted in 30% FBS in PBS to minimize background. Autoantibody data was also provided by both the Mathews and Thomas groups.

2.3.14 Statistical analysis

The difference between mean values was assessed by Student's *t* test, with $p<0.05$ considered significant. All experiments were performed at least three times with data ± SEM obtained in triplicate in each experiment. For LAG-3 FACS analysis, data are representative of at least three independent experiments and fold change of expression is calculated as indicated. Survival analysis was done using the product-limit (Kaplan-Meier) method with the endpoint defined as disease. Data on animals that did not develop type 1 diabetes were censored. The *p* values were determined by Log-Rank test.

2.4 RESULTS

2.4.1 CA treatment delays spontaneous diabetes

CA treatment disrupts innate immune-mediated proinflammatory signals (48) and delays islet allograft rejection (297), prompting us to determine the effects of its long-term administration on

type 1 diabetes onset. NOD females (4 wks old) implanted with CA pellets demonstrated delayed diabetes onset compared to control mice ($p<0.0001$). Furthermore, stopping CA pellet implantation at 29 weeks afforded protection against diabetes until 40 weeks of age (**Figure 11**), suggesting that redox modulation imparts inhibition of autoreactive processes and delays end-organ autoimmunity.

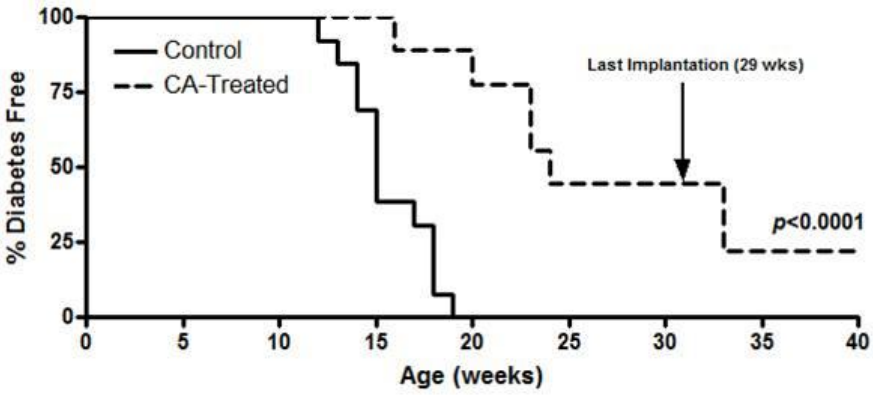


Figure 11: Spontaneous diabetes is reduced upon systemic CA treatment. NOD females (n=7) were implanted with a 14-day sustain release CA pellet (2.1 mg/kg/day) bi-weekly, and control NOD mice (n=14) were left untreated. Pellet implantation was stopped at 29 weeks of age. Diabetes was monitored by blood glucose, with 2 consecutive readings of >300 mg/dl indicating overt disease, ****p<0.0001*.

2.4.2 Redox modulation decreases T_H1 effector function

T_H1 T cells play a key role in mediating type 1 diabetes (338; 339). To mechanistically determine how modulation of the redox state affects diabetogenic CD4+ T_H1 adaptive immune effector responses, BDC-2.5.TCR.Tg splenocytes were stimulated +/- M +/- CA *in vitro*. CA treatment diminished T cell activation, shown by decreased frequency of CD4+CD69+ cells ($p<0.05$ at 72 and 96h) (**Figure 12A**) and reduced CD4+ T cell proliferation ($p<0.05$ at 96h) (**Figure 12B**). In conjunction with previous results (48), redox modulation significantly lowered IFN-γ production ($p<0.05$) and reduced Tbet protein expression (**Figure 12C, D**). These data

indicate that CA diminishes T cell activation and T_H1 effector function, likely contributing to the diabetes protection observed above (**Figure 11**).

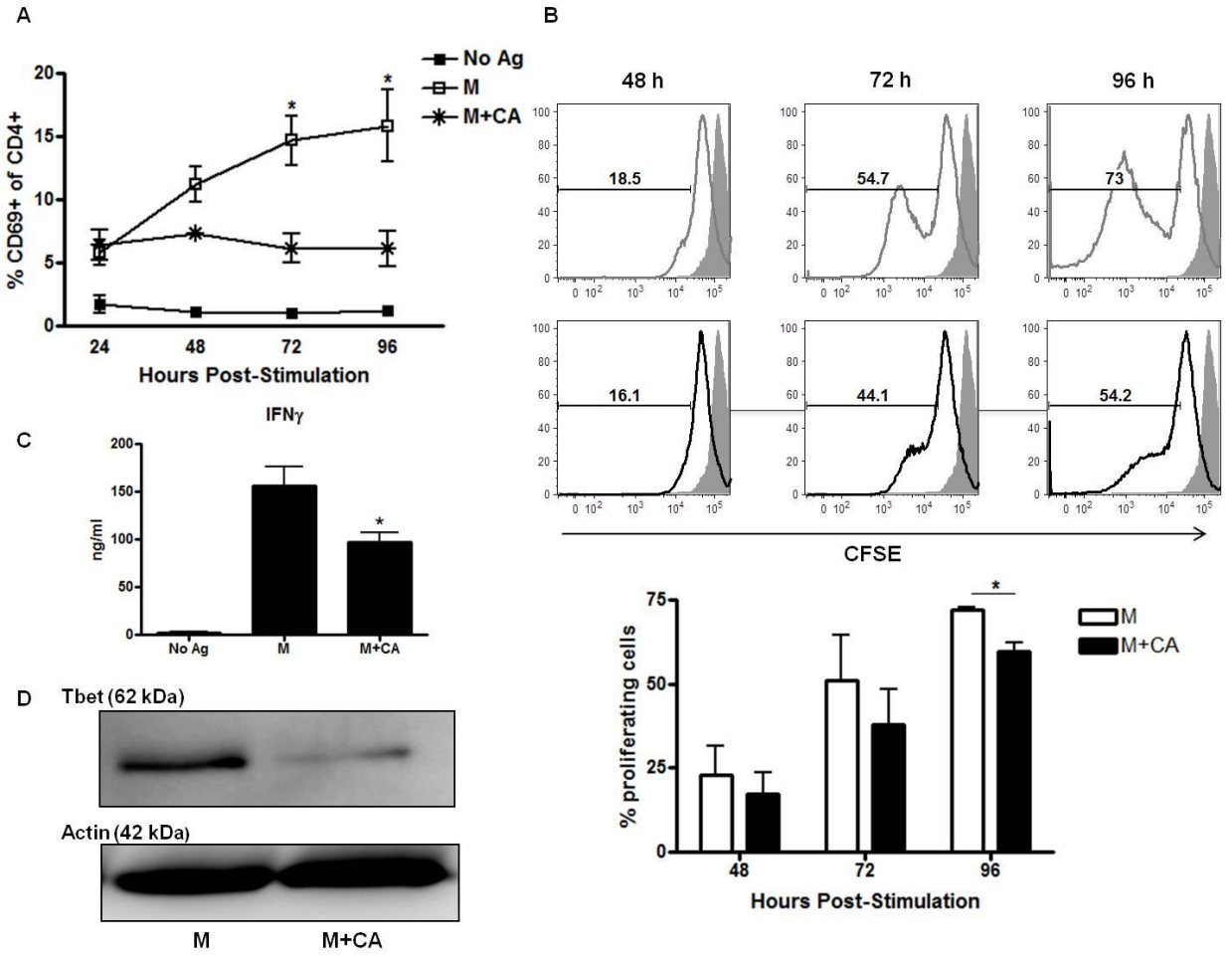


Figure 12: Redox modulation promotes a reduction in T cell activation and effector function. (A) BDC-2.5.TCR.Tg splenocytes were left untreated or stimulated with M +/- CA. At 24-96h, cells were stained for and gated on CD4+ cells, and CD69 was analyzed by flow cytometry, n=3 independent experiments, *$p<0.05$. (B) CFSE-labeled splenocytes were treated with M +/- CA. At 48-96h, cells were stained and gated on CD4+CFSE+ cells. Grey lines = M, Black lines = M+CA, Filled histograms = CFSE+ cells at time point 0. % proliferating cells = average of 3 independent experiments, *$p<0.05$ (C) Supernatants from 96h cultures were used in an IFN-γ ELISA, n=3 independent experiments performed in triplicate, *$p<0.05$. (D) Whole cell lysates from 96h cultures were probed for Tbet by immunoblot. Actin was probed as a loading control. Data are representative of 3 independent experiments.

2.4.2.1 BDC-2.5 T cells have reduced activation/memory markers following CA treatment

In addition to CD69+ expression, BDC-2.5.TCR.Tg splenocytes were also analyzed for other activation and memory markers upon redox modulation. CD40 interaction with CD40L on T cells is known to aid in the activation of APCs and B cells. Furthermore, CD40 has been detected on diabetogenic BDC-2.5.TCR.Tg T cells, with putative roles in inducing NF-κB activation and binding directly to other T cells in the periphery for faster responses (340). Moreover, adoptive transfer of $CD40^{hi}$ diabetogenic T cells rapidly cause type 1 diabetes in a NOD.*scid* mouse model (341). CA treatment of BDC-2.5.TCR.Tg splenocytes stimulated with M reduced the amount of CD4+CD40+ cells to almost significance at 96h (**Figure 13A**), potentially correlating with lower diabetogenicity. CD40L expression was also measured after CA treatment. CD4+CD40L+ cells, conversely, were increased upon redox modulation (**Figure 13B**). Although this result seems to pose a conundrum for reduced activation, studies have described CD40L as a homeostatic surface marker (342) that is downregulated by CD40 ligation (343). If CD40 is decreased *in vitro*, less ligation would occur, resulting in sustained levels of CD40L. Further studies would need to be conducted in a more physiological system *in vivo* or with isolated T cells to definitively comprehend the differences in this costimulatory pathway.

In addition to activation markers, the memory surface molecule CD44 was also analyzed. CA treatment decreased the number of CD4+CD44+ cells (**Figure 13C**), indicating a possible reduction in CD4+ effector memory T cells as CD44 is known to be critical for sustaining the memory phenotype (344). Another molecule that was analyzed upon redox modulation was Fas ligand (FasL), which is important for binding Fas on target cells and inducing apoptosis. In the

context of T cell activation, FasL expression is initially low but is increased as stimulation continues. Eventually, FasL-Fas interaction will instigate activation-induced cell death, ultimately returning the immune response to homeostasis. Upon stimulation of BDC-2.5.TCR.Tg splenocytes, CD4+FasL+ cells are lowered after CA treatment (**Figure 13D**). Such decreases may be related to the overall activation delay seen with CD69 expression and proliferation (**Figure 12**); however, reports indicate that memory CD4+ T cells can obtain cytotoxic characteristics (345; 346), such as FasL expression, and this mechanism may be used to directly lyse beta cells, as was shown for CD8+ T cells (347). Reduced FasL after CA treatment may indicate a less cytotoxic phenotype of memory diabetogenic T cells.

The last set of molecules analyzed after redox modulation were two negative T cell regulators, CTLA-4 and PD-1. CTLA-4 is necessary for T cell contraction, and expressed at later stages after T cell activation. CA treatment results in slight reduction of CTLA-4 expression, especially at 72h post-stimulation (**Figure 13E**). In reference once again to the general delay in T cell activation seen above, this effect may be a kinetic phenomenon. On the other hand, PD-1, another molecule implicated in negatively regulating T cells, was not affected by redox modulation (**Figure 13F**). Overall, these data suggest additional facets of redox modulation-dependent reductions in T cell activation, which would be beneficial in decreasing diabetogenicity. More studies are needed to obtain significant conclusions.

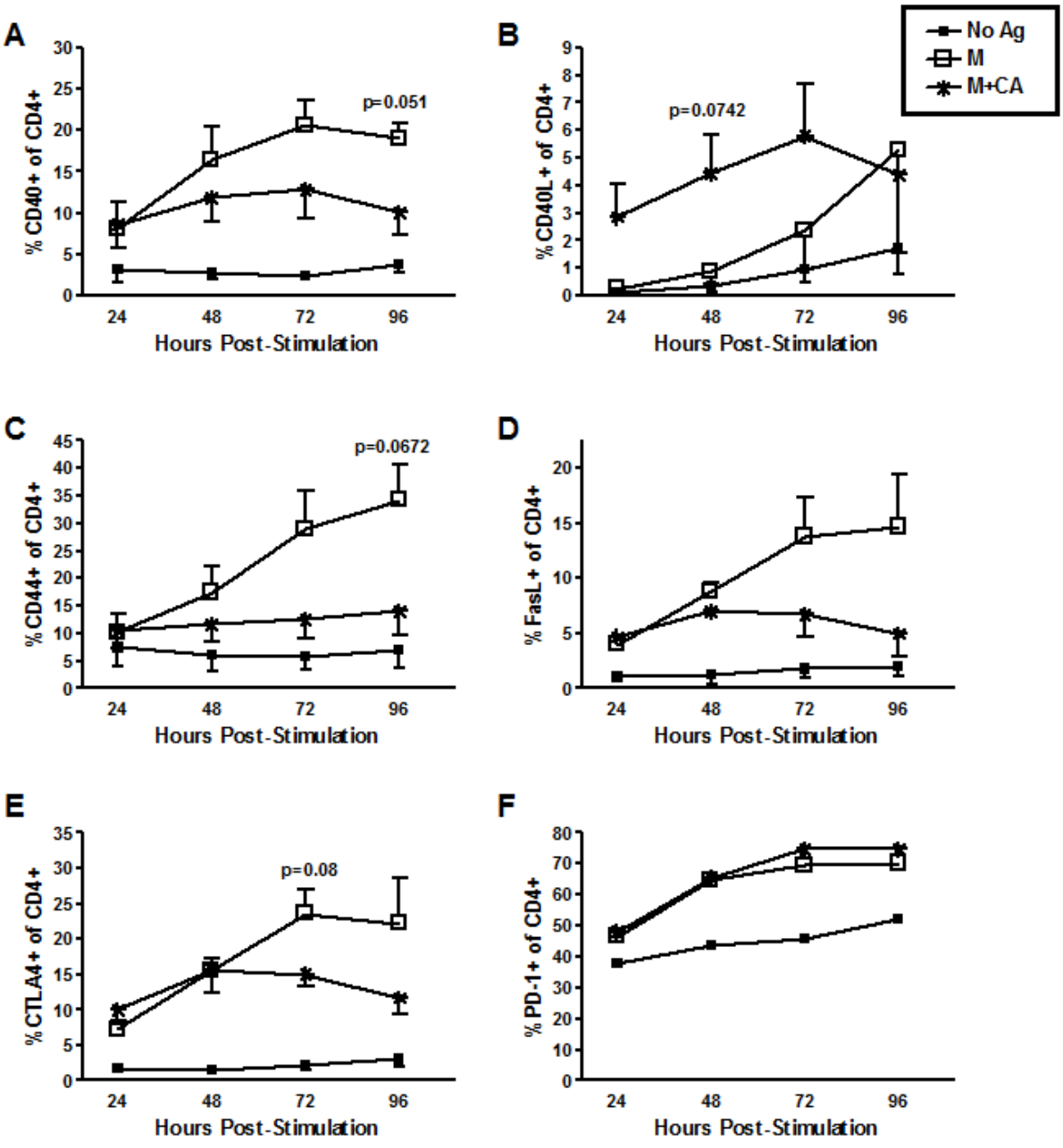


Figure 13: Redox modulation decreases diabetogenicity of BDC-2.5.TCR.Tg CD4+ T cells through various activation and memory markers. BDC-2.5.TCR.Tg splenocytes were left untreated or stimulated with M +/- CA. At 24-96h, cells were stained for and gated on CD4+ cells, and CD40L, CD40, CD44, CTLA-4 (n=3), FasL (n=2), or PD-1 (n=1) was analyzed by flow cytometry.

2.4.3 CA treatment limits antigen-specific T cell frequency

To determine if CA treatment affected the frequency of antigen-specific T_H1 cells *in vivo*, we immunized NOD mice (6-8 wks old) with a known autoantigen, insulin, and utilized inguinal LN cells on day 6 for primary intracellular IFN-γ detection and recall ELISPOT assay (**Figure 14**). IFN-γ-expressing CD4+ T cells were reduced after CA treatment compared to control animals ($p<0.005$) (**Figure 14A**). Furthermore, LN cells from CA-treated animals displayed decreased IFN-γ secreting cells compared to control animals ($p<0.05$), with a significant reduction in insulin-specific effector function after recall stimulation (**Figure 14B**). The frequency of antigen-specific cells in control animals was ~1 in 19,000, whereas in CA-treated animals, the frequency diminished to 1 in 46,000. These results suggest that redox modulation disrupts expansion of insulin-specific T cells, which may lead to delays in autoimmune-mediated beta cell destruction and in type 1 diabetes onset.

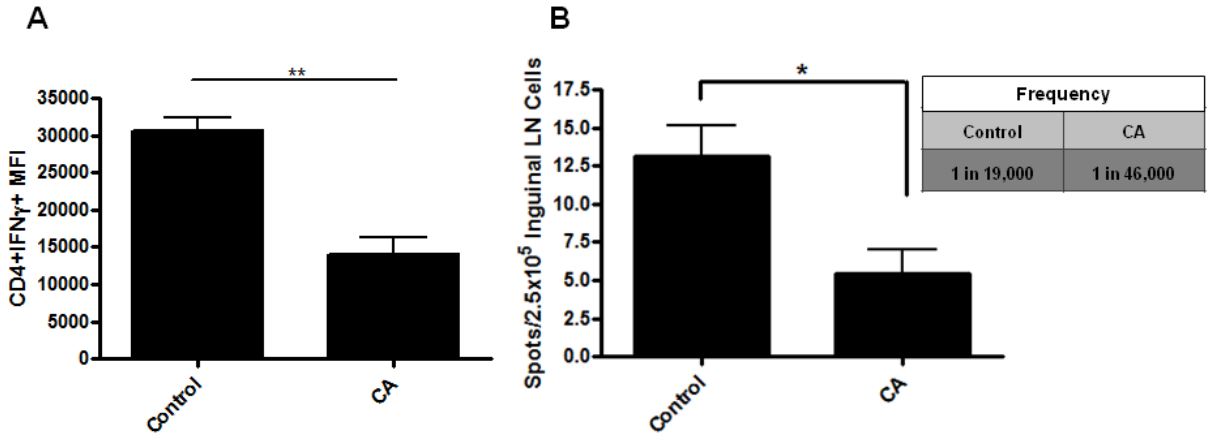


Figure 14: CA treatment reduces insulin-specific T cell effector function and frequency. NOD mice were treated with CA or HBSS daily. Mice were immunized with insulin in CFA. (A) Inguinal LNs were removed at day 6 post-immunization, and surface stained for CD4 as well as intracellularly stained for IFN-γ for flow cytometric analysis. Cells were gated on CD4+ cells, n=3 independent experiments with 2 mice/group, *$p<0.005$. (B) Inguinal LNs were also isolated on day 6 and stimulated with insulin in a recall IFN-γ ELISPOT. Two days after stimulation, ELISPOT plates were developed, and spots were counted using the Zeiss KS Elispot Imaging system. Frequency = $2.5x10^5$/avg # of spots per treatment. Graph shows the average of 3 independent experiments performed in triplicate (*$p<0.05$).

2.4.3.1 Redox modulation reduces IFN-γ production following stimulation of NOD T cells

Further support for CA-mediated reductions in IFN-γ was obtained through both *in vitro* stimulation and *in vivo* immunization experiments using the open TCR repertoire NOD mouse model. NOD splenocytes stimulated with the mitogen ConA in the presence of CA showed significant reductions in IFN-γ production at 48h (**Figure 15A**). Moreover, immunizing NOD mice with whole islet cells or the non-autoantigen HEL demonstrated similar reductions in IFN-γ secretion after 7 days of *in vivo* CA treatment followed by 2-3 days of recall antigen stimulation of LN cells *in vitro* (**Figure 15B, C**). Unlike insulin immunization, however, the levels of IFN-γ were not significantly reduced, although the trend is apparent.

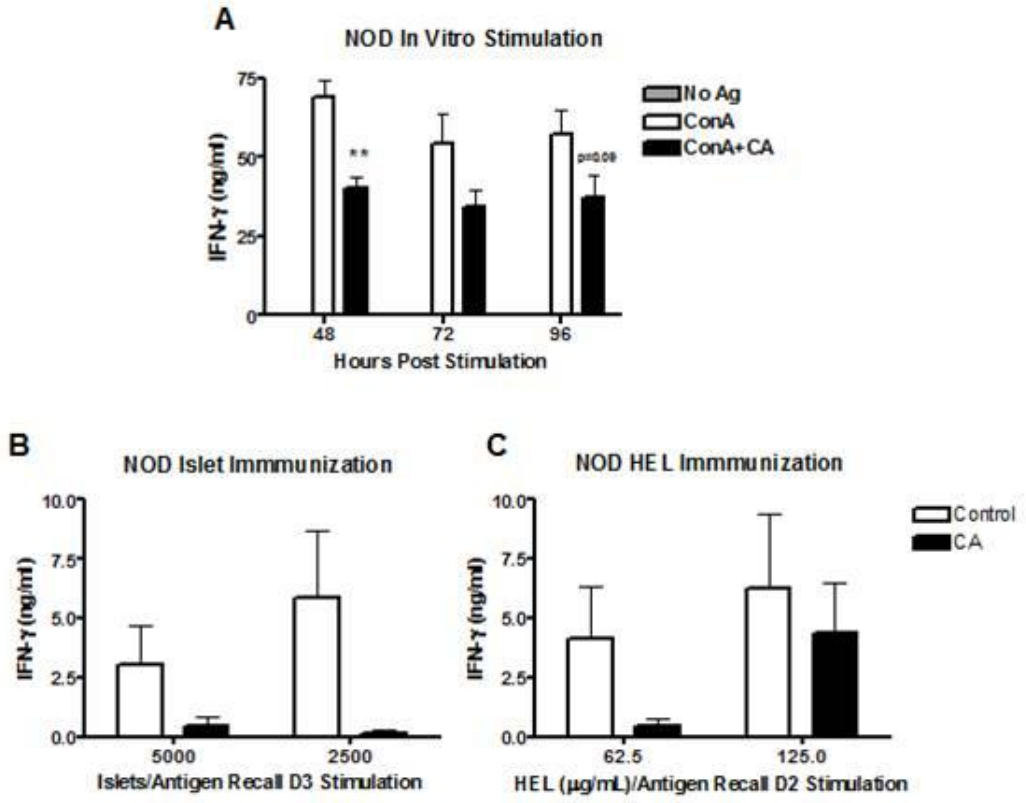


Figure 15: CA treatment decreases IFN-γ production following stimulation of NOD T cells. (A) NOD splenocytes were left unstimulated or stimulated with ConA +/- CA for 96h. Supernatants from 48-96h cultures were used in an IFN-γ ELISA, n=4 independent experiments performed in triplicate, ***p<0.005*. (B-C) NOD mice were treated with CA or HBSS daily. Mice were immunized with 5000 NOD.*scid* islet cells or 50 µg HEL in CFA. Inguinal LNs were removed at day 8 post-immunization and stimulated with (B) 5000 or 2500 NOD.*scid* islet cells for 3 days or (C) 62.5 or 125 µg/mL HEL for 2 days. Supernatants were used in an IFN-γ ELISA, n=3 independent experiments.

2.4.4 CD4+LAG-3+ T cell frequency is enhanced following CA treatment

LAG-3 is important in negatively regulating T cell responses, and thus, may play a role in mediating decreased T cell activation following CA administration (328; 348; 349). We first measured LAG-3+ T cell frequency following M +/- CA stimulation of BDC-2.5.TCR.Tg splenocytes *in vitro*. M+CA treatment resulted in a higher frequency of LAG-3+ T cells

compared to M-alone stimulated samples (**Figure 16A**). The MFI of LAG-3 did not differ between groups. Because LAG-3 is not constitutively expressed (350; 351), unstimulated cells +/- CA treatment expectedly demonstrated low LAG-3+ T cell frequencies. Upon quantification of the *in vitro* results, the fold change in LAG-3+ T cell frequency reached significance at 24h and 48h ($p<0.05$) post-stimulation (**Figure 16B**), indicating kinetically-delayed T cell activation.

We next looked in vivo for LAG-3 kinetics following insulin immunization and CA administration of NOD mice (6-8 wks old). As shown by others (321; 352-355) and similar to our *in vitro* results (**Figure 16A, B**), control-treated animals exhibited a lower peak of LAG-3+ cells by day 3 post-immunization in comparison to CA-treated mice; however, redox modulation resulted in an enhanced trend toward LAG-3+ cells at day 3 post-immunization ($p=0.07$) (**Figure 16C**). No difference in LAG-3 MFI was seen between the groups (data not shown). These data demonstrate that CA treatment can affect LAG-3+CD4+ T cell frequency *in vivo*, albeit not to significance, suggesting slight obstruction of T cell activation following autoantigen immunization in the presence of redox modulation.

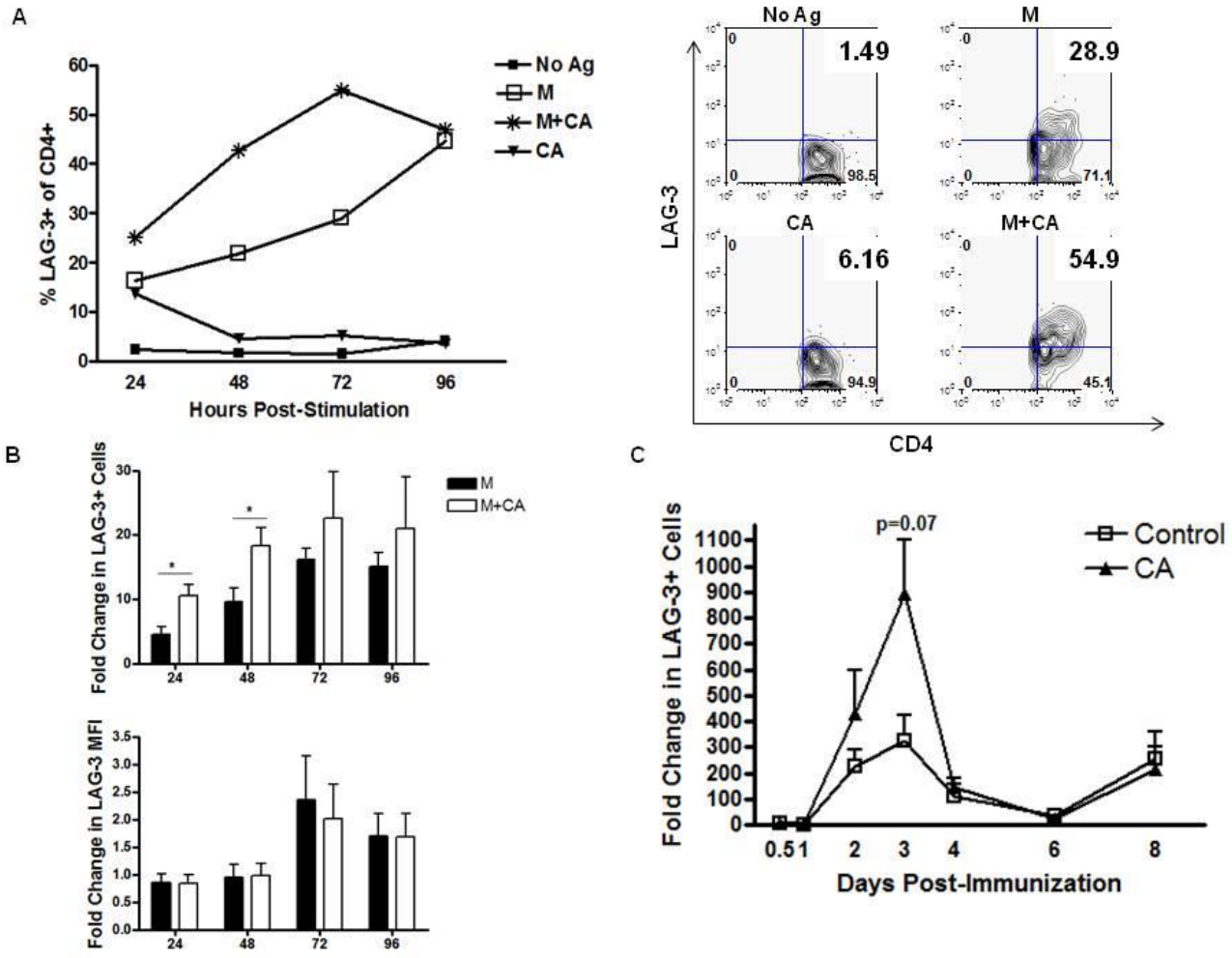


Figure 16: LAG-3+ T cell frequency is enhanced upon stimulation plus CA. BDC-2.5.TCR.Tg splenocytes were left untreated or stimulated with M +/- CA in vitro. (A) At 24-96h, cells were stained and gated on CD4+, and LAG-3 expression was analyzed by flow cytometry. Graph is representative of 4 independent experiments. Dot blots representative of 72h stimulation. (B) Fold change in % CD4+LAG-3+ cells and LAG-3 MFI was calculated as Ag/No Ag vs. Ag+CA/No Ag and averaged from the 4 in vitro independent experiments represented in A, *$p<0.05$. (C) NOD mice were split into two groups (n=14/group) and treated daily with CA or HBSS as a control. On day 0, mice were immunized with insulin in CFA. Inguinal LNs were removed and pooled at the indicated days post-immunization (days 0.5, 1, 2, 3, 4, 6, 8) from 2 mice/group, stained for and gated on CD4+ cells, and LAG-3 was analyzed by flow cytometry. Graph shows the average of 3 independent experiments.

2.4.4.1 Autoantigen stimulation plus CA increases the frequency of LAG-3+ T cells

In addition to cognate mimotope stimulation, BDC-2.5.TCR.Tg splenocytes were stimulated with more physiological autoantigens, whole islet cells and beta-membrane, which is an isolated preparation of the insulin-containing secreted beta granules. Following redox modulation, splenocytes stimulated with either antigen demonstrated significant increases in the frequency of LAG-3+ cells compared to splenocytes stimulated without CA (**Figure 17A**).

BDC-2.5.TCR.Tg T cells are considered primed autoreactive cells, as they mature *in vivo* in the presence of their cognate antigen (277), chromogranin A, which is part of the beta granule membrane (115). Redox modulation was additionally tested in a naïve, autoreactive strain called the 6.9TCR/NOD.C6 mouse. The T cells in the C6.6.9 model express the BDC-6.9 transgenic TCR, which is specific for an unknown beta granule membrane antigen. Antigen for the BDC-6.9 TCR is encoded on chromosome 6 in the NOD mouse; however, in the NOD.C6 model, this chromosomal region is replaced by the BALB/c locus, making the T cells naïve autoreactive since they are not matured *in vivo* in the presence of their cognate antigen (356). In this system, redox modulation during stimulation with beta-membrane significantly enhanced the numbers of LAG-3+ T cells (**Figure 17B**). These data demonstrate the ability of CA to affect LAG-3 in both a primed and naïve T autoreactive cell state.

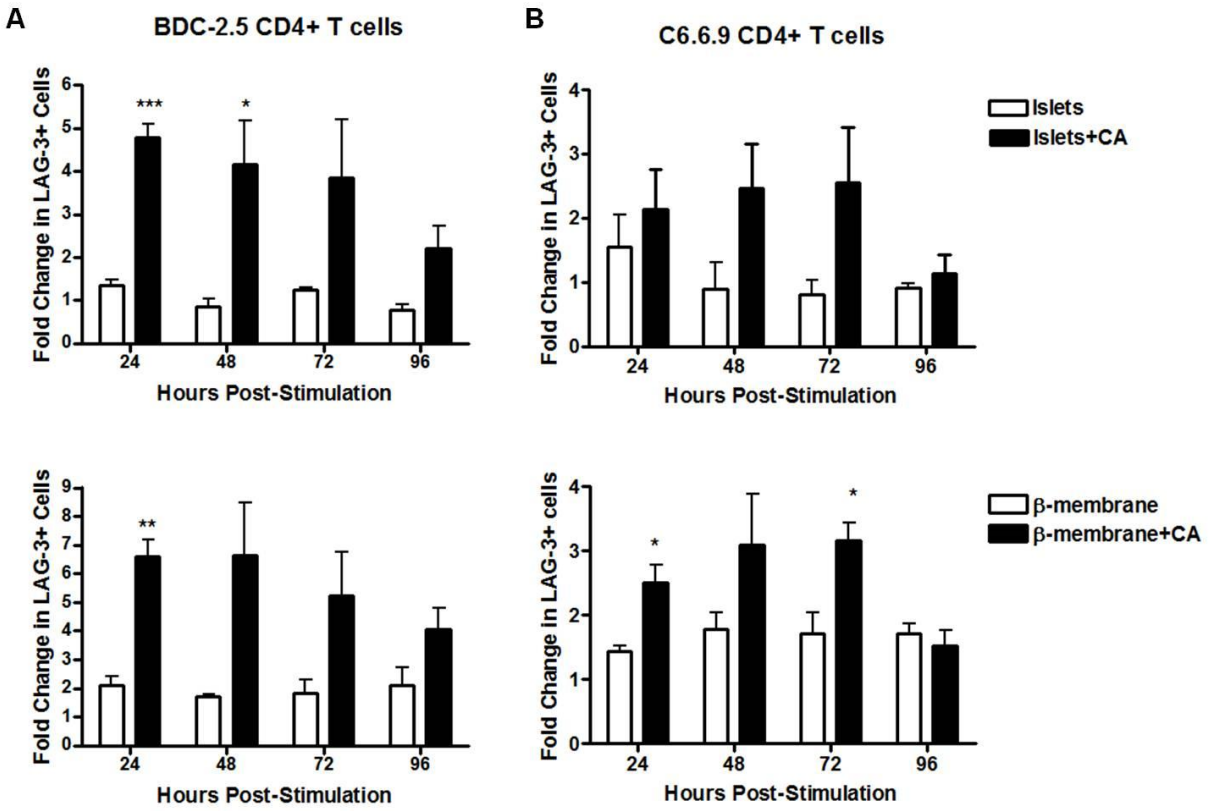


Figure 17: LAG-3+ T cell frequency is enhanced following autoantigen stimulation plus CA. (A) BDC-2.5.TCR.Tg splenocytes or (B) C6.6.9 splenocytes were stimulated with NOD.*scid* islet cells or beta-membrane +/- CA in vitro. At 24-96h, cells were stained and gated on CD4+, and LAG-3 expression was analyzed by flow cytometry. Fold change in % CD4+LAG-3+ cells was calculated as Ag/No Ag vs. Ag+CA/No Ag and averaged from 3 in vitro independent experiments. *$p<0.05$, **$p<0.005$, ***$p<0.0005$.

2.4.5 LAG-3 shedding is reduced upon CA treatment

In addition to surface levels, sLAG-3 was also analyzed following M stimulation of BDC-2.5.TCR.Tg splenocytes. LAG-3 contains four extracellular domains (D1-D4), a connecting peptide region (CP), a transmembrane domain (321), and a cytoplasmic tail (335). Within the immunological synapse, an antigen-mediated respiratory burst activates TACE by oxidizing Cys522 and Cys600 to release the TACE prodomain (54; 55). Active TACE then cleaves the 70 kDa full-length LAG-3 within the CP, shedding D1 through D4 domains, a 54 kDa fragment (321). sLAG-3 shed into the serum can be measured as a marker of T cell activation (334) **(Figure 18)**.

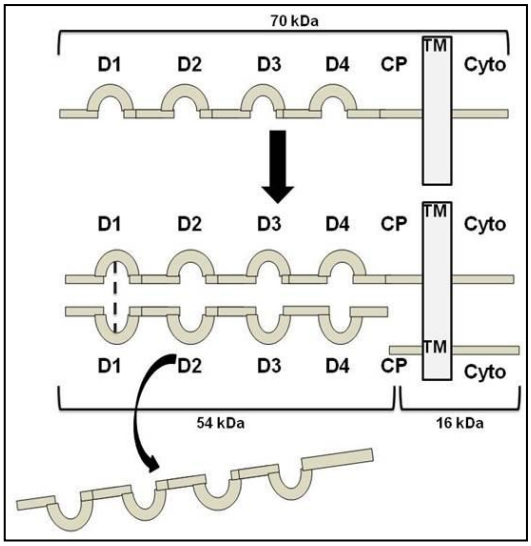


Figure 18: LAG-3 cleavage schematic. LAG-3 is expressed on the cell as a 70 kDa molecule. TACE cleavage occurs within the CP region, releasing a 54 kDa subunit that transiently dimerizes with other full-length LAG-3 left on the cell surface. Eventually, the cleaved portion is shed into the serum as sLAG-3 and eventually excreted out of the body (321).

Immunoprecipitation and subsequent western blotting of BDC-2.5.TCR.Tg splenocyte culture supernatants demonstrated reduced sLAG-3 after CA treatment compared to control samples (**Figure 19A**), illustrating decreased LAG-3 cleavage upon redox modulation. Notably,

sLAG-3 was undetectable in the 'No Ag' sample, which did not undergo antigenic stimulation. From these results, we postulate that CA can reduce LAG-3 shedding by modulating TACE enzymatic function.

2.4.6 CA exposure decreases sLAG-3 in a TACE-dependent manner

We next wanted to test the dependence, as a result of T cell activation, of redox-mediated TACE modifications on LAG-3 cleavage. In order to attribute the reduction of LAG-3 shedding to CA-regulated TACE, we compared M vs. M+CA vs. M+TAPI, a known TACE inhibitor. M+CA decreased sLAG-3 levels in comparison to M alone ($p<0.05$) as detected by ELISA (**Figure 19B**) and consistent with **Figure 19A.** Both CA and TAPI reduced the amount of detectable sLAG-3 to a similar extent ($p<0.05$) in comparison to M alone. Furthermore, a comparison between M+TAPI and M+CA+TAPI demonstrated no significant difference in reducing sLAG-3 levels. These data suggest that CA treatment is likely inhibiting TACE-dependent LAG-3 cleavage.

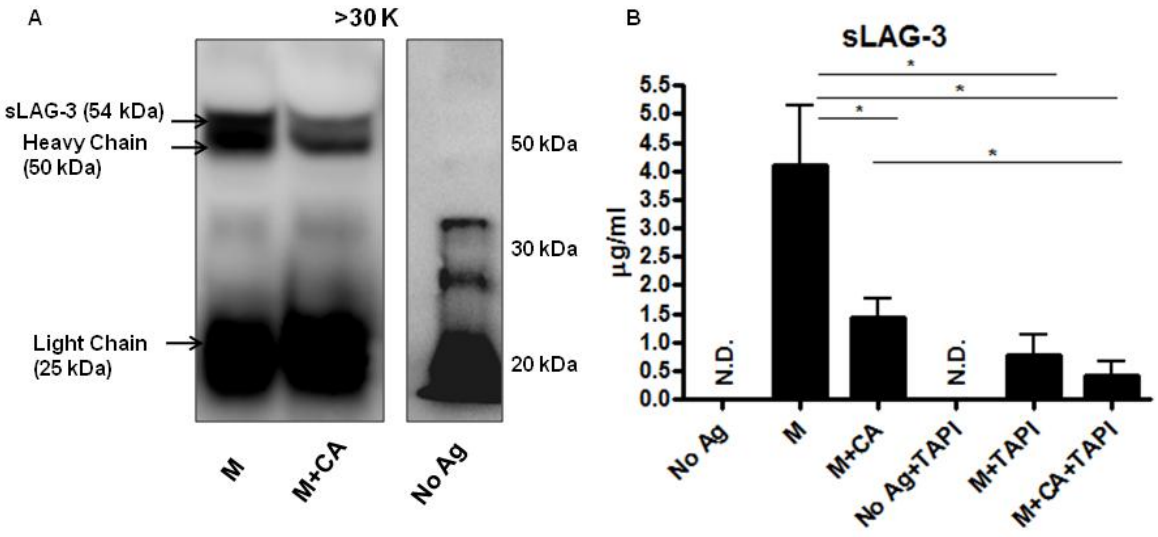


Figure 19: Soluble LAG-3 protein is decreased following CA treatment. (A) Supernatants from BDC-2.5.TCR.Tg splenocytes stimulated +/- M +/- CA for 48h were concentrated using Amicon Ultra Centrifugal Filters at a 30K cutoff. The >30K portion was then immunoprecipitated with anti-LAG-3 Ab, separated on an SDS-PAGE gel, and probed for LAG-3 by western blot. Data representative of 3 independent experiments. (B) BDC-2.5.TCR.Tg splenocytes were stimulated with M +/- CA +/- TAPI-1 for 72h. Supernatants were collected and used in sLAG-3 ELISAs. N.D.= none detected. Graph shows the average of 4 independent experiments performed in triplicate. *$p<0.05$.

2.4.6.1 LAG-3 transcription and protein levels are reduced following CA treatment

In addition to reductions in sLAG-3 detected in supernatants after stimulation plus CA, LAG-3 transcription and translation were also measured in whole splenocytes and isolated CD4+ T cells. At 72h post-stimulation, LAG-3 mRNA is significantly reduced in BDC-2.5.TCR.Tg splenocytes activated with M+CA (**Figure 20A**). Likewise, both full-length and cleaved LAG-3 protein levels are also reduced in CA-treated splenocytes and isolated CD4+ T cells (**Figure 20B, C**). These data may seem to contradict the flow cytometric results which show no detectable differences in the amount of LAG-3 per cell (MFI) after redox modulation (**Figure 20C**). However, the flow cytometric studies only measured the amount of LAG-3 on the cell surface,

not what was contained inside the cell. Previous reports have suggested the storage of preformed LAG-3 in intracellular vesicles (353), which could be the source of the differences detected in whole cell lysates by western blot. Overall, the reductions in LAG-3 mRNA and protein would likely contribute to the significant decrease in cleaved sLAG-3. This outcome, together with the effects of CA on TACE activity, would further delay diabetogenic T cell activation.

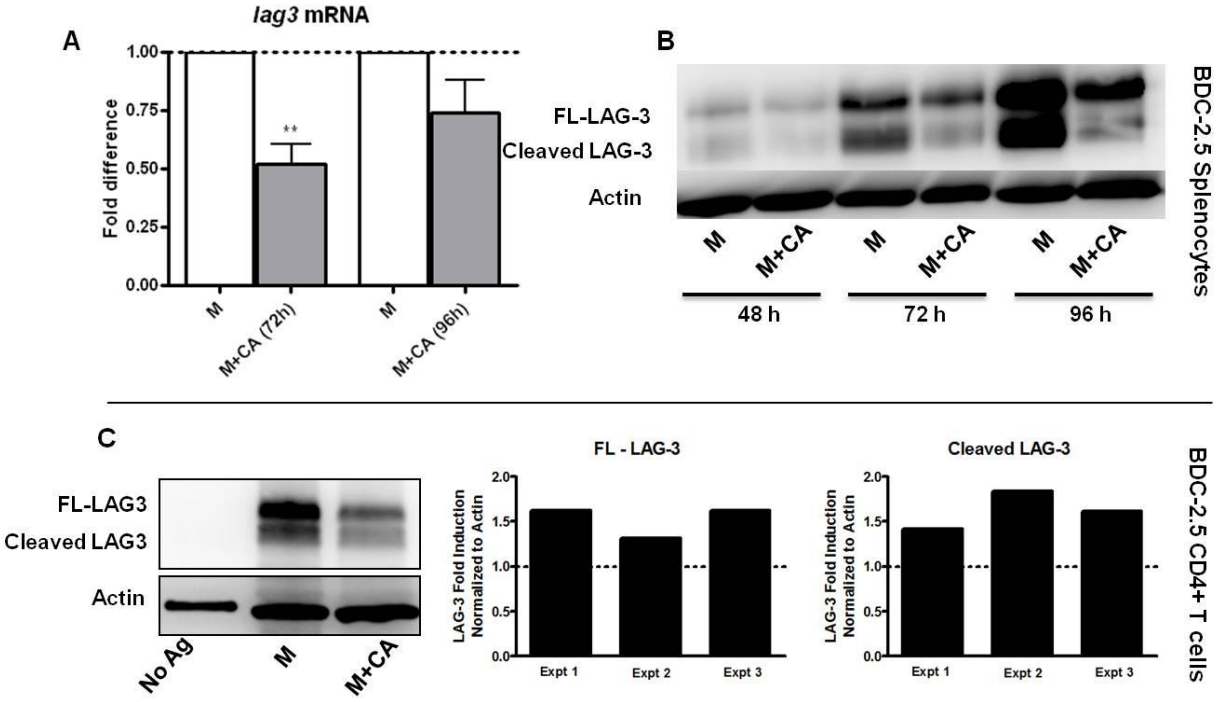


Figure 20: LAG-3 transcription and protein levels are reduced upon redox modulation. (A) BDC-2.5.TCR.Tg splenocytes were stimulated with M +/- CA for 72-96h. mRNA was isolated, cDNA was transcribed, and relative lag3 mRNA levels were measured by qRT-PCR. The fold change of M samples were set arbitrarily to 1 and compared to M + CA treatment. All samples were normalized to the endogenous GAPDH control. n=3 independent experiments. ***p<0.005*. (B-C) BDC-2.5.TCR.Tg splenocytes were treated with M +/- CA for 48-96h. (B) Whole cell lysates were made and probed for LAG-3. Actin was used as a loading control. Graph representative of n=3 independent experiments. (C) CD4+ T cells were isolated via MACS separation after 72h stimulation. Whole cell lysates were made and probed for LAG-3. Actin was used as a loading control. Densitometry was determined by normalizing M and M+CA to actin and then dividing M/M+CA for 3 independent experiments.

2.4.7 Redox modulation reduces TACE levels and enzymatic activity

We also wanted to determine if CA treatment specifically decreased TACE enzymatic activity. Utilizing BDC-2.5.TCR.Tg splenocytes in an in vitro TACE-specific fluorogenic assay, enzymatic activity in CA-treated cells was significantly decreased compared to M-stimulated cells ($p<0.005$) (**Figure 21A**). As a positive control, TAPI-treated cells also demonstrated a significant reduction in TACE activity ($p<0.0005$). To delineate whether the difference in enzymatic activity corresponded with decreased levels of TACE protein, we performed western blots for the TACE prodomain and active isoforms. TACE is formed as a latent/inactive enzyme containing a disulfide linkage, whereby oxidation of the bond promotes autocatalytic cleavage of the prodomain (20 kDa) from the active subunit (80 kDa) (54; 55). Under CA exposure, cleavage of the TACE prodomain was reduced compared to control samples (**Figure 21B**), indicating less oxidation of the critical cysteine switch and likely resulting in decreased enzymatic activity. Membrane lysates also exhibited diminished levels of active TACE following CA treatment (**Figure 21C)**. Interestingly, immature TACE was also reduced upon redox modulation, suggesting less overall activation-induced expression of TACE (54; 357).

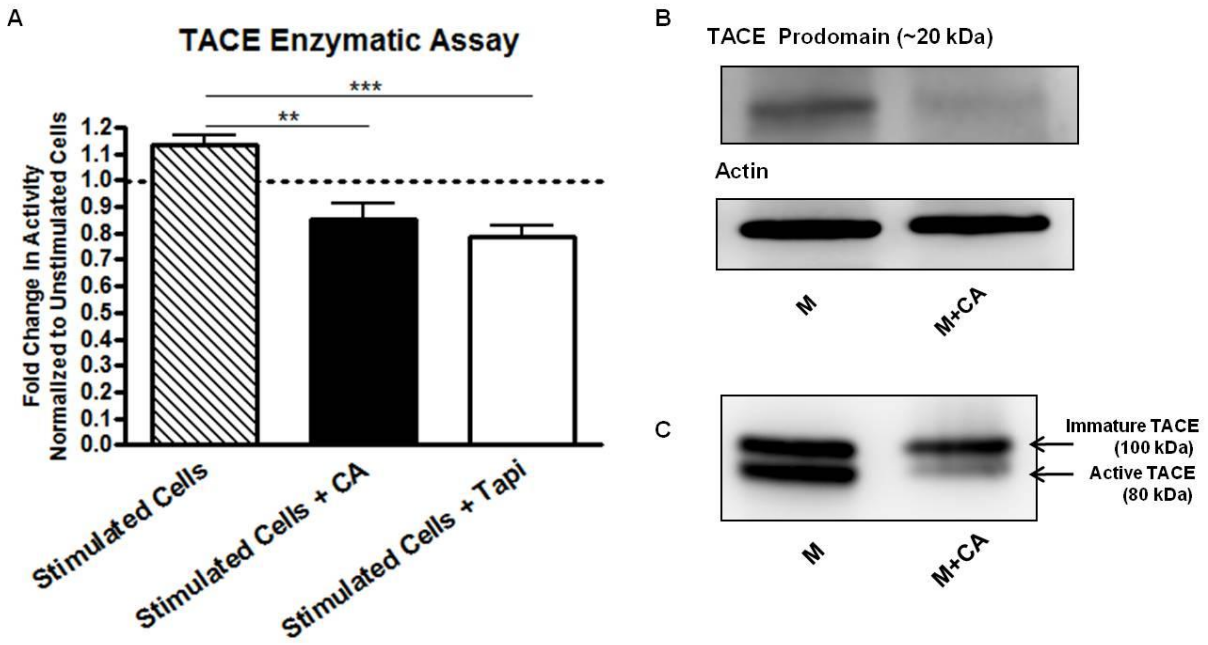


Figure 21: Redox modulation diminishes active TACE levels and enzymatic function. (A) BDC-2.5.TCR.Tg splenocytes were stimulated with M +/- CA +/- TAPI for 24h and supplemented with TACE-specific fluorogenic substrate. Fluorescence was measured at 6h post substrate addition. The fold change in activity was calculated by Stimulated Cells/Unstimulated vs. Stim + CA/Unstimulated vs. Stim + TAPI/Unstimulated Cells. Graph shows the average of 3 independent experiments performed in triplicate. *p<*0.05,**0.005, ***0.0005*. (B-C) BDC-2.5.TCR.Tg splenocytes were stimulated with M +/- CA for 72h and probed for TACE by western blot. (B) Whole cell lysates were used. Actin was probed as a loading control. (C) Membrane lysates were utilized. Data are representative of 3 independent experiments.

2.4.7.1 CA is able to inhibit protein oxidation, leading to less TACE activity

As a proof of principle, BDC-2.5.TCR.Tg splenocytes were stimulated +/- CA to test the overall oxidation of proteins as well as the ability of TACE to cleave pro-TNFα, its canonical shedding target. Membrane lysates were isolated and stained with F5M, which labels sulfhydryl-containing reduced proteins. Redox modulation showed a general decrease in protein oxidation, contributing to reduced TACE activation (**Figure 22A**). Furthermore, BDC-2.5.TCR.Tg splenocytes stimulated with islet cells + CA demonstrated significant reductions in TNFα secretion, once again indicating effects on TACE enzymatic activity (**Figure 22B**).

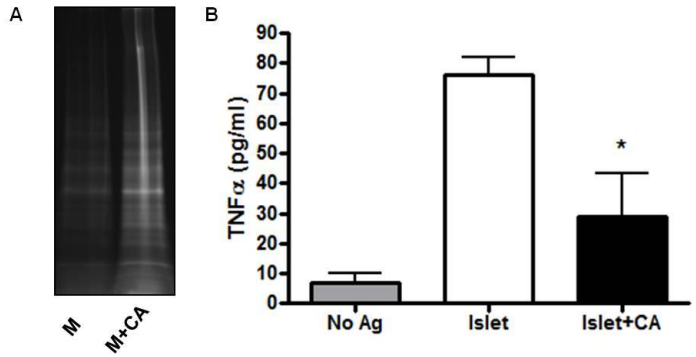


Figure 22: Redox modulation reduces protein oxidation and TNF*α* shedding. BDC-2.5.TCR.Tg splenocytes were stimulated with (A) M +/- CA or (B) NOD.*scid* islet cells +/- CA. (A) Membrane lysates were labeled with F5M, run on an SDS-PAGE gel, and visualized by fluorescence. Gel representative of 3 independent experiments. (B) Supernatants were collected after 72h stimulation and utilized in a TNFα ELISA. n=3 independent experiments, **p<0.05*.

2.4.8 CA treatment prevents diabetes transfer in correlation with reduced sLAG-3 serum levels

We next monitored LAG-3 in conjunction with diabetes progression upon adoptive transfer of disease-causing T cells. Measurement of sLAG-3 has been used as an index of breast cancer prognosis, with greater levels corresponding to better anti-tumor CTL responses and patient survival (334). Underlying these observations is an increase in T cell activation, and we therefore propose that diabetogenic T cell activation can be indirectly ascertained by serum sLAG-3. To assess this possibility, NOD.*scid* mice (10 wks old) were adoptively transferred with BDC-2.5.TCR.Tg splenocytes and treated daily with CA. Control animals all developed diabetes by day 15 post-transfer, whereas CA-treated mice remained disease-free until the end of the study at day 28 ($p<0.0001$) (**Figure 23A**). Furthermore, serum levels of sLAG-3 steadily increased in control animals over time, yet sLAG-3 from CA-treated mice was significantly lower at days 12 and 16 post-transfer ($p<0.05$) **(Figure 23B)**. Splenocytes were isolated either at diabetes onset (day 16) or at the end of the experiment (day 28) and stained for LAG-3. CA-treated animals had a higher frequency of LAG-3+CD4+T cells versus control animals ($p<0.05$) (**Figure 23C**).

Additionally, in vivo CA treatment decreased active TACE protein levels compared to control animals (**Figure 23D**). sLAG-3, therefore, serves as a biomarker of type 1 diabetes progression in this model and correlates with enhanced LAG-3+ T cells, decreased active TACE levels, and inhibition of disease following CA treatment.

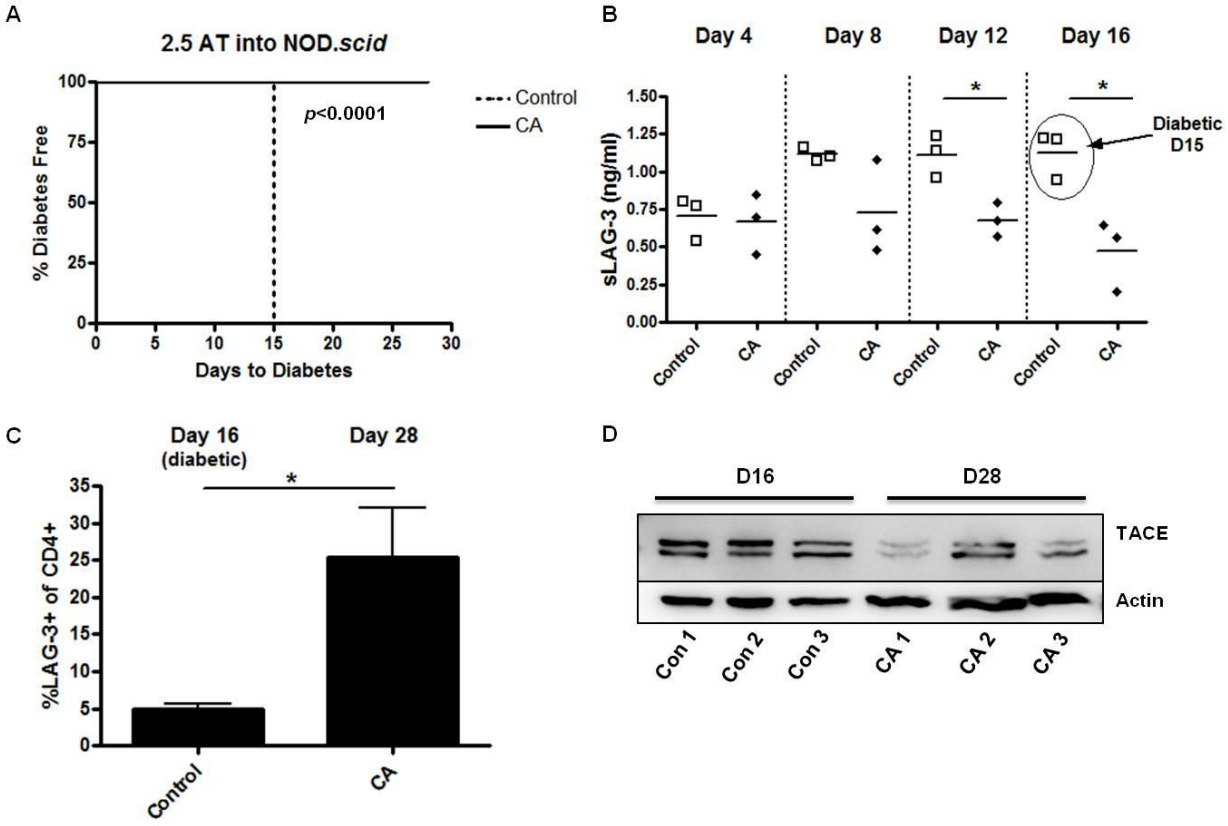


Figure 23: Redox modulation delays diabetes onset, which correlates with decreased sLAG-3 and enhanced LAG-3+ cells. NOD.*scid* mice (n=3/group) were treated on day -1 through day 28 i.p. with or without CA. BDC-2.5.TCR.Tg splenocytes were i.v. transferred on day 0. (A) Mice were monitored by glucosuria for the onset of type 1 diabetes and considered diabetic after two consecutive blood glucose readings of >300 mg/dl,****p<0.0001*. (B) Blood was collected retroorbitally every 4 days to day 16, and serum was isolated for sLAG-3 ELISA. Open squares = control, filled diamonds = CA-treated.**p<0.05. (*C) On day 16 and 28 post-transfer, splenocytes were stained for and gated on CD4+ cells. LAG-3 was detected by flow cytometry. Graph shows the average of 3 mice/group, **p<0.05*. (D) Lysates were made from splenocytes and run on an SDS-PAGE gel. Western blots were performed to measure TACE expression.

2.4.9 sLAG-3 levels correlate with diabetes incidence in NOD mice

Historically, NOD females are known to have an earlier onset and greater diabetes incidence than NOD males. Therefore, sLAG-3 was measured over several weeks of age to determine if it correlated with diabetes onset. In **Figure 24A**, sLAG-3 is significantly increased in NOD females early in life (6-8 weeks of age) in comparison to NOD males. After 10 weeks of age, the differences in sLAG-3 between genders are less pronounced. NOD female mice in this particular cohort succumbed to disease starting at 13 weeks of age, significantly earlier than male disease onset and 7 weeks following the significant differences in sLAG-3 observed between female and male mice (**Figure 24B**). This delay in disease after T cell activation between the sexes may be attributed to differences in the development of autoantibodies, which are known to precede diabetes onset (358) and to depend on T cells for their formation. This possibility was further investigated utilizing human serum samples.

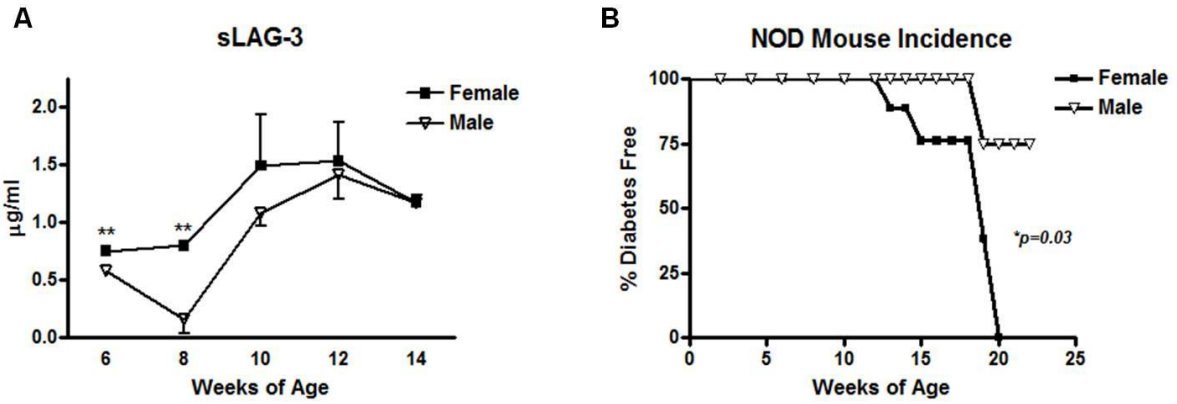


Figure 24: Early detection of sLAG-3 correlates with greater type 1 diabetes incidence in NOD females. (A) NOD females and males were bled for serum every 2 weeks starting at 6 weeks of age. Sera were quantified using a sLAG-3 ELISA. ***p<0.005*. (B) NOD mice were monitored for diabetes onset weekly, starting at 12 weeks of age. Two subsequent blood glucose readings >300 mg/dl were considered diabetic. n=4 mice/group, **p<0.05*.

2.4.10 First-degree relative sLAG-3 levels inversely correlate with the number of autoantibodies

Upon T cell activation, sLAG-3 is shed from the cell surface, enabling the measurement of serum levels prior to disease onset, as depicted in **Figures 23** and **24**. sLAG-3 was first quantified from the serum of type 1 diabetes patients ranging from bleeds at onset to bleeds almost 6 years after onset (**Figure 25**). No differences in sLAG-3 were detected regardless of the date of collection, suggesting the time point of serum evaluation may be too late and heterologous immunity may have masked any distinction.

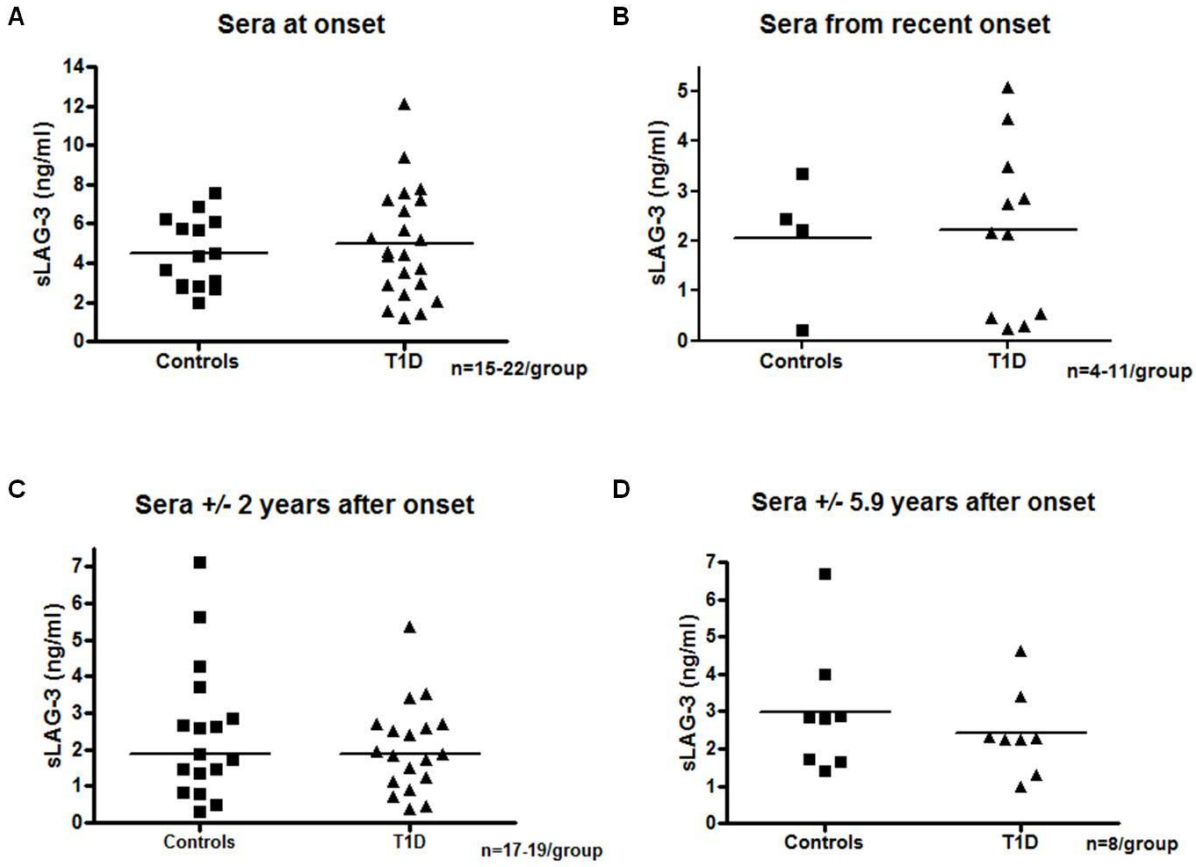


Figure 25: sLAG-3 levels do not vary between healthy controls and type 1 diabetes patients at onset or beyond. Sera from different bleed collection times (A: at onset, B: recent onset, C: +/- 2 years post-onset, D: +/- 5.9 years post-onset) were utilized in a hLAG-3 ELISA. The number of serum samples per collection time is indicated on the graphs.

The autoantigens identified in type 1 diabetes are T cell-dependent antigens, which require antigen presentation and TCR binding. T cell help through MHC-peptide interaction with the B cell and costimulation via CD40L-CD40 is necessary for plasma cell activation and antibody secretion (359). Consequently, autoantibody production is secondary to T cell activation. The kinetics of sLAG-3 would most likely precede the secretion of autoantibodies for type 1 diabetes prediction and onset. As a genetic disease, first-degree relatives of type 1 diabetes patients have a high susceptibility to disease (4; 5; 358). In this population, HLA testing can be performed to predict onset; additionally, autoantibodies are often measured to determine risk. The number of autoantibodies is a reliable predictor of type 1 diabetes, with 3 being the highest (GAD, IAA, IA2) and 0 being the lowest risk (360). Therefore, sLAG-3 was measured in first-degree relatives who contained various numbers of autoantibodies. In comparison to type 1 diabetes patients, sLAG-3 levels in antibody-negative (Ab-) first-degree relatives were almost significantly higher (**Figure 26A**), indicating the beginning stages of T cell activation and possibly predicting progression to disease. Furthermore, the number of autoantibodies was determined compared to sLAG-3; 0-1 autoantibody correlates with greater sLAG-3 levels, whereas 2-3 autoantibodies correlates with very low levels of sLAG-3 (**Figure 26B**). This phenomenon demonstrates an inverse correlation of sLAG-3 with the number of autoantibodies, both contributing to type 1 diabetes risk.

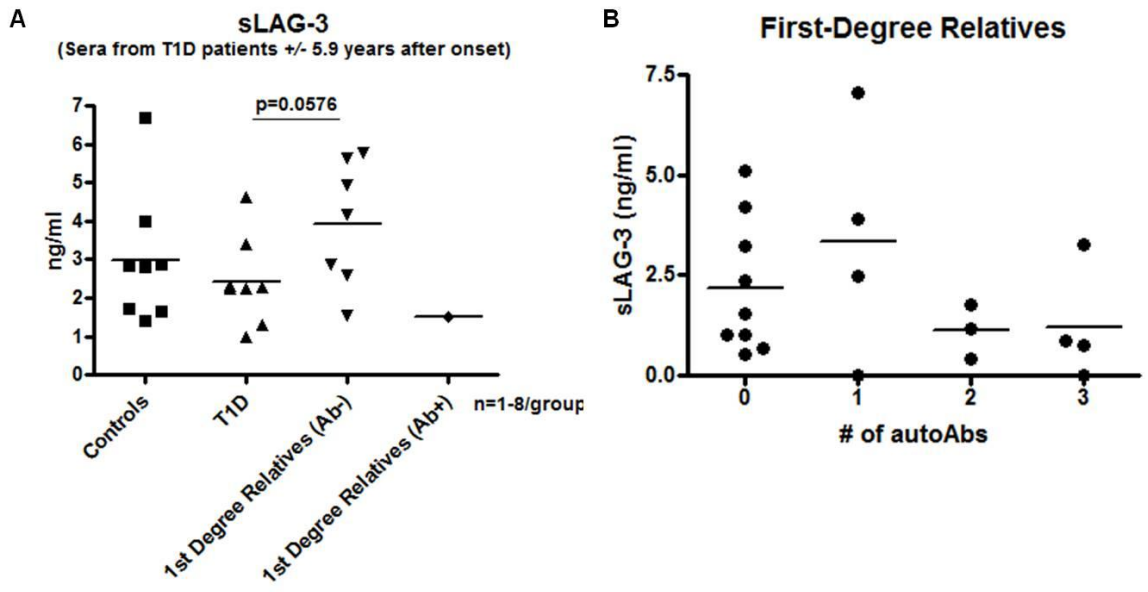


Figure 26: sLAG-3 inversely correlates with autoantibody levels. Sera were utilized in a hLAG-3 ELISA. (A) Sera were collected at +/- 5.9 years post-onset (reanalyzed from Figure 25), from first-degree relatives that were antibody-negative or positive, or from healthy controls. n=1-8 samples/group. (B) sLAG-3 from first-degree relatives was compared to the number of autoantibodies present. n=20.

2.5 DISCUSSION

Because CA treatment directly affects innate immune cells and proinflammatory third signal synthesis (48) as well as NF-κB and NF-κB-dependent gene transcription (64), we sought to understand how modulating redox balance could influence activation and function of diabetogenic T_H1 cells. In particular, we hypothesized that CA administration would decrease TACE-dependent LAG-3 shedding, leading to autoreactive T cell hyporesponsiveness and reduced type 1 diabetes.

In this study, long-term modulation of the redox state resulted in significantly delayed type 1 diabetes onset, illustrating the importance of ROS in promoting autoreactive immune responses. With the correlation of 1 mouse year = 34 human years (361), CA was able to extend

diabetes onset for about 10 years, and if the mouse model truly depicts human type 1 diabetes, CA treatment would result in a human onset time of 22 years old instead of 12 years old in the controls. However, stopping the CA treatment at 29 weeks does not seem to afford absolute enduring protection against disease onset. This may be due to blood clearance of the modulator as a result of troughing of the CA level below the effective concentration and consequently, loss of therapeutic efficacy. Under this circumstance, CA treatment alone may require chronic administration to inhibit diabetes onset; however, CA administration in combination with an antigen-specific therapeutic approach targeting self-reactive T cells might afford long-lasting protection by inducing T cell-specific ignorance or tolerance. Nonetheless, CA treatment has marked effects on early T cell responses, resulting in a delay in diabetes onset. BDC-2.5.TCR.Tg T cells demonstrated decreased activation, proliferation, and effector function upon CA treatment, which correlated with enhanced LAG-3+CD4+ T cells *in vitro* and a trend towards significant increases *in vivo*. The effect of CA on LAG-3 is T cell specific in our system, as double-staining for CD3 and CD4 overlapped 100%, excluding the possibility of pDC expression of LAG-3 (data not shown). Absence of T cell activation, coupled with greater LAG-3+CD4+ T cell frequency, indicates two possible consequences of redox modulation: (1) less activation/progression to effector function of antigen-specific autoreactive T cells and/or (2) obstruction of LAG-3 shedding. Notably, T cells from insulin immunized mice exhibited reduced T_H1 effector responses (decreased IFN-γ synthesis and a lower frequency of antigen-specific T cells), suggesting that regulation of LAG-3 may be responsible for this phenomenon. With recent reports demonstrating accelerated diabetes in LAG-3(-/-) NOD mice (332; 333), our redox modulation results may be reflective of T cell ignorance (362; 363). Diabetogenic T cells in NOD mice already have an advantage of efficiently expanding from a greatly reduced precursor

pool (364). If CA delays or prevents the autoantigen-specific T cell pool necessary for reaching the threshold at which a break in tolerance to self-antigen occurs, disease onset should be reduced.

Collectively, our results also suggest that an overall delay in T cell activation kinetics may be the main cause of redox modulation anomalies. Impaired TACE activity, via LAG-3, offers new insights into a novel mechanism of autoreactive T cell activation thus far unknown, and redox-dependent modifications together can contribute and feed-forward to prevent diabetogenic immune responses.

Redox modulation also reduced the transcription and translation of LAG-3. We have previously identified NF-κB as another target of redox modulation (64). Notably, redox-dependent NF-κB is a predicted transcription factor responsible for LAG-3 expression (SABiosciences' Text Mining Application). Therefore, it is tempting to speculate that new LAG-3 protein synthesis is retarded as a result of CA-mediated NF-κB inhibition, contributing to the reduction in sLAG-3 observed. CA was also able to affect TACE activation and enzymatic activity, further contributing to decreased sLAG-3. Immature TACE levels were also reduced after treatment. Because oxidants activate signaling kinases and GTPases to drive the expression of metalloproteases (54; 357), scavenging of oxidants by CA (64; 269; 313) may inhibit proper signal transduction and cause decreased expression of immature TACE. Another metalloprotease, ADAM10, unlike TACE, is responsible for constitutive LAG-3 shedding (321) and may also be partially inhibited by CA treatment (54; 315; 316; 320). However, our experiments using TAPI blocked LAG-3 shedding by both metalloproteases, ruling out enzyme-specific differences (365). Both TAPI and CA demonstrated similar reductions in sLAG-3 versus M alone, supporting the notion that CA can directly modify TACE-dependent cleavage.

Based on the current data, redox-modulated LAG-3+ cells may have altered signal transduction pathways and/or are functioning as suppressive T cells (355; 366; 367). Upon TCR recognition of peptide-MHC, LAG-3 colocalizes with the TCR in the lipid raft and binds with greater affinity to MHC class II molecules than does CD4 (331; 368; 369). However, the mere blockade of CD4 binding to MHC class II is not sufficient to decrease T_H1 activation; therefore, LAG-3 may signal in a negative manner to downregulate T cell activation (351; 370). Although LAG-3-MHC class II ligation stimulates SHP-1 and ERK activation in the DC, signaling through LAG-3 into the T cell is currently undefined (371). The cytoplasmic tail of LAG-3 contains a KIEELE motif (351) and an EP motif which binds to the protein LAP (LAG-3-associated protein) as determined by yeast two-hybrid system, though the function of LAP is unidentified (372). By mutating the cytoplasmic KIEELE motif of LAG-3, proliferation is increased, indicating the importance of inhibitory downstream signaling in control of expansion (354). Therefore, the KIEELE motif is thought to mediate negative signaling, whereas the EP motif may prevent LAG-3 from acting as a coreceptor (349). Nonetheless, the EP motif is rare in human proteins, and since LAG-3 lacks an immunoreceptor tyrosine-based inhibitory motif (ITIM), this unique EP motif may be critical for full LAG-3 function (372). LAG-3-TCR cocapping studies performed by Hannier et al. show inhibition of calcium fluxes, induction of CD25, but lack of apoptosis, following LAG-3 cross-linking (368). These results provide evidence that LAG-3 is necessary for immune response control, yet inhibits cell death and induces IL-2R, most likely for eventual proliferation or the maintenance of regulatory T cells. Retention of LAG-3 on CD4+ T cells could potentially decrease the frequency of activated autoreactive T cells and, based on recent findings, may act as regulatory T cells. Natural CD4+CD25+ Tregs have been shown to express LAG-3 upon activation (355), and

CD4+CD25+FOXP3+LAG-3+ adaptive Tregs have been detected in tumor draining lymph nodes, requiring cell-to-cell contact for suppression (373). Furthermore, IL-10 secreting CD4+CD25-FOXP3-LAG-3+ Tregs have been isolated from the Peyer's Patches and express Egr2, a transcription factor necessary for anergy (366). Contrary to kinetics in effector cells, regulatory T cells retain the expression of LAG-3 despite activation status, and require LAG-3, as their suppressive activity is decreased upon antibody blockade of the molecule (367). Additionally, ectopic expression of LAG-3 is able to bestow suppressor activity upon CD4+ T cells (321; 355), illustrating the possible effects of non-physiological manipulation and preservation of functional LAG-3. Ultimately, the conservation of cell surface LAG-3 may lead to early contraction of T cell activation, either by directly affecting the T cell on which it is expressed, or by imparting regulation via modulation of accessory cells (371). Both signal transduction and regulatory potential will be determined in future studies.

sLAG-3 shedding coincides with in vivo SEB-mediated T cell activation (335) as well as T cell activation in breast cancer screens (334). At present, there is a paucity of serum biomarkers that measure T cell activation prior to overt diabetes (374), and little is known about the endogenous role of LAG-3 in autoimmune settings. We therefore, wanted to determine if we could monitor diabetes progression via serum sLAG-3. Redox modulation prevented type 1 diabetes onset in the rapid adoptive transfer model, similar to previous reports (36), but more importantly, sLAG-3 directly correlated with T cell activation and autoimmunity. Serum sLAG-3 was enhanced in control animals before and upon disease onset, suggesting greater activation of diabetogenic T cells. sLAG-3 may, therefore, serve as a T cell-specific diagnostic marker for initiation of beta cell destruction, and along with surface LAG-3 expression, may additionally function as surrogates of immunomodulation. Furthermore, sLAG-3 was higher in NOD females

than males at early time points, which tracks with diabetes risk and indicates a delay between T cell activation and disease onset. However, human sLAG-3 did not differ between type 1 diabetes patients and healthy controls; instead, sLAG-3 inversely correlated with the number of autoantibodies present in first-degree relatives highly susceptible to disease. This suggests that sLAG-3 may be a potential biomarker for the early progression of at-risk individuals to help predict disease manifestation a priori autoantibody appearance, but larger first-degree relative studies need to be conducted in order to definitively make this conclusion. Additionally, therapies that utilize prophylactic drugs, as well as attempts at reversal of autoimmunity, may result in the modification of adaptive immune responses, and measurement of sLAG-3 would allow for determination of treatment efficacy on autoreactive T cells in a noninvasive manner.

Taken together, our data suggest that redox modulation arrests LAG-3 shedding by impeding expression kinetics and decreasing TACE activity. Through redox manipulation, LAG-3 surface expression is maintained at levels adequate to attenuate TCR-mediated T_H1 cell activation and effector function. This would be beneficial for maintaining diabetogenic effector cells in a quiescent state or in preventing their activation entirely. In concert with the recently reported mechanisms of direct and indirect actions of CA on immune cells and diabetes progression (36; 48; 64; 154; 278; 297), the identification of LAG-3-mediated immunoregulation adds another layer to the control of autoimmunity. Our discovery also supports the use of sLAG-3 as a novel surrogate marker of type 1 diabetes progression in preclinical situations and possibly as a means to monitor the effectiveness of T cell-directed immunotherapy.

3.0 REDOX MODULATION PROMOTES METABOLIC QUIESCENCE AND REGULATION OF IMMUNE CELLS

3.1 REVIEW: CHANGING THE ENERGY OF AN IMMUNE RESPONSE

The breakdown of nutrients into the critical energy source ATP is the general purpose of cellular metabolism and is essential for sustaining life. Similarly, the immune system is composed of different cell subsets that are indispensable for defending the host against pathogens and disease. The interplay between metabolic pathways and immune cells leads to a plethora of different signaling pathways as well as cellular activities. The activation of T cells via glycolysis-mediated upregulation of surface markers, for example, is necessary for an appropriate effector response against an infection. However, tight regulation of immune cell metabolism is required for protecting the host and resuming homeostasis. An imbalance of immunological metabolic function and/or metabolic byproducts (reactive oxygen species) can oftentimes lead to diseases. In the case of cancer, overactive glucose metabolism can lead to hyperproliferation of cells and subsequent decreases in cytotoxic T cell activity, which attack and destroy the tumor. For this reason and many more, targeting metabolism in immune cells may be a novel therapeutic strategy for treatment of disease. The metabolic pathways of immune cells and the possibilities of immunometabolic therapies will be discussed.

3.1.1 Aerobic respiration

Cellular metabolism is necessary for generating energy and sustaining life. Through a series of steps involved in glycolysis (glucose), fatty acid (fat) oxidation, and amino acid (protein) oxidation, cells can break down ingested products into critical energy sources. This energy, better known as adenosine triphosphate or ATP, is synthesized as a result of the degradation of nutrients. Oxygen (O_2) plays a key role in enabling reactions required for the formation of ATP. In human cells, oxidative phosphorylation is the main process leading to the generation of ATP (375). The degradation of nutrients through the glucose oxidation, fatty acid oxidation, or amino acid oxidation pathways converge to all produce acetyl-CoA, a key molecule that provides a carbon source for fueling the TCA cycle. The oxidation of acetyl-CoA to carbon dioxide (aerobic respiration) then allows for the subsequent reduction of nicotinamide adenine dinucleotide (NAD+) and flavin adenine dinucleotide (FAD) via the TCA cycle. The intermediate products, NADH and $FADH_2$, serve as electron transport chain coenzymes for oxidative phosphorylation. For efficient respiration, electrons must be transferred from NADH and $FADH_2$ to oxygen via the mitochondrial complexes along the electron transport chain within the inner mitochondrial membrane. To ensure proper oxidative phosphorylation, electrons must be strictly allocated down the electron transport chain while protons must be pumped across the mitochondrial membrane. This movement of electrons not only facilitates the production of H_2O, but also drives a proton gradient that causes the phosphorylation of adenosine diphosphate to adenosine triphosphate **(Figure 27)**. ATP can then be utilized for a number of events including DNA/RNA/protein synthesis, cell signaling, cytoskeletal rearrangement, cell proliferation, and metabolic pathways. In addition to its necessity for energy production, the electron transport chain is also responsible for the formation of mitochondrial reactive oxygen species (ROS)

through continuous 'leakage' of electrons, causing partial reduction of O_2 molecules (376). Such events lead to the generation of superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-), which can both stimulate physiological actions as well as damage important molecules in the body (377), depending on the quantity. At low levels, ROS help control normal cellular functions, as demonstrated by the immune system's reliance on this type of signaling for regulation, activation, T cell proliferation (378; 379), NF-κB activation (380), and signal transduction (381-383). Conversely, high levels of ROS lead to oxidative stress, which has been linked to a variety of diseases, aging, and cell death (22; 384-386). ROS production is, thus, a necessary evil for functional aerobic metabolism.

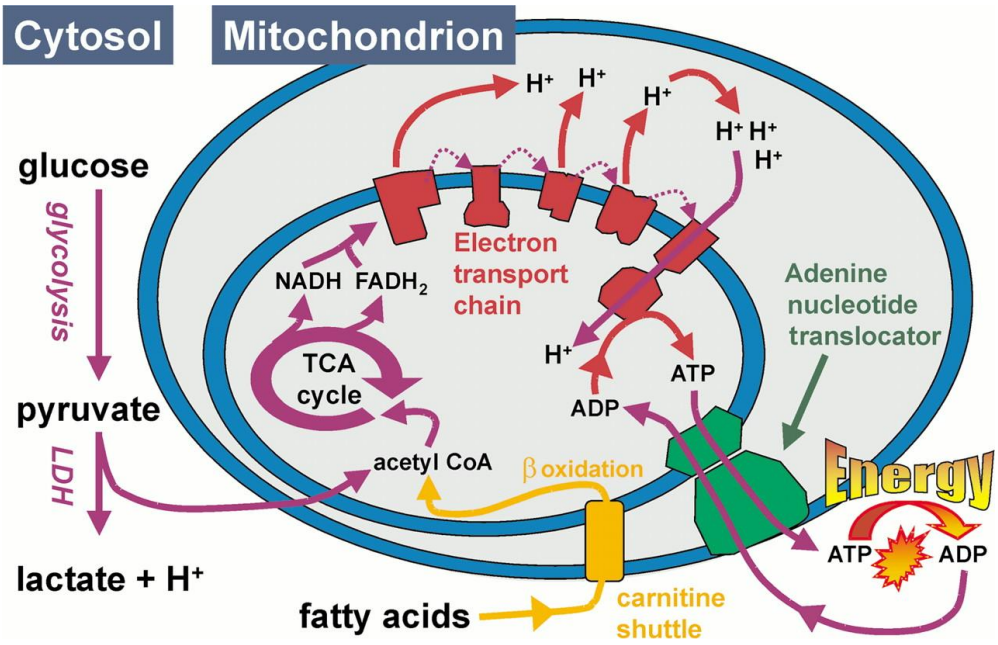


Figure 27: Energy generation in mitochondria. Mitochondria generate energy by oxidative phosphorylation. Alternatively, energy may be generated by glycolysis alone, where pyruvate is converted to lactate. *Used with permission from Sex Transm. Infect. (387).*

3.1.2 Aerobic glycolysis

Although aerobic respiration is the main source of ATP in most mammalian cells, an alternative form of metabolism, glycolysis, is crucial in both immunity and disease states. Glycolysis enables the conversion of one glucose molecule to 2 pyruvate molecules, with subsequent production of lactate, NAD+, and ATP. The utilization of glycolysis is a less efficient form of metabolism. During mitochondrial respiration, a cell is able to produce 38 ATP molecules; however, during glycolysis, only 2 molecules of ATP are generated. Obviously, the 19-fold increase in ATP via oxidative phosphorylation seems more advantageous to the cell, especially since both metabolic pathways can use glucose as the starting material. However, glycolysis is especially important in times of hypoxia (anaerobic) and can occur even in the presence of oxygen (aerobic), such as with tumors and immune cells. Both anaerobic and aerobic glycolysis are crucial for the maintenance of tumor cells (388-390). Tumor cells switch their energy production from oxidative phosphorylation to glycolysis upon transformation to malignancy (391; 392). This enables the tumor to rapidly grow in hypoxic environments and evade host immune cell defense mechanisms (393-395). In oxygenated environments, tumor cells display augmented glucose transport and glycolysis (396; 397). This counterintuitive metabolic programming has been attributed to over-adaptation to hypoxic environments, a greater need for macromolecules during unrestrained proliferation (glycolysis affords better protein/nucleotide synthesis), and elevated expression of the glycolytic enzyme hexokinase (398; 399). This high aerobic glycolysis seen in cancer cells is called the Warburg effect (388; 400) and has led to the development of anti-glycolytic drugs for cancer treatment (401-403). During proliferation, T cells and cancer cells show similar metabolic programming. Thus T cells, even in the presence of sufficient oxygen, also choose to ferment glucose, as further discussed below.

3.1.3 Immune cell metabolism

The immune system is made up of two different arms: the innate and adaptive immune system. The innate system is a first-line of defense against pathogens and foreign substances. Unlike the adaptive arm, the innate response is non-specific and is mediated by antigen-presenting cells and granulocytes. Examples of innate cells include dendritic cells, macrophages, and neutrophils. After successful priming by the innate cells, the adaptive immune response, made up primarily of T and B cells, provide antigen-specific protection against the insult, either through the release of cytotoxic granules, cytokines, or antibodies. Immune cells, like most other cells in the body, utilize nutrients via cellular metabolism. At rest, immune cell metabolism is able to regulate cell volume, ion integrity and growth (404). However, in addition to housekeeping proliferation and sustenance, ATP within the immune cells must be ready to carry out various functional activities such as phagocytosis, activation, antigen presentation/processing, migration, phosphorylation, differentiation, and effector responses (404; 405). Most of these actions are thermodynamically taxing, requiring notable and rapid changes in metabolism (404; 406). Furthermore, immune cells must facilitate cytoskeletal changes, increased ion signaling, enhanced phospholipid turnover, and greater macromolecule synthesis in a very short time during rapid energy consumption (407). Importantly, resting immune cells, especially those of the adaptive arm, contain little glycogen stores, resulting in the dependence of imported glucose to uphold metabolic needs (408-410).

Because of the diverse functionality of immune cells, several important differences exist between their metabolism and that of other cells within the body. Alveolar cells, for example, are reliant primarily on oxidative phosphorylation for the generation of sufficient ATP (411). Those cells which are in constant contact with oxygenated blood are especially formulated for

mitochondrial respiration. On the other hand, immune cells travel through the body to monitor the peripheral tissues. Once a foreign antigen is detected, APCs migrate to the draining secondary lymphoid organs where they can process and present the antigen to lymphocytes. APCs are present in strategic areas of the body as resident phagocytes. These cells, therefore, are not necessarily exposed to normoxic conditions at all times. For instance epidermal dendritic cells reside within the deep tissue layers of the skin (412), where oxygen tension is lower than the dermis (413). Such conditions create a slightly hypoxic environment in which key immune cells must be able to utilize alternate forms of metabolism in order to survive and function properly (414). Similarly, lymphocytes, upon activation within the secondary lymphoid organs, travel to the site of inflammation by traversing the endothelial cell wall into the target area (415). Once again, the migration of lymphocytes away from the source of oxygen causes slight hypoxia and a resultant loss of dependence on oxidative phosphorylation. Most sites of inflammation are also areas of lowered oxygen, with innate phagocytes clogging the blood vessels (416-418). Localization of immune cells thus requires adaptation to different oxygen levels and promotes more glycolytic pathways (419; 420).

Although influential, the environment is not the only element dictating the metabolic choices within immune cells. The activation of both innate and adaptive immune cells is absolutely critical for protecting the body from pathogens and insults. Consequently, the cells cannot afford to be inefficient in their nutrient metabolism. With this being said, it would seem likely that immune cells should generate ATP via oxidative phosphorylation, fostering the most energy from the nutrients provided. However, this is not entirely the case. Although some mitochondrial respiration does occur in immune cells, the level at which it is used depends significantly on the cell's specificity and state of reactivity. Activated (and some inactive)

immune cells prefer to utilize glycolysis, as it is 100-times faster than oxidative phosphorylation for macromolecule synthesis and proliferation (421) (**Figure 28**).

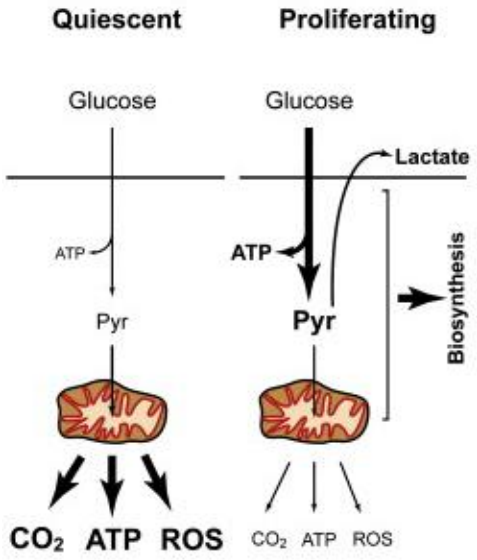


Figure 28: Metabolic pathways of resting vs. activated immune cells. In quiescent cells, glucose is oxidized within mitochondria generating high levels of CO_2, ATP, and ROS. Conversely, proliferating cells partially oxidize glucose by the glycolytic pathway to generate lactate, ATP, and metabolic intermediates for biosynthesis of macromolecules. *Used with permission from Biochim. Biophys. Acta (422).*

As mentioned, naïve APCs monitor the body for foreign substances. In this resting state, myeloid cells and granulocytes favor glycolysis (423; 424). Homeostatic protein turnover, degradation, and synthesis all occur via growth factor utilization (425; 426). Once antigen is phagocytosed, APCs immediately upregulate costimulatory molecules and process and present antigen on their cell surface. Such events require greater ATP; however, APCs retain their dependence upon glycolysis (423; 424). Dendritic cells, for example, are known to undergo metabolic changes towards greater glycolysis upon toll-like receptor (TLR) stimulation (427). Moreover, classically activated macrophages (known as M1), which promote proinflammatory cytokines are known to be regulated by glycolysis; however, alternatively activated macrophages

(M2), which are anti-inflammatory, rely more so on mitochondrial respiration (428; 429). Glycolysis is, therefore, important for inflammatory responses and can be detrimental in the face of chronic inflammatory diseases. Granulocytes, such as neutrophils, also favor glycolysis (423; 430). Neutrophils are the first mediators at the site of foreign entry. Their immediate degranulation and pyrogenic secretion lead to subsequent cell death (431). In accordance with the glycolytic reliability of APCs, quick responses are needed by neutrophils, but survival via greater ATP production is not necessary. Fast reactivity and turnover rates of both APCs and granulocytes, therefore, make sense with their choice to generate ATP through the more rapid glycolytic pathway.

Lymphocytes, on the other hand, rely heavily on oxidative phosphorylation during resting states but switch their metabolic needs to glycolysis during activation. Subsequently, some lymphocytes return to oxidative phosphorylation after clearance of an antigen to generate memory (432-434). Therefore, there is a cyclical pattern of metabolic pathways, fluctuating between anabolism and catabolism, as reviewed by Pearce *et al.* (435). At rest, T cells need a constant supply of nutrients as well as TCR stimulation (436). The idea of TCR 'tickling' has long been thought to prevent deletion of mature T cells (437). The active metabolism behind the quiescent state is a relatively novel idea. Circulating naïve lymphocytes undergo oxidative phosphorylation to generate a surplus of ATP reserves via catabolic metabolism, the breakdown of nutrients (410). In a sense, quiescent immune cells remain 'at attention' in order to quickly mobilize following antigen stimulation (438). Preservation of quiescence is mediated by turnover of cell cycle proteins, a very active event which requires a lot of ATP (425; 426), and by upregulation of cyclin-dependent kinase inhibitors (439). Quiescent cells not only utilize glucose, amino acids, and lipids for ATP generation, but can also extract nutrients from those

proteins which are degraded, via autophagy or self-eating (404; 440; 441). Engagement of the TCR as well as growth factors and homeostatic cytokines, like IL-4, IL-5, IL-7, and IL-3 (442-444), also all play roles in keeping naïve cells alive. Cytokine-receptor signaling, specifically, can activate protein kinases that are necessary for the uptake of adequate ATP to preserve homeostatic processes (410), whereas a lack of TCR interaction will downregulate glucose transport, ATP, and mitochondrial potential (436). Insufficiencies in glucose uptake by T cells will lead to BAX induction and apoptosis (445); however, this stringency serves to control the naïve T cell population, ensuring the turnover of existing cells as new cells are produced to avoid over accretion (436).

Lymphocytes also need to become activated upon antigen stimulation, albeit more rapidly than innate immune cells. Such changes in metabolism are well-documented in the case of mitogen-stimulated lymphocytes (435; 438; 446). Within the first 24h post-mitogen stimulation, lymphocytes considerably enlarge their size (435). During this time, new macromolecules are being synthesized, including nucleotides and proteins. Following the growth, T cells then divide every 4-6 hours (446) and eventually will differentiate into effector cells. Effector functions such as cytokine production and cytotoxic granule release then enable the cell to attack the infected target tissue. This quick change in cell size and function relies primarily on obtaining nutrients from the environment and driving glycolysis (410). Instead of initiating the long process of oxidative phosphorylation, T cells convert glucose to pyruvate via the more rapid glycolysis (421), and in the process, generate ATP and lactate, which allows for the conversion of NADH back to NAD+ to retain glycolysis. The dependence of T cells on glucose is so great, that even in the presence of excess glutamine, which is another carbon source that can be metabolized by lymphocytes, proliferation is stunted (442). T cells are not able to enhance mitochondrial

respiration to a level that meets their energy needs. Moreover, T cells require high levels of NADH as macromolecular precursors; therefore, greater lactate production equals more NAD generation (406). Notably, following mitogen-stimulation, an excess of lactate can be measured from T cells (447; 448). Aerobic glycolysis ensures that enough energy is made to propel macromolecule synthesis (anabolic metabolism), which is ultimately crucial for clearance of a pathogen, as well as to keep the cells alive and functional (410; 449; 450). Some oxygen consumption does still occur (451), yet is typically only utilized in situations where glucose is limited (452; 453). Mitogenic-stimulation of peripheral blood mononuclear cells, for example, causes oxygen consumption attributed to ATPase activity, protein synthesis, and nucleic acid synthesis (405; 438). Moreover, activation of lymphocytes promotes a calcium flux, which will also drive the upregulation of mitochondrial enzyme activity for sufficient oxidative phosphorylation (454; 455). In the case of infection, T effector cells promote clearance of the pathogen and primarily utilize glycolysis for rapid growth. By the time pathogen has been cleared, the mitochondrial capacity of effector cells is reduced in such a way that they can only sustain viability through glycolysis. In the absence of adequate nutrients and IL-2 present during infection, effector cells are unstable and eventually will undergo apoptosis. Certain clones, nonetheless, will retain their ability to switch back to oxidative phosphorylation. Such cells will contain greater mitochondrial mass, either through differences in biogenesis or asymmetric division (432). In autoreactivity (456) as well as graft-versus-host disease (457), chronically stimulated T cells rely on oxidative phosphorylation in contrast to acutely activated cells (those discussed above) which depend on glycolysis. Oftentimes, such illnesses and metabolic outcomes correlate with mitochondrial dysfunction and/or increased mitochondrial mass present during the disease (458). Lipid oxidation, specifically, is important for the generation of both

regulatory T cells (Tregs) (459) and memory CD8+ T cells (460), countering glycolytic effector CD4+ and CD8+ T cells. Following T cell activation, not only will the clearance of antigen help generate memory, but remaining T effector cells will die via apoptosis due to decreased growth factors and metabolism (389). Memory T cell metabolism is similar to naïve, in that both populations require oxidative phosphorylation; however, some critical changes occur over the course of an immune response to ensure memory T cell survival. Those that become memory cells will have higher TCR affinities, which allows for advantageous survival in conditions of nutrient limitation and low homeostatic engagement (436). Furthermore, CD8 T cells, described to have substantial spare respiratory capacity after clearance of infection, will be long-lived memory cells (434). Spare respiratory capacity is especially critical for producing sufficient ATP under times of stress and for boosting long-term cell survival (461; 462), which would be necessary for memory response. Similarly, CD4 T cell memory is enhanced via blockade of a gene called *Noxa* that is responsible for driving apoptosis under conditions of limiting glucose (463; 464).

3.1.4 Connecting immune signaling and metabolism

Stimulation of T cells via the TCR requires proper engagement by MHC-peptide on the APC. Additionally, costimulation during T cell activation is critical for downstream signaling and effector function. Conversely, a lack of costimulation can lead to T cell anergy and deletion (465; 466). Appropriate T cell activation is not only governed by mere mechanistic interaction and a cascade of signaling molecules, but there are important links to metabolism. CD28 is the quintessential costimulatory molecule for T cell activation. Its ability to bind CD80/CD86 on APCs enables downstream signaling and promotes T cell differentiation (467; 468). Analogous

to insulin-receptor signaling, CD28 has been shown to enhance glucose metabolism by triggering an accumulation of glycolytic intermediates (409), stimulating glycolysis, and increasing glucose transporter expression (469). CTLA-4, on the other hand, offsets the effects of CD28, reducing glycolysis and rendering cells quiescent (470). One of the most essential downstream signaling cascades bridging the gap between T cell activation and metabolism is the PI3K-Akt-mTOR pathway. CD28, IL-2 and TCR engagement leads to PI3K-dependent Akt activation, which in turn increases the amount of glucose transporters on the plasma membrane as well as elevates activities of glycolytic enzymes i.e. hexokinase and phosphofructokinase (406; 410; 444). PI3K as well as MAPK and NF-κB can all activate Myc, which is responsible for inducing glucose transporters as well as glycolytic enzymes (471; 472). Akt, in conjunction with STAT5, also plays a role in glucose uptake in resting T cells (473). Unlike other cell types, lymphocytes only express the Glut1 glucose transporter (406; 436). In the absence of adequate TCR and/or cytokine stimulation during both the resting and activated states, Glut1 will be internalized, leading to downregulation of surface expression, reduced transport of glucose across the plasma membrane, and decreased viability of the cell (474). CD28-mediated Akt signaling is especially important for glucose uptake as it is necessary for expression and trafficking of Glut1 to the cell surface (409; 475). Comparable to T cells, B cells also increase Glut1 expression following BCR engagement (408). PI3K similarly plays a critical role in B cell proliferation and immunoglobulin synthesis through regulation of glycolysis and Glut1 (476). Although Glut1 expression is critical for lymphocyte activation, a balance must exist; otherwise, overexpression of Glut1 can manifest into hyperactive lymphocytes and pathologies (475).

mTOR is another critical regulator of metabolism in immune cells (477). The ability of mTOR to sense nutrient availability (478; 479) leads to the induction of mRNA translation and

protein synthesis (480). Without proper mTOR signaling, T cell proliferation will be blocked (481) and anabolic storage processes will be decreased (482; 483). Inhibition of the PI3K-Akt-mTOR pathway can also lead to T cell anergy (484; 485), whereas mTOR-deficient T cells do not differentiate into effector T cells, but instead regulatory T cells (486). Additionally, mTOR has been linked to chemokine-dependent signaling, resulting in T cell migration (487-489) and cancer metastasis (490; 491). These data highlight the importance of proper metabolic signaling in initiating an effective adaptive immune response and reveal possible targets for therapeutic intervention.

Cytokine binding and cytokine receptor expression have also been connected with metabolism. Although immune cells primarily utilize glycolysis, some oxidative phosphorylation still occurs and is necessary for functionality. If mitochondrial respiration is blocked at different complexes of the electron transport chain, both TNF binding to its receptor on cells (492) and IL-2R expression on lymphocytes are reduced (379). Interestingly, TNFα-deficient mice are actually protected from obesity-induced insulin resistance, highlighting the importance of proinflammatory cytokines in metabolic signaling (493), (494). Other innate immune cytokines, such as IL-1, IL-6, IL-3 and IL-7, also contribute to metabolism. IL-1 can prevent fatty acid synthesis (495), whereas IL-6 can both increase the levels of lipid and glucose metabolism (496). IL-3, which is known to support the growth of myeloid and lymphoid cells, is important for sustaining Glut1 on the surface of lymphocytes (444) and has been directly shown to shift metabolism from oxidative phosphorylation to glycolysis (497). In order for activated Akt to sustain glucose uptake in both resting and activated T cells, IL-7 must be present (442). Overall, each cytokine binds a specific receptor and coordinates T cell function with metabolic needs.

Similar to macrophages, adipocytes can also release cytokines/chemokines (i.e. IL-1, IL-6, IFNγ, TNFα, MCP1) to bridge the gap between immunity and metabolism (469; 498; 499). Adipokines can recruit monocytes and lymphocytes into the adipose and promote proinflammatory and anti-inflammatory functions. Overnutrition can induce adipocyte hypertrophy, creating a hypoxic core and MCP-1 production, which facilitates macrophage entry into the adipose (500). Lymphocytes associated with adipose tissue are oftentimes modulators of the infiltrating macrophages (501). For example, Tregs are present in greater abundance in the adipose of lean mice, correlating with an anti-inflammatory macrophage phenotype (502). Furthermore, proinflammatory effector T cells have been detected in the fat of obese mice, leading to the recruitment of even more proinflammatory macrophages (503) and contributing to insulin resistance (504; 505).

Hormone secretion, from adipose as well as other tissues, is also important in regulating lymphocyte function. Leptin, a hormone released from the adipose, along with insulin, which is secreted from the pancreatic beta cells, both play critical roles in connecting metabolism to the immune system. Leptin, which regulates food intake by inhibiting appetite, is low in times of starvation, resulting in decreased metabolism to maintain vital organs. Consequently, low leptin levels lead to immunosuppression (469). In a well-nourished environment, leptin can modulate both the innate and adaptive arms of the immune system to promote greater cytokine production (506), decreased apoptosis (507) and skew T cells towards the T_H1 lineage (508; 509). The effects of leptin on T_H1 responses have been especially documented in the context of autoimmunity. Leptin has been shown to accelerate type 1 diabetes onset in NOD mice via enhancement of IFNγ-producing T cells (510). Furthermore, higher leptin levels have been detected in female animals that are susceptible to EAE induction versus resistant males,

positively correlating with an increase in T_H1 responses (511). Heightened immune responses following leptin signaling may be damaging in autoimmunity; however, in obesity, failure of proper immunity, resulting in increased infections, has been linked to greater leptin insensitivity in severely overweight individuals (512). In general, the immune system cannot function properly in times of over- or under-nutrition (513), revealing the many intricacies between metabolism and immunity. Similarly, insulin, which promotes cellular metabolism by stimulating the uptake of glucose and storage as glycogen, can play a role in modulating the T cell response. Beyond acting as a lymphocyte-specific antigen in type 1 diabetes, insulin helps shape T cell growth and function. Upon activation, insulin receptor is expressed on T cells. Insulin signaling then facilitates glucose uptake, amino acid transport, lipid metabolism, and protein synthesis (514). Stimulating CD4+ and CD8+ T cells in the presence of insulin can induce more T_H2-type cells and cytokines (515), leading to a more anti-inflammatory environment. These data strongly suggest why lack of insulin signaling in both type 1 and type 2 diabetes can lead to both enhanced T_H1 cells and uncontrolled inflammation (516).

3.1.5 Modulators of metabolism and potential immunometabolic therapeutic implications

Mitochondrial activity has been implicated as a cause of aging, and metabolic dysfunction and ROS production have been linked to neurodegeneration, cancer, and autoimmunity (22; 384-386). An accumulation of ROS and redox-damaged byproducts eventually leads to cell dysfunction and death. (517). Indeed, mutations of the electron transport chain can diminish ROS production and thus elongate life (518; 519). Although immune cells contain higher levels of antioxidants than other cells (520) and rely on both glycolysis and respiration, aging immune cells show accrued impairment, causing reduced lymphocyte proliferation. Functional decline of

immune cells, or senescence, often correlates with age, as free radical production overwhelms antioxidant defenses and the risk of infections/tumors is enhanced (521-523). During a normal mammalian lifetime, metabolic pathways are kept in check via a number of endogenous mediators such as hypoxia-inducible factor-1 and uncoupling proteins, which prevent oxidative phosphorylation by partially dissipating the mitochondrial proton gradient. Pertaining to aerobic respiration, the existence of antioxidants particularly protects against oxidative stress and damage. Additionally, endogenous mechanisms do exist to restrict ROS production so as not to damage neighboring tissues. For example, Kupffer cells, macrophages residing within the liver, do not undergo respiratory bursts, thus protecting the surrounding parenchyma from any ROS-mediated destruction (524; 525). Conversely, peritoneal macrophages, which are more involved in clearance of infection, can experience an oxidative burst, with less threat of damage to surrounding tissue (526). Despite these many mechanisms, improper metabolism of immune cells can result in disease. The strict dependence of immune cells on glucose for survival and activation, however, may make them good targets for metabolic therapeutics (435). Such therapeutics could potentially better control autoimmunity, transplantation rejection, neurodegeneration and cancer.

3.1.5.1 Hypoxia-inducible factor 1

Hypoxia-inducible factor-1 (HIF-1) is especially important for modulating metabolism under low oxygen conditions. HIF-1 inhibits the progression of pyruvate into the TCA cycle by redirecting it to lactate production, via activation of pyruvate dehydrogenase kinase (527-529). Furthermore, HIF-1 can induce glycolytic enzymes while reducing mitochondrial oxygen consumption (529). Such a switch exists to preserve the viability of cells in times of hypoxia. Interestingly, a similar

change occurs in activated lymphocytes, as discussed above. The metabolic similarity between hypoxic cells and lymphocytes begs the question of whether or not HIF-1 plays a role in modulating T cell activation. Hypoxic areas within the body create a need for immune cells to survive and function properly in all environments, hence the importance of HIF-1 activation (414). Under hypoxic conditions and anaerobic glycolysis, specifically during wound healing, T cells will shift from T_H1 to T_H2 type responses, directing less inflammatory function in the absence of oxygen (530). In mice prone to type 2 diabetes, decreased levels of HIF-1 indeed lead to impaired wound healing (531).

Besides hypoxic conditions, HIF-1 is able to induce expression of genes that improve immune cell viability during aerobic glycolysis (449). The expression of HIF-1 is initially provoked by insulin, IGF1 (insulin-like growth factor 1), and angiotensin, all of which play roles in growth and survival (532; 533). HIF-1 is increased in activated T cells and promotes expression of Glut1, aiding in T cell survival (414; 534). In addition to its importance in maintaining T cell viability, HIF-1 also helps regulate T cell subset differentiation. T_H17 cell differentiation requires enhanced glycolysis and expression of the transcription factor RORγT, both of which are increased via HIF-1 activation (535; 536). Furthermore, HIF-1 is known to directly repress Foxp3, the transcription factor critical for Treg induction (535). Tregs, unlike other T cell subsets, are primarily powered through lipid oxidation (459). Likewise, lipid metabolism, which would mainly utilize oxidative phosphorylation, can inhibit glycolytic-dependent T_H17 differentiation (537).

HIF-1 also plays important roles in controlling innate cell functions (527). ATP, glycolytic enzymes and Glut1 expression are all regulated by HIF-1 in macrophages and neutrophils. Under hypoxic conditions, APC phagocytosis and antigen presentation as well as

granulocyte responses are weakened (538; 539). Upon HIF-1-deficiency, innate cell motility, invasiveness, pathogen killing and T cell-stimulating abilities decrease further (424; 540). Without effective HIF-1 expression, APCs and granulocytes suffer dysfunctional host defenses. On the contrary, chronic inflammation and HIF-1 may together instigate tissue fibrosis, autoimmunity and tumor progression by affecting both innate and adaptive immune cells.

In chronic kidney disease and obesity, HIF-1 can switch from its proangiogenic function to promote fibrosis (541; 542). Relevant to HIF-1's ability to modulate T_H17 differentiation, mice deficient for HIF-1 are resistant to inducible experimental autoimmune encephalomyelitis (EAE) (535; 536), a rodent model of multiple sclerosis in which disease is largely mediated by T_H17 cells. Furthermore, HIF-1 has been shown to play major negative roles in prostate cancer tumorigenesis (543), breast cancer prognosis (544), and many other cancer outcomes (545), through induction of genes responsible for cell proliferation, angiogenesis, survival, migration, and glucose metabolism (546). Immune cells play crucial roles in mediating appropriate wound healing, tolerating self-antigens, and cytotoxic killing of tumor cells. Therefore, blocking HIF-1 may allow for appropriate immunity and alleviation of disease. The list of HIF-1 inhibitors is expanding (545). For example, digoxin, which inhibits HIF-1 gene and protein expression, can block tumor growth (547) as well as RORγt-dependent T_H17 differentiation (548), yet does not affect other T cell lineages. It is tempting then to speculate that while blockade of HIF-1 may stunt proliferation and development of T_H17 cells, cytotoxic CD8 T cells may still be active to allow for killing of tumors. Conversely, since HIF-1 is known to repress Treg differentiation, inhibition of this molecule may augment suppressive T cells. In the context of autoimmunity, this side effect may be beneficial in protecting against self-antigen recognition. However, in the case

of cancer, combinatorial therapies of digoxin along with chemotherapy and radiation may be necessary for complete regression.

3.1.5.2 Uncoupling proteins

For oxidative phosphorylation to occur properly, collaboration between electron transfer and proton pumping is a necessity. A disturbance in the 'coupling' of electrons to protons would lead to increased futile proton current, decreased ATP production, and diminished ROS levels. In the context of immune cell mitochondrial dysfunction, such as in Alzheimer's and diabetes, an interruption of oxidative phosphorylation may be beneficial for reduction of ROS byproducts. Endogenously, certain proteins exist to manifest this disruption. Uncoupling proteins (UCP) are known proton uniporters that, in the context of a proper activator, can uncouple mitochondrial respiration in a controlled way (549; 550). Such processes are used for thermogenesis from brown adipose tissue (551) and for reducing the production of free radicals from mitochondria (552). UCP2 has been suggested to decrease pyruvate entry into the TCA cycle (553; 554), overall limiting ROS production and age-related damage (555-558). Overexpression of UCP3 in a high-fat diet fed mouse was able to rescue insulin signaling (493) and knockout of UCP2 drives persistent NF-κB activation as well as heightened ROS production in immune cells, resulting in resistance to certain infections (559; 560). These data reveal UCP2 as a plausible immunometabolic therapeutic target. T cells produce high levels of mitochondrial UCP2 following activation; this has been attributed to the need for rapid proliferation via glycolysis as well as the necessity of low level ROS production for adequate gene expression and signaling activity (561). In an oxidative stress environment, where interplay between innate immune cells and T cells generates high ROS, uncoupling the electron transport chain may be useful in

resetting homeostasis. Moreover, UCP2 decreases glucose-stimulated insulin release (562), highlighting its potential for preventing the release of self-antigen and thus controlling autoreactive T cell responses. To date, uncouplers have not been utilized in the treatment of autoreactive T cells. However, chemical uncouplers can reduce oxidative stress. Rottlerin, a mitochondrial uncoupler, can reduce apoptosis of alveolar macrophages in a model of systemic autoimmune disease (563). A study utilizing a mitochondrial fission inhibitor, which led to greater uncoupling, normalized oxidative stress levels in hyperglycemia (564). Another drug, 2,4-dinitrophenol, leads to uncoupling through dissipation of the proton gradient, resulting in decreased hepatic insulin resistance in a non-alcoholic fatty liver disease model (565). Furthermore, 2,4-dinitrophenol has been shown to enhance the adhesion phenotype (increased collagen and VEGF) for post-peritoneal surgical wound healing (566), a process in which macrophages play an important role (567). Uncouplers targeting specific cells, therefore, may be a potential therapeutic for immune diseases where oxidative stress is high, whereas uncoupling inhibitors may be utilized for some cancers, especially those which are resistant to chemotherapy (568; 569).

3.1.5.3 Nutrient limitation

The immune system is highly dependent on the glucose levels available. Everyday physiological nutrient limitation throughout the periphery protects from the over accumulation of naïve T cells, allowing for the turnover of older and development of new cells (436). Likewise, the adaptive immune response also relies on the availability of sufficient amino acids. Innate immune cells can control the supply of amino acids and thus regulate T cell responsiveness. Upon CD40 ligation or LPS stimulation, APCs can increase their cysteine production and share this with

interacting T cells, which cannot make their own (570; 571). Cysteine is critical for T cell survival due to its necessity in glutathione production (572; 573). Similarly, indoleamine 2,3-dioxygenase (IDO) expression by macrophages can reduce levels of extracellular tryptophan, which is also needed, but not directly produced, by T cells. A lack of tryptophan will eventually cause T cell anergy or apoptosis (574; 575), again suppressing an immune response.

In a similar manner, environmental nutrient limitation may also affect an immune response. Caloric restriction causes greater lipid metabolism, lessening the dependence on glucose and thus decreasing an immune response (576). Lipid metabolism is the chief mechanism of energy production in immune-privileged sites, such as the eye, brain, and placenta. Fatty acid utilization leads to lower costimulatory molecule expression, resistance to apoptosis, and less damage by free radicals (576-578), correlating with blunted immune responses. In contrast, immune-sensitive areas that depend on glucose metabolism are more susceptible to infection and death following an effective immune response (443; 445; 579). Notably, saturated and unsaturated fatty acids also differ in their abilities to stimulate an immune response. Saturated fatty acids, which are considered more detrimental to health, induce greater activation of TLR2 and TLR4 on myeloid cells, whereas unsaturated fatty acids can inhibit TLR signaling and NF-κB activation (580-582). In particular, polyunsaturated acids can alter the T cell membrane, negatively impacting signaling and activation of lymphocytes (583; 584). Moreover, less caloric intake also correlates with better DNA repair, reduced antioxidant decline, diminished cancer rates, and an increase in mouse lifespan (585-588). In autoimmunity and metabolic syndrome, such constraints may be highly effective in quelling inflammation (589; 590). Specifically, obesity has been associated with dysfunctional phagocytosis and respiratory burst in macrophages (591; 592). Similar to leptin insensitivity in obesity, continuous ingestion

of saturated fatty acids eventually will lead to a reduction in innate and adaptive immune responses, making an individual more susceptible to cancer and infections (593) and reducing wound healing capacity (594; 595). Caloric restriction of obese mice, which were at-risk for breast cancer, significantly reduced tumor growth and mimicked mTOR inhibition for regulating cell proliferation (596). Nutrient limitation via endogenous and exogenous factors may therefore ameloriate a number of immune diseases with metabolic components.

3.1.5.4 Anti-glycolytics

Because of the high dependence of immune cells on glucose metabolism, anti-glycolytics have been implemented to limit immunity and treat disease. One of the most studied drugs is rapamycin. Rapamycin is able to inhibit glucose metabolism via blockade of mTOR, downstream of PI3K-Akt. Such inhibition leads to decreased T and B cell activation and function as well as decreased cellular proliferation. Rapamycin has been studied extensively for treatment of advanced cancer (597; 598) and in transplant patients to mitigate rejection and graft-versus-host disease (599-601). At the immune cell level, rapamycin can affect T cell differentiation and memory. Rapamycin treatment enhanced the quality and quantity of CD8 T cell memory responses by switching metabolism from glycolysis to oxidative phosphorylation (460). Rapamycin can also mimic dietary restriction, increasing life span (602) as shown additionally in memory T cells (433; 460). Thus, caloric reduction and fat metabolism discussed above may be used as alternatives to rapamycin and may also improve T cell memory (435; 603). In CD4 T cells, rapamycin can promote CD4 Treg development (604-606) as well as modulate chemokine receptors for mobilizing effector cells out of the periphery and back to the lymphoid organs (607). The immunosuppressive effects of rapamycin, however, have been associated with higher

infection rates (608; 609) as well as hyperglycemia, due to inhibition of the PI3K-Akt-mTOR signaling pathway required for Glut1 expression and translocation to the plasma membrane (610; 611), making it more suitable as a metabolic treatment for chronic inflammation and organ transplantation.

3-bromopyruvate is another anti-glycolytic drug primarily used for the treatment of cancer. During glycolysis, a series of enzymes are necessary for the breakdown of glucose into pyruvate. 3-bromopyruvate is able to inhibit the activity of the first enzyme in the glycolytic pathway, hexokinase (612). Hexokinase is often overexpressed in tumors and contributes to the high glycolytic activity seen in cancerous cells (398; 399). Treatment of tumor cells with 3-bromopyruvate drains intracellular ATP levels, resulting in cell death (613). Although systemic treatment may be detrimental to actively proliferating and cytokine-secreting T cells, intra-tumoral treatment may allow for the induction of anti-tumor CD8 memory T cells, since a reversal of metabolism from glycolysis to oxidative phosphorylation is necessary for the maintenance of adaptive memory (434; 463). Additionally, 3-bromopyruvate is quite specific for cancer cells, with little to no toxicity of healthy tissue (614; 615). Therefore, eradication of the cancerous cell growth with 3-bromopyruvate may diminish the suppressive microenvironment surrounding the tumor, allowing for greater infiltration of cytotoxic killer cells and subsequent tumor immunity – an area of research that still requires investigation.

3.1.5.5 Anti-mitochondrial drugs

Blocking different mitochondrial complexes along the electron transport chain can manifest in phagocytic defects (616) and NF-κB inactivation (617). These types of treatments are standard in lab settings; however, drugs that can be used in the clinic need to be better characterized.

Metformin, for example, is an anti-oxidative phosphorylation drug and is predominantly used as an anti-diabetic drug. It enhances glucose disposal in muscle and reduces hepatic gluconeogenesis. Unlike rapamycin, which directly blocks mTOR, metformin can activate AMP-activated protein kinase (AMPK), which in turn blocks mTOR function, inhibiting cell proliferation (618; 619). AMPK activation induces glucose uptake from the blood, increasing glycolysis and lessening the dependence of cells on oxygen (620). Metformin can specifically impede complex I of the electron transport chain and inhibit oxygen consumption in cancer cells (621; 622). Particularly, metformin usage for cancer therapy has been widely studied in those with type 2 diabetes (623; 624), a known risk factor for tumor formation (625). Like the anti-glycolytics mentioned previously, reductions in cancer cell growth may permit the cytotoxic killing of tumor cells by T cells, albeit when treatment is administered directly to the tumor. The impact of metformin has also been characterized in a mouse model of cancer vaccination. Upon metformin treatment of ova-specific T cells, a significant enhancement of memory CD8 T cells was detected along with better tumor regression. These results demonstrate the ability of metformin to modulate the immune system outside of its anti-proliferative effects. Additionally, in the context of proinflammatory cytokines, metformin is able to block the activation of NF-κB, resulting in diminished cytokine-induced endothelial cell adhesion molecule expression (626; 627). Such an effect may lead to decreases in chronic macrophage infiltration into the adipocytes of type 2 diabetics as well as lower the damaging consequences of autoimmunity. Of course, better understanding of metformin's selective effects on the immune system is necessary for these types of indications.

As another approach to metabolic control for immune regulation, antioxidants have been utilized in a plethora of disease models, including autoimmunity, infections, neurodegeneration,

and cancer (154; 628-631). Although antioxidants do not specifically block oxidative phosphorylation, they are important for decreasing ROS produced from the electron transport chain during mitochondrial respiration, thus reducing damaging side effects. During aerobic respiration, the glutathione transported from the cytosol into the mitochondrial membrane is the only antioxidant available for metabolizing H_2O_2 (632). Therefore, the augmentation of antioxidants present in immune cells may alleviate certain cellular dysfunctions. Specifically, antioxidants are able to show improvement of immune-mediated disorders, such as lymphocyte and macrophage function in the face of aging, septic shock, asthma, and type 1 diabetes (154; 633-636). The mechanisms by which antioxidants improve immune responses vary greatly. An antioxidant present in green tea called epigallocatechin-3-gallate can reduce T cell signaling via downregulation of cytokine receptors (637) and ameliorate EAE through an enhancement of regulatory T cells (638). Other studies demonstrated the antioxidant ability of resveratrol to decrease collagen-induced arthritis by suppressing T_H17 responses (639) and to enhance B cell lymphoma recognition by CD4+ T cells through upregulation of HLA class II molecules (640). Consistently, other antioxidants have shown promise in restricting NF-κB activation (64; 641; 642), leading to many anti-inflammatory effects. On the metabolic side, a recent study utilizing α-tocopherol, the antioxidant component of Vitamin E, demonstrated a reduction in glycolysis in lymphoma cells through blockade of lactate dehydrogenase activity (643).

Our group focuses on the usage of manganese metalloporphyrins as catalytic antioxidants for the scavenging of ROS (hydrogen peroxide, superoxide, peryoxynitrite) and the mimicking of superoxide dismutase (154; 268). Unlike other antioxidants, the metalloporphyrins are catalytic and can repetitively eliminate ROS, resulting in many immunological effects. These antioxidants have shown promise in reducing type 1 diabetes incidence through autoreactive T_H1 cell

modulation (36; 154; 644) and in protecting islets during isolation for transplantation (79; 297). Furthermore, NF-κB activation (64) and CD8 T cell effector function (278) are reduced upon metalloporphyrin treatment. Inhibition of NF-κB after metalloporphyrin administration has also shown promise in decreasing acute central nervous system injury, effectively enhancing neurologic function following ischemic stroke (645). Additionally, metalloporphyrins can protect lungs from radiation-induced injury (646-648) and kidneys from ischemia/reperfusion injury (649). Interestingly, metalloporphyrins display oxidoreductase abilities, where they can act as scavengers in the cytoplasm, as in the context of reduced TACE oxidation (644), yet work as oxidizers in the nucleus, inhibiting the reduction of the p50 subunit of NF-κB and effectively blocking its DNA binding (64). In the context of cancer, metalloporphyrins are also able to block HIF-1 activation, decrease hypoxia, reduce tumor-protective cytokine release and ultimately suppress tumor growth (650). HIF-1, as mentioned above, is critical for facilitating glycolysis in times of low oxygen; moreover, tumor cells rely heavily on glycolysis to survive, making them an obvious target of metalloporphyrin-induced regulation. The wide scope of metalloporphyrin effectiveness allows for their usage in a range of immunologic diseases, all centered around restoring redox balance; yet the effect of metalloporphyrins on fundamental immune cell metabolism has yet to be described. In conjunction with the cancer studies, we have observed promotion of hyporesponsive T cells after antioxidant treatment (48; 644), which further instigated curiosity regarding metabolism. Preliminary studies suggest decreases in both glycolysis and oxidative phosphorylation following metalloporphyrin administration. An overall metabolic reduction may then decrease T cell differentiation and return cells to stasis or quiescence, all while retaining viability, since metalloporphyrins are not toxic (79; 268; 278). If T cells are in fact displaying lowered metabolism, the potential for treating chronic inflammatory

conditions, such as autoimmunity, is widespread. With frontline therapeutics, such as anti-metabolites, rapamycin, and antibodies against costimulatory molecules, either failing in the clinic or leading to unwanted side effects, agents that modulate immune function, are reversible, and show no toxicity are highly desirable. Metalloporphyrins meet all of these demands and therefore, may be beneficial for reducing inflammatory disorders/potentiating cancer regression and should elicit greater attention in the search for alternative metabolic therapies.

3.1.6 Summary

The immune system plays a vital role in maintaining a fine balance in the battle against infections and cancer, but requires rigorous control in order to walk the fine line of regulator and menace leading to self-reactivity and autoimmunity. Both oxidative phosphorylation and glycolysis are critical for fulfilling the metabolic needs of immune cells. In innate immune cells, glycolysis is the predominant form of metabolism, whereas adaptive immune cells fluctuate between oxidative phosphorylation and glycolysis, depending on their activation status. Nonetheless, the heavy reliance of immune cells on glucose utilization makes them good targets for immunometabolic therapies. A number of endogenous molecules can be pursued, including HIF-1 and UCP2. Alternatively, caloric restriction, anti-glycolytics, and antioxidants all exhibit potential in resetting homeostasis in chronic inflammation while possibly enhancing immunity in cancer models. Overall, metabolic regulation should be an active line of research for the control of immune-mediated disorders.

3.2 ABSTRACT

The immune system is critical for protecting the body against infections and cancer, but needs scrupulous regulation to limit self-reactivity and autoimmunity. Our group has utilized a metalloporphyrin catalytic antioxidant (CA) as a potential immunoregulatory therapy in the context of type 1 diabetes. We have previously reported CA-mediated effects, such as a decrease in proinflammatory cytokine production from APCs and T cells as well as reduced diabetes onset in NOD mice and upon adoptive transfer of diabetogenic T cells. However, it is unclear whether or not CA can promote immunoregulation outside of T_H1 cell surface molecules and cytokines. Upon CA treatment, T_H1 cells do not skew towards a T_H2 lineage, based on cytokine profiles. Furthermore, CD25+Foxp3+ cells are not enhanced following CA treatment during *in vitro* stimulation of BDC-2.5.TCR.Tg CD4+ T cells. In contrast, adoptive transfer of a mixture of control:CA-treated splenocytes does impart extended protection against diabetes onset in comparison to CA-treated splenocytes alone, highlighting the possibility of some type of regulation following *in vivo* redox modulation, possibly beyond classical Treg suppresion. The reliance of immune cells on specific metabolic pathways during different activation states makes them good targets for immunometabolic therapies. Although antioxidants do not specifically block oxidative phosphorylation, they are known to decrease ROS produced from the electron transport chain during mitochondrial respiration, thus reducing damaging side effects. Therefore, the ability of redox modulation to affect the bioenergetics of diabetogenic splenocytes was tested. In the context of diabetogenic splenocytes, CA treatment mildly decreases overall oxidative phosphorylation. Upon further subdivision of oxidation from two different substrates, CA administration demonstrated enhancement of glucose oxidation and reduction of fatty acid oxidation; however, these alterations were not due to a decrease in mitochondrial complex and

biogenesis molecules. CA also displays anti-Warburg effect characteristics, in which it decreases aerobic glycolysis needed for immune cell proliferation, demonstrated by reduced lactate production, deactivation of mTOR, and lowered Glut1 expression. Despite this outcome, CA does not cause cell death, as the effects of redox modulation are reversible. The reduction in aerobic glycolysis-mediated metabolism seen after administration correlates with decreased immune cell reactivity and therefore, CA treatment promotes cell stasis or quiescence and can impede diabetogenic autoimmune responses.

3.3 INTRODUCTION

The control of self-reactivity is critical for prevention and potential reversal of autoimmunity. In particular, regulating immune cells in type 1 diabetes can lead to reduced beta cell damage. Both the innate and adaptive immune cells should be targeted for complete protection. T cells, specifically, can be controlled in many ways. Different T cell subsets have specific functions for mediating protection against disease. T_H1 cells are known to promote immunity against intracellular pathogens, whereas T_H2 cells drive allergic and anti-parasitic responses. Notably, the cytokines produced from each subset can block the induction of a different subset. For example, IL-4 produced by T_H2 cells can inhibit Tbet, the transcription factor necessary for the T_H1 cytokine IFN-γ. This intrinsic regulatory mechanism is important for endogenous control of immune responses; however, it can also be exploited for protection against chronic inflammation. T_H1 cells are the primary subset responsible for type 1 diabetes pathology. Although T_H1 cells are necessary for progression of disease, the T_H2 compartment has been credited with a protective role due to its ability to cause a cytokine shift away from the T_H1

cytokines (172; 651; 652). Strategies to instigate this skewing are sought after and highly promising for regulating autoreactive T cell responses.

In addition to skewing, Treg-mediated suppression is another mechanism for potentially inhibiting autoreactive T cell responses. Regulatory T cells are formed endogenously within the thymus (natural Tregs) to prevent self-reactive T cell responses (653). Moreover, adaptive Tregs can be induced in the periphery during inflammatory processes (204). Despite different origins, each class of Tregs has the common ability to downregulate effector function and inflammation. The classic phenotypic marker for Tregs is Foxp3, the transcription factor necessary for their development. Beyond expression of Foxp3, the diversity of Tregs is substantial. Certain subsets can secrete IL-10 and TGF-β (654; 655), whereas others can mediate suppression via a contact-dependent mechanism, such as cytolysis (656) and CTLA-4 ligation (657). Deficiencies in Treg numbers and function have been reported in a number of autoimmune disorders, including type 1 diabetes (210). A lack of endogenous regulation in collaboration, with genetic HLA susceptibility and high oxidative stress, is the perfect storm for a break in tolerance to self-antigen and autoimmune destruction of beta cells. Treg promotion in the face of type 1 diabetes may be beneficial for preventing frank disease and/or the regeneration of beta cells.

Another mechanism of T cell regulation is the production of indoleamine 2,3-dioxygenase (IDO) from APCs. IDO is the enzyme responsible for tryptophan catabolism, leading to a depletion of this essential amino acid. T cells are reliant on tryptophan for proliferation, and thus, IDO has the ability to hinder their expansion (658). Physiologically, IDO is produced to help contract an immune response, limiting inflammation and preventing bystander damage. IFN-γ is essential for IDO induction (659; 660), illustrating the interplay

between APCs and T cells in immune cell regulation and the ability of innate cells to control adaptive populations.

One of the most understudied mechanisms of immune cell control is metabolic modification. This type of regulation is relatively unexploited but promising in the context of autoreactivity. Both innate and adaptive immune cells depend on specific metabolic pathways for survival. Naive and central memory T cells are highly dependent on oxidative phosphorylation, and although mitochondrial respiration does increase, glycolysis is the primary metabolic pathway utilized during both T cell and APC activation. Resting T cells undergo oxidative phosphorylation as they traverse the body, building up reserves of ATP in preparation for an upcoming response (410). Cells that have successfully made it through thymic selection wait for stimulation by activated APCs. In NOD mice, which spontaneously develop type 1 diabetes, self-reactive T cells are not efficiently deleted in the thymus, allowing for the escape of autoreactive cells into the periphery (661; 662). Thus, self-reactive T cells that target the beta cells might not be using oxidative phosphorylation, but instead may switch over to glycolysis, since they have a strong enough signal to drive activation without deletion and survive selection. During the initiation of an immune response, rapid macromolecule synthesis and cell growth are essential for productive effector function. Instead of relying on the slow process of oxidative phosphorylation, immune cells use the much faster (100X) glycolytic pathway to meet their energy demands (421). Notably, the inhibition of glycolysis through blockade of HIF-1, for example, reduces T_H17 differentiation (535; 663). Moreover, blocking mTOR via rapamycin treatment also elicits glycolytic reduction and can augment Treg development (604; 606). On the innate cell side, DCs upregulate glycolysis upon TLR stimulation (427), and proinflammatory macrophages require glycolysis for cytokine production (428; 429). The metabolic demands of

immune cells highlight the importance of both glycolysis and oxidative phosphorylation in activation and survival.

An imbalance of immunological and/or metabolic function can often lead to diseases, such as autoimmunity. In previous studies, we demonstrated the ability of a metalloporphyrin catalytic antioxidant (CA) to prevent T cell activation through the inhibition of LAG-3 cleavage, a negative regulator of T cell function (644). Furthermore, CA can block the activation of NF-κB, decreasing proinflammatory cytokine production (64). Both of these effects contribute to the prevention of spontaneous diabetes onset in the NOD mouse and disease progression upon adoptive transfer diabetogenic T cells (36; 644). Other groups have demonstrated the ability of antioxidants to suppress T_H17-mediated arthritis (639), enhance Treg for the resolution of EAE (638), and reduce cancer cell glycolysis and growth (643). However, it is unknown whether CA treatment causes: A) a skewing of diabetogenic T_H1 cells to a different subset; B) an increase in immunoregulatory functions; or C) immune cell metabolic quiescence. In this study, each of these possibilities is addressed in the context of redox modulation, ultimately contributing to the prevention of an autoimmune response.

3.4 RESEARCH DESIGN AND METHODS

3.4.1 Materials

NOD.BDC-2.5.TCR.Tg and NOD.*scid* mice were bred and housed under specific pathogen-free conditions in the Animal Facility of Rangos Research Center at Children's Hospital of Pittsburgh of UPMC (Pittsburgh, PA). Female mice at 4-10 wks of age were used in all experiments. All animal experiments were approved by the Institutional Animal Care and Use Committee of the

Children's Hospital of Pittsburgh and were in compliance with the laws of the United States of America. Foxp3-FITC (eBioscience, San Diego, CA), Rat IgG2α isotype-FITC, Fc Block, CD4-PE, and CD25-APC (BD Biosciences, San Diego, CA) were used for flow cytometry. Anti-CD3 and anti-CD28 (BD Biosciences) were utilized for T cell stimulation. Mouse anti-mMitoProfile Total OXPHOS Rodent Antibody Cocktail, rabbit anti-mGlut1 (Abcam, Cambridge, MA), rabbit anti-mphospho-mTOR (Ser2448), rabbit anti-mmTOR (Cell Signaling, Danvers, MA) were used for Western blots. Primary antibodies rat anti-mIFN-γ (BioLegend, San Diego, CA) and mouse anti-mIDO (EMD Millipore, Billerica, MA) and secondary antibodies goat anti-rIgG-Cy3 and goat anti-mIgG-FITC (Sigma, St. Louis, MO) were used for confocal microscropy. Antibody pairs for IFN-γ, IL-10, IL-4 and IL-2 ELISAs were purchased from BD Biosciences; mouse Duoset for IL-17 ELISAs were purchased from R&D Systems (Minneapolis, MN). L-Lactate Assay Kit was purchased from Abcam. MnTE-2 catalytic antioxidant (CA) was a generous gift from James Crapo, MD at National Jewish Health. CA was prepared as previously described (48) and used at 10 mg/kg in all *in vivo* experiments and at 68 μM in all *in vitro* experiments.

3.4.2 In vitro T cell assay

BDC-2.5.TCR.Tg splenocytes were seeded in 96-well round-bottom plates with 0.5 μM of BDC-2.5 mimotope (M) (EKAHRPIWARMDAKK) in supplemented DMEM (48) (Invitrogen Life Technologies). At 48-96h post-stimulation, all cells were counted with a hemocytometer, and supernatants were harvested for ELISA.

3.4.3 Cytokine measurements by ELISA

IFN-γ, IL-4, IL-10, IL-17, and IL-2 ELISAs were performed according to manufacturer's instructions. All ELISAs were read on a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA), and data was analyzed using SoftMax Pro v5.4.2 (Molecular Devices).

3.4.4 In vitro Treg phenotype assessment

BDC-2.5.TCR.Tg CD4+ T cells were isolated from mouse spleen via MACS CD4+ T cell Isolation Kit (Miltenyi Biotec, Auburn, CA). Cells were plated at $1x10^5$ in supplemented DMEM and stimulated +/- CA with anti-CD28 (1 µg/ml) and a titration of pre-coated anti-CD3 (0.1-1 µg/ml). After 5 days of incubation, cells were collected, surface stained for CD4 and CD25, and intracellular stained for Foxp3 or isotype to determine Treg phenotype by flow cytometry. Supernatants were used for IFN-γ ELISA.

3.4.5 Surface/intracellular staining and flow cytometric analysis

Cells were surface stained as previously described (278). For intracellular Foxp3 staining, FC block (BD Biosciences) was first applied to the cells. Cells were then surface stained followed by fixation/permeabilization using BD Cytofix/Cytoperm (BD Biosciences). Intracellular Foxp3 or isotype staining was then conducted in BD PermWash. Fluorescence was measured on a FACSAria (BD Biosciences). Flow cytometric analysis was done using FlowJo Software v6.4 (Tree Star, Ashland, OR). All samples were gated on CD4+ cells.

3.4.6 In vivo 7 day treatment

BDC-2.5.TCR.Tg mice were treated with CA or HBSS (10 mg/kg) intraperitoneally for 7 days. On day 8, spleens were collected for further analysis, described below.

3.4.7 Adoptive transfer of diabetogenic splenocytes

Following 7 day *in vivo* treatment, splenocytes were isolated from control or CA-treated mice. A total of $1x10^7$ splenocytes was then adoptively transferred intravenously into NOD.*scid* mice; transfers included control alone, CA alone, and control:CA ratios of 1:6, 1:4, and 1:1. Diabetes was monitored starting at 10 days post-transfer. Overt diabetes was measured by a positive glucosuria test followed by two consecutive blood glucose readings of $\geq$ 300 mg/dL.

3.4.8 Confocal microscopy

Following 7 day *in vivo* treatment, splenocytes were isolated and plated in 96-well glass-bottom plates (MatTek Corporation, Ashland, MA) at the same ratios as mentioned above (excluding 1:1). Cells were stimulated with 5000 NOD.*scid* islet cells for 24h. After stimulation, cells were fixed in 4% paraformaldehyde for 15 min at RT, permeabilized in 0.5% Triton-X-100 in PBS for 10', and washed with PBS in between all steps. Plates were stored at 4^oC O/N. On the second day, cells were stained with IDO (1:50) and IFN-γ (1:50) in BD PermWash for 1h at 37^oC. Secondary antibodies were then used alone or with double-stained cells: goat anti-mouse-IgG-FITC (1:64) and goat anti-rat-IgG-Cy3 (1:250). Cells were visualized via an Olympus Fluoview FV1000 confocal microscope (Olympus Imaging, Center Valley, PA). Digital images (n=3-4) were acquired from each well using a 40X objective. Staining was quantified via MetaMorph

(Molecular Devices, Sunnyvale, CA). Results are expressed as number of positively stained cells per nuclei per image.

3.4.9 Splenocyte respiration rates

On day 8 post CA or HBSS treatment, splenocytes were harvested, and $2x10^7$ cells were measured by an Oroboros High Resolution Respirometer (Innsbruck, Austria) in a stirred 2 mL chamber in supplemented DMEM. Oxygen sensor was calibrated at each experiment according to the manufacturer's instructions. Calculations of respiratory rates were performed by software built into the instrument. Basal oxygen consumption was measured followed by 1) oligomycin (1 µM), to inhibit ATP production by blocking complex V (ATPase) of the electron transport chain; 2) at least 8 additions of 1 µM FCCP, to uncouple oxidative phosphorylation; and 3) rotenone (1 µM), to measure non-mitochondrial oxygen consumption via inhibition of complex I in the electron transport chain.

3.4.10 Preparation of cell lysates and western blotting

BDC-2.5.TCR.Tg splenocytes were harvested after 7 days of *in vivo* treatment. Whole cell lysates were prepared as described (337). Protein concentration of all lysates was determined by BCA protein assay (Thermo Fisher, Rockford, IL). Protein lysates were separated on 4-20% SDS–PAGE gels. Western blots were performed as described (64) (with the exception of no boiling, 150 mAmp transfer for 1.5h, and blocking O/N in 5% non-fat dry milk in PBS for those samples probed with MitoOXPHOS antibody) with antibodies to MitoOXPHOS (1:250) (Santa Cruz), Glut1 (1 µg/ml) (Abcam), mTOR (1:1000), phospho-mTOR (1:1000), phospho-S6K

(1:1000), phospho-PHAS (1:1000) (Cell Signaling) and β-actin (1:10,000). All primary antibodies were diluted in 5% BSA in TBST, except for MitoOXPHOS in 1% non-fat dry milk in PBS. Secondary antibodies were from Jackson ImmunoResearch, West Grove, PA. Chemiluminescence was detected using ECL Plus reagent (Amersham Pharmacia Biotech, Buckinghamshire, UK). Blots were analyzed using Fujifilm LAS-4000 imager and Multi Gauge software (Fujifilm Life Science, Tokyo, Japan).

3.4.11 Mitochondrial mRNA quantification

BDC-2.5.TCR.Tg splenocytes were isolated after 7 days of *in vivo* treatment. Cells were collected, pelleted, and used for RNA isolation. RNA was isolated via the RNAeasy Kit (Quiagen, Valencia, CA), followed by cDNA synthesis using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). The qRT-PCR was performed using the following primer pair: PCG-1 (forward) 5'-ACCCACAGGATCAGAACAAACCCT-3', (reverse) 5'-TGGTGTGAGGAGGGTCATCGTTT-3'; TFAM (forward) 5'-AGTCTTGGGAAGAGCAGATGGCT-3', (reverse) 5'-AGACCTAACTGGTTTCTTGGGCCT-3'; Nrf1 (forward) 5'-AACGGAAACGGCCTCATGTGTTTG-3', (reverse) 5'-GAGTACAATCGCTTGCTGTCCCA-3'; Complex I (NDUFB8) (forward) 5'-TTCGGCTTTGTGGCTTTCATGGT-3'; (reverse) 5'-AAAGCCCATCAAGCCTCCTCAGAT-'; Complex II (SDHB) (forward) 5'-AATGCAGACGTACGAGGTGGATCT-3'; (reverse) 5'-TTGCCTCCGTTGATGTTCATGGC-3'; Complex III (UQCRC2) (forward) 5'-ATCGGCTTGTTCGTTAAAGCAGGC-3'; (reverse) 5'-TGCCTTCTACAGTGTACGCCATGT-3'; Complex IV (COX1) (forward) 5'-CTCACAGTGCGGTCCAAC-3'; (reverse) 5'-CCAGCACCTGGTACTTAAG-3'; Complex V

(ATP5A) (forward) 5'-GGGCGTGTGTTAAGCATTGGTGAT-3'; (reverse) 5'-ATTGTCGGGTTCCAAGTTCAGGGA-3'; GAPDH (forward) 5'-GCATCCTGCACCACCAACT-3', (reverse) 5'-CTGGCATGGCCTTCCGTGTT-3'. Quantitative RT-PCR was performed using a Light Cycler 2.0 (Roche, Indianapolis, IN). The reaction mixtures containing SYBR Green were generated following the manufacturer's protocol. The cycling program was: initial denaturation at 95°C for 10 min, 40 cycles of amplification with a denaturation step at 95°C for 5 sec, an annealing temperature of 60°C for 15 sec, and an extension step at 72°C for 20 sec. All samples were normalized to GAPDH, and CA-treated samples were compared to control samples arbitrarily set to 1.

3.4.12 Glucose and palmitate uptake assay

BDC-2.5.TCR.Tg splenocytes were isolated after 7 days of *in vivo* CA treatment. Glucose oxidation was determined as described previously (664). Briefly, 1μC_i of D-[6-^{14}C] glucose and 2.5 μM cold glucose in 0.2% BSA-Hanks buffer was added to splenocytes. Tubes were incubated at 37^{o}C in water bath with rotation for 1 hour. To terminate metabolic reactions, 200 μl 2 N HCl was added and 500 μl Hyamine (PerkinElmer Life Sciences) was added. $^{14}CO_2$ generated was then detected using a beta counter. Palmitate oxidation was measured as described previously (665). Briefly, splenocytes were resuspended in sucrose/Tris/EDTA buffer and incubated for 1 hour in reaction mixture (pH 8.0) containing [1-^{14}C] palmitic acid. Measurements of acid-soluble metabolites and trapped CO_2 were then detected.

3.4.13 ATP determination

ATP determination kit was used following the manufacturer's instructions (Invitrogen Life Technologies). Spleens were collected on day 8 following 7 days of *in vivo* treatment, weighed, homogenized in 1X reaction buffer, and centrifuged. 1 ml boiling water was added to each cell pellet, vortexed, centrifuged at 12,000 rpm for 5 min at 4^{o}C, and supernatants were utilized in the assay. Background was measured for each standard and sample and subtracted accordingly. Luciferase was measured on a Victor^{3} Multilabel Counter 1420 (PerkinElmer, Waltham, MA) and ATP values were normalized to spleen weight.

3.4.14 Lactate assay

L-Lactate Assay Kit was used following the manufacturer's instructions (Abcam). Spleens were collected on day 8 following 7 days of *in vivo* treatment, weighed, sonicated in lactate assay buffer, and diluted 1:5 in buffer.

3.4.15 Statistical analysis

The difference between mean values was assessed by Student's *t* test, with $p<0.05$ considered significant. All experiments were performed at least three times. Data are mean ± SEM. Survival analysis was done using the product-limit (Kaplan-Meier) method with the endpoint defined as disease. Data on animals that did not develop type 1 diabetes were censored. The *p* values were determined by Log-Rank test.

3.5 RESULTS

3.5.1 Redox modulation does not skew T cells to the T_H2 or T_H17 subset

Diabetogenic T cells stimulated in the presence of CA were previously demonstrated to have significantly decreased IFN-γ production, in comparison to control cells (644). Therefore, we wanted to determine whether or not cells were being skewed to a different T helper cell subset following redox modulation. BDC-2.5.TCR.Tg splenocytes were treated with M +/- CA for 72h, and supernatants were analyzed for IL-4, IL-10, IL-17 and IL-2 by ELISA. All cytokines were reduced in the presence of CA, except for IL-2, which is actually increased and necessary for T cell proliferation (**Figure 29**). CA has been previously reported to diminish expansion (644) yet keep cells alive (278; 304), suggesting a delayed usage of IL-2, which may attribute to its elevation after treatment. These data indicate that diabetogenic cells are not driven to a T_H2 or T_H17 subset following redox modulation.

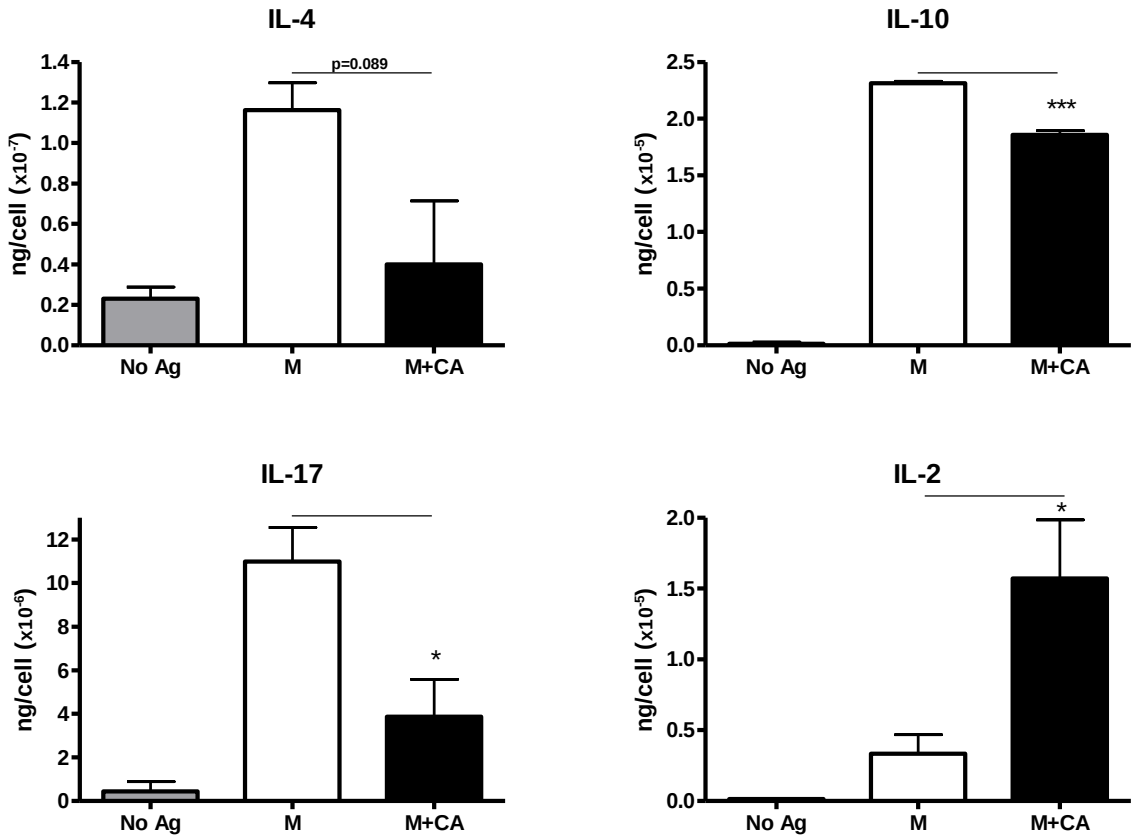


Figure 29: CA administration decreases T_H17 and T_H2 subset cytokines. BDC-2.5.TCR.Tg splenocytes were left untreated or stimulated with M +/- CA for 72h. Cells were counted and supernatants were utilized in IL-4, IL-10, IL-17, and IL-2 ELISAs. Cytokine per cell = ng cytokine/# of total cells after 72h stimulation. n=3 independent experiments performed in triplicate, *$p<0.05$, ***$p<0.0005$.

3.5.2 CA treatment does not enhance a regulatory T cell phenotype in vitro

With no push towards a different T helper cell subset, we next wanted to determine if CA could promote regulatory T cell differentiation *in vitro*. Utilizing BDC-2.5.TCR.Tg isolated CD4+ T cells, CD25+Foxp3+ phenotype was measured following static anti-CD28 and a range of anti-CD3 concentrations +/- CA. After 5 days of stimulation, CA-treated cells did not demonstrate a significant increase in regulatory T cell phenotype through a range of anti-CD3 concentrations

(**Figure 30A**). However, CA was still able to significantly decrease IFN-γ production at anti-CD3 concentrations of 0.1-0.2 µg/ml (**Figure 30B**). Despite the reduction in T_H1 phenotype, stimulating diabetogenic T cells under non-polarizing conditions in the presence of CA did not enhance a regulatory T cell phenotype *in vitro*.

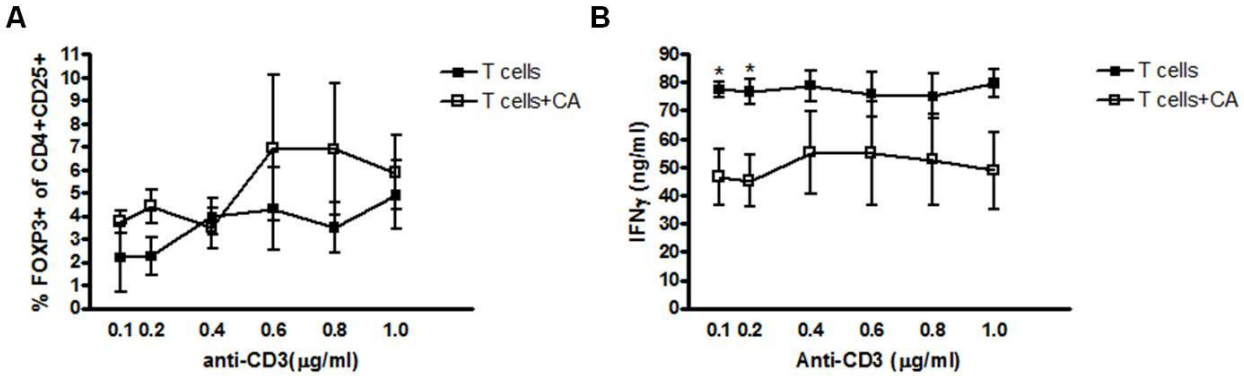


Figure 30: CA treatment does not increase Treg phenotype of CD4+ diabetogenic T cells. BDC-2.5.TCR.Tg CD4+ T cells were isolated from spleens. Cells were stimulated with pre-coated titration of anti-CD3 and soluble anti-CD28 (1.0 µg/ml) +/- CA. (A) After 5 days of incubation, cells were collected and stained for CD4, CD25, and Foxp3. Samples gated on CD4+CD25+ cells by flow cytometry. n=3 independent experiments. (B) Supernatants from cultures were used in an IFN-γ ELISA. n=3 independent experiments, *$p<0.05$.

3.5.3 Redox modulation of donor mice delays diabetes transfer

Although no skewing to a different T helper cell subset and no enhancement of Treg phenotype was demonstrated following CA addition *in vitro*, we conducted an *in vivo* suppression assay with varying ratios of control and CA-treated cells. CA or HBSS (control) was administered to BDC-2.5.TCR.Tg mice for 7 days, and on day 8 cells were transferred alone or in control:CA ratios of 1:1, 1:4, and 1:6 into NOD.*scid* recipients. Control transfers succumbed to diabetes by day 10, consistent with historical data (36; 644), whereas CA transfers exhibited significantly delayed diabetogenic potential ($p<0.0005$) (**Figure 31A**). Strikingly, the majority of the 1:6 adoptive transfer ratios surpassed the diabetes onset point of the CA transfers (>23 days),

illustrating *in vivo* cross-regulation between the two populations (**Figure 31B**). Conversely, ratios of 1:4 and 1:1 did not display reduced diabetogenic potential, with 1:1 actually accelerating disease (**Figure 31C, D**), indicating a need for higher numbers of CA:Control cells to alter diabetogenic potential. The contribution of the CA-treated cells within the 1:6 ratio transfers is therefore enough to prevent the autoimmune attack of diabetogenic BDC-2.5.TCR.Tg splenocytes up to the end of the study, ~40 days post-transfer.

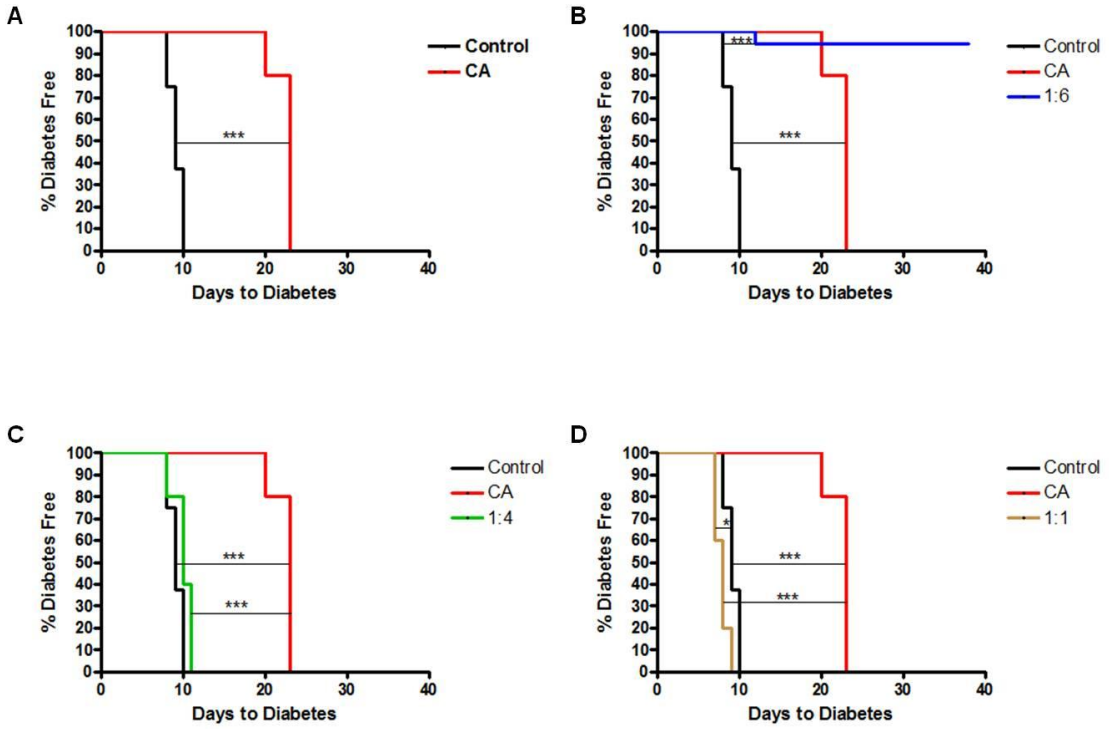


Figure 31: CA treatment of donor mice leads to in vivo regulation of diabetogenic potential upon adoptive transfer. BDC-2.5.TCR.Tg mice were treated for 7 days i.p. with CA or HBSS at 10 mg/kg. On day 8, splenocytes were harvested and adoptively transferred i.v. into NOD.*scid* recipients. Mice were monitored by glucosuria for the onset of type 1 diabetes and considered diabetic after two consecutive blood glucose readings of >300 mg/dL. (A) Control vs. CA, ****p<0.0001*; (B) Control vs. 1:6, ****p<0.0001*; (C) Control vs. 1:4, no significance, CA vs. 1:4, ****p<0.0001*; (D) Control vs. 1:1, **p<0.05*, CA vs. 1:1, ****p<0.0001*. n=3 mice/transfer.

3.5.4 IDO induction correlates with diabetogenic potential of adoptive transfer ratios

To elucidate the mechanism responsible for the delay in diabetes after 1:6 control:CA adoptive transfers, splenocytes were plated *in vitro* in the presence of islets. IDO and IFN-γ induction were visualized via confocal microscropy. CA-treated cells demonstrated dramatically increased levels of IDO in comparison to control cells (p=0.0588) (**Figure 32**). The 1:6 ratio also showed moderately increased IDO production in comparison to control cells (p=0.061); however, the IFN-γ detected in the 1:6 ratio was trending higher (p=0.2007) than the levels in CA-treated cells. This difference in IFN-γ secretion may be important for the longer diabetes delay in the 1:6 transferred mice, as IFN-γ is necessary for IDO induction (659; 660).

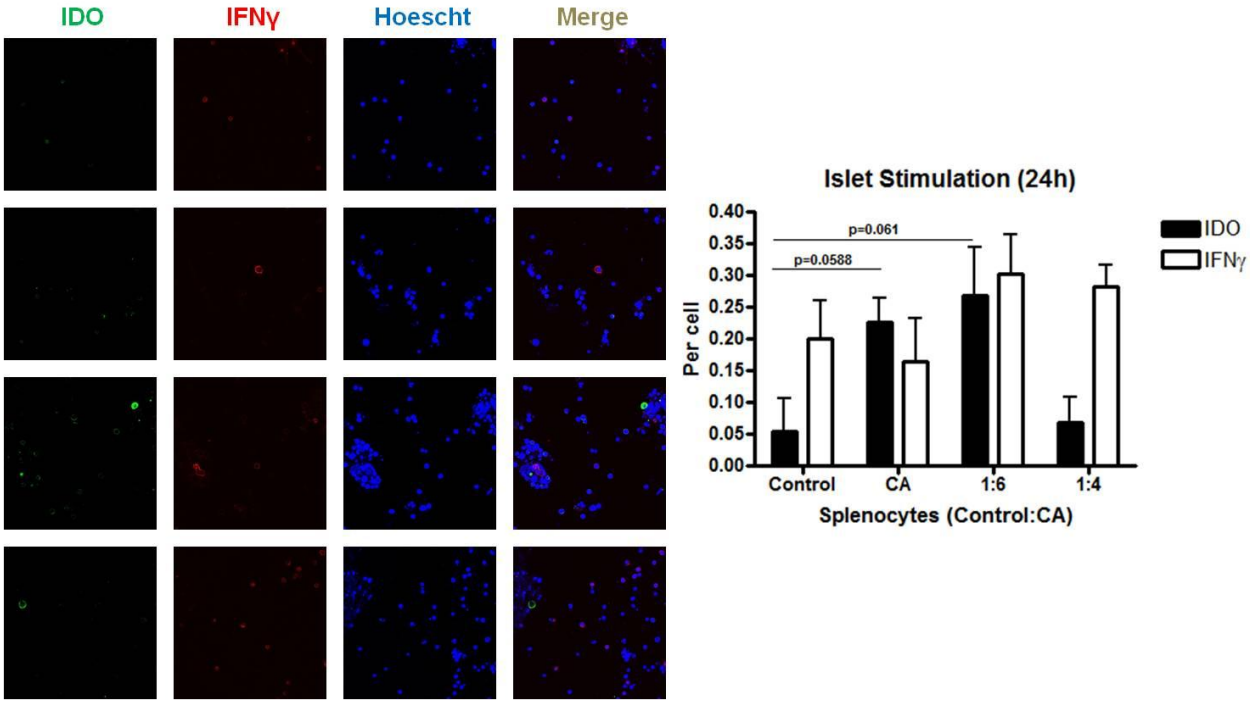


Figure 32: IDO induction correlates with decreased diabetogenic potential. Following 7 day CA or HBSS in vivo treatment, splenocytes were harvested and plated as control alone, CA alone, or control:CA ratios of 1:6 and 1:4. Cells were then stimulated with islets for 24h. Following stimulation, cells were immunostained for IDO and IFNγ. Confocal images were obtained at a 40X objective and quantified. IDO and IFN-γ per cell were calculated by dividing by the nuclei (Hoescht) per image. A total of 3 images/condition was averaged ± SEM.

3.5.5 Redox modulation mildly decreases overall oxygen consumption of diabetogenic splenocytes

In addition to the 1:6 extension in diabetes onset delay, we also wanted to further deduce why CA treatment alone failed to afford durable protection against disease onset past 20 days post-transfer. Based on previous studies, redox modulation can inhibit T_H1 effector function and block immune cell NF-κB activation (48; 64; 644). These effects are important for preventing disease, but may not be the sole mechanisms for reducing immune cell activity. Because CA can scavenge ROS, which are a constant byproduct of cellular respiration, another level of regulation may be contributing to the delayed but eventual beta cell destruction (**Figure 31A**). Therefore, we wanted to determine if CA-treated autoreactive cells also displayed a reduced metabolic

signature, thus regulating their ability to generate enough energy to push toward effector function. To study this, we first calculated respiration by measuring overall oxygen consumption in control versus CA-treated splenocytes (**Figure 33A**). Although uncoupling respiration from ATP synthesis with FCCP titration elicited statistically significant decreases following CA treatment, redox modulation did not significantly reduce respiration at baseline (Basal), during maximal uncoupling (FCCP 8.0 μM), or from non-mitochondrial oxygen consumption sources (Rot), the major indicators of oxidative phosphorylation (**Figure 33B**). Therefore, overall oxidative phosphorylation was only mildly affected upon CA treatment.

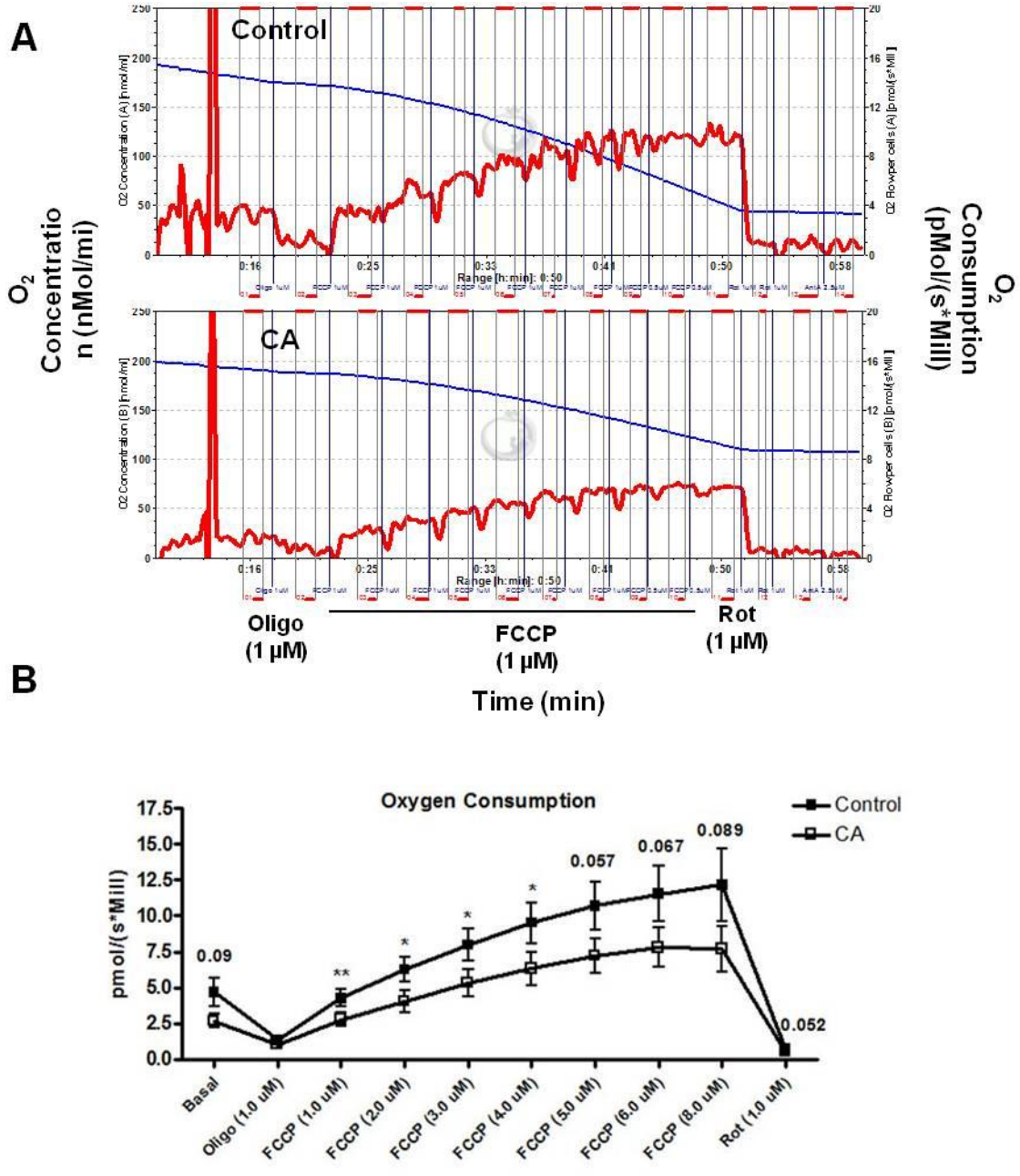


Figure 33: Respiration is reduced following *in vivo* redox modulation. BDC-2.5.TCR.Tg mice were treated for 7 days i.p. with CA or HBSS at 10 mg/kg. On day 8, splenocytes were harvested and analyzed in an Oroboros Respirometer. Oxygen consumption was measured as pmol/second/million cells (pmol/(s*Mill)). (A) Respiration was determined at basal conditions and after the addition of mitochondrial inhibitors, oligomycin (Oligo) and rotenone (Rot), and additive amounts of the mitochondrial uncoupler FCCP. (B) n=5 independent experiments, *$p<0.05$, **$p<0.005$.

3.5.6 CA treatment does not significantly alter mitochondrial complex/biogenesis molecules

Based on the minor decrease in respiration, we hypothesized that mitochondrial biogenesis may be unchanged after CA treatment. To first assess this possibility, we measured all mitochondrial complexes (666) from whole cell splenocyte lysates via western blot, using an antibody cocktail, after 7 days of *in vivo* treatment. The cocktail contains antibodies to complex subunits that are labile if not assembled properly; therefore, an accurate depiction of the mitochondrial complexes should be feasible with this probe. In general, mitochondrial complex proteins are not significantly decreased after CA treatment (**Figure 34A, B**). Furthermore, mRNA levels of these same complexes are similarly unaltered (**Figure 34C**). Lastly, mitochondrial biogenesis transcription factors, which regulate the production of the mitochondrial complexes, were also measured by qRT-PCR. *Pgc-1* and *Tfam* were not statistically different following CA treatment (**Figure 34D**). PGC-1 stimulates mitochondrial biogenesis through regulation of nuclear respiratory factor 1 (Nrf1) (667). Together, PGC-1 and Nrf1 can initiate mitochondrial transcription factor A (TFAM), which activates mitochondrial DNA replication and transcription (668). These results indicate similar mitochondrial abundance in control versus CA-treated splenocytes, despite the slight reduction in oxygen consumption. However, redox modulation did significantly decrease *Nrf1* mRNA (**Figure 34D**). Nrf1 not only contributes to mitochondrial biogenesis, but it also plays roles in a wide range of cells, including binding to the antioxidant response element (ARE) for gene transcription during times of oxidative stress (669). Reduced Nrf1 after redox modulation may therefore correlate with a reduced need for antioxidant transcription, as CA acts as an effective scavenger of ROS and reduces oxidative stress on its own.

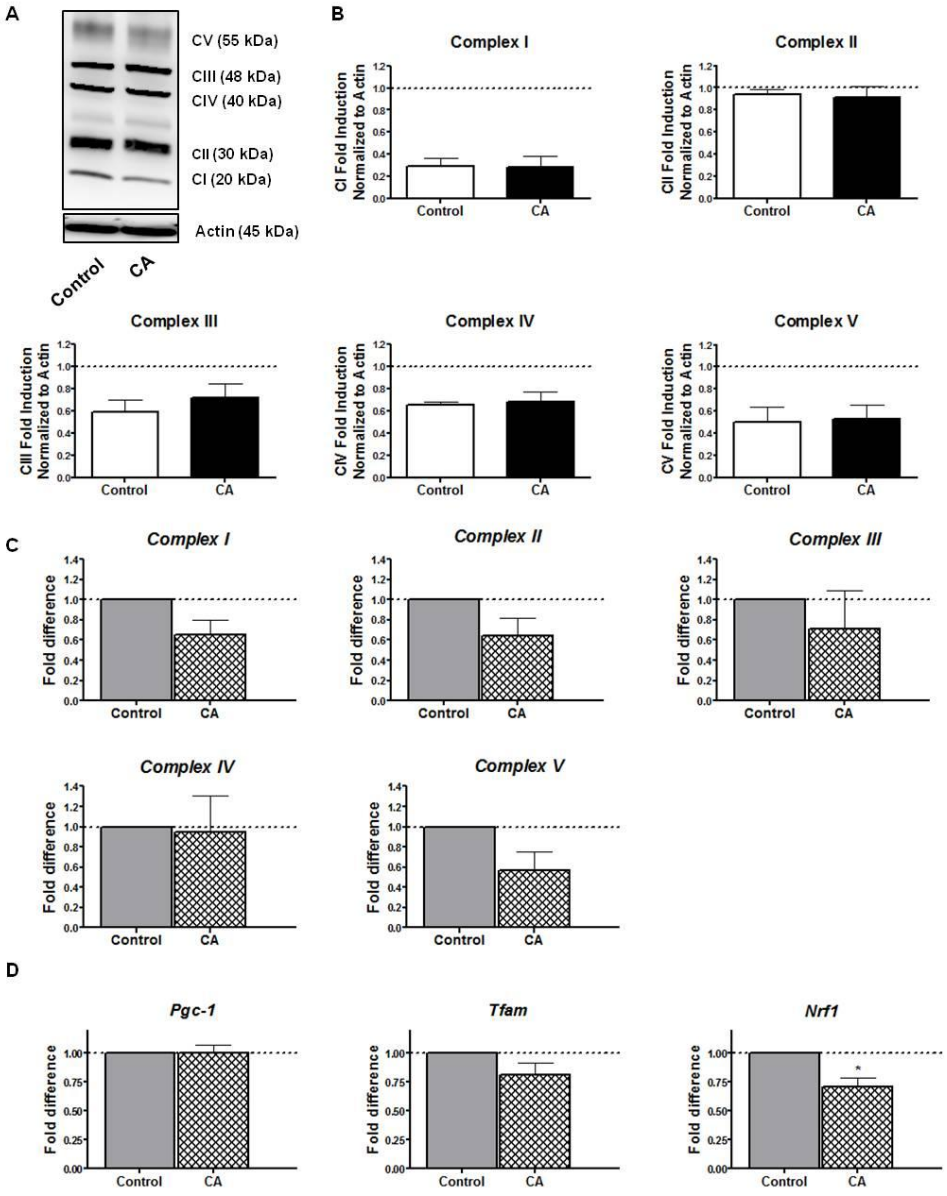


Figure 34: CA treatment does not significantly alter mitochondrial complex and biogenesis molecules. BDC-2.5.TCR.Tg mice were treated for 7 days i.p. with CA or HBSS at 10 mg/kg. On day 8, splenocytes were harvested for protein lysates and mRNA isolation. (A) Whole cell lysates were probed for MitoOXPHOS antibody cocktail by western blot. Actin was probed as a loading control. Each complex is indicated based on its molecular weight. Data are representative of 3 independent experiments. (B) Densitometry was quantified for each complex by normalizing control and CA-treated cells to actin. Fold induction of complex protein from 3 independent experiments. (C-D) Relative mitochondrial complex or biogenesis mRNA levels were measured by qRT-PCR. The fold change of control samples were set arbitrarily to 1 and compared to CA treatment. All samples were normalized to the endogenous GAPDH control. n=4-5 independent experiments.

3.5.7 Redox modulation decreases aerobic glycolysis of diabetogenic splenocytes

Activated immune cells undergo aerobic glycolysis as their main form of metabolism (421; 423; 424). Based on the results indicating a non-significant decrease in oxidative phosphorylation (**Figure 33**), we next wanted to ascertain the level of glycolysis following CA treatment. To determine glycolytic dominance, we measured lactate production in mouse spleens after 7 days of *in vivo* treatment (**Figure 35A**). Lactate levels were significantly decreased in CA-treated spleens ($p<0.05$). Based on these data, aerobic glycolysis is not enhanced as compensation for the observed mild decrease in oxidative phosphorylation; instead, aerobic glycolysis is significantly reduced, which may contribute to immune cell quiescence and stunted diabetogenic potential.

3.5.8 CA treatment induces more efficient glucose oxidation while reducing fatty acid oxidation

Glucose utilization via aerobic glycolysis is the standard pathway for activation of immune cells. However, glucose and other substrates can be used for the TCA cycle and oxidative phosphorylation. To parse out differences in substrate oxidation, in contrast to overall oxygen consumption (**Figure 33**), glucose and the fatty acid palmitate were used in an uptake assay. In CA-treated splenocytes, glucose oxidation, as measured by radiolabeled CO_2 production, was significantly enhanced ($p<0.005$) in comparison to control cells (**Figure 35B**). Conversely, fatty acid oxidation, which would increase in the absence of sufficient glucose oxidation, was decreased following redox modulation (**Figure 35C**). Moreover, spleen ATP levels remain unchanged between the groups (**Figure 35D**), suggesting an augmentation of TCA cycle

efficiency after CA administration, resulting in more glucose oxidation but less aerobic glycolysis necessary for driving activation of diabetogenic splenocytes.

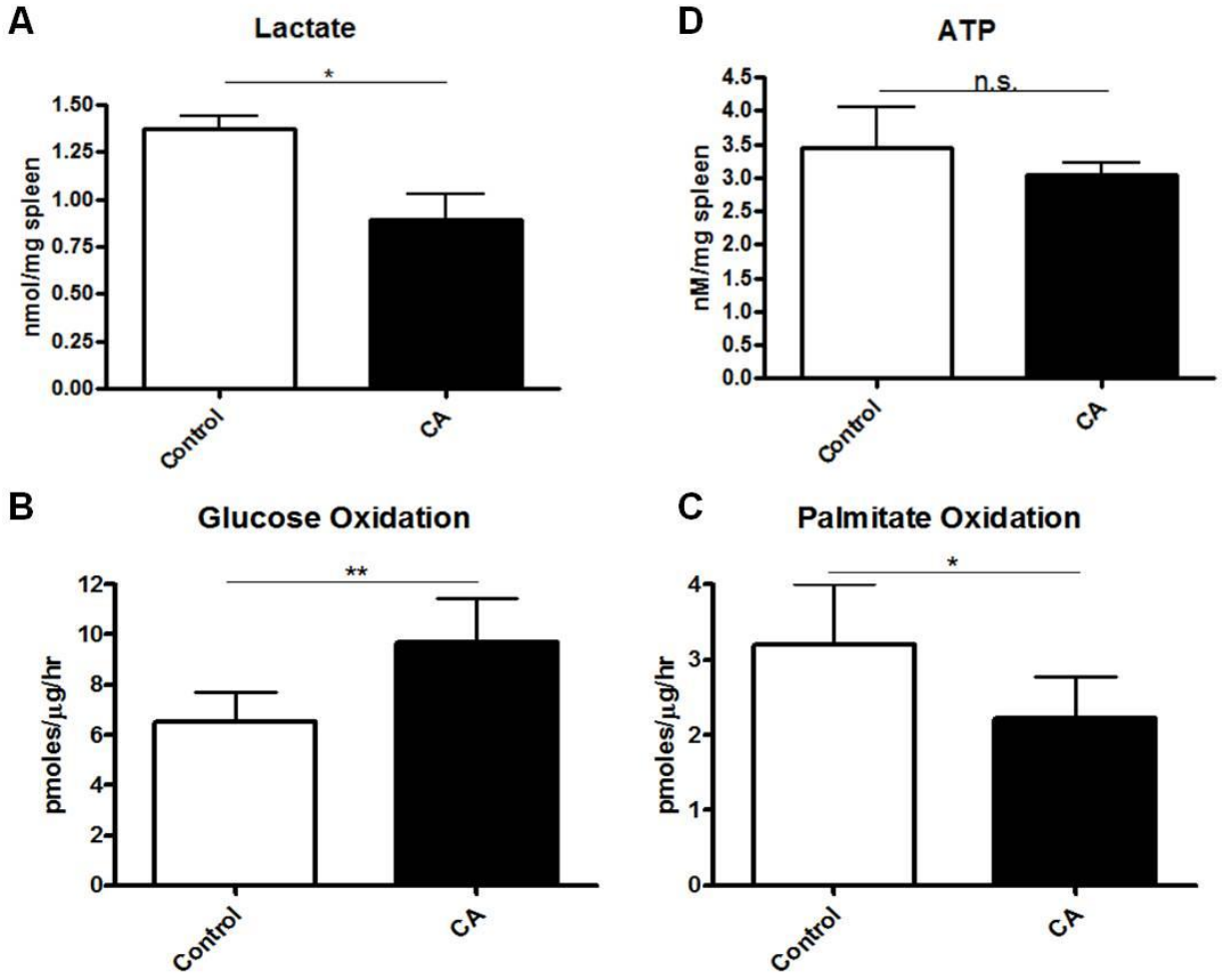


Figure 35: Glucose oxidation efficiency is enhanced after redox modulation. BDC-2.5.TCR.Tg mice were treated for 7 days i.p. with CA or HBSS at 10 mg/kg. On day 8, spleens were harvested. (A) Spleens were weighed, homogenized, and used in a lactate assay. Lactate (nmol) was quantified per mg of spleen. (B) Isolated splenocytes were given D-[6-^{14}C] glucose and 2.5 µM cold glucose, incubated for 1 hour, and $^{14}CO_2$ was measured. (C) Isolated splenocytes were given [1-^{14}C] palmitic acid, incubated for 1 hour, and $^{14}CO_2$ was measured. (D) Spleens were weighed, homogenized, boiled and used in an ATP determination assay. ATP (nM) was quantified per mg of spleen. n=3 mice/group for all assays. *$p<0.05$, **$p<0.005$.

3.5.9 Redox modulation leads to less mTOR activity and Glut1 expression

In accordance with the decrease in lactate production, we sought to determine CA-mediated aerobic glycolytic signaling effects, specifically through mTOR and Glut1 expression. mTOR activation is essential for driving cell cycle. Decreased activation of mTOR blocks expansion of cells, especially in times of starvation or when glycolysis is low. After CA treatment, mTOR phosphorylation is increased (**Figure 36A**), which has conflicting interpretations. Phosphorylation at serine 2448 can activate mTOR (670); however, removal of serines 2448-2450 can actually enhance mTOR activity, suggesting that serine 2448 is part of a repressor domain (671), indicating a potential reduction in glycolysis and a blockade in splenocyte expansion. To further investigate mTOR activity, downstream signaling targets, S6 kinase and PHAS were measured. Upon CA treatment, phosphorylation of both S6 and PHAS is decreased (**Figure 36B**), indicating a reduction in mTOR activity and confirming previously reported data (644), where CD4+ T cell proliferation was also blunted by redox modulation. Additionally, Glut1 protein was assessed in whole cell splenocyte lysates. Glut1 is a glucose transporter protein upregulated by cytokines and glycolytic enzymes. Redox modulation can decrease cytokine production from both innate and adaptive immune cells (48; 64; 644). Glut1 expression is decreased following CA administration (**Figure 36C**), suggesting diminished glycolysis in response to lowered cytokine stimulation. These data along with those showing reduced aerobic glycolysis together highlight the potential of CA treatment to lower immune cell bioenergetics, which most likely contributes to decreased activation and delayed diabetes onset upon transfer.

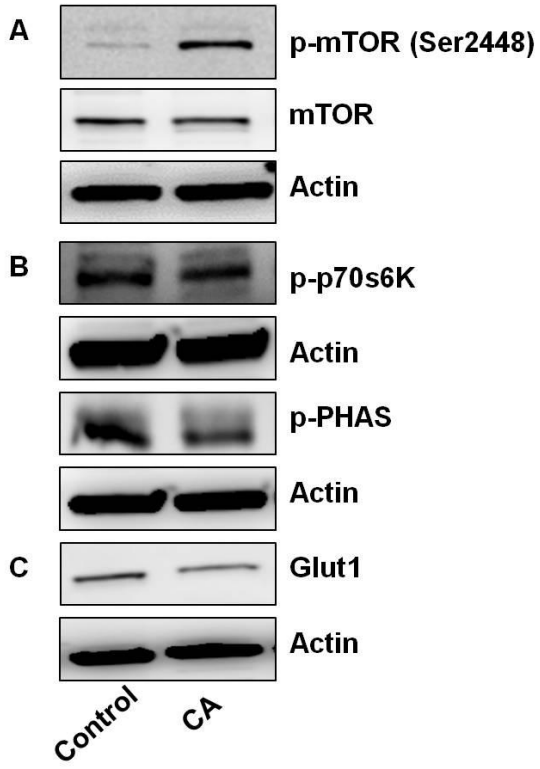


Figure 36: Redox modulation leads to increased phospho-mTOR and decreased Glut1 expression. BDC-2.5.TCR.Tg mice were treated for 7 days i.p. with CA or HBSS at 10 mg/kg. On day 8, splenocytes were harvested for protein lysates. (A) Whole cell lysates were probed for (A) phospho-mTOR and mTOR (B) phospho-p70s6K and phosphor-PHAS and (C) Glut1 by western blot. Actin was probed as a loading control for both panels. Data are representative of (A, C) 3-4 independent experiments, (B) 1-2 independent experiments.

3.5.10 CA-mediated immunomodulatory alterations are reversible

CA treatment affects both immune and metabolic functions of diabetogenic immune cells. Although both of these parameters clearly contribute to the reduction in diabetogenic potential of autoreactive splenocytes, determining the reversibility of these effects, as seen during the adoptive transfer in which CA-treated autoreactive cells eventually cause disease (**Figure 31A**), is important for establishing redox modulation as a potential clinical agent. Therefore, we measured the reversibility of CA administration in an immunological ELISA assay. Following 7

days of *in vivo* treatment, transgenic autoreactive splenocytes were stimulated *in vitro* with cognate peptide. By day 4 post-stimulation, CA-treated IFN-γ production returned to control levels (**Figure 37**). Although this reversal, i.e. towards a control cell phenotype, demonstrates the inability of CA treatment to chronically block diabetogenic function, explaining the eventual diabetes onset after adoptive transfer, it demonstrates no global immunosuppression after treatment has ceased, resulting in a lack of long-term, unwanted side effects.

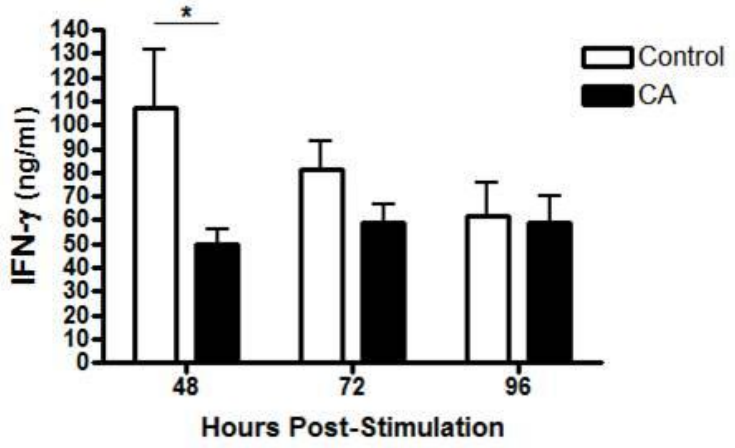


Figure 37: Diabetogenic immune function blockade is reversible after ending CA treatment. BDC-2.5.TCR.Tg mice were treated for 7 days i.p. with CA or HBSS at 10 mg/kg. On day 8, splenocytes were harvested for *in vitro* stimulation with M +/- CA. At 48-96h, supernatants were collected and used in an IFN-γ ELISA, n=5 independent experiments performed in triplicate, *$*p<0.05$*.

3.6 DISCUSSION

The alteration of undesired immune responses can be beneficial in the context of a plethora of diseases, including autoimmunity. Unlike cancer and infections, controlling self-reactivity requires a reduction in innate and adaptive immune cell activities. A number of endogenous pathways exist to directly or indirectly adjust immune responses. Such modifications of T cells

include skewing towards a different T helper subset, Treg-mediated suppression, and APC-derived IDO induction for the inhibition of T cell proliferation and survival. Feed-forward mechanisms and interplay between the two arms of the immune response will extend these T cell adjustments to eventually downregulate innate cell functions (672), thus widely alleviating any aberrant responses. Besides strictly immunological mechanisms, another means of immune cell regulation is metabolic restraint. This type of control can theoretically affect both innate and adaptive immune cells. Anti-glycolytics and treatments to inhibit oxidative phosphorylation have been utilized in cancer, transplantation, and neurodegenerative diseases (547; 597; 600; 613; 629). In autoimmunity, for example, HIF-1 blockade leads to decreased glycolysis, resulting in diminished T_H17-mediated murine EAE (535). Similarly, antioxidant treatments, which effectively lower oxidative stress propelled by mitochondrial respiration, can relieve T_H17-mediated murine arthritis (639) and induce regulatory T cells (673; 674). In previous studies, we demonstrated reduced type 1 diabetes through antioxidant treatment; however, the experiments were limited to T_H1 response measurements (644). We therefore wanted to determine whether CA treatment affected the specific regulators of T cell function listed above and/or if redox modulation could influence immune cell metabolism.

CA treatment is known to decrease T_H1 responses via reductions in IFN-γ (48; 278; 644). Through detection of cytokine secretion, we investigated whether CA administration skews diabetogenic T cells to a different T helper subset, such as T_H17 or T_H2 cells. The induction of T_H2 cells can inhibit T_H1 cell responses, successfully biasing the autoreactive T cell pool away from beta cell destruction (172; 675; 676). Cytokines were measured on a per-cell basis to exclude any differences that may be acquired from overall decreased cell proliferation upon *in vitro* stimulation in the presence of CA, shown previously using BDC-2.5.TCR.Tg splenocytes

(644). In the present studies, we conclude that CA treatment does not skew T_H1 cells towards another T helper subset, as cytokine decreases were detected for IL-17, IL-4, and IL-10.

Next, we measured the ability of CA treatment to promote Treg differentiation. Regulatory T cells are important for suppressing autoreactive T cells that have escaped thymic selection. Moreover, generation of Tregs may afford long-term tolerance to self-antigen and/or reversal of immune-mediated tissue damage. After CA administration *in vitro*, we did not observe an enhancement of Treg differentiation following anti-CD3/CD28 stimulation under non-polarizing conditions. Low anti-CD3 concentrations are known to drive CD4+Foxp3+CD25+ Tregs *in vitro* (677); CA treatment, even with the lowest dose of anti-CD3, does not significantly increase Treg development over control cells. However, polarizing conditions may have to be implemented in future studies in order to rule out this possibility completely.

Despite these data, adoptive transfers of CA-treated diabetogenic splenocytes yielded *in vivo* suppression of autoreactive responses. Transfer of CA-treated cells alone caused a significant delay in diabetes onset; however, the control:CA ratio of 1:6 was able to extend this delay of diabetes onset, suggesting regulatory interactions between the two populations. Notably, CA-treated cell transfers ultimately succumbed to disease at day 23, whereas the 1:6 ratio remained diabetes free until the end of the study (40 days). This difference between the transfers was particularly interesting on two levels: 1) the mechanism in play between the two cell populations in the 1:6 ratio transfer and 2) the eventual disease manifestation after CA-treated cell transfer.

IDO induction in APCs causes the catabolism of tryptophan, an essential amino acid for T cell proliferation and survival. The enzymatic activity of IDO is useful for contraction of an

immune response and has been exploited in the setting of transplantation. IDO released from DCs can inhibit alloreactive T cells, blocking transplant rejection (678; 679). In autoimmunity, IDO can potentially have a similar effect, restraining autoreactive T cell responses (680). Utilizing the 1:6 transfer ratio *in vitro*, we detected an increase in IDO levels in comparison to control, CA-treated, and 1:4 transfer ratios. IDO production has been postulated to follow ligation of LAG-3 on T cells to MHC class II on DCs (204; 371; 681). In our previous study, CA enhanced the frequency of LAG-3+ T cells and also decreased its cleavage from the cell surface (644), suggesting a possible mechanism for this study's results. Moreover, IDO is controlled by IFN-γ; IFN-γ can bind to its receptor on APCs and signal through JAK/STAT pathways to initiate IDO expression (682). Because CA can decrease IFN-γ production (48; 278; 644), it is possible that redox modulation alone may lower the T_H1 cytokine level too much, not allowing for sufficient IDO activity and resulting in the eventual onset of type 1 diabetes after adoptive transfer. CA treatment during islet transplantations likewise results in too little IFN-γ, and in the absence of any other immunosuppression, transplants are eventually rejected (unpublished data). A delicate balance of IFN-γ levels is thus necessary for reducing diabetogenic potential, and the contribution between both control and CA-treated cells in the 1:6 ratio may be sufficient for inducing IDO, restraining disease onset.

With the ability of CA to scavenge ROS produced during cellular respiration (64; 683), it is plausible for redox modulation to extend beyond immunological control. Adoptive transfers of CA-treated diabetogenic splenocytes significantly impede type 1 diabetes in comparison to control cells. However, the loss of regulation around 23 days post-transfer led us to test whether or not immune cell metabolism is restrained by redox modulation until the amount of CA troughs, consequently reversing self-reactive control. Adequate bioenergetics levels are crucial

for directing activation and expansion of immune cells. Resting and memory T cells rely primarily on oxidative phosphorylation, whereas activated T cells depend on mainly on glycolysis. Innate cells, on the other hand, predominately utilize glycolysis during all activation states (423; 424). Oxidative phosphorylation is important for ATP storage in immune cells, preparing them for efficient function upon activation and some increases are detected upon activation (410). Conversely, the macromolecule synthesis necessary for immune cell expansion is a result of the quicker metabolic route of glycolysis (421). In general, both metabolic pathways are necessary for priming and activating autoreactive immune cells. Redox modulation demonstrated only a mild reduction in overall oxygen consumption of diabetogenic splenocytes. This effect on mitochondrial respiration was not due to an alteration in primary mitochondrial biogenesis or complexes of the electron transport chain. However, *Nrf1*, a transcription factor important for biogenesis as well as activating the ARE, was significantly decreased after CA treatment. This result is potentially because of the antioxidant actions of CA. The need for more antioxidant genes to be transcribed following Nrf1 binding to the ARE is likely lowered in the presence of redox modulation. Moreover, scavenging of ROS by CA reduces oxidative stress, which is known to upregulate *Nrf1* gene transcription (684; 685). The significant reduction in *Nrf1* may therefore be related to the stress level of the cell and not necessarily an impact on mitochondrial abundance.

We next measured lactate production and signaling molecule activation to determine if compensatory increases in aerobic glycolysis resulted from the slight decrease in respiration after CA treatment. During times of dominant glycolysis, the metabolic intermediate pyruvate is fermented to lactate, producing NAD+ for macromolecule synthesis and for the continuation of glycolysis. Lactate levels increase during T cell activation (409; 447), again illustrating how

immune cells rely heavily on aerobic glycolysis to transition from a resting state. Lactate levels in the spleen, a secondary lymphoid organ, were significantly lowered in CA-treated mice, suggesting that aerobic glycolysis is decreased upon redox modulation.

In addition to lactate levels, we also measured the glycolytic signaling molecules phospho-mTOR and Glut1. mTOR is activated downstream of the PI3K-Akt pathway, which is stimulated during T cell activation and hence, glycolysis (409). For efficient cell cycle progression and proliferation, mTOR is activated, allowing for the activation of downstream signaling targets p70s6K and phospho-PHAS1 (480; 686), which drive ribosome biogenesis and translation initiation, respectively. During times of starvation or low glycolysis, phosphorylated mTOR is decreased, repressing its own catalytic activity and promoting hyporesponsive T cells (485; 671), yet protecting against apoptosis (687). Furthermore, dephosphorylated mTOR has been postulated to augment the function of p21 and p27 cyclin-dependent kinase inhibitors (688), effectively blocking cell cycle progression. CA treatment boosted the amount of phosphorylated mTOR in comparison to control splenocytes, highlighting conflicting interpretations. Serine 2448 phosphorylation may both activate mTOR (670) yet contribute to an mTOR repressor domain (671). Further studies on several phosphorylation sites within mTOR will need to be conducted to definitively interpret our results. However, reductions in phosphorylation of s6K and PHAS indicate a potential reduction in glycolysis and a blockade in splenocyte expansion due to mTOR inactivation. This result also positively correlates with previous studies displaying decreased CD4+ T cell proliferation following redox modulation (644) and provides more evidence of reduced aerobic glycolysis.

Glut1, the only glucose transporter expressed on immune cells (406), was also measured. CA-treated splenocytes showed lower Glut1 levels than in control cells. Glucose uptake through

Glut1 is critical for metabolic processes; reduced Glut1 levels are indicative of an environment lacking the growth factors/cytokines necessary for perpetuating PI3K-Akt signaling pathways necessary for Glut1 expression (409; 475). CA can decrease cytokine levels from T cells and APCs (64; 278; 644); this lack of cytokine production may also act to reduce Glut1 expression. CA-treated cells do, however, remain viable (278), and therefore, the reduction in Glut1 is not harmful to splenocyte health. Indeed, regulation of Glut1 expression is actually essential for controlling hyperresponsive lymphocytes so as not to cause pathology (475). Additionally, HIF-1, a molecule especially critical for glycolysis during both aerobic and anaerobic respiration, can control the induction of Glut1 (414). With the reduction in Glut1 expression, CA may be downregulating HIF-1. In a radiation-induced pulmonary injury model, HIF-1 activation is decreased after CA treatment (646). Similarly, HIF-1 deactivation via redox modulation can enhance tumor radiosensitivity (689). These data demonstrate the importance of glycolysis for immune responsiveness and support our experiments depicting reduced Glut1 and decreased mTOR activity after CA administration.

Aerobic glycolysis is primarily used during tumor cell proliferation, a phenomenon known as the Warburg effect. This metabolic pathway is critical for effectively synthesizing new macromolecules for cell expansion and is characterized by HIF-1 activation, Glut1 expression, and lactate production. Although CA treatment only mildy decreased overall oxidative phosphorylation and reduced aerobic glycolysis, we parsed out different oxidation substrate pathways (glucose and palmitate) to determine any differences. Glucose oxidation efficiency is enhanced following CA administration, whereas fatty acid oxidation is decreased. While glucose-driven oxidative phosphorylation is necessary for preparing the cell for activation (410), fatty acid oxidation is critical for driving chronic inflammation, as in the case of EAE (690).

Furthermore, overall ATP production within the spleen is unchanged between control and CA treatment. These results do not necessarily mean that glucose is oxidized faster or more than control cells, but may instead indicate better TCA cycle function and possibly better coupling in the electron transport chain. ROS produced during the electron transport chain are able to damage TCA enzymes, such as aconitase (691; 692). When these enzymes are harmed by ROS, their activity is decreased (693), resulting in less efficient glucose oxidation. Importantly, reduced fatty acid oxidation may indicate less autoimmune-mediated chronic activation, further controlling self-reactivity. Overall, redox modulation leads to better glucose breakdown, yet reduced immune cell activation via aerobic glycolysis.

With the current antibodies and immunosuppressive agents in the clinic, negative side effects are almost always a cause for concern. For example, blocking immune cell activation for prevention of transplantation rejection usually requires long-term drug usage, which often instigates unavoidable damage. On the other hand, for treatment of autoimmunity, certain immunosuppressive agents may be too toxic or result in bystander injury that only worsens the disease. Cyclosporine A, for example, can block T cell activation and has been used outside of transplantation in severe cases of rheumatoid arthritis and psoriasis (694; 695). However, hepatotoxicity and nephrotoxicity are common side effects of this drug, which can lead to chronic organ disease or failure (666; 696; 697). A new class of small molecule agents is needed to better control autoreactivity yet protect against lasting side effects. Unlike certain immunosuppressants, antioxidant treatment can benefit autoimmunity as well as preserve organ function (79; 304), with little to no toxicity at the recommended amount. The toxic dose of the CA is 9-times the effective dose used in our studies (698), illustrating its bioavailability and low risk of injury. Importantly, CA treatment is reversible, as measured by the return of IFN-γ after

clearance of the agent, confirming the maintenance of cell viability following administration. The reversibility of redox modulation could especially be useful for acute illnesses or for tumor therapies. Furthermore, CA treatment could serve as a means to reset the threshold of hyperresponsive immune cells. This would then allow for subsequent administration of a more targeted immunotherapy, such as an antibody that specifically affects T cells, to effectively prevent or reverse autoimmunity.

In conclusion, redox modulation does not promote skewing of T helper subsets or induction of classical Treg cells. IDO production may be the mechanism behind the extension of diabetes delay in competitive adoptive transfers; however, the 1:6 ratio of control:CA-treated cells is not physiologically relevant, unless only subsets of cells could be targeted during systemic CA administration. Therefore, metabolic control by redox modulation may be attributable to the reduction in diabetogenic potential, highlighting this agent as not only immunomodulatory and cytoprotective, but also reversible, with anti-Warburg effect characteristics, overall promoting immune cell quiescence. Bioenergetic regulation is thus a possible therapeutic option for controlling self-reactivity and may hold promise for prevention or reversal of type 1 diabetes.

4.0 REDOX MODULATION IMPROVES INSULIN SENSITIVITY AND INFLAMMATION IN A HIGH-FAT DIET-INDUCED TYPE 2 DIABETES MODEL

4.1 ABSTRACT

In type 2 diabetes, a collaboration of impaired insulin signaling and decreased insulin secretion leads to hyperglycemia and long-term health complications. Insulin resistance is further exaggerated through chronic dietary fat intake and inflammation. The liver and adipose tissue are two sites of abnormal fat deposition and immune cell infiltrate upon obesity-induced diabetes. Through utilization of a metalloporphyrin catalytic antioxidant (CA), we sought to determine whether redox modulation could assuage liver and adipose complications as well as enhance insulin sensitivity and glucose tolerance in a high-fat diet-induced mouse model of type 2 diabetes. In addition to scavenging reactive oxygen species and mimicking superoxide dismutase, CA can decrease proinflammatory cytokine secretion through inhibition of NF-κB activation. Moreover, CA is not toxic and can preserve beta cell function. In the present study, CA delayed weight gain, reduced serum leptin and insulin levels, alleviated hepatic steatosis, decreased adipose immune cell infiltrate, and enhanced the acute phase IL-6 response for tissue repair. Overall, CA treatment is a potential therapeutic strategy for impeding type 2 diabetes pathogenesis and reducing chronic diabetic side effects.

4.2 INTRODUCTION

Unlike type 1 diabetes, in which a loss of beta cells contributes to the absence of insulin, type 2 diabetes is not autoimmune-mediated, but instead induces poor glucose tolerance as a result of insulin resistance. Insulin is important for regulating energy metabolism by facilitating glucose uptake in skeletal muscle, while reducing gluconeogenesis in the liver and lipolysis in the adipose tissue. As the amount of blood glucose and lipid metabolites tips into excess in the context of obesity, the liver, muscle, and adipose are unable to compensate and become the targets of fat deposition and inflammatory cell infiltrate. In particular, global insulin receptor signaling becomes desensitized, resulting in abnormal translocation of glucose transporters and impaired glucose metabolism (699). The pancreatic beta cells attempt to restore glucose tolerance by secreting more insulin; however, as peripheral insulin resistance increases, the beta cell secretory capacity is unable to re-establish insulin sensitivity (700; 701). Type 2 diabetes manifests when loss of glucose tolerance supersedes insulin resistance.

Control of blood glucose levels is critical. A combination of hyperglycemia, obesity and insulin resistance can enhance oxidative stress and lead to chronic complications. In type 2 diabetes, long-term complications include cardiovascular disease, hypertension, dyslipidemia, and poor circulation, together termed ‘metabolic syndrome’ (702; 703). Unlike type 1 diabetes, type 2 diabetes often can be treated through caloric restraint and exercise (704; 705). However, with approximately 2/3 of U.S. adults considered overweight or obese, type 2 diabetes is a prevalent epidemic and requires further inquiry into alternative treatment options for the initial disease and the costly diabetic complications (706).

The mechanisms leading to insulin resistance and complications are highly dependent on oxidative stress and inflammation. Hyperglycemia and lipotoxicity can lead to abundant ROS

generation and inflammation (707). Rodent models of type 2 diabetes and human patients display high ROS in the islets (708). Islets, in comparison to other tissues, have low antioxidant levels (91), making them extremely susceptible to oxidative stress and hyperglycemia (709). High glucose levels can impair glucose-stimulated insulin secretion, whereas H_2O_2, a potent oxidizer, can damage glucose metabolism, together resulting in dysfunctional insulin release (95; 710). Defects in glucose metabolism lead to deficient ATP production, further lending to diminished glucose-stimulated insulin secretion (711). For this reason, insulin resistance and beta cell mitochondrial dysfunction are both thought to play a role in type 2 diabetes.

In addition to hyperglycemia, an overall increase in dietary fat leads to an energy surplus, overworking islet mitochondria and generating an abundance of ROS (712). Mitochondria derived ROS have been implicated in enhancing the expression of insulin signaling inhibitors, leading to less glucose uptake. Specifically, ROS can directly inhibit insulin signaling through activation of stress kinases like JNK and p38 MAPK (713; 714), leading to serine/threonine phosphorylation of insulin receptor substrate 1 (IRS1), and inhibition of PI3K activation downstream of insulin ligation. In the context of inactivated PI3K, the glucose transporter Glut4 in skeletal muscle does not translocate to the plasma membrane and glucose uptake is reduced (713). Moreover, H_2O_2 can directly inhibit Akt and glucose transporter activity (715) as well as reduce IRS1 and IRS2 protein levels after chronic exposure (716). These ROS-mediated modifications can augment insulin resistance and play critical roles in promoting disease complications.

As a result of heightened ROS levels, redox-dependent transcription factors such as NF-κB further exacerbate type 2 diabetes via proinflammatory cytokine secretion. Activation of NF-κB leads to the production of MCP-1, IL-1β, IL-6, and TNFα, all of which are detrimental to

insulin tolerance if chronically produced (717). During obesity and type 2 diabetes, macrophages infiltrate the fat and liver, where they worsen insulin resistance through proinflammatory cytokine production (718; 719). In healthy individuals, insulin is able to bind insulin receptor and signal through PI3K and Akt to block proinflammatory cytokine production. It does so by phosphorylating FOXO proteins (720; 721), removing them from the nucleus and disabling their ability to facilitate NF-κB activation. Therefore, insulin acts as an endogenous anti-inflammatory agent (722; 723), and improper insulin signaling results in an exaggerated inflammatory environment. Aberrant proinflammatory cytokine production positively correlates with insulin resistance in type 2 diabetes (724), and anti-inflammatory therapies have shown promise in improving insulin sensitivity (725). IL-1R-antagonist or anti-IL-1β can preserve beta cell function in type 2 diabetes (726; 727). Furthermore, blockade of NF-κB through resveratrol and curcumin administration reduces adipocyte cytokine production, suggesting improved insulin sensitivity through anti-inflammatory strategies (728).

In our previous studies, we utilized a manganese metalloporphyrin catalytic antioxidant (CA) to block proinflammatory cytokine production and inhibit type 1 diabetes onset (36; 48; 278; 644). The CA we used is able to scavenge a broad range of ROS, including superoxide, hydrogen peroxide, and peryoxynitrite, and acts a SOD mimetic (269). Importantly, we have detected reductions in both oxidative stress and NF-κB activation following administration of CA (64). Based on these studies, we wanted to determine whether CA treatment could modulate high-fat diet-induced inflammation in the male, C57BL/6J model of type 2 diabetes and if this treatment could assuage insulin resistance as well as obesity-induced liver steatosis and inflamed-adipose complications.

4.3 RESEARCH DESIGN AND METHODS

4.3.1 Materials

C57BL/6J mice were purchased from Jackson Labs (Bar Harbor, MN) and housed under specific pathogen-free conditions in the Animal Facility of Rangos Research Center at Children's Hospital of Pittsburgh of UPMC (Pittsburgh, PA). Male mice at 6-8 wks of age were used in all experiments. All animal experiments were approved by the Institutional Animal Care and Use Committee of the Children's Hospital of Pittsburgh and were in compliance with the laws of the United States of America. High-fat diet (HFD) chow was purchased from Research Diets, Inc (New Brunswick, NJ). Mice were placed on either standard chow diet (n=12) or HFD chow consisting of 60% kCal fat (n=22) for 12 weeks. Mice were weighed weekly until the end of the experiment. MnTE-2 catalytic antioxidant (CA) was a generous gift from James Crapo, MD at National Jewish Health. CA was prepared as previously described (48) and used at 5 mg/kg subcutaneously every 3 days in all in vivo experiments.

4.3.2 Comprehensive diagnostic panel

At 12 weeks of chow, blood samples were collected from each mouse for toxicity profiling using a VetScan Comprehensive Diagnostic Profile with a VetScan Chemistry Analyzer (Abaxis, Union City, CA). Liver, kidney, and pancreatic dysfunction were tested through evaluation of alanine aminotransferase (ALT), albumin (ALB), alkaline phosphatase (431), amylase (649), calcium (Ca), creatinine, globulin (GLOB), phosphorus, potassium, sodium (Na), total bilirubin, total serum protein (TP), and blood urea nitrogen levels.

4.3.3 10-week IPGTT

A 20% glucose solution (2.0 g/kg) was intraperitoneally administered to fasted mice at 10 weeks of HFD feeding. Blood samples were collected at 0, 30, 60, 90 and 120 min after glucose injection. Blood glucose levels were measured using a glucometer (Ascensia Breeze 2, Bayer).

4.3.4 Serum adipokine measurement

Fasting serum insulin and leptin was evaluated at 5 weeks, 10 weeks, and 12 weeks. Blood samples were collected and serum adipokines were determined by a Milliplex Mouse Serum Adipokine Panel, according to manufacturer's instructions (Millipore, Billerica, MA).

4.3.5 Liver and fat histology

Liver and adipose tissue of sacrificed animals were harvested, fixed in 10% formalin, and embedded in paraffin. Cut paraffin sections were stained with hematoxylin, eosin, an IL-6 antibody or an F4/80 antibody (Abcam, Cambridge, MA). Sections were visualized via an Axioplan 2 microscope (Zeiss). Digital images (n=3-4) were acquired from each slide using a 20X objective with at least 3 mice/group. Staining was quantified via MetaMorph (Molecular Devices, Sunnyvale, CA). Results are expressed as percent of positively stained area/total measured area.

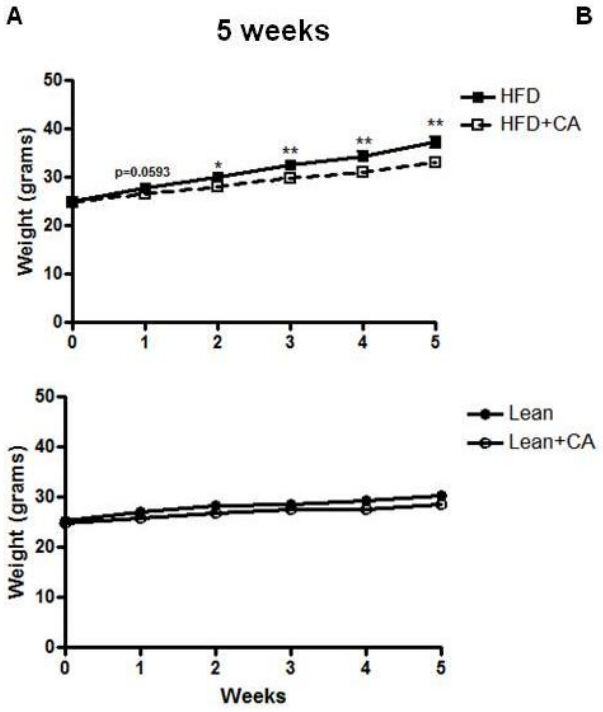
4.3.6 Statistical analysis

The difference between mean values was assessed by Student's *t* test, with $p<0.05$ considered significant. All experiments were performed on at least 6 mice/group. Outliers were excluded from final result calculations. Data are mean ± SEM.

4.4 RESULTS

4.4.1 Redox modulation delays early weight gain in HFD-fed mice

Historically, high-fat diet fed mice gain weight exponentially over time (729), making this a good model for studying type 2 diabetes. HFD mice did gain weight as expected; however, at early time points (5 weeks) following the onset of HFD chow, CA treatment significantly reduced weight gain (**Figure 38A**). Standard chow fed mice, termed 'Lean' and 'Lean+CA', exhibited no difference in weight gain, but remained lighter than their HFD fed counterparts. Weight gain past 5 weeks did not vary between HFD and HFD+CA treated mice or Lean and Lean+CA treated mice (data not shown). By the end of the study after 12 weeks of chow, no significant differences were detected in the weights of HFD and HFD+CA treated mice (**Figure 38B**). These data indicate that early onset of obesity is delayed upon redox modulation; however, elongated high calorie intake and subsequent weight gain are not altered with CA treatment.



B

12 weeks

Group A: Weight, g	Group B: Weight, g	*p* value
Lean	**Lean+CA**	
32.4 +/- 0.77	31.0 +/- 0.97	NS
Lean	**HFD**	
32.4 +/- 0.77	45.9 +/- 1.13	<0.0001
Lean	**HFD+CA**	
32.4 +/- 0.77	43.3 +/- 1.27	<0.0001
Lean+CA	**HFD+CA**	
31.0 +/- 0.97	43.3 +/- 1.27	<0.0001
HFD	**HFD+CA**	
45.9 +/- 1.13	43.3 +/- 1.27	NS
HFD	**Lean+CA**	
45.9 +/- 1.13	31.0 +/- 0.97	<0.0001

Figure 38: CA treatment delays early-onset obesity in HFD-fed mice. (A) Over 5 weeks of chow administration, weights were measured for HFD, HFD+CA, Lean, and Lean+CA treated mice. n=6-11 mice/group. *$p<0.05$, **$p<0.005$. (B) At the conclusion of the study (12 weeks), mice were weighed. Data from n=5-6 mice/group are displayed as averages +/- SEM. *p* values less than 0.05 are considered significant. NS = not significant.

4.4.2 Redox modulation does not promote hepatotoxicity, nephrotoxicity, or pancreatic toxicity

To confirm that CA treatment does not harm liver, kidney, or pancreatic health during HFD administration, a comprehensive diagnostic profile was conducted on each mouse group. All mice were measured for each analyte, but only those demonstrating significant differences are listed in **Table 1**. No differences were seen amongst all groups in phosphorus, potassium, total bilirubin, creatinine and blood urea nitrogen levels. Overall, HFD chow enhanced the amount of each analyte tested in comparison to Lean mice. However, CA treatment did not augment HFD-mediated levels or impart toxicity in either group. The only analyte significantly increased after HFD+CA treatment was sodium (Na); however, the escalation was not substantial in comparison

to Lean or Lean+CA sodium levels (data not shown), indicating that metalloporphyrin in conjunction with high fat may affect salt concentration. CA administration also significantly increased albumin in Lean+CA treated mice compared to Lean mice, although the values of albumin did not exceed those detected in HFD fed mice. Moreover, redox modulation enhanced alkaline phosphatase levels in Lean+CA treated mice compared to Lean mice. HFD+CA treated mice also displayed increased alkaline phosphatase levels compared to HFD mice, though they did not reach statistical significance and have not been included in this table. CA may be elevating ALP in the blood by unknown mechanisms, but treatment did not consistently increase ALT or total bilirubin, which are often used as secondary tests for monitoring liver dysfunction. Therefore, HFD chow did boost levels of analytes to levels typically detected in obesity, but CA treatment did not synergize to cause major hepatotoxicity, nephrotoxicity, or pancreatic toxicity, making it a safe therapeutic strategy in the HFD model. We are confident that the delay in weight gain from CA treatment (**Figure 38A**) in the HFD+CA group was not a result of toxicity, but instead a true effect of therapeutic efficacy.

Analyte	Group A	Group B	*p* value
ALB (g/dL)	*Lean*	*Lean+CA*	
	3.37 +/- 0.09	4.08 +/- 0.03	0.003
	Lean	*HFD*	
	3.37 +/- 0.09	4.13 +/- 0.03	0.0012
	Lean+CA	*HFD+CA*	
	4.08 +/- 0.03	4.48 +/- 0.11	0.0125
	Lean	*HFD+CA*	
	3.37 +/- 0.09	4.48 +/- 0.11	0.0007
ALP (U/L)	*Lean*	*Lean+CA*	
	40.0 +/- 2.30	57.25 +/- 2.50	0.0045
	Lean+CA	*HFD*	
	57.25 +/- 2.50	42.0 +/- 5.50	0.0386
ALT (U/L)	*Lean*	*HFD*	
	35.33 +/- 2.60	113.7 +/- 15.30	0.0072
	Lean+CA	*HFD+CA*	
	33.67 +/- 1.76	86.0 +/- 3.48	<0.0001
	Lean+CA	*HFD*	
	33.67 +/- 1.76	113.7 +/- 15.30	0.0065
	Lean	*HFD+CA*	
	35.33 +/- 2.60	86.0 +/- 3.48	0.0001

Analyte	Group A	Group B	*p* value
AMY (U/L)	*Lean*	*HFD*	
	1086 +/- 14.84	1221 +/- 43.11	0.0418
	Lean	*HFD+CA*	
	1086 +/- 14.84	1194 +/- 23.0	0.0156
Ca (mg/dL)	*Lean+CA*	*HFD+CA*	
	9.77 +/- 0.05	10.05 +/- 0.05	0.0073
	Lean	*HFD+CA*	
	9.73 +/- 0.03	10.05 +/- 0.05	0.0047
Na (mmol/L)	*HFD*	*HFD+CA*	
	147.7 +/- 0.067	151.3 +/- 0.48	0.0024
TP (g/dL)	*Lean+CA*	*HFD+CA*	
	5.93 +/- 0.03	6.25 +/- 0.09	0.0113
	Lean+CA	*HFD*	
	5.93 +/- 0.03	6.03 +/- 0.03	0.0446
	Lean	*HFD+CA*	
	5.67 +/- 0.15	6.25 +/- 0.09	0.0145
GLOB (g/dL)	*Lean*	*HFD+CA*	
	2.23 +/- 0.15	1.80 +/- 0.06	0.0266

Table 1: CA treatment does not cause toxicity. Blood was collected from each mouse at the end of 12 weeks of chow administration. Blood was utilized in a comprehensive diagnostic profile using a VetScan Chemistry Analyzer. Data table includes only those values demonstrating significant differences. ALB = albumin, ALP = alkaline phosphatase, ALT = alanine aminotransferase, AMY = amylase, Ca = calcium, Na = sodium, TP = serum total protein, GLOB = immunoglobulin. g/dL = grams/deciliter; U/L = units/liter; mg/dL = milligrams/deciliter; mmol/L = millimoles/liter. n=5-6 mice/group. *p* values less than 0.05 are considered significant.

4.4.3 CA treatment reduces fasting blood glucose levels, but does not improve postprandial glucose tolerance

After 10 weeks of HFD chow, fasting blood glucose levels were measured for all mouse groups. HFD+CA treatment statistically decreased fasting blood sugar in comparison to HFD mice ($p<0.05$) (**Figure 39A**). Lean mice with or without treatment did not vary in their blood glucose levels, whereas both HFD fed groups exhibited higher blood glucose levels in comparison to Lean and Lean+CA treated mice. Although fasting glycemia was reduced after CA treatment, redox modulation did not alleviate glucose intolerance in HFD mice during an IPGTT (**Figure 39B**). Lean and Lean+CA treated mice normalized blood glucose levels by 120 min post-glucose challenge, whereas HFD and HFD+CA treated mice failed to normalize. Therefore, fasting blood

glucose is alleviated after redox modulation, but postprandial glucose tolerance does not improve after CA treatment.

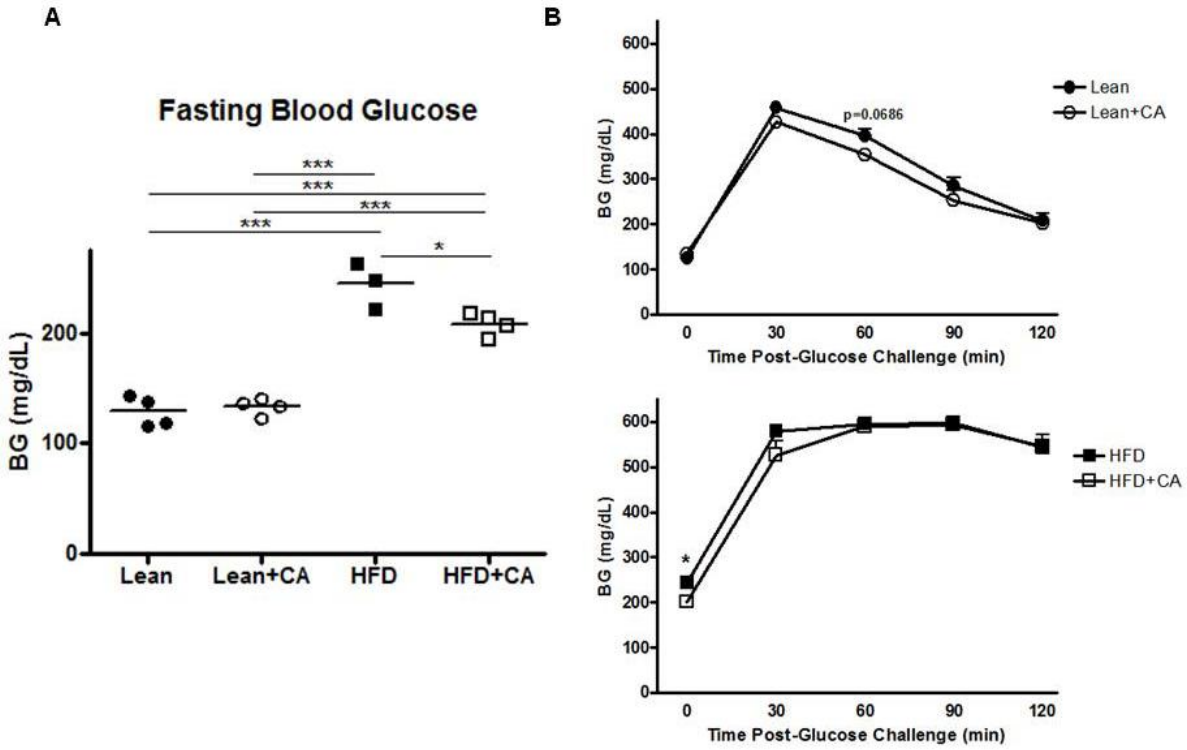


Figure 39: CA treatment improves fasting but not postprandial blood glucose levels. (A) At 10 weeks of chow administration, mice were fasted overnight, and blood glucose was measured. (B) Mice were administered 2.5 g/kg glucose solution intraperitoneally. Every 30 minutes, blood glucose was measured until 120 minutes post-glucose challenge. n=3-4 mice/group, **p<0.05, ***p<0.0005*.

4.4.4 Fasting leptin and insulin levels are reduced following CA treatment

The serum adipokines leptin and insulin were measured after overnight fasting at different time points during the 12 week chow comparison. CA treatment significantly reduced fasting leptin levels in HFD mice at 5 weeks (**Figure 40A**). Leptin is an important hormone for regulating appetite. Furthermore, leptin resistance has been associated with obesity, resulting in greater serum leptin levels and a decreased satiated feeling (730; 731). These observations are consistent

with the weight gain delay detected in HFD+CA treated mice (**Figure 38A**), suggesting that lower leptin resistance may obviate polyphagia during early obesity onset. Serum insulin levels were also measured after fasting. At 10 and 12 weeks, redox modulation significantly reduced insulin levels in comparison to HFD mice ($p<0.05$) (**Figure 40B**), indicating improved insulin sensitivity despite postprandial glucose intolerance detected above (**Figure 39B**).

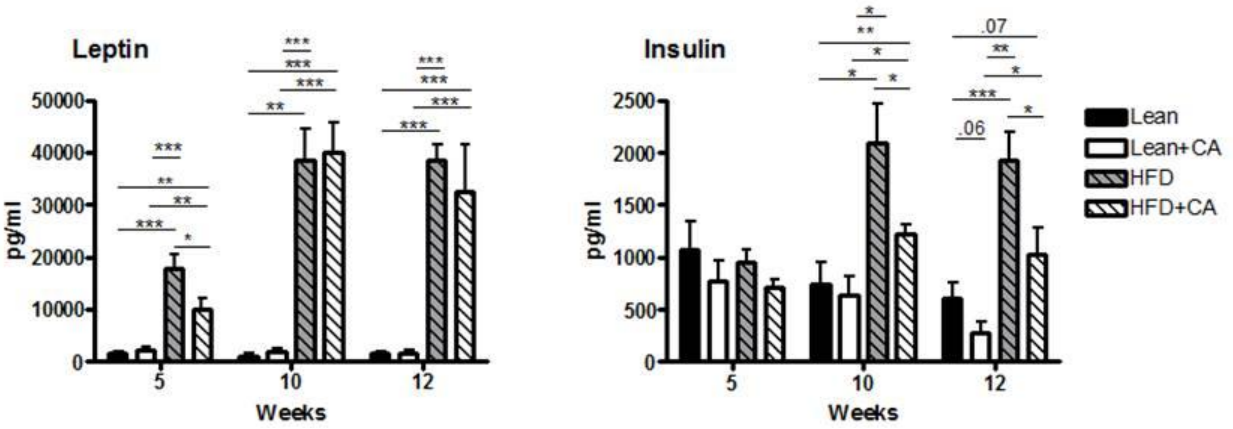


Figure 40: Adipokine levels were reduced in HFD mice administered CA treatment. Mice were fasted overnight at 5, 10, and 12 weeks, and serum was collected from each mouse. (A) Leptin and (B) insulin were measured via an adipokine panel. Data obtained in triplicate for n=5-6 mice/group. *$p<0.05$, **$p<0.005$, ***$p<0.0005$.

4.4.5 CA treatment reduces cellular infiltrate of adipose tissue in HFD mice

Adipose tissue is a site of insulin-dependent glucose disposal. As adiposity increases, adipokine release is also enhanced, attracting macrophages into the adipose tissue. Immune cell infiltration leads to greater proinflammatory cytokine production and adipokine release, effectively worsening insulin resistance. Despite a lack of difference in weight at the end of the study, HFD+CA treated mice displayed a slight reduction in overall visual obesity in comparison to HFD mice (**Figure 41A**). Furthermore, CA treatment was able to reduce infiltration into the adipose in comparison to untreated HFD mice (**Figure 41B**). Accumulation decreases in the adipose may contribute to the lower insulin levels detected in HFD+CA mice (**Figure 40B**).

Moreover, CA treatment has previously been reported to significantly reduce MCP-1, IFNγ, and TNFα (48; 64; 79; 644), which serve to attract and activate macrophages. In the HFD model of type 2 diabetes, similar mechanisms may be occurring following redox modulation.

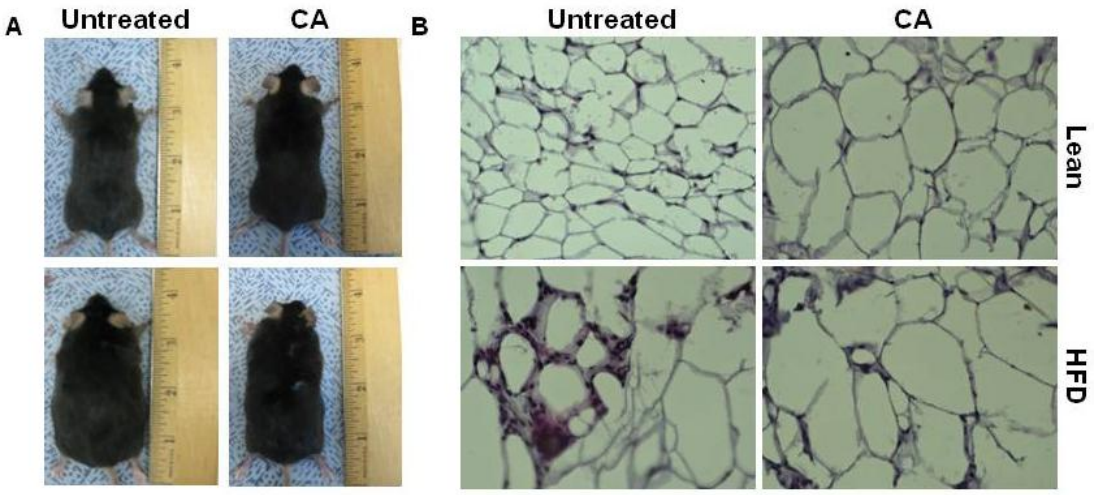


Figure 41: CA treatment reduces immune cell infiltration in the adipose tissue. (A) Upon sacrifice, mice were photographed to visualize obesity. (B) Visceral fat was removed from each mouse, fixed in 10% formalin, and embedded in paraffin. Fat sections were cut at 0.7 µm and stained with hematoxylin and eosin (H&E). Sections were visualized with a 20X objective. Histology representative of 5-6 mice/group.

4.4.6 Redox modulation reduces liver steatosis in HFD mice

Liver, the site of gluconeogenesis and glycogen storage, regulates hepatic glucose production. Insulin inhibits the release of FFA from adipocytes. In the context of insulin resistance, lipolysis from the adipose tissue liberates FFA, which can then be stored in peripheral sites, such as the liver, causing steatosis or fatty liver. Immediately after sacrifice, liver was weighed from all mouse groups. CA treatment significantly reduced liver weights in comparison to untreated HFD mice (**Figure 42A**). Lean and Lean+CA liver weights were both decreased compared to HFD fed mice, although CA treatment also significantly reduced Lean liver weights in comparison to untreated Lean mice. Liver sections were also visualized for fat droplets by H&E staining. CA

treatment diminished fat accumulation in HFD mouse livers in comparison to untreated HFD mice (**Figure 42B**). Sections of Lean and Lean+CA did not display significant differences in fat droplets by microscopy. Instead, lowered liver weights in Lean+CA treated mice suggests that redox modulation not only lowers liver steatosis but may also reduce other accessory cell infiltrate; however, reduced weight was not due to liver dysfunction or disease, as no toxicity was detected in any mouse group (**Table 1**).

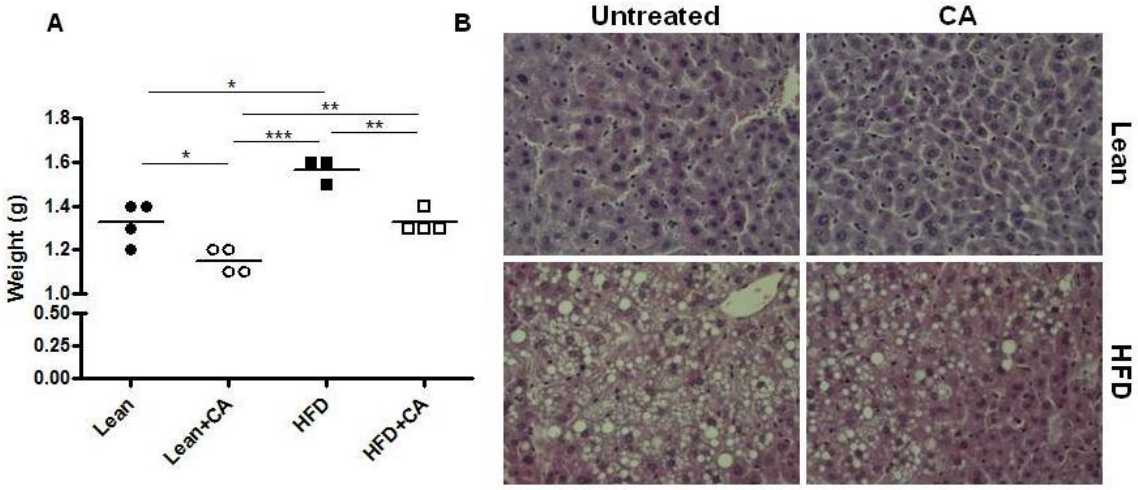


Figure 42: Liver steatosis is diminished following CA treatment of HFD mice. (A) After sacrifice at 12 weeks, livers were removed and weighed. n=3-4 mice/group, **p<0.05, **p<0.005, ***p<0.0005*. (B) Livers were fixed in 10% formalin, embedded in paraffin, and 0.5 µm sections were stained with H&E. Sections were visualized with a 20X objective. Histology representative of 3-4 mice/group.

4.4.7 CA administration enhances liver IL-6 production coincident with macrophage infiltrate in HFD mice

IL-6 is thought to contribute to the inflammatory environment that promotes insulin resistance in type 2 diabetes, although IL-6 has also been characterized as a necessary cytokine for the acute phase response, which helps to resolve tissue damage and assuage insulin resistance (337). After 12 weeks of HFD administration, CA treatment augmented liver IL-6 production in comparison to untreated HFD mice (**Figure 43**). Notably, IL-6 was also enhanced in Lean+CA treated mice

compared to HFD mice. Conversely, no differences were detected in Lean versus Lean+CA treated mice. These data indicate a positive role for IL-6 after redox modulation in both HFD and Lean mice. IL-6 may correlate with the lower fasting blood glucose, fasting serum insulin, adipose infiltrate, and liver steatosis in HFD+CA treated mice.

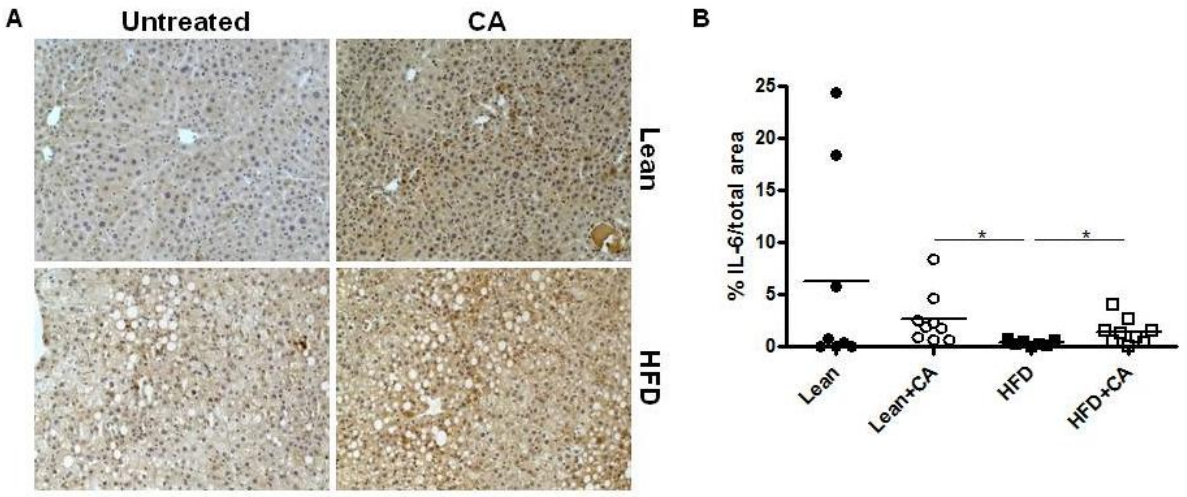


Figure 43: Liver IL-6 is augmented in CA treated mice. (A) After sacrifice at 12 weeks, livers were fixed in 10% formalin, embedded in paraffin, and 0.5 µm sections were stained with IL-6 and counterstained with hematoxylin. Sections were visualized with a 20X objective. Histology representative of 3-4 mice/group. (B) Quantification of %IL-6/total measured area from n=3 images/mouse/group. *$p<0.05$.

In correlation with IL-6 levels, macrophage infiltrate into the liver was determined by F4/80 histological staining. CA-treated Lean and HFD mice exhibited the highest amounts of macrophage infiltrate compared to untreated animals (**Figure 44**). Macrophages are a known source of IL-6 production, making them a plausible candidate for the enhanced IL-6 production following CA treatment (**Figure 43**). Additionally, hepatocytes can also produce IL-6 to instigate tissue repair. The contribution of both cell types may synergize to reduce liver steatosis and fasting blood glucose levels.

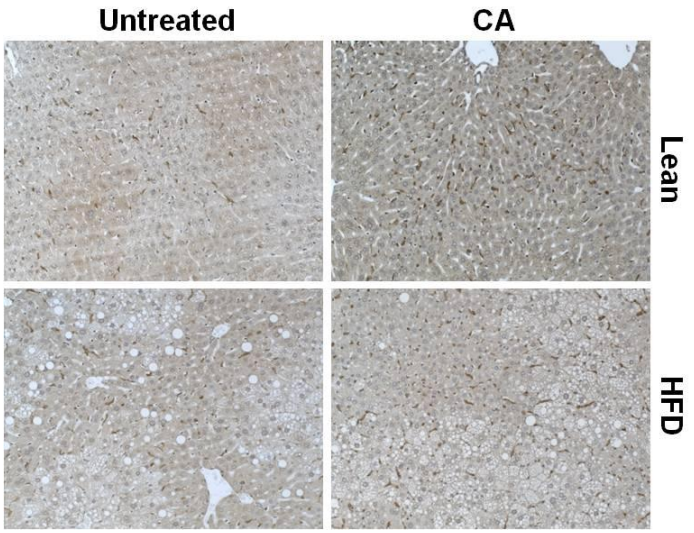


Figure 44: Macrophage infiltrate into the liver is enhanced following CA treatment. After sacrifice at 12 weeks, livers were fixed in 10% formalin, embedded in paraffin, and 0.5 µm sections were stained with F4/80 and counterstained with hematoxylin. Sections were visualized with a 20X objective. Histology representative of 3-4 mice/group.

4.5 DISCUSSION

Obesity-induced type 2 diabetes and its complications are a widespread problem amongst U.S. individuals as well as a cost burden on the healthcare economy. Treatment options to reduce the risk of insulin resistance and/or diabetic complications are of major importance. During the course of chronic high fat intake, glycemic control may fail as a result of compounded peripheral insulin resistance. The mechanisms triggering insulin resistance include oxidative stress and inflammatory-mediated cellular infiltrate, signaling defects, and reduced glucose uptake. Specifically, the liver and adipose tissue are two sights of abnormal lipid deposition and immune cell infiltrate. We sought to determine whether redox modulation using a metalloporphyrin catalytic antioxidant could assuage liver and adipose complications as well as enhance insulin sensitivity and glucose tolerance.

Early in the course of HFD chow administration, CA treatment was able to delay weight gain in comparison to untreated mice. This result was not due to toxicity of the CA, as multiple diagnostic parameters displayed no hepatoxicity, nephrotoxicity, or pancreatic toxicity. However, leptin levels were also reduced in redox modulated HFD mice after 5 weeks of chow. In order to signal to the body that satiety has been met, leptin signals through leptin receptor on neuronal tissues, regulating appetite and body weight. In obesity-induced type 2 diabetes, leptin signaling is thought to be defective, mimicking what occurs with insulin resistance. Specifically, leptin signals through PI3K, like insulin, and oxidative stress-mediated inhibition of this pathway may contribute to leptin resistance (732). Additionally, ROS can decrease IRS protein levels after chronic exposure (716), and deletion of IRS2 resulted in leptin resistance in a mouse model (733). Under fasting conditions, elevated serum leptin levels are proportional to the mass of adipose tissue and indicate obesity-induced leptin resistance (734; 735). Therefore, our treatment can delay weight gain early after chow administration, potentially as a result of lowered leptin concentrations.

Notably, redox modulation was able to reduce fasting blood glucose levels, but could not alter glucose intolerance during an IPGTT. This result is in line with early type 2 diabetes development, where normoglycemia is detected under fasting conditions, but postprandial hyperglycemia manifests (700). Although the IPGTT was conducted at 10 weeks post chow administration, a delay in type 2 diabetes is likely based on the delay in weight gain and leptin resistance detected early in the course of CA treatment. Additionally, fasting serum insulin levels were significantly decreased in CA-treated HFD mice at both 10 and 12 weeks. Despite the failed glucose tolerance test, redox modulation was able to maintain insulin sensitivity in HFD mice. This regulation may be due to (A) an inhibition of stress kinases such as JNK, which

would improve insulin signaling; (B) a reduction in proinflammatory cytokine production; and/or (C) a decrease in beta cell mitochondrial dysfunction. We previously demonstrated that CA diminishes TNFα, and MCP-1 levels through the inactivation of NF-κB (48; 64; 79). Furthermore, CA can improve glucose metabolism (**Figure 34**) by reducing ROS damage of mitochondrial enzymes and thereby enhance the efficiency of the TCA cycle. All of these improvements would allow for both healthier beta cell function and better insulin signaling, ultimately lowering hyperinsulinemia in HFD mice.

The generation of greater adipose tissue causes the secretion of more proinflammatory adipokines, and macrophage infiltration into the adipose tissue increases with the severity of obesity (736). MCP-1, for example, is significantly increased in adipose and in circulation following HFD-induced obesity (737). This chemokine serves to attract macrophages into the adipose and under conditions of adipocyte stress and necrosis, macrophages display a 'crown' arrangement surrounding fat cells, suggesting increased phagocytic activity towards dying cells (738). Moreover, depletion of T_H1 CD4+ cells or CD8+ cells ameloriates systemic insulin resistance by decreasing macrophage infiltration into adipose tissue (503; 505). CA has previously been shown to not only decrease MCP-1, but also reduce CD4 T cell proliferation and IFNγ secretion (644), which is known to trigger macrophage activation. The reduction of infiltrate detected in the fat of HFD+CA treated mice may therefore result from diminished chemoattractant and/or accessory cell-mediated macrophage localization and activation.

As adipose expands, an increase in FFA release can instigate peripheral accumulation in the liver. In correlation with reduced infiltrate into the adipose, CA treatment may reduce the stress and MCP-1 secretion from the adipose, which in turn, would diminish lipolysis and hepatic steatosis in comparison to untreated HFD mice (737). Fat deposition in the liver can be attributed

to several mechanisms. One known regulator liver triglycerides is sirtuin 6 (SIRT6). In human fatty livers, SIRT6 levels are low (739), indicating a need for this molecule to decrease fat accumulation. Similarly, signaling through JNK1 is able to protect against liver steatosis (740). An accretion of long-chain polyunsaturated fatty acids within the liver is known to regulate the metabolism of lipids, and in steatosis, these beneficial fatty acids are also down, causing a reduction in beta oxidation as well as Glut4 receptor expression (741). Hepatic SREBP-1c is responsible for lipogenesis, and in both mouse models of obesity and human patients, SREBP-1c expression is increased (742; 743). Additionally, the buildup of FFA in the liver, through either lipogenesis or adipocyte lipolysis, stimulates ROS production and mitochondrial dysfunction, further promoting systemic insulin resistance (744). All of these factors are possible targets of redox modulation and delineating their involvement in reducing steatosis will be investigated in future studies.

Our most intriguing observation in this study was the augmentation of IL-6 production in CA-treated HFD and Lean mice. Although IL-6 is thought to be detrimental by feeding in to the inflammatory environment during insulin resistance, other studies also indicate a beneficial role for the cytokine. IL-6-/- mice, for example, spontaneously become obese (496), and ablation of IL-6 in the liver exacerbates obesity-induced insulin resistance (745). As a possible mechanism of these results and our observations, IL-6 contributes to the acute phase response (746) for wound healing (746). NF-κB-dependent cytokines, such as IL-1β, IL-6, and TNFα, constitute the APR (747), which helps to resolve inflammation by driving tissue repair as well as hepatocyte growth factor (HGF) production. HGF mimics insulin signaling through PI3K-Akt activation and suppresses NF-κB activation through GSK3β inactivation (748; 749). HGF is therefore a potent anti-inflammatory molecule. Our group has demonstrated that IL-6 can induce HGF for

resolution of inflammation (337), suggesting its protective role for liver repair after hyperglycemia and lipid accumulation in type 2 diabetes. Moreover, IL-6 is thought to regulate secretion of glucagon-like peptide-1 (GLP-1), a digestive protein necessary for stimulating beta cell insulin secretion. In obesity, IL-6 enhances GLP-1, which in turn augments insulin secretion to try and prevent diabetes onset (750). A fine balance of IL-6 can therefore suppress hepatic inflammation and improve systemic insulin sensitivity. The relative production of IL-6 from both hepatocytes and F4/80+ macrophages will need to be further investigated to determine the dominant source in CA-treated HFD livers. IL-6 elevation upon redox modulation of HFD fed mice may then correlate with the early acute phase response, essentially attempting to restore liver functionality and delaying fulminant diabetes onset.

Antioxidant administration is warranted as a means of reducing inflammation and secondary oxidative stress for control of type 2 diabetes pathology and complications. Regulating inflammation is thought to benefit insulin-stimulated glucose uptake at peripheral sites. High-density lipoprotein (HDL) ("good cholesterol") and glutathione, both of which have inherent antioxidant properties, are impaired in type 2 diabetes (751-754). These results highlight the importance of reinstating antioxidant levels for control of blood glucose and prevention of diabetic complications. Antioxidants such as resveratrol, inhibit NF-κB activation in adipocytes, whereas epigallocatechin gallate, an antioxidant found in green tea, enhances glucose uptake in adipose tissue (755). Moreover, salsalate treatment, a form of salicylate, improves glucose tolerance, and reduces circulating FFA through inhibition of NF-κB (756). Antioxidant administration can not only improve insulin sensitivity, but can also delay or decrease diabetic complications. HDL, as mentioned above, can act as an antioxidant and anti-inflammatory, preventing diabetes-induced atherosclerosis (752). Indirect elevation of HDL, through the usage

of statins, is a potential therapy for reducing risk of long-term complications (757). Similarly, administration of exogenous glutathione was able to reduce ICAM-1 levels, an important endothelial marker of inflammation, and subsequently, lower vascular complications (758). Evidence suggests that scavenging ROS, through the usage of Vitamin E for example, is not potent enough to impede diabetic complications; instead, SOD and/or catalase mimics hold more potential for improving mitochondrial function and lowering DNA damage in the context of type 2 diabetes (759), justifying the use of CA treatment.

Through redox modulation of HFD mice, we observed delayed obesity and reductions in fasting blood glucose, insulin, and leptin levels. Furthermore, liver and adipose lipid accumulation and cellular infiltrate, respectively, were also decreased after CA treatment, indicating greater systemic insulin sensitivity and lowered inflammation. Augmented IL-6 production in the liver is a potential mechanism of redox-modulated steatosis prevention and may be a direct consequence of the acute phase response. CA treatment is therefore a potential therapeutic strategy for impeding type 2 diabetes pathogenesis and reducing chronic diabetic side effects.

5.0 CONCLUSIONS AND FUTURE DIRECTIONS

5.1 CONCLUSIONS

An imbalance of ROS and antioxidant levels generates oxidative stress. In autoimmune type 1 diabetes and obesity-induced type 2 diabetes, high ROS triggers heightened proinflammatory responses, which can manifest into beta cell dysfunction or ablation. To first test whether control of ROS can overcome self-reactivity, a catalytic antioxidant was used in diabetogenic murine models. Specifically, CA was able to delay type 1 diabetes onset through blocking T_H1 responses. The mechanism of CA-mediated regulation involved reducing activation of TACE, a redox-dependent metalloprotease found in immune cells. Subsequently, the TACE ligand, LAG-3, displayed decreased shedding from CD4+ T cells. As a negative regulator of T cell activation, the retention of LAG-3 was then able to inhibit autoreactive T cell stimulation and effector function, contributing to the delayed diabetes observation. Moreover, soluble LAG-3 can be utilized as a serum biomarker of diabetes progression in mice. Soluble LAG-3 may also serve as a biomarker of disease risk in first-degree relatives of type 1 diabetes patients a priori of autoantibody detection.

The effects of CA on immune cell metabolism were next assessed to identify a second layer of autoreactive regulation and to better understand the limitation of redox modulation. CA treatment prevented uncoupled respiration of diabetogenic splenocytes, resulting in lowered

experimental oxidative phosphorylation. Additionally, aerobic glycolysis, often utilized by tumor cells and termed the 'Warburg effect', was also reduced following CA treatment. These observations suggested improved metabolic efficiency, yet no enhancement of autoreactive immune cell activation, contributing to weakened diabetogenic potential. Importantly, CA treatment does not impart long-term side effects, as the inactivation of immune cells was reversible.

The ability of CA to affect metabolism led to a deviation from autoimmunity and experimentation in a high-fat diet model of type 2 diabetes. As a final objective, redox modulation of obesity-induced inflammation, insulin resistance, and diabetic complications was characterized. CA treatment delayed early weight gain in correlation with reduced serum leptin levels. Additionally, CA effectively lowered fasting insulin and blood glucose levels throughout the course of the study; however, this effect was not sustained postprandially. Redox modulation did decrease liver steatosis and immune cell infiltrate into adipose tissue. Notably, IL-6 was enhanced in the liver after CA treatment. This result is likely attributed to an increase in the acute phase response for liver repair, suggesting an overall delay in the chronic damage observed with a loss of glucose tolerance.

Overall, CA can impair autoreactive immune cell activation through (1) reduced metalloprotease-dependent shedding of LAG-3; (2) decreased bioenergetics and subsequent metabolic quiescence; and (3) from previous studies, inhibition of NF-κB activation. Effects on oxidative stress and inflammation in autoimmunity transcend into alleviation of insulin resistance and glucose tolerance in non-autoimmune type 2 diabetes. Therefore, redox modulation can affect cellular function through immunomodulatory, anti-inflammatory, and cytoprotective pathways, which culminate with negative regulation of Warburg effect

characteristics. This opens up redox modulation as a potential therapeutic for those pathological conditions that have an inflammatory component, such as type 1 and type 2 diabetes.

5.2 FUTURE DIRECTIONS

Several future studies will need to be conducted in order to further develop the work of this thesis project. As a follow-up to Chapter 2, the importance of LAG-3 signaling in T cells and on APC activation will be deduced. LAG-3-/- mice are currently housed at the University of Pittsburgh animal facility. As expected, *in vitro* stimulation of these splenocytes and *in vivo* immunization of these mice results in enhanced T_H1 responses (**Figure 45**). Delineation of the signaling cascade downstream of LAG-3 will enable a better understanding of the molecule's role in prevention of T cell activation and will allow for establishment of targeted molecular agonists (or antagonists, for chronic infection and cancer). Moreover, the importance of soluble LAG-3 in APC activation is also currently undefined. Surface LAG-3 interaction with MHC class II will stimulate ERK-mediated SHP-1 recruitment and inhibit DC activation (371). However, studies using non-physiological sLAG-3, such as recombinant fusion sLAG-3 or LAG-3-Ig, demonstrate activation of DCs (760-762). Therefore, delineating the physiological role of sLAG-3 is necessary. Experiments utilizing bone marrow-derived macrophages and dendritic cells in transwell cocultures with LAG-3-/- or WT T cells may facilitate greater comprehension of this biological mechanism. To definitively assess the ability of soluble LAG-3 to serve as a biomarker for type 1 diabetes risk in first-degree relatives, greater numbers of human serum samples need to be obtained and tested. Furthermore, correlations between the

number of autoantibodies and soluble LAG-3 levels must be concretely established in a larger sampling population.

For the metabolic studies in Chapter 3, more work needs to be done to verify the enhanced efficiency of the TCA cycle following CA treatment. Enzymes important for glucose metabolism, such as aconitase, may first be measured. NADPH and glutathione levels can also be quantified to determine any deviation towards the pentose phosphate pathway after CA administration. Other experiments that should be incorporated into this project are a full-body glucose uptake study using a PET scan, for assessing global glucose metabolism efficiency, and measurement of ATP levels, to characterize final energy output. These studies would augment evidence of metabolic quiescence. Reversal of the effects imparted by CA treatment was demonstrated by both a recovery of IFNγ secretion and diabetes onset after CA administration was stopped. Short-term CA treatment may thus reset immune cell homeostasis, necessitating long-term use of the agent or a combinatorial therapy approach for complete autoreactive prevention. A range of other agents, such as rapamycin, will be administered along with CA to define appropriate kinetics, duration, and long-term outcomes in protection from type 1 diabetes.

The high-fat diet study reported in Chapter 4 requires the most persistence to confirm the mechanisms behind the observed results. First, the enhanced IL-6 after CA treatment needs to be attributed to the hepatocytes and/or macrophages. Co-immunostaining will be conducted on liver sections. Liver lysates must also be tested for: 1) anti-inflammatory HGF levels, as IL-6 is known to increase HGF for tissue repair, reconciling the contradictory cytokine augmentation and 2) the PI3K-Akt pathway constituents, as insulin signaling and sensitivity relies on these specific molecules. In addition, another HFD vs. HFD+CA study is currently underway, and the same outputs will be measured as above. However, several other experiments will also be

performed prior to and after sacrifice. Most importantly, IPGTTs and an insulin tolerance test will be conducted. Although the IPGTT from Chapter 4 showed no improvement of glucose tolerance after CA treatment, a caveat exists in the measurement technique. Mice were initially injected with 2.0 g/kg of a glucose solution, causing a spike in blood glucose that was out of range (>600) of the glucometer. As a means of bypassing this restriction, glucose will be administered at a lower dose (1.0 g/kg). At 5 weeks of HFD chow, the new dose of glucose exhibited measurable differences in HFD vs. HFD+CA mice (**Figure 46**), which correlates better with the reduced fasting blood glucose and insulin observed in Chapter 4. Moreover, an insulin tolerance test will be conducted to confirm enhanced insulin sensitivity after treatment. Before sacrifice, the difference in lean and fat mass will also be measured in these animals, as the total weight gain did not vary between groups in the reported findings. Total HDL ("good cholesterol") will also be assessed from serum. Finally, insulin signaling with or without CA treatment will be studied more in-depth using HepG2 cells in culture. These experiments should illuminate observations and provide further support for the use of CA in type 2 diabetes treatment.

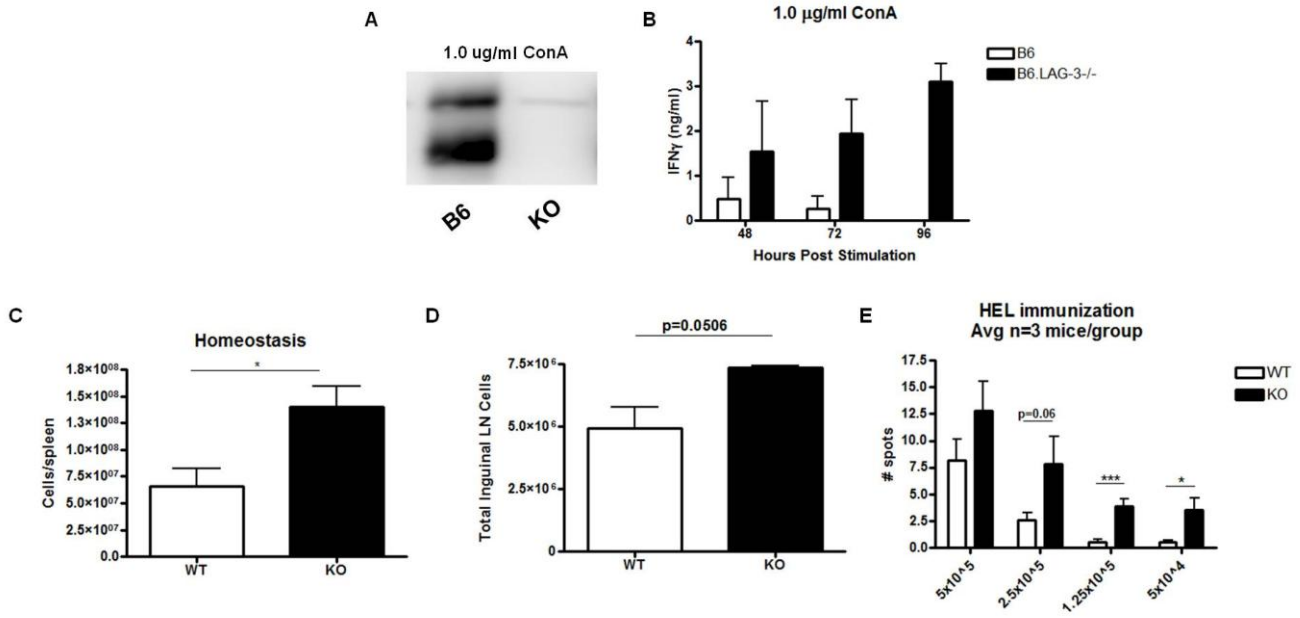


Figure 45: LAG-3-/- mice exhibit enhanced T_H1 responses. (A) Whole cell lysates from B6 WT vs. KO splenocytes stimulated with 1.0 µg/ml ConA for 72h were probed for LAG-3. (B) Splenocytes were stimulated with 1.0 µg/ml ConA, and supernatants were collected at 48-96h. IFNγ was measured by ELISA. n=2 independent experiments. (C) Spleens were harvested at homeostasis and cells were quantified by trypan blue exclusion. (D-E) Mice were immunized s.c. at the base of the tail with HEL in CFA. Draining inguinal lymph node cells were quantified and titrated in an IFNγ recall ELISPOT assay at 8 days post-immunization. n=3 mice/group. **p<0.05, ***p<0.0005*.

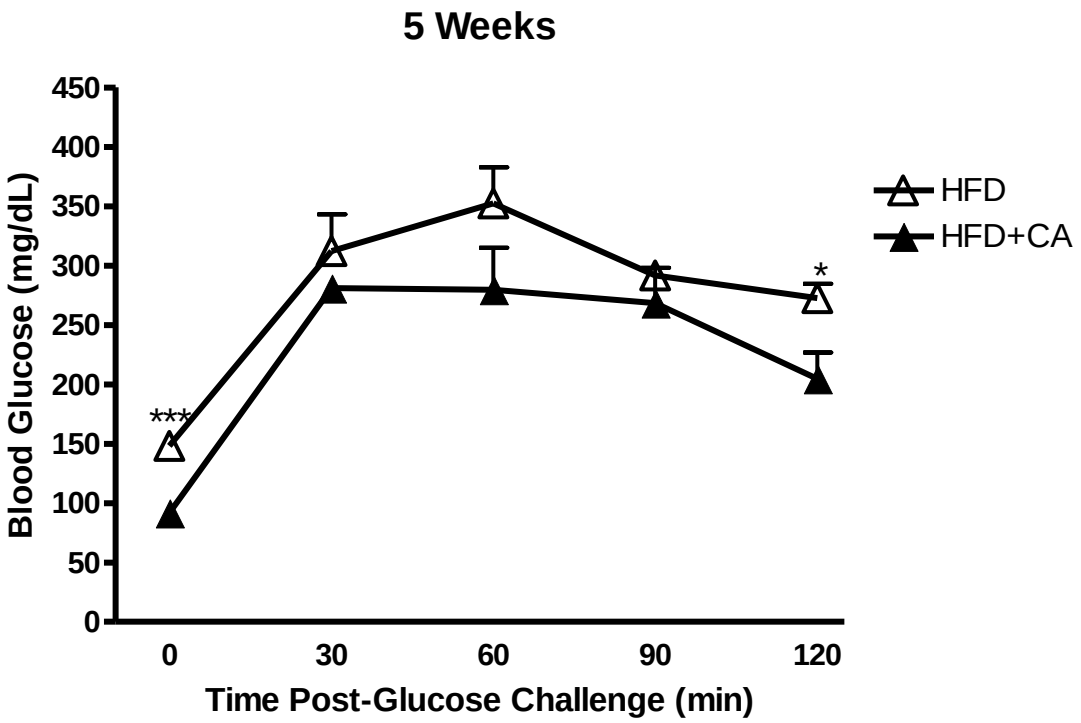


Figure 46: Glucose tolerance is improved after CA treatment. An IPGTT was conducted on mice fed HFD or HFD+CA for 5 weeks. Mice were fasted overnight beforehand and serum was obtained at t=0 for basal glucose levels. Glucose was injected at a dose of 1.0 g/kg and blood glucose was measured every 30 min until 120 min post-glucose challenge. n=4 mice/group. **p<0.05, ***p<0.005*.

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