MECHANISMS GOVERNING THE BIOENERGETICS OF NAÏVE AND EFFECTOR CD4^{+} T CELLS AS A MEANS OF CONTROLLING AUTOIMMUNITY

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Dana M. Previte, Ph.D.

University of Pittsburgh, 2017

CD4$^+$ T cells are a critical component of the adaptive immune system as they generate large amounts of cytokines that help shape the immune milieu. Additionally, they are the primary contributors to the immunopathology exhibited in Type 1 Diabetes. The field of immunometabolism has elucidated that the cellular metabolic profile of immune cells has a significant impact on their function, and ultimately the fate of the overall response. Naïve CD4$^+$ T cells rely primarily on mitochondrial oxidative phosphorylation, but upon antigen encounter, reprogram their metabolism to aerobic glycolysis. Understanding the mechanisms that govern these programs could be critical in developing new therapies for limiting aberrant T cell responses in autoimmunity. Here we examined the contributions of two different molecules, Lymphocyte Activation Gene 3 (LAG-3) and reactive oxygen species (ROS) in controlling T cell metabolism. LAG-3 is an inhibitory receptor expressed on the surface of CD4$^+$ T cells, and deficiency in naïve T cells leads to enhanced homeostatic expansion. Our results indicate that LAG-3 expression on naïve CD4$^+$ T cells serves to restrain cellular metabolism and mitochondrial biogenesis as a means of maintaining quiescence. These results are compelling as loss of LAG-3 expression in a model of Type 1 Diabetes results in accelerated disease progression, potentially due to T cell metabolic enhancements, as our data would suggest. Single nucleotide polymorphisms in the LAG-3 gene have also been linked to autoimmune disease

susceptibility. With regards to ROS, Type 1 Diabetes is known to be highly driven by oxidative stress, and $CD4^+$ T cells require acute doses of ROS to drive optimal activation. Therefore, we sought to understand if ROS signaling contributes to the metabolic transition that occurs during T cell activation. Indeed, ROS inhibition resulted in reduced mTOR signaling and aerobic glycolysis. Altering metabolism in this manner also delayed Type 1 Diabetes progression in an adoptive transfer model of disease. Collectively, this work demonstrates that both LAG-3 and ROS regulate $CD4^+$ T cell metabolism, which, in turn, greatly impacts T cell activation potential and ability to drive disease.

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PREFACE

"Don't give up, don't ever give up."

- Coach James "Jimmy V" Valvano

Growing up in the Previte household, sports were always a constant, whether it was cheering on a Pittsburgh sports team or watching the sheer excitement of March Madness. This quote by Coach Valvano has always been one that resonated with me, as it was so poignant given his unwavering optimism and courage in the face of cancer. Graduate school has been quite the rollercoaster ride, and there were many times where giving up would have been easier than staying the course. However, along with this quote, some very special people helped me to persevere and finally reach my goal.

First I would like to thank Dr. Jon Piganelli for taking a chance on me, and allowing me to join his laboratory. You have allowed me to learn and think on my own and gave me the reigns to drive this work myself. For that I am truly grateful. I hope you feel that the gamble paid off. To the Piganelli lab members, both past and present, thank you for all the help and laughter along the way, especially when things were not working scientifically. Specifically, I would to thank Drs. Gina Coudriet and Meghan Marre. You ladies have helped me so much in navigating my way through graduate school with your wisdom and experience. Also, you have

been excellent examples of women scientists and mothers. I am so proud to not only call you my colleagues, but also my friends.

To my wonderful grandparents, Martin and Margaret Sholtis, and Peter and Imogene Previte, for instilling in me that a strong work ethic can help you achieve your goals. They worked hard to provide better lives for their children and grandchildren, and it is through their drive and determination that I have been blessed with the opportunities I have had. Moreover, they taught me the importance of education, and were always a source of unconditional love. Sadly, I lost two of my grandparents during this process, and my only regret is that they are not here today to see this dream come to fruition.

To my sister, Marissa Previte, thank you for being my roommate, running partner, and fellow Housewives lover. It has been great growing into adulthood with you over the past five years, and you have been a great source of levity, love, and fun. I am proud of how close we have become.

Next, I would like to thank my parents, Mark and Laverne Previte. Thank you, Mom, for being the glue that holds this family together. Your strength and love are unmatched by anyone I know. Growing up, you showed me that a woman can be a successful professional, mother, and wife, and I could not have asked for a better female role model. I hope I can be at least half as successful as you have been. To my dad, the first Dr. Previte – thank you for paving the way for me in academia, but also allowing me to blaze my own trail into science. I can honestly say that you were the first person to teach me how to think critically, whether that was discussing politics, curriculum, or zone versus man-to-man defense. I cannot thank both of you enough for being constant sources of love and support.

To my wonderful husband, Dr. Matthew Brown. I do not think any of this would have been possible without you. You have seen me at my best and my worst, and have loved and supported me regardless. You are one of the most driven and intelligent people I have ever met, and I am so lucky to have you. Thinking back, I am so thankful for that first BGSA happy hour where we met, and I look forward to what the future holds for us. I love you so much.

Lastly, I would like to end with this:

> "…And to all the little girls who are watching this, never doubt that you are valuable and powerful and deserving of every chance and opportunity in the world to pursue and achieve your own dreams."
>
> --Hillary Rodham Clinton

This is for those girls, as I was once one of them.

ABBREVIATIONS

2-DG – 2-Deoxyglucose

2-NBDG - 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2

7AAD – 7-Aminoactinomycin D

ADAM10 - A disintegrin and metalloproteinase domain-containing protein 10

ADAM17 - A disintegrin and metalloproteinase domain-containing protein 17

AMPK – AMP-activator protein kinase

AntA – Antimycin A

APC – Antigen presenting cell

APC/C – Anaphase promoting complex cyclosome

ASCT2 – Alanine-Serine-Cysteine Transporter 2

ATP – Adenosine triphosphate

Bcl-2 – B cell lymphoma 2

Cdh1 – Cadherin 1

CFSE – Carboxyfluorescein succinimidyl ester

CHgA – Chromagranin A

CTLA-4 – Cytotoxic T lymphocyte antigen 4

DC – Dendritic cell

DHE - Dihydroethidium

DN – Double negative

DUOX – Dual oxidase

EAE – Experimental autoimmune encephalomyelitis

ECAR – Extracellular acidification rate

ETC – Electron transport chain

FCCP - Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone

FOXO – Forkhead box O

FoxP3 – Forkhead box P3

Fyn - Proto-oncogene tyrosine-protein kinase

HIV – Human immunodeficiency virus

HK2 – Hexokinase 2

IFNγ – Interferon gamma

IL-1β - Interleukin 1β

IL-2 – Interleukin 2

IL-7 – Interleukin 7

IL-10 – Interleukin 10

IL-15 – Interleukin 15

JAK3 – Janus kinase 3

KLF – Kruppel-like factor

LAG-3 – Lymphocyte activation gene 3

LAT – Linker for activation of T cells

LCMV – Lymphocytic choriomeningitis virus

Lck – Lymphocyte-specific protein tyrosine kinase

LDHA – Lactate dehydrogenase A

MAPK – Mitogen activated protein kinase

Mcl-1 – Induced myeloid leukemia cell differentiation protein 1

mDNA – Mitochondrial DNA

MHC – Major histocompatibility complex

MLR – Mixed lymphocyte reaction

MnP – Manganese metalloporphyrin; MnTE-2-PyP^{5+}

MnSOD – Manganese superoxide dismutase

MOTC – Microtubule-organizing center

mROS – Mitochondrial reactive oxygen species

MS – Multiple Sclerosis

mTOR – Mammalian target of rapamycin

NAC – N-acetylcysteine

NADPH – Nicotinamide adenine dinucleotide phosphate

nDNA – Nuclear DNA

NF-κB – Nuclear factor kappa B

NK – Natural Killer cell

NO – Nitric Oxide

NOD – Non-obese diabetic

NOX – NADPH oxidase

OCR – Oxygen consumption rate

Oligo - Oligomycin

OXPHOS – Oxidative Phosphorylation

PD-1 – Programmed cell death-1

PFKFB3 - 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3

PI – Propidium iodide

PI3 – Phosphoinositol 3

PKC – Protein kinase C

PMA - Phorbol 12-myristate 13-acetate

pS6 – Phospho-S6 ribosomal protein

PTEN – Phosphatase and tensin homolog

RA – Rheumatoid arthritis

RAG-1 – Recombination-activating gene-1

RNS – reactive nitrogen species

ROS – reactive oxygen species

Rot - Rotenone

SCID – Severe immunocompromised deficiency

SEB – *Staphylococcus aureus* Enterotoxin B

Sirt-1 – Sirtuin-1

sLAG-3 – Soluble lymphocyte activation gene 3

SLE – Systemic Lupus Erythematosus

SNAT1 – Sodium-coupled neutral amino acid transporter 1

SNAT2 - Sodium-coupled neutral amino acid transporter 1

SNP – Single nucleotide polymorphism

SOD – Superoxide dismutase

SP – Single Positive

SRC – Spare respiratory capacity

STAT5 – Signal transducer and activator of transcription 5

T1D – Type 1 Diabetes

TCA – Tricarboxylic acid cycle

TCR - T cell receptor

T_{eff} – Effector T cell

TGFβ – Transforming growth factor beta

T1D – Type 1 Diabetes

TCA – Tricarboxylic acid cycle

Th1 – T helper cell type 1

Th2 – T helper cell type 2

TIL – Tumor infiltrating lymphocytes

TIM-3 – T-cell immunoglobulin domain and mucin domain

T_{mem} – Memory T cell

TNFα – Tumor necrosis factor alpha

T_{reg} – Regulatory T cell

UCP – Uncoupling protein

1.0 INTRODUCTION

1.1 TYPE 1 DIABETES

The immune system protects against pathogens, in part by distinguishing self from non-self. While this self/non-self recognition is usually maintained, in some instances the immune system can mount a response against self-antigens, leading to organ-specific autoimmunity. Tolerogenic mechanisms, including T regulatory cells, are in place to combat ensuing autoimmunity; however, these mechanisms can fail due to inherent dysfunction or unrestrainable inflammation. This subsequent autoimmune attack is responsible for numerous diseases including rheumatoid arthritis, multiple sclerosis, and type 1 diabetes, to name a few.

Specifically, type 1 diabetes (T1D) occurs when autoreactive T cells target pancreatic β cells for destruction. The loss of β cell mass and function results in reduced insulin secretion and diminished blood glucose regulation, culminating in hyperglycemia. By the time T1D patients present with hyperglycemia, 80-90% of the β cell mass has already been lost [1, 2]. Therefore, in order to maintain blood glucose levels, patients must administer exogenous insulin for the remainder of their lives. If poorly controlled, hyperglycemia can lead to multiple complications including cardiovascular issues, nephropathy, and retinopathy, to name a few. While insulin therapy is efficacious for most, there are a percentage of patients who cannot manage blood glucose with insulin alone, and islet transplantation has become a potential therapy for these

patients [3]. However, with transplantation, patients must be placed on rigorous immunosuppressive regimens to minimize islet graft rejection, and several of these therapies have been shown to have direct negative effects on β cells [4, 5].

CD4$^+$ and CD8$^+$ T cells contribute to the immunopathology exhibited in T1D and demonstrate islet infiltration [6-8]. Additionally, activated T cells mediate auto-autoantibody secretion by B cells [9, 10], further targeting islet β cells. While the initiating events that trigger immune activation against the β cell are not fully elucidated, it is well accepted that genetic and environmental factors may be responsible for promoting the initial break in tolerance. These factors include: viral infection [11], family history [12], ER stress [13, 14], chemical exposure [15], and pancreatic inflammation and ROS [16].

The most widely used animal model of T1D is the non-obese diabetic or NOD mouse, that spontaneously develops the disease, closely mimicking human progression [17-20]. With regards to T cell biology, the NOD.BDC-2.5 T cell receptor (TCR) transgenic animal has been most critical in further dissecting antigen-specific T cell responses relevant to T1D [21-23]. Here, all CD4$^+$ T cells recognize a peptide formed by covalent cross-linking of two β cell peptides: proinsulin and Chromogranin A (CHgA) [24]. Adoptive transfer of isolated BDC-2.5 T cells into NOD.*scid* recipients is sufficient for T1D induction; yet, very rarely do BDC-2.5 mice demonstrate T1D incidence themselves, owed to an inherent regulatory T cell population [25, 26].

1.2 METABOLIC PATHWAYS UTILIZED IN T CELLS

With the recent interest in immune cell metabolism, it has become better appreciated that cellular metabolic pathways are a contributing factor in dictating T cell function and fate. As the functions of naïve, effector, and memory T cells differ, so do their nutrient requirements, resulting in differential bioenergetic profiles. Two predominant metabolic pathways utilized by T cells are glycolysis and oxidative phosphorylation (OXPHOS) (Figure 1). Glycolysis is the breakdown of glucose to generate ATP and reducing equivalents that maintain cellular redox balance. Once glucose is transported into the cell, it undergoes multiple enzymatic reactions that ultimately result in the formation of two pyruvate molecules for each molecule of glucose [27]. At that point, the presence of oxygen dictates the fate of pyruvate. If oxygen is present, pyruvate will move into the mitochondria to support oxidative metabolism. Alternatively, in the absence of oxygen, pyruvate is converted to lactate that is secreted by the cell in a process known as anaerobic glycolysis [27, 28]. However, there are instances in which glucose is converted to lactate even in the presence of oxygen [27, 29-31]. This process, known as aerobic glycolysis, or the Warburg effect, is demonstrated by activated T cells [32-34].

If pyruvate is transported into the mitochondria, it is converted to acetyl-CoA and enters the tricarboxylic acid (TCA) cycle [28, 35]. Here, a series of chemical reactions occur that result in the production of more energy (ATP), reduction of NAD^+ to NADH, and release of carbon dioxide [36]. Glucose is not the only carbon source that can be utilized for acetyl-CoA generation and metabolism, as fatty acids and glutamine can also be shuttled into the TCA cycle.

The NADH produced by the TCA cycle is used to support the electron transport chain (ETC), also located in the mitochondria, thus coupling respiration with ATP synthesis [37]. The ETC is comprised of five mitochondrial proteins, complexes I-V, that drive electron movement

via redox reactions [35, 38, 39]. In addition, protons (H^+) are translocated from the mitochondrial matrix to the intermembrane space, creating a concentration gradient. Complex V, or ATP synthase, serves as a proton pump, moving protons down the concentration gradient back into the matrix, driving phosphorylation of ADP to ATP [40-42]. Oxygen serves as the final electron acceptor in the ETC generating superoxide (O_2^-), and is thus consumed by the process. Superoxide cannot easily cross membrane barriers; therefore, mitochondrial-expressed manganese superoxide dismutase (MnSOD) acts to dissipate superoxide to hydrogen peroxide (H_2O_2) as a means of eliminating the species [43, 44]. Alternatively, hydrogen peroxide is easily diffusible across membranes, and mitochondrial glutathione peroxidase and cytoplasmic catalase facilitate the decomposition of hydrogen peroxide to water and oxygen (O_2) [45]. These enzymes together serve as a means of protecting the cell against free radical damage.

Work in tumor and T cell biology have elucidated that aerobic glycolysis is the preferred pathway for highly proliferative cells [30, 31, 46]. This is in part due to glycolysis shuttling carbon molecules towards biosynthetic pathways that enable lipid membrane and nucleotide formation. Phosphorylated glucose (Glucose-6-phosphate) can either be shuttled through glycolysis or into the Pentose Phosphate Pathway (PPP), which aids in nucleotide synthesis (Figure 1) [47]. Additionally, citrate can be shuttled out of the TCA cycle to build lipids [27, 28]. Both metabolic shunts are upregulated in activated T cells to support daughter cell formation [34, 48, 49].

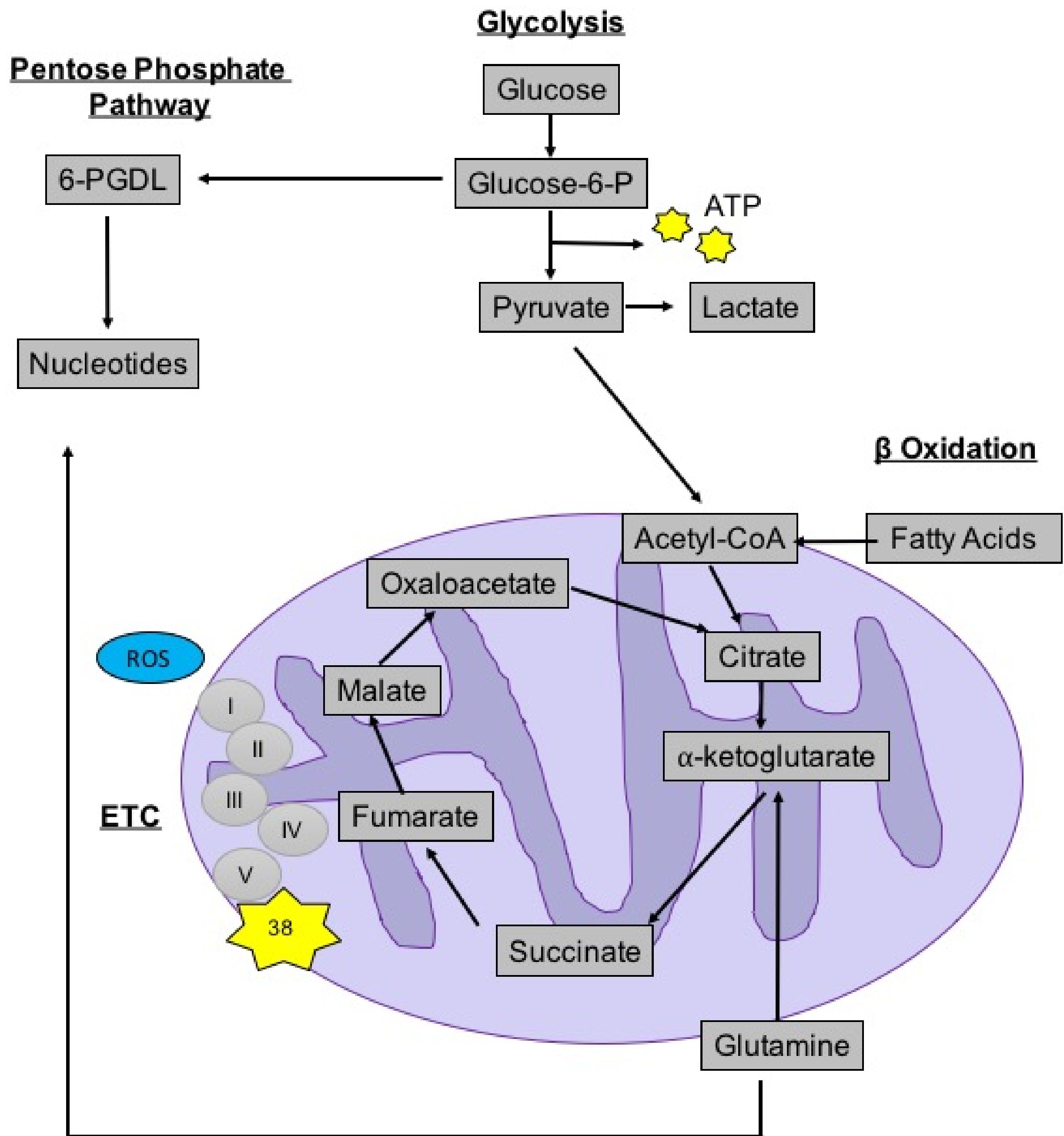

Figure 1. Metabolic Pathways in T cells.
T cells have been shown to utilize various metabolic pathways to generate ATP, drive proliferation, balance cellular redox or maintain longevity. Glycolysis and the Pentose Phosphate Pathway (PPP) take place in the cytosol, while β oxidation (Fatty Acid Oxidation) and glutaminolysis feed directly into the Tricarboxylic Acid Cycle (TCA) that occurs in the mitochondria. The Electron Transport Chain (ETC) also occurs in the mitochondria, resulting in ATP formation and oxygen consumption.

1.3 NAÏVE T CELLS

1.3.1 Actively maintained quiescence

Following development in the thymus, naïve T cells constantly traffic through the lymphatics as a means of immune surveillance. Therefore, naïve T cells must generate enough ATP to power cytoskeletal rearrangement for migration. To do so, they rely on oxidative metabolism, which provides maximal ATP production from nutrients such as glucose, glutamine, and fatty acids [48-50]. Naïve T cells are considered relatively quiescent as they demonstrate very low levels of homeostatic proliferation [51, 52]. With this, their biosynthetic needs are greatly reduced compared to actively proliferating cells, which is reflected in their considerably lower levels of glycolysis [48-50].

Studies examining naïve T cell quiescence have elucidated that it is a much more active process, rather than simply a default state [53]. Quiescence is dynamically preserved by a transcriptional program that not only upregulates quiescence-associated genes, but simultaneously inhibits activation genes. The FOXO (Forkhead box O) family of transcription factors are highly active in naïve T cells and other quiescent cell types, and regulate pathways associated with proliferation, apoptosis, and metabolism [54, 55]. With regard to proliferation, FOXOs stabilize expression of cell cycle inhibitors, including p27 and p21 [54, 55]. FOXO1 specifically enhances expression of Kruppel-Like Factors (KLFs), another family of quiescence regulators. In particular, LKLF is known to suppress Myc expression in T cells to control metabolism and cell cycle entry [56]. KLFs also promote expression of multiple homing molecules, including CD62L, which are necessary for T cell lymphatic trafficking [57]. More recently, Tsc1 (Tuberous sclerosis 1), a known tumor suppressor, was shown to actively suppress

mammalian target of rapamycin (mTOR) signaling in naïve T cells as a means of preserving quiescence [58]. Interestingly, loss of Tsc1 resulted in increased apoptosis of peripheral naïve T cells and defective antigen-specific responses *in vivo* [58]. This work highlights that alterations in T cell homeostasis can have dramatic effects on activation potential and antigen clearance.

Low-level T cell receptor (TCR) stimulation by self-peptides presented by major histocompatibility complex (MHC), or tonic signaling, is necessary for protection against apoptosis as TCR blockade during this process results in holes in the T cell repertoire [59-61]. T cells deficient in TCR signaling components like Fyn and Lck, demonstrated reduced survival [62-64], suggesting that the signals required by naïve T cells mimic those used to promote activation. Microarray studies comparing tonic signals to those from foreign antigens revealed similar gene expression profiles induced by both self and antigenic peptides [65]; however, the degree of induction was much lower in self-stimulated T cells. These results suggest that signaling to maintain homeostasis versus induce activation are the same, but it is the strength of signal that ultimately determines T cell fate.

1.3.2 Interleukin-7

As with all immune cells, growth factors and signaling are necessary for maintaining the immune system. For naïve T cells, Interleukin-7 (IL-7) plays a significant role in sustaining homeostasis and cellular metabolism [66-68]. IL-7 is produced by various non-hematopoietic cells including thymic epithelial cells, lymphatic endothelial cells [69], and fibroblastic reticular cells [70, 71]. IL-7 signals via the IL-7 receptor which is composed of the common γ chain subunit CD132 and the unique IL-7Rα (CD127) subunit. Global deletion of the IL-7R results in a severe combined

immunodeficiency (SCID) phenotype, further highlighting its requirement in T cell survival [72-74]. IL-7 promotes naïve T cell survival and population maintenance by mediating expression of the anti-apoptotic proteins, Bcl-2 [73, 75] and Mcl-1 [76], while simultaneously reducing expression of pro-apoptotic proteins Bid, Bim, and Bad [52, 77, 78]. IL-7 has been shown to be a critical growth factor for developing thymoctyes [79] and memory T cells [80-82] as well.

IL-7/IL-7R signaling is mediated by downstream activation of the JAK3/STAT5 and PI3K/Akt signaling pathways [68, 79]. Activation of STAT5 and Akt by phosphorylation leads to increased survival and glucose metabolism in naïve T cells [83, 84]. In several instances increased IL-7 signaling results in downregulation of surface and mRNA expression of IL-7R, suggesting a negative feedback loop to protect against over stimulation and breaks in quiescence [85]. This downregulation has also been suggested as a mechanism to prevent overutilization by specific T cell clones, resulting in atrophy of others, and thus creating a less diverse T cell population.

The PI3/Akt pathway regulates FOXO transcription factors via phosphorylation, resulting in translocation from the nucleus to the cytoplasm. Nuclear localization of FOXOs drives expression of the cell cycle inhibitors like p27 Kip1. Thus, inhibition of FOXOs by Akt via IL-7 signaling can lead to cell cycle entry and proliferation. The phosphatase PTEN regulates Akt activation [86] and is highly expressed in quiescent cells, including hematopoietic stem cells [87, 88] and naïve T cells [89]. PTEN-deficient T cells demonstrate extensive hyperproliferation and do not require IL-7 to maintain either proliferation or survival, due to constitutively active Akt [89, 90].

Expression and surface trafficking of the passive glucose transporter 1 (Glut1), which is essential for glucose uptake in T cells, increases upon IL-7 signaling, and knockdown of either

STAT5 or Akt reduces this response [68]. Interestingly, *in vitro* experiments where T cells were cultured in high or low glucose demonstrated that glucose concentration correlated with the degree of responsiveness to IL-7, indicating that glucose uptake aids in perpetuating IL-7 signaling [68]. The development of conditional deletion of the IL-7R in T cells allowed for further understanding of how IL-7 regulates naïve T cell metabolism and survival *in vivo* [91]. These studies indicated that loss of IL-7 signaling led to decreased cell size and frequency of both $CD4^+$ and $CD8^+$ T cells, which was due to reduced pSTAT5 signaling, glucose uptake, and cellular glycolysis [91]. While the main role of IL-7 is to maintain glycolysis in naïve T cells, there is some evidence that it also supports amino acid transport in $CD8^+$ T cells, which is necessary for T cell growth [92].

1.3.3 Homeostatic proliferation of naïve T cells

Naïve T cells are successful at maintaining steady numbers in the periphery. This sense of space is controlled by competition for survival signals from self-peptide/MHC and IL-7. Yet, low levels of homeostatic proliferation of naïve T cells do occur. Due to the utilization of IL-7 in the periphery, levels of this cytokine in lymphoreplete animals remain quite low [93]. During instances of lymphopenia, serum IL-7 levels increase due to lack of utilization, and can therefore trigger homeostatic expansion of the remaining T cells. This is exemplified by robust proliferative responses of naïve T cells adoptively transferred into Rag-deficient animals, whereas transfer into wildtype syngeneic animals results in very low, if any, proliferation. Several surface markers have been reported to alter homeostatic expansion such as CD24, which is necessary for proliferation [94] and B and T lymphocyte attenuator (BTLA) which inhibits

proliferative potential [95]. CD5 has also been identified as a marker of increased tonic signaling and proliferation on naïve T cells [96, 97]

Work by *Min et al.* further identified two different types of proliferative responses that occur upon T cell transfer into lymphopenic hosts. The slower, more limited proliferation was termed homeostatic, which was shown to be highly dependent upon IL-7 [98]. Alternatively, some T cells underwent more vigorous, IL-7-independent expansion, termed spontaneous [98]. These spontaneously proliferative T cells, while naïve in the sense that they have not experienced foreign antigen, demonstrated memory-like characteristics, including increased CD44 expression and a lowered activation threshold [99, 100].

1.4 T CELL ACTIVATION AND METABOLIC REPROGRAMMING

Upon TCR:peptide/MHC (Major histocompatibility) engagement, naïve T cells become activated, and with activation different functions ensue, including clonal expansion and effector function acquisition. With these new functions, the cellular metabolic profile of T cells also drastically changes [48, 49, 101]. Cellular division is an energetically demanding process, as the cell must replicate DNA and build new plasma membranes. To support the heightened level of macromolecule synthesis and ATP demand, T cells must transition from a slower, oxidative metabolism, to a faster program heavily dependent upon aerobic glycolysis (Figure 2). This metabolic transformation has been extensively characterized in tumor cells, and is often referred to as the “Warburg effect,” as it was first observed/described by Dr. Otto Warburg [31].

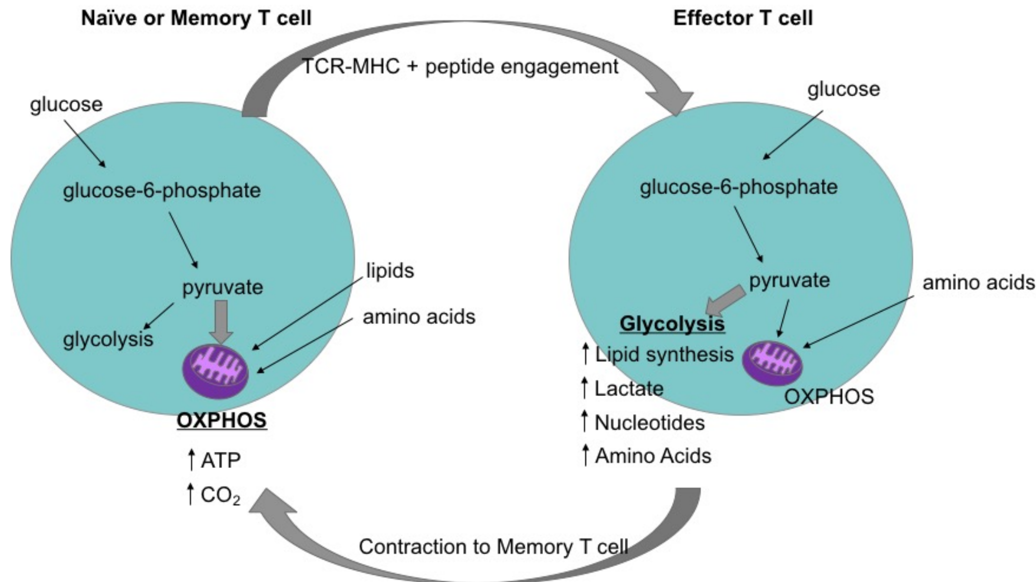

Figure 2. T cell metabolic reprogramming during the transition from naïve to effector and effector to memory.
Naive T cells are predominantly oxidative, breaking down glucose, fatty acids, and amino acids via the TCA cycle and electron transport chain. Antigenic stimulation results in a transition to aerobic glycolysis, where glucose is metabolized to lactate or shuttled into biosynthetic pathways. Contraction to a memory T cell population results in transition back to a more oxidative phenotype.

The metabolic reprogramming of activated T cells can be broken down into two stages: 1. cell growth and preparation for division, and 2. clonal expansion. During the early stages of T cell activation, mitochondrial metabolism is critical for supporting cellular growth in preparation for division. Specifically, calcium (Ca^{2+}) signaling mediated by TCR engagement drives increased activation of the energy sensor AMP-activated protein kinase (AMPK) [102]. Once the requirements for cellular division have been met and division begins, aerobic glycolysis dominates. It seems counterintuitive that aerobic glycolysis would be preferred as it only generates two net ATP, in comparison to the thirty-eight generated via OXPHOS. However, as explained earlier, aerobic glycolysis results in increased carbon shuttling into the pentose phosphate pathway, which is responsible for lipid and nucleotide synthesis, along with production of reducing equivalents to maintain cellular redox balance.

1.4.1 T cell "Myc-tabolism"

To support the new requirement for aerobic glycolysis, activated T cells undergo a major alteration in their transcriptome. The transcription factor Myc has been well-characterized as an important mediator of this process. Naïve T cells express little to no Myc; however, 24 hours post-stimulation, Myc expression levels are highly upregulated [103]. Signaling via the PI3/Akt and mTOR pathways enable this increased expression. Myc induces expression of various glycolysis-supporting genes, including the glucose transporter Glut1, hexokinase 2 (HK2), and lactate dehydrogenase (LDHA) (Figure 3). With this new metabolic machinery in place, activated T cells are poised to utilize glucose via glycolysis to support their rapid proliferation.

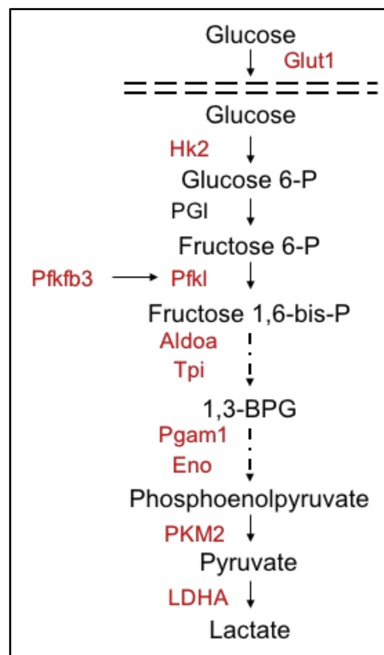

Figure 3. Myc-dependent genes of glycolysis.
The transcription factor Myc has been shown to upregulate expression of various glycolysis-associated genes (indicated in red) during T cell metabolic reprogramming.

Myc is a key oncogene, and similar to its role in T cells, is able to drive high levels of glycolysis in tumors [30, 104]. In some instances, it has even been shown to result in glucose addiction [29]. Also in tumors, Myc has also been shown to support cell cycle progression, thus making it a key linchpin in coordinating rapid proliferation with the metabolic programming necessary to support it. This is also true for T cells during activation. Unlike tumors, activated T cells do not demonstrate sustained Myc expression. As stated previously, levels of Myc reach a peak at 24 hours post-stimulation in T cells; however, T cells are able to maintain glycolysis well past this point. A recent study by *Chou et al.* indicated that another transcription factor AP4, is upregulated during $CD8^+$ T cell activation and maintains expression of glycolysis-associated genes following downregulation of Myc expression [105]. In their model, $CD8^+$ T cells that lacked AP4 were unable to sustain expression of Myc-driven genes and glycolysis, resulting in reduced immunity against West Nile Virus [105].

Aerobic glycolysis is not the only metabolic pathway powered by Myc. Myc has also been shown to increase expression of genes associated with the pentose phosphate pathway and glutaminolysis, both of which aid in increased macromolecule synthesis during T cell activation [103, 106, 107]. Interestingly, Myc has also been shown to drive increased OXPHOS and mitochondrial biogenesis in some cell types [108-110]; yet, it remains to be determined if this is true in T cells.

1.4.2 Glutamine – a key amino acid during activation

While aerobic glycolysis is the predominant pathway utilized in effector T cells, oxidation via the mitochondria is not completely terminated. Some pyruvate generated via glycolysis does

translocate into the mitochondria for oxidation via the TCA cycle and electron transport chain, resulting in ATP production and ROS generation. In addition, uptake and oxidation of the amino acid glutamine via glutaminolysis is highly upregulated in activated T cells, indicating that glucose is not the only energy source required by activated T cells [46, 50]. Glutamine metabolism supports ATP generation, redox regulation, and TCA cycle intermediate restoration [46, 49]. Transport of glutamine into the T cell occurs in large part via the Alanine-Serine-Cysteine transporter 2 (ASCT2) [111], but use of the sodium-coupled neutral amino acid transporters (SNAT) 1 and 2 can also facilitate glutamine uptake [112, 113]. Upregulation of ASCT2 and thus increased glutamine uptake is highly dependent upon CD28 signaling [111, 112]. Once in the cell, glutamine is rapidly converted to glutamate by the enzyme glutaminase. Glutamate can then be utilized for lipid, purine, and pyrimidine synthesis, enabling cell membrane formation and DNA replication. Additionally, in conjunction with cysteine and glycine, glutathione is produced, which is critical for maintaining cellular redox balance, especially with increased ROS being generated via increased OXPHOS [114].

Studies have indicated that the requirement for glutamine occurs in the later stages of T cell activation, primarily to support proliferation and cytokine production. Depriving T cells of glutamine during activation resulted in reduced Interferon-γ (IFNγ) and Interleukin-2 (IL-2) production [112, 115, 116]. Also, the concentration of glutamine present during T cell activation *in vitro* was directly proportional to the degree of proliferation observed [112, 115]. Glutamine concentration had no effect on CD69 expression on T cells following stimulation with αCD3, but did modulate CD25 upregulation in the same cells, again highlighting its requirement for proliferation and not for activation [115].

Further studies have demonstrated that the upregulation of these metabolic pathways during T cell activation is a highly-coordinated event. For example, not only is Myc critical for driving expression of glycolytic genes, but it also mediates transcription of glutamine transporters and glutaminolytic enzymes [103, 117]. Moreover, the inhibition of the anaphase-promoting complex-cyclosome (APC/C)-Cdh1 during T cell activation allows for upregulation of both 6-phosphofructo-2-kinase/fructose-2,6- biphosphatase 3 (PFKFB3) and glutaminase, each rate limiting enzymes of glycolysis and glutaminolysis, respectively [118]. This upregulation correlated with increased T cell movement into S phase of the cell cycle, further delineated the interplay between cellular proliferation and metabolism.

1.4.3 T cell asymmetric division and metabolism

Asymmetric division is a process whereby two phenotypically different daughter cells result from a single mitotic event. This process has been demonstrated not only by mammalian cells, but also those from *Drosophila* and *C. elegans* [119]. In particular, stem cells are the most well studied cells that demonstrate these properties. With stem cells, this mechanism is in place to ensure self-renewal of a pluripotent progenitor while also providing a cell for terminal differentiation [120]. During activation, T cells have shown similar asymmetric division [121-124]. Following APC encounter, the T cell divides for the first time, with the proximal daughter demonstrating a more effector-like phenotype (CD62L^{lo}, CD25^{hi}, CD44^{hi}, CD69^{hi}, IFNγ^{hi}), while the distal daughter exhibits memory T cell characteristics (CD62L^{hi}, CD25^{lo}, CD44^{lo}, CD69^{lo}) [123]. Adoptive transfer experiments indicated that both proximal and distal daughters provided protection against an acute infection directly after transfer. However, distal daughters provided

more robust protection during a secondary challenge 30 days post-transfer, further solidifying their memory potential [123]. TCR affinity has been shown to influence asymmetry as T cell stimulation with more antigenic peptides results in more distinct asymmetric populations [125]. With regards to disease potential, $CD8^{hi}$ proximal daughters showed increased diabetogenicity and immunopathology in a Rip-mOVA model of Type 1 diabetes (T1D) [125].

More recently metabolism and mTOR signaling were shown to be segregated asymmetrically during T cell division [126, 127]. *Verbist et al.* reported that APC proximal T cells expressed higher levels of Myc and were more glycolytic in comparison to distal daughters [127]. These results corroborated studies performed by *Pollizzi et al.,* which indicated that mTOR and its signaling are inherited asymmetrically as well, with $CD8^{hi}$ $mTOR^{hi}$ T cells demonstrating greater size and glycolytic capacity [126]. Interestingly, both studies indicated that $CD8^{hi}$ T effectors and $CD8^{lo}$ memory precursors showed no difference in mitochondrial respiration, suggesting that the enhanced oxidative potential memory cells exhibit [128-130] may require further shaping by extrinsic factors, like Interleukin-15 (IL-15) [131]. It is important to note that this is certainly not the only way in which memory T cells are formed, but may explain differences between central memory (those from asymmetric division) versus effector memory (those generated during effector contraction).

1.5 MEMORY T CELLS AND FATTY ACID OXIDATION

Following antigen clearance, T effector cells undergo one of two fates – apoptosis or transition to a long-lived memory T cell (T_{mem}) [132-134]. Cellular metabolism has been shown to be a crucial factor in mediating the transition from effector to memory, in that T cells must transition

from aerobic glycolysis back to a more oxidative phenotype [50, 107, 130, 135, 136]. Specifically, fatty acid oxidation via the mitochondria is the predominant pathway utilized by T_{mem} cells, and inhibition of this pathway drastically reduces the T_{mem} cell pools [129, 130, 136].

To understand why fatty acid oxidation would be advantageous, one must consider the characteristics of a T_{mem} cell. First, memory T cells must be long-lived, as their primary function is to protect against potential secondary antigen challenge [137]. With this comes the responsibility of maintaining basal processes that require ATP. Fatty acid oxidation, in contrast to aerobic glycolysis, generates more ATP per molecule of substrate, thus providing the cell with more energy to power basal processes. As stated earlier, aerobic glycolysis is important for shuttling carbons towards macromolecule synthesis, but as memory T cells do not rapidly proliferate, biosynthesis is less of a concern. With secondary challenge, memory T cells mount a faster and more robust response in comparison to naïve T cells. Since the propensity for activation is tightly coupled to cellular metabolism, memory T cells require a bioenergetic advantage over naïve T cells to support this functionality [128, 129].

The bioenergetic advantage exhibited by memory T cells has been well characterized over the last several years. This advantage includes increased mitochondrial biogenesis and mitochondrial mass, allowing for greater ATP production. Additionally, T_{mem} possess a greater mitochondrial spare respiratory capacity (SRC) [128]. Most cell types only respire at a basal rate, which is considered only a fraction of their actual metabolic potential. The SRC is the difference between the maximal, or the highest possible rate of oxygen consumption during cellular stress, and its basal rate. $CD8^+$ memory T cells have demonstrated enhanced SRC in comparison to both naïve and effector T cells, and this characteristic is highly dependent on fatty acid oxidation as treatment with the fatty acid oxidation inhibitor etomoxir results in decreased

SRC magnitude [128]. As IL-7 is necessary for survival and metabolism of naïve T cells, memory T cells rely heavily upon IL-15, another member of the common γ chain cytokine family. Through *in vitro* and *in vivo* analyses, IL-15 is necessary for driving increased fatty acid oxidation, SRC, and mitochondrial biogenesis in memory T cells.

1.6 LYMPHOCYE ACTIVATION GENE-3

1.6.1 LAG-3 as a CD4 homolog

Lymphocyte activation gene 3 (LAG-3; CD223), is a 70kDa type I transmembrane protein expressed by various immune cells, including $CD8^+$ and $CD4^+$ T cells [138, 139], T regulatory cells [140, 141], B cells [142], NK cells [138], and dendritic cells [143]. Structurally, LAG-3 consists of four extracellular Ig domains, a connecting peptide region, a transmembrane domain, and a cytosolic tail [144, 145]. LAG-3 is considered a CD4 homolog, as they both share four Ig domains, yet there is only about 20% amino acid homology between the two proteins. CD4 and LAG-3 share the common ligand, MHC class II, which is predominantly expressed on antigen presenting cells (i.e. macrophages, dendritic cells). While their ligand is shared, CD4 or LAG-3 ligand engagement results in opposing positive or negative signaling, respectively. LAG-3 binds to MHC class II with a much higher affinity as compared to CD4 and at a different site, suggesting ligand competition as one regulatory role for LAG-3 [146].

The genes encoding LAG-3 and CD4 are located adjacently on the human chromosome 12 [139]. Both genes share similar intron positioning, including an intron located in the first

immunoglobulin superfamily domain. This, coupled with the homologous structure suggests they were derived from a common ancestor [139, 147]. Whole organ genetic studies indicated that LAG-3 expression was limited to lymphoid organs in humans, with similar results in mice [147, 148]. Yet, in mice detectable levels of LAG-3 mRNA were also found in the brain [148]

Low levels of LAG-3 are expressed on the surface of naïve T cells in comparison to the high surface expression of CD4 on T cells [149]. LAG-3 is mainly located in large intracellular stores located near the microtubule-organizing center, poising LAG-3 to be quickly trafficked to the T cell surface upon activation [149]. This trafficking has been shown to be dependent upon protein kinase C signaling [149, 150]. At the T cell surface, LAG-3 is localized to the immunological synapse, co-localizing in lipid rafts with the TCR and CD4 [149, 151].

1.6.2 Inhibitory receptor role of LAG-3 in T cell activation

CD4 and LAG-3 play opposing roles by mediating or dampening activation, respectively, during T cell activation. In order for LAG-3 to inhibit T cell activation, it requires downstream signaling via its cytoplasmic tail, as ectopic expression of tailless LAG-3 in T cell hybridomas did not inhibit cellular proliferation as effectively as wildtype LAG-3 [145]. Located in the cytoplasmic tail is the KIEELE motif, which studies using site-directed mutagenesis determined was required for LAG-3's inhibitory function [144, 145]. Interestingly, the exact downstream signaling has yet to be elucidated; however, some studies have indicated that cross-linkage of surface LAG-3 resulted in calcium flux inhibition during T cell activation [152].

Numerous studies have helped to elucidate the inhibitory role LAG-3 plays during T cell activation. In these studies, LAG-3:MHC class II engagement during activation resulted in

decreased CD69 expression, clonal expansion, and IFNγ and IL-2 production *in vitro* using antigen specific and nonspecific models, including SEB and OVA. Similar results were observed *in vivo* using infection and nominal antigen models. Alternatively, blockade of LAG-3 using monoclonal antibodies *in vivo* and *in vitro* reversed its inhibitory effects [151]. These studies were the first steps towards understanding the potential for targeting LAG-3 in human diseases like chronic viral infections and cancer, to restore functional T cell responses. While another inhibitory receptor, CTLA-4 was shown to modulate T cell signaling by blocking localization of signaling proteins to TCR-associated lipid rafts [153, 154], LAG-3 did not demonstrate similar effects [151].

While it might be inferred that the inhibitory effects of LAG-3 would only be demonstrated in $CD4^+$ T cells, as they directly interact with its ligand MHC class II, studies have shown that LAG-3 expression does inhibit $CD8^+$ T cell responses as well [151, 155, 156]. This begs the question as to whether LAG-3 ligation is absolutely required for T cell inhibition. The fact that LAG-3 is only upregulated to the T cell surface during activation highlights its role in fine-tuning the immune response. Furthermore, as LAG-3 inhibits clonal expansion and effector function, two processes that require T cell metabolic reprogramming, we postulate that LAG-3 plays an important role in tempering this response to protect against apoptosis and preserve the T_{mem} pool.

To more fully understand the role of LAG-3 in disease models and immune cell function, animal global and conditional knockouts have been developed. Not only has LAG-3 been shown to inhibit activated T cell responses, but it also regulates naïve T cell homeostatic expansion. *Workman et al.* showed that LAG-$3^{-/-}$ animals demonstrate enhanced expansion of both naïve $CD4^+$ and $CD8^+$ T cells, as compared to their wildtype counterparts [157]. Additionally, LAG-3-

deficient OT.II CD4$^+$ T cells repopulated lymphopenic hosts to a greater extent following adoptive transfer [157]. LAG-3 has also been implicated as a negative regulator in several autoimmune disease models. For example, global knockout of LAG-3 in non-obese diabetic (NOD) animals resulted in accelerated progression to T1D [155, 158].

1.6.3 LAG-3 cleavage and soluble LAG-3

For optimal CD4$^+$ T cell activation to ensue, LAG-3 must undergo proteolytic cleavage from the cell surface, thus allowing CD4's activation signal to proceed. The disintegrin/matrix metalloproteases ADAM10 and ADAM17 have been shown to mediate LAG-3 cleavage in its connecting peptide region [144, 159]. ADAM10 and ADAM17 have also been shown to mediate membrane shedding of TNFα [160], Notch [161, 162], CD62L (L-Selectin) [163, 164], and CD44 [165], to name a few. Specifically, ADAM17 activity is redox-dependent, as it requires alterations by ROS to mediate conformational changes [166, 167]. Maintenance of LAG-3 on the surface of CD4$^+$ T cells either by metalloprotease inhibition via chemical inhibitor [159] or redox modulation [168], or mutating the cleavage site within LAG-3 [144], resulted in dampened T cell proliferation and effector function.

The resulting cleaved portion of LAG-3, or soluble LAG-3 (sLAG-3), can be detected in both human and murine serum during immune responses. sLAG-3 has been shown to be a robust measure of T cell activation *in vivo*, as high serum levels correlated with positive prognosis and survival in progesterone or estrogen receptor positive breast cancer patients [169]. In a study examining Th1 and Th2 responses in *Mycobacterium tuberculosis* patients, those who received antimycobacterial treatment and had better prognostic outcome, exhibited higher levels

of serum sLAG-3 as compared to untreated patients [170]. Previous work from our laboratory demonstrated that sLAG-3 may also serve as an early stage biomarker for Type I Diabetes (T1D) onset in animal studies [168], and work examining sLAG-3 levels in samples from T1D patients and first degree relatives have been promising (*Delmastro-Greenwood et al.;* unpublished work).

1.6.4 LAG-3 as a marker of T cell exhaustion

One of the main areas of research that has further elucidated the inhibitory role of LAG-3 is through the study of T cell exhaustion. T cell exhaustion is a phenomenon that occurs in cancer and chronic viral infection, where due to the constant bombardment with antigen and persistent inflammation, T cell responses wane. Not only do exhausted T cells demonstrate a unique transcriptional program, but overexpression of specific surface markers is one of the key determinants in exhaustion [171]. These surface markers include not only LAG-3, but also programmed cell death-1 (PD-1), cytotoxic T lymphocyte antigen-4 (CTLA-4), and T-cell immunoglobulin domain and mucin domain (TIM-3), to name a few [171, 172]. Expression of these receptors in various combinations has been observed, depending on the disease. For example, in models of murine B16 melanoma, MC38 colon adenocarcinoma, and Sa1N fibrosarcoma, dual expression of LAG-3 and PD-1 was observed on exhausted $CD8^+$ and $CD4^+$ tumor-infiltrating lymphocytes (TIL) [173]. LAG-3 has also been observed on exhausted $CD8^+$ T cells in a murine model of lymphocytic choriomeningitis virus (LCMV) [156] and in human HIV patients [174]. While exhaustion has been studied predominantly in $CD8^+$ T cells, there is evidence of $CD4^+$ T cells experiencing a similar fate. In models of *Plasmodium* malaria [175]

and B16 melanoma [176] dual expression of LAG-3 and PD-1 identified hyporesponsive $CD4^+$ T cells, and targeting both molecules via antibody blockade restored $CD4^+$ T cell responses.

1.6.5 Regulatory T cell function via LAG-3-mediated inhibition

As activated T cells have been shown to be high expressers of LAG-3, so have some $CD4^+$ $CD25^+$ $Foxp3^+$ T regulatory (T_{reg}) cells. Naturally occurring T_{regs} make up 5-10% of the human $CD4^+$ T cell repertoire and are required for maintaining immune homeostasis, protecting against autoimmunity, and controlling chronic inflammation [177]. T_{regs} utilize several mechanisms for suppressing immune responses, including secretion of inhibitory cytokines like IL-10 and TGFβ, sequestration of IL-2 and adenosine, two necessary factors for optimal T cell activation, and direct cytolysis of T_{eff} by granzyme B/perforin release [177]. Increased frequencies of T_{regs} have been demonstrated in several solid tumor models and correlate with negative prognostic outcomes [178, 179]. Alternatively, defects in T_{reg} populations correlate with increased autoimmune disease [180, 181].

Subsets of T_{regs} in both murine and human studies have been shown to express LAG-3 on their surface, supporting LAG-3 as a marker for these cells [182, 183]. Also, LAG-3 has been implicated as a mechanism utilized by T_{regs} to mediate immunosuppression, either by targeting dendritic cells (DCs) or T_{eff} directly. $LAG\text{-}3^+$ T_{regs} can engage immature DCs via LAG-3 binding to MHC class II on the DC, thereby suppressing DC maturation and altering DC differentiation toward a tolerogenic phenotype [184]. Studies by *Camisaschi et al.* identified a $LAG\text{-}3^+$ $CD4^+$ $CD25^{hi}$ $Foxp3^+$ T_{reg} population in both healthy donors and melanoma and colorectal cancer patients [140]. The frequency of this T_{reg} population was increased in the

peripheral blood, lymph nodes, and tumor microenvironment of the cancer patients. These LAG-3^+ T_{regs} produced higher levels of IL-10 and TGFβ, as compared to LAG-3^- T_{regs}, and their suppressive capacity was dependent upon cell-to-cell contact. Additionally, in an *in vitro* suppression assay, LAG-3^+ T_{regs} induced greater T_{eff} suppression as compared to LAG-3-deficient T_{regs}, further implicating LAG-3 as a marker for potent T_{regs} [140].

1.7 AUTOIMMUNITY AND IMMUNOMETABOLISM

1.7.1 Immunometabolism and insight into autoreactivity

As with T1D and other autoimmune diseases, there is still a gap in our understanding regarding autoreactive T cells. However, understanding how cellular bioenergetics and biosynthesis shape both immune cell differentiation and function provide an avenue for expanding our knowledge regarding what is inherently different about autoreactive T cells, and how to exploit these differences to control autoimmunity. Work reported thus far has revealed that metabolic differences do exist between autoreactive T cells and their non-autoreactive counterparts [185-187]. For instance, studies from *Yin et al.* have shown that autoreactive T cells from patients with systemic lupus erythematosus (SLE) demonstrate enhanced aerobic glycolysis and OXPHOS during activation, enabling increased IFNγ production [188]. By targeting these pathways with the chemicals 2-DG and metformin, respectively, T cell bioenergetics were reset to the levels of T cells from healthy controls. This metabolic-based treatment also normalized T cell metabolic dysfunction in animal models of SLE, resulting in significant reductions in disease

pathology [188]. Nevertheless, treatment cessation led to flare ups in animals, suggesting that while metabolism-targeted treatment ameliorated disease, it did not generate durable tolerance [189].

Patients with multiple sclerosis (MS) exhibit increased concentrations of glutamine and glutamate in their cerebrospinal fluid and brain biopsies[190, 191]. As increases in glutamine and glutamate are associated with disease severity, concentrations of these metabolites have been suggested as potential biomarkers for MS [190, 191]. Earlier, it was discussed that glutamine metabolism via glutaminolysis is crucial for $CD4^{+}$ T cell differentiation and cytokine production (see section 1.3.2). Studies in experimental autoimmune encephalomyelitis (EAE), the murine model of MS, have shown that knockout of the glutamine transporter ASCT2 resulted in reduced Th17 cytokine production, diminished lymphocyte infiltration into the central nervous system, and lessened disease severity [111]. Together, these results suggest that glutamine is a critical metabolite for pathogenic T cells in MS, and targeting this pathway in patients could be efficacious.

As described earlier, mitochondria are at the center of metabolic function in T cells, and mitochondrial dysfunction is known to contribute to autoreactivity [185-187]. SLE T cells demonstrate elevated mitochondrial membrane potential and increased ROS production [192, 193]. This is in part due to an increased reliance on glucose oxidation, which occurs in the mitochondria, rather than aerobic glycolysis [189]. In Rheumatoid Arthritis (RA), hypoxia and increased pro-inflammatory cytokine production in synovial joints are associated with increased mitochondrial DNA mutations [194, 195]. Of note, hypoxia led to reduced expression of respiratory chain subunits, thereby contributing to mitochondrial dysfunction [195].

Overall, autoreactive T cells do not play by the same metabolic rules as other T cells. These metabolic differences do contribute to pathogenicity, and metabolic-based therapies could be the next frontier in treatment for autoimmunity.

1.7.2 Immunometabolism and T1D

While work in other autoimmune diseases has furthered our understanding of immunometabolism, there remains a gap in knowledge concerning T cell metabolism in T1D. Studies in the NOD mouse have shown that T regulatory cells are present in the pancreas in the early stages of disease, and they can suppress early effector T cell responses [196]. However, there is a tipping point where effector T cells become refractory to T_{reg}-mediated suppression. This is not simply a numbers game because at low T_{reg} to effector T cell ratios, effector activation and function prevails, and it is not due to defects in the T_{regs} themselves as they can suppress non-diabetogenic T cells [196]. These studies beg the question of what is inherently different about these autoreactive T cells, and examining T cell bioenergetics could provide crucial insight. The body of work presented here was developed to further explore the mechanisms that govern metabolic pathways in $CD4^+$ T cells as a means of potentially controlling autoimmunity in T1D.

LAG-3 has been shown to have a critical role in modulating autoreactive T cell responses in T1D. Studies by *Bettini et al.* indicated that LAG-3-deficient NOD animals exhibited accelerated T1D progression compared to wildtype controls, and LAG-3 inhibition by antibody blockade yielded similar results [155]. Studies from our laboratory corroborated these findings by indicating that inhibition of LAG-3 cleavage from NOD T cells delayed T1D onset [168].

Moreover, in non-autoimmune strains, LAG-3 is a negative regulator of naïve T cell homeostatic expansion [157]. As described earlier, the ability for any cell to proliferate is controlled by nutrient availability, and converting those nutrients to energy and macromolecules. Other inhibitory receptors have also shown to modulate T cell metabolism [197, 198]. Therefore, we hypothesized that LAG-3 regulates T cell responses by modulating their bioenergetic profile.

Furthermore, previous reports from our laboratory and others demonstrated that inhibiting ROS production during T cell activation resulted in reduced effector function transition, clonal expansion, and T1D progression, in conjunction with maintenance of LAG-3 surface expression [168, 199-202]. Additionally, ROS have been implicated as critical signaling molecules in mediating metabolic changes in other disease models [43, 185, 203, 204]. With this, and that IFNγ production and proliferation are dependent upon T cells transitioning to aerobic glycolysis, we hypothesized that ROS are critical for enabling metabolic reprogramming in T cells during T1D induction.

1.8 REACTIVE OXYGEN SPECIES MEDIATE T1D IMMUNOPATHOLOGY

Decades of research have demonstrated that T1D pathology and inflammation is highly mediated by ROS and oxidative stress [16, 205-207]. This includes ROS being necessary for activating both the innate and adaptive immune responses. In particular, it has been reported that T cells require acute doses of ROS to facilitate proliferation and differentiation [208-210]. The doses of ROS present during T cell activation have a dramatic effect on the outcome of the immune response generated, as too little ROS results in antigen hyporesponsiveness, and high doses lead

to oxidative stress-induced apoptosis [211-213]. Therefore, controlling cellular redox is of utmost importance in controlling T cell responses.

T1D progression, and autoimmunity in general, occurs when central and peripheral tolerance mechanisms break down. Central tolerance refers to elimination of autoreactive T cells in the thymus during development; whereas, peripheral tolerance refers to mechanisms outside of the thymus, like suppression by regulatory T cells. While the predominant focus has been on the influence of ROS during T cell activation, redox has also been shown to play a significant role during thymocyte development and in T regulatory cell formation and function. Therefore, to fully understand the broad influence of cellular redox on T cell function in the context of T1D, a systematic approach must be conducted to fully dissect the direct and indirect effect these signals have on modulating T cell responses.

Although the etiology of T1D is still not completely understood, it is well accepted that T1D is driven, in part, by oxidative stress [16, 205-207]. In fact, murine β cells are highly susceptible to oxidative stress since they demonstrate low antioxidant enzyme expression [214], yet generate high levels of mitochondrial-derived ROS via glucose oxidation [215-217]. These ROS are necessary for activating both the innate and adaptive immune responses. In the context of T1D, β cell ROS production and antigen release can activate islet-resident macrophages and dendritic cells (DCs) (Figure 4). These innate cells, also known as antigen presenting cells (APC), phagocytose β cell antigens and ferry them to the pancreatic draining lymph nodes [16]. In the draining lymph node, activated APCs present β cell antigens to self-reactive $CD4^+$ and $CD8^+$ T cells, in the context of co-stimulation, along with pro-inflammatory cytokines and ROS. Together, these signals result in T cell activation and trafficking to the pancreas. Both $CD4^+$ and $CD8^+$ T cells are present in pancreatic islet infiltrates during T1D progression [7, 8, 218]. T cells

induce β cell destruction by either direct killing ($CD8^+$ T cells) or production of inflammatory cytokines like Interferon γ (IFNγ) and tumor necrosis factor α (TNFα) ($CD4^+$ T cells) [16, 219]. This induced killing results in more β cell antigen and ROS release, thereby activating more APCs and continually driving this vicious cycle. With respect to $CD4^+$ T cells, their importance in T1D pathogenesis was exemplified by studies demonstrating that $CD4^+$ T cell depletion completely prevented disease progression [218, 220]. In particular, T cells have been shown to require acute doses of ROS to facilitate activation and effector function [208-210]. These ROS can come from multiple sources and are further explored below.

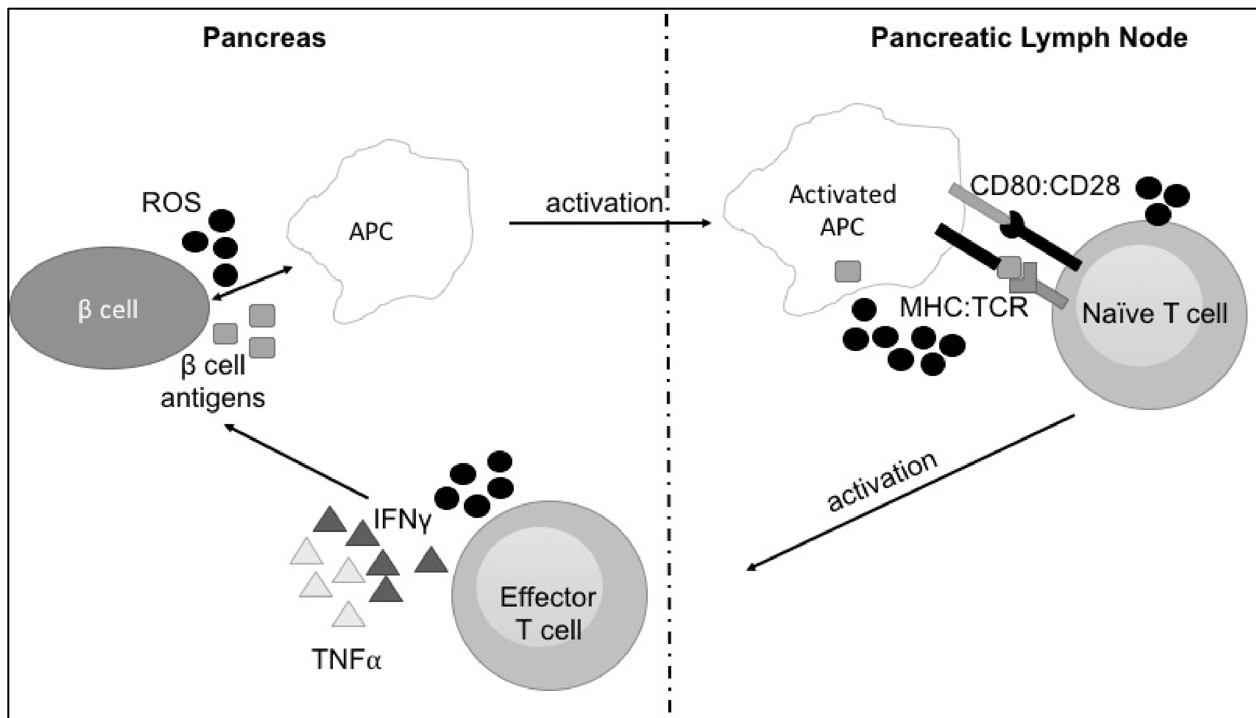

Figure 4. The role of ROS in driving the immunopathology of Type 1 Diabetes (T1D).
Reactive oxygen species (ROS) have been shown to be critical mediators of β cell destruction in Type 1 Diabetes. Not only do β cells themselves produce high levels of ROS that can induce cell death, but these ROS can also prime activation of infiltrating antigen presenting cells (APCs) like macrophages. APC-derived ROS can also contribute to β cell loss directly. Once activated, APCs then traffic to the pancreatic lymph node, where ROS serve as third signal during T cell activation. Activated T cells then traffic to the pancreas and drive β cell apoptosis via inflammatory cytokines, direct β cell lysis, and ROS production via NADPH oxidase expression.

1.9 SOURCES OF ROS THAT INFLUENCE T CELL FUNCTION

Once naïve T cells receive all three signals to become activated (antigen presentation, co-stimulation, and cytokines/ROS), undergo two major processes -- clonal expansion and differentiation . ROS are known to modulate both processes, and there are multiple sources, both intrinsic and extrinsic, of ROS that T cells can encounter.

1.9.1 NOX-derived ROS

NADPH oxidases (NOX) are intermembrane enzymes, which are the primary source of respiratory burst across many cell types. NOXs are multimeric enzymes that consist of different combinations of subunits, depending on the specific isoform expressed. Of all immune cells, expression of a functional NOX is most highly associated with innate immune cells, including macrophages, neutrophils, and eosinophils [221, 222]. These cells express a phagocyte NOX, or NOX-2 isoform, comprised of the subunits cytochrome b_{558} (complex formed by $p22^{phox}$ and $gp91^{phox}$), $p40^{phox}$, $p47^{phox}$, and $p67^{phox}$ (encoded by the genes *Cyba*, *Cybb*, *Ncf4*, *Ncf1*, and *Ncf2*, respectively) [223]. Of the NOX-2 subunits, the cytochrome b_{558} complex is membrane bound, while a complex of the remaining subunits exists in the cytosol. Upon activation via phosphorylation of $p47^{phox}$, the cytosolic complex translocates to the membrane, enabling a fully functional enzyme. Cytochrome b_{558} is then able to facilitate electron transfer from NADPH to oxygen, forming superoxide. The factors flavin and heme associate with NOX-2 to enable electron transfer as well [223, 224].

Further studies in T cells have demonstrated that they too express a phagocyte-type NOX (Figure 5). Specifically, mRNA studies comparing murine primary T cells and T cell lines to macrophages indicated similar expression levels of the NOX genes *Cyba*, *Cybb*, *Ncf1*, and *Ncf2,* [225], and TCR-mediated activation of purified T cells resulted in generation of both superoxide and hydrogen peroxide [213, 225]. Of note, these studies were completed using APC-independent mechanisms of T cell stimulation, indicating that ROS generated by T cells themselves are sufficient for mediating activation [213]. Upon knockout of $p47^{phox}$, generation of hydrogen peroxide by T cells was severely blunted; yet, superoxide production remained intact. Further manipulation using the flavin-dependent inhibitor diphenylene iodonium (DPI),

yielded similar results [213], suggesting that T cells may express other NOX isoforms that are non-flavin dependent. Indeed, later studies confirmed T cell expression of DUOX-1, a calcium-dependent NOX isoform that functions independently of $p47^{phox}$ [226]. Studies from *Kwon et al.* further demonstrated that DUOX-1 expression in T cells was responsible for very early hydrogen peroxide generation upon TCR engagement [226]. Examination of NOXs and DUOXs in other cell types have shown that the magnitude of ROS generation greatly differs, with NOXs providing more robust ROS production. Therefore, the signals propagated by NOX and DUOX may result in different phenotypic outcomes in T cells, and further dissection in disease states, like T1D, is necessary.

As both macrophages and T cells express functional NOXs, animals on the NOD background have been generated to study the contribution of these ROS to T1D pathogenesis. Specifically, the NOD.$Ncf1^{m1J}$ contain a point mutation in the *Ncf1* gene encoding for the $p47^{phox}$ subunit of NOX-2; thus, quenching global NOX-2 function and ROS production [200, 202]. These animals demonstrate delayed T1D incidence as compared to their wildtype counterparts, which was attributed to reduced activation of both macrophages and T cells [200, 202]. However, some $CD4^+$ T cell responses do develop, yet rather than being Th1 (*i.e.* IFNγ), like that elicited in T1D, they are skewed to a Th17 phenotype, characterized by secretion of the cytokine IL-17 [202]. Consistent with these changes, the animals develop experimental autoimmune encephalomyelitis (EAE), the animal model of multiple sclerosis (MS). Together, these results suggest that NOX-derived ROS enable autoreactive T cell activation in T1D. However, lowering the oxidative threshold via NOX-2 inhibition allows for the activation of other autoreactive T cell clones, further solidifying that genetic predisposition to autoimmunity is ultimately paramount.

1.9.2 Macrophages

One of the most well-characterized sources of ROS encountered by T cells is the macrophage. Under homeostatic conditions, low levels of tissue-resident macrophages are present in the pancreas, and upon an environmental trigger can become activated by β cell-derived ROS (discussed below) and peptides [16, 207]. Macrophages represent the predominant infiltrate during the very early stages of insulitis in T1D, as their increased ROS and pro-inflammatory cytokine production mobilizes and activates more macrophages [8, 227]. As stated earlier, macrophages are known to express NOX, which transport electrons across membranes, resulting in superoxide formation [221, 223, 224]. As superoxide itself cannot freely move across cellular membranes, dismutation to hydrogen peroxide allows for a potent ROS that can diffuse across the T cell membrane. During macrophage-mediated activation of T cells, a tight immunological synapse is formed. ROS are known to be very proximal signaling molecules; therefore, the tight synapse formed between the macrophage and T cell creates a spatial arrangement that fosters ROS signaling between the two cells. Concentrations of hydrogen peroxide ranging from 10-100 uM produced by macrophages are capable of initiating signaling cascades, including the mitogen-activated protein kinase (MAPK) [213], PI3/Akt [228, 229], and NF-κB [230] pathways in T cells. These ROS can also perpetuate downstream TCR signaling [231], and together, result in T cell-mediated pro-inflammatory cytokine production [232, 233].

In T1D, macrophages are the early islet infiltrators, and their activation is required for generating T cell responses and ensuing disease [234-236]. Not only can these ROS signal to T cells during MHC:TCR engagement, but they also activate the MAPK and NF-κB pathways in macrophages [237]. Both MAPK and NF-κB activation result in increased production of pro-inflammatory cytokines like TNFα and Interleukin-1beta (IL-1β), which shape the T cell

response in addition to ROS signaling [238, 239]. Studies using chemical NOX inhibitors or NOX-deficient macrophages have demonstrated that inhibition of ROS in the macrophage can alter $CD4^+$ T cell activation, resulting in delayed T1D progression [200, 202, 240]. Yet, reconstituting ROS production by either the T cell or macrophage is sufficient for restoring optimal crosstalk and activation, indicating that the source of ROS is not as crucial as the presence of ROS.

1.9.3 Mitochondrial-derived ROS

Mitochondria are also cellular sources of ROS, including in T cells. Mitochondria are necessary for generating ATP via oxidative phosphorylation to power cellular processes. Substrates such as glucose, fatty acids, and glutamine, can be oxidized via the Tricarboxylic acid (TCA) cycle and electron transport chain (ETC) in the mitochondria [35, 39]. The ETC consists of five mitochondrial complexes (I-V) which are important for coupling the transport of electrons with the transport of protons from the mitochondrial matrix into the intermembrane space [35, 39]. NADH dehydrogenase (Complex I), succinate dehydrogenase (Complex II), and cytochrome c reductase (Complex III) are all capable of generating superoxide due to electron leak [38, 44]. Superoxide produced by Complexes I and III is released into the mitochondrial matrix, where superoxide dismutase-2 (MnSOD) converts it to hydrogen peroxide [43]. Superoxide formation by complex III is released into both the mitochondrial matrix and intermembrane space and has been shown to be a potent signaling intermediate [241, 242]. Mitochondria have also been shown to migrate towards the immunological synapse (the TCR:MHC junction) within the T cell during activation. This migration is dependent upon TCR-mediated calcium signaling and

results in increased mROS production [243]. While it has been shown that mitochondrial migration is important for maintaining calcium signaling during activation, it remains to be understood what role this juxtaposition plays for mROS signaling on T cell activation.

During T cell activation, mitochondrial oxidation increases dramatically within the first 24 hours of TCR engagement [244]. This increase, driven by calcium and adenosine monophosphate-activated protein kinase (AMPK) signaling, is necessary for cellular growth in preparation for cell division [102]. With increased oxidation, increased mitochondrial-derived ROS (mROS) also increases. Work by *Sena et al.* demonstrated that mitochondria-derived ROS are essential for mediating the transition from naïve to effector in CD4^{+} T cells [244]. Specifically, Complex III-deficient T cells demonstrated reduced activation marker expression (CD69 and CD25) and growth factor production (IL-2), in comparison to wildtype T cells [244]. *In vivo* experiments utilizing T cell-specific Complex III knockout animals demonstrated similar results in both CD4^{+} and CD8^{+} T cells. However, results from *Tse et al.* indicated that compensatory superoxide production from the APC should be sufficient for facilitating T cell activation in the instance that the T cell itself is deficient [202]. These conflicting results highlight that further delineation of superoxide production and signaling is necessary, and there may be strain-specific differences as *Tse et al.* utilized the NOD model whereas *Sena et al.* used C57Bl/6 animals. Additionally, the source of ROS in these instances, the APC versus the T cell's mitochondria, could mediate differential signaling.

Mitochondrial dysfunction has been characterized in T cells from other autoimmune diseases [185-187]; however, similar studies remain to be performed using T1D-specific T cells. For example, T cells from Systemic Lupus Erythematosus (SLE) patients demonstrate elevated mitochondrial membrane potential and increased ROS production [192, 193]. This is in part due

to increased reliance on glucose oxidation, which occurs in the mitochondria, rather than aerobic glycolysis (which occurs in the cytosol), during activation [189]. Moreover, in rheumatoid arthritis (RA), hypoxia and increased pro-inflammatory cytokine production in synovial joints are associated with increased mitochondrial DNA mutations [194, 195]. Of note, hypoxia led to reduced expression of respiratory chain subunits, thereby contributing to mitochondrial dysfunction [195]. These studies set a precedence for mitochondrial dysfunction in enabling autoreactive T cells, and studies in T1D could provide further insight into inherent differences between autoreactive and non-pathogenic T cells.

1.9.4 β cells

Pancreatic β cells are responsible for dynamic glucose sensing and insulin secretion to maintain blood glucose homeostasis. Acute doses of ROS are required for supporting insulin secretion [215-217]; therefore, β cells have mechanisms in place to provide the necessary oxidative signals. First, β cells, like macrophages and T cells, express multiple NOX isoforms (NOX 1, 2, 4, NOXA1, and NOXO1) [245-248]. Moreover, β cells demonstrate high levels of glucose oxidation in the mitochondria, thus resulting in increased mitochondrial-derived ROS [249-251]. Interestingly, murine β cells demonstrate low antioxidant enzyme expression, making it difficult to maintain redox balance [214].

In T1D, β cell-derived ROS, coupled with antigen release, are responsible for activating and mobilizing APCs [16]. A positive feedback loop exists in that pro-inflammatory cytokine and ROS release by tissue resident APCs can induce more β cell ROS production. T cell

activation in T1D occurs in the pancreatic draining lymph node; therefore, β cell derived ROS would not have a direct impact on this process. However, an indirect effect does exist as APC activation is dependent upon β cell interactions, and how the APC is activated can then influence T cell mobilization. Activated T cells that traffic to the pancreas can encounter β cell generated ROS, which can promote increased T cell NF-κB activation and pro-inflammatory cytokine secretion.

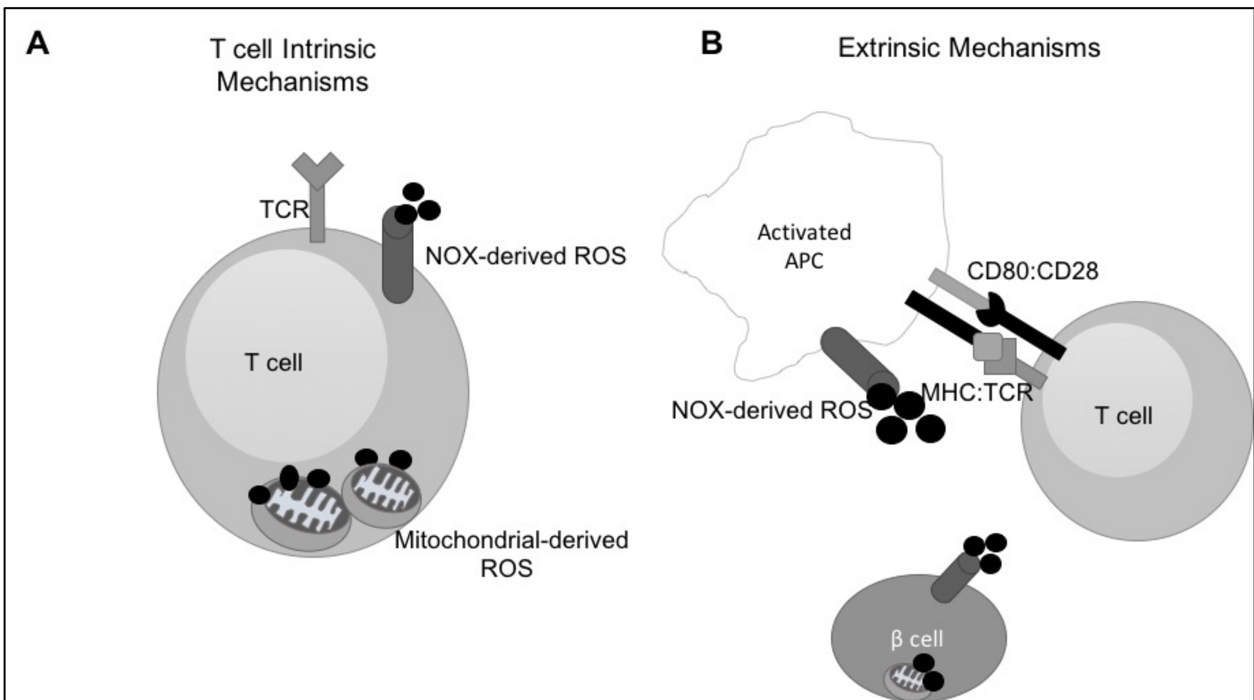

Figure 5. T cell intrinsic and extrinsic sources of ROS.
A. T cells express functional NADPH oxidases (NOX), which can generate ROS. Additionally, oxidative phosphorylation in the mitochondria can result in ROS leak. B. During T cell – APC interaction, activated APCs, like macrophages, generate NOX-derived ROS that coupled with inflammatory cytokines, enable optimal T cell activation.

1.10 ROS AND THYMOCYTE DEVELOPMENT

T cell development is an intricate process whereby thymocyte precursors move through various stages of differentiation in the thymus. All early thymocytes start as CD4-CD8- double negative precursors [252]. The double negative stage is where TCR-β receptor rearrangement occurs and cells express a pre-TCR comprised of a β chain and a pre-Tα chain. From there, double negative cells differentiate to double positive (DP) $CD4^{+}CD8^{+}$ thymoctyes. At this time, TCR α chain rearrangement occurs and a mature αβ TCR is expressed. DP thymocytes then undergo two critical developmental processes – repertoire selection and lineage commitment. T cell repertoire selection includes both positive and negative selection where thymocytes interact with MHC loaded with self-peptides [252-254]. Low affinity for self-peptide/MHC results in maturation to a single positive T lymphocyte. Interestingly, 90% of thymocytes die by neglect at this stage, as their TCR is inert [253]. Alternatively, high affinity for self-peptide/MHC results in apoptosis as a way of protecting against potentially autoreactive T cells escaping into the periphery [253, 254]. This is known as central tolerance. It is here, during negative selection, that disease progression initiates in autoimmune-prone individuals, although the mechanisms by which self-reactive T cells escape deletion are not fully understood. Lineage commitment, or commitment to either a $CD8^{+}$ or $CD4^{+}$ single positive T cell occurs based on successful engagement of either MHC class I or II, respectively. Once matured, T lymphocytes then exit the thymus and begin to seed the periphery.

Cellular redox is critical for thymocyte development and selection. Specifically, thymocytes express a family of proteins, known as the uncoupling proteins (UCP) 1-3 [255-258]. This group of proteins reside in the mitochondria and serve as proton transporters, thereby

dissipating the proton gradient created in the mitochondrial matrix. Thus, oxidative phosphorylation is uncoupled from ATP production, and uncoupling can lead to increased electron leak and ROS production. Several groups have shown that expression levels of UCP 1-3 in the thymus fluctuate with age, and murine studies using individual UCP knockouts have shown alterations in percentages of double and single positive populations [259, 260], further indicating their requirement at different stages of thymocyte development. Loss of UCP-1 expression specifically, results in increased mitochondrial oxygen consumption, ROS and ATP production, and lowered apoptotic potential [259, 261].

Another way in which redox regulates thymocyte development is through the control of the redox-sensitive transcription factor NF-κB, as activation in the cytoplasm can be initiated by ROS. This upstream activation can result in phosphorylation of either IκB or IκBα, allowing for release of p50/p65 and its translocation to the nucleus [262, 263]. Alternatively, in the nucleus, reduction of the cysteine 62 residue of the p50 subunit is required for optimal DNA binding [264]. Antioxidant treatment [265] and Cu/Zn SOD overexpression [266] have both been shown to result in dysfunctional thymocyte development, due to defective NF-κB signaling. This is explained by the fact that NF-κB can also serve as a survival signal, and as demonstrated in developing T lymphocytes [267]. These studies underscore that there is a fine balance between ROS generation and antioxidant capacity, and alterations could set the stage for the formation of autoreactive T cells and autoimmunity.

It has been well-established that breakdowns in central tolerance in the thymus do occur and lead to T1D progression [268]. Some studies have demonstrated that reduced insulin transcript levels in the thymus correlated with increased susceptibility to T1D [269, 270]; thereby, providing direct evidence that alterations in thymic selection influences autoimmune

progression. Further studies in the NOD model have indicated that self-reactive T cells and thymocytes are genetically programmed to be more resistant to apoptosis [271-273], exemplified by studies showing NOD thymocytes were more resistant to apoptosis induced by dexamethasone (Dex) treatment [274, 275]. Thymocytes protected from Dex-induced apoptosis demonstrated increased SOD expression, indicating that increased antioxidant enzyme expression is beneficial in this context [276]. The NOD mouse has also been shown to demonstrate increased NF-kB signaling in various cell types in comparison to non-autoimmune strains [277, 278]. While this has not been shown specifically in thymocytes, we would postulate that thymocytes from these animals demonstrate a similar phenotype. This increase could also contribute to thymocyte resistance to apoptosis, reduced negative selection, and increased pro-inflammatory cytokine production in the periphery, contributing to disease progression.

1.11 REDOX REGULATION OF T CELL ACTIVATION

Balancing cellular redox has a critical impact on T cell activation and longevity. Acute doses of ROS, either generated by the T cell itself or from an extrinsic source (*i.e.,* macrophages) are necessary for driving the transition from naïve to effector (Figure 6) [213, 226, 244, 279]. However, like other cell types, suboptimal or excessive concentrations of ROS can result in hyporesponsiveness [168, 199, 200] or oxidative stress, respectively, which can have profound implications on T cell functionality and immunity. Studies have shown that resistance to oxidative stress increases with differentiation from naïve to effector [209, 280, 281]. This is likely due to effector T cells traveling to highly inflamed and hypoxic tissues; whereas naïve T

cells remain in the normoxic lymph nodes and lymphatics. Therefore, the influence of ROS on T cells is highly dependent upon the concentration of ROS and the context in which these signals are presented.

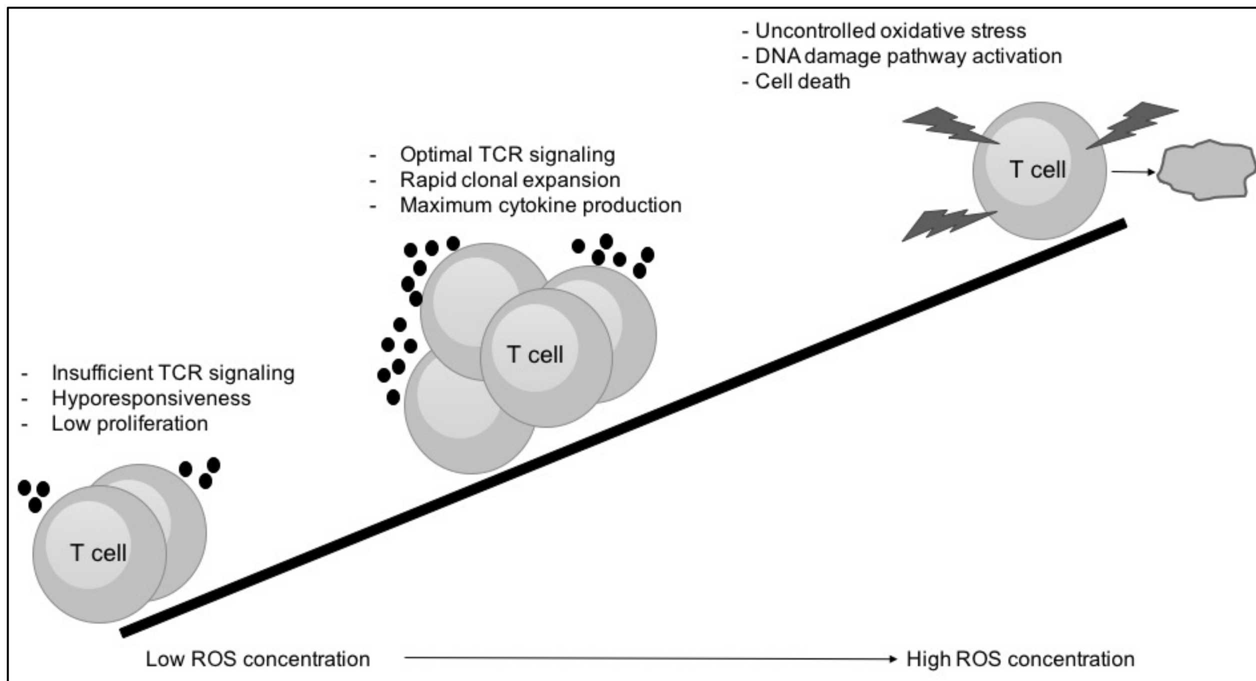

Figure 6. The influence of ROS on T cell outcome during activation.
The dose of ROS present during T cell priming and activation can have a dramatic effect on effector T cell outcome. High levels of ROS can induce oxidative stress and DNA damage, resulting in T cell death, while low levels will be inefficient at driving optimal activation. Moderate levels, either produced by APCs or T cells themselves, ensure proper TCR signaling, clonal expansion, and effector differentiation.

1.11.1 Redox modulation of TCR signaling

Downstream signaling initiated by the TCR upon MHC-peptide recognition is essential for antigen-specific T cell activation. This signaling is propagated by numerous adaptor proteins, kinases, and calcium signaling [282]. In addition, protein tyrosine phosphatase (PTP) inhibition

aids in signal maintenance [283]. With respect to TCR signaling, several adaptor proteins and downstream signaling cascades have been shown to be redox sensitive (Figure 7). For instance, lymphocyte-specific protein tyrosine kinase (Lck), a member of the Src family of kinases, which interacts with the cytoplasmic tail of both CD4 and CD8, is required for potentiating intracellular TCR signaling [280, 284]. Low doses of hydrogen peroxide are capable of inducing Lck phosphorylation (Try394), resulting in increased catalytic activity [285, 286]. Activation of Lck results in phosphorylation of both the CD3 ζ chains and another kinase Zap70. Micromolar concentrations of hydrogen peroxide also stimulate Zap70 activation [287]. Furthermore, increased protein kinase C (PKC) and MAPK signaling and intracellular calcium levels have all been shown to be stimulated by hydrogen peroxide [288, 289].

LAT, or linker for activation of T cells, is an adaptor protein associated with the TCR [282]. With TCR engagement, LAT serves as an anchor for formation of multiprotein signaling complexes, aiding in amplifying TCR signaling. Work by *Gringhuis et al.* demonstrated that LAT's localization to the plasma membrane is highly redox sensitive. Specifically, LAT contains a cysteine residue that upon oxidative modification, results in a conformational change that inhibits interaction with glycolipid-enriched microdomains of the plasma membrane [290]. This modification increases during times of oxidative stress and results in reduced Interleukin-2 (IL-2) production by T cells [291]. This specific modification was demonstrated by T cells isolated from RA patients, due to high levels of inflammation [291]. Like RA, T1D is also highly driven by ROS and inflammation, and inflamed islets demonstrate high levels of oxidative stress [16, 206, 292]. It is interesting that with this highly oxidative environment, T cell responses are still occur, resulting in T1D progression. These results highlight that while both RA T cells and T1D T cells share autoreactivity, their resistance to oxidative stress may greatly

differ. Side-by-side comparison studies may help elucidate potential differences. A reducing environment between the APC and T cell is also critical for facilitating TCR signaling as T cells increase reduced thiols at the plasma membrane [293], and APCs support this milieu by secreting cysteine into immunologic synapse [294]. Together, these studies exemplify that while ROS drive optimal TCR signaling, there are also mechanisms in place to protect redox balance.

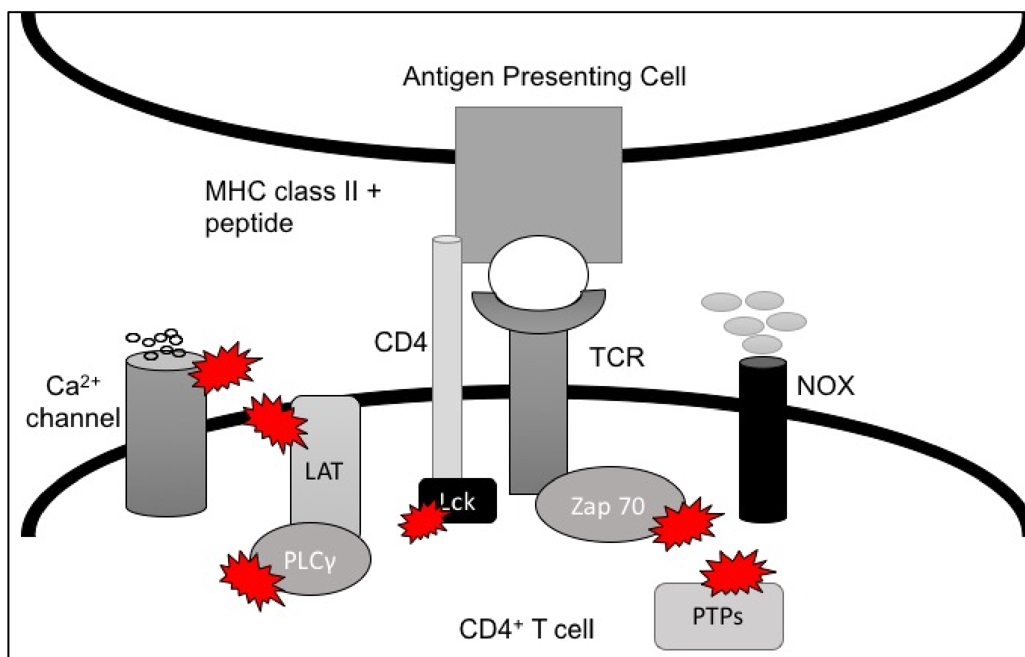

Figure 7. TCR signaling is highly redox dependent.
Many components downstream of the TCR have been shown to be redox dependent, and alterations in redox status can result in suboptimal or completely inhibited TCR signaling. Calcium channel signaling that occurs during TCR stimulation has also been shown to be redox dependent. Red stars indicate those proteins/channels that are known to be influenced by redox. Protein tyrosine phosphatases (PTPs), Protein lipase C (PLC), Linker for activation of T cells (LAT).

1.11.2 Redox cycle within the cell cycle

As naïve T cells are relatively quiescent, they primarily reside in G_0 phase of the cell cycle [52, 59]. ROS are potent cell cycle entry signals in T cells and other cell types alike [295]. To enter the cell cycle during activation an oxidative event, like hydrogen peroxide or superoxide signaling, must occur [296, 297]. Additionally, ROS are required for S phase entry and DNA replication, as without ROS, cells arrest at the G1/S checkpoint [298].

Mitochondrial metabolism, a major producer of ROS, increases in the early stages prior to cell cycle entry potentially ensuring that the signals for progression are present, and enabling necessary cell growth prior to division [43]. Mitochondrial ROS can activate the protein Romo1, which in turn impairs the cell cycle inhibitor p27 Kip1, allowing proliferation to progress [299]. This increase occurs simultaneously with reduced manganese superoxide dismutase (MnSOD) expression during the later stages of the cell cycle (S, G2, and M), to allow for oxidative signals to prevail [43].

With balancing ROS to drive proliferation, the antioxidant glutathione is also balanced within the cell. The positioning of glutathione is a direct indicator of cell cycle phase in that a more cytoplasmic location is indicative of G_0/G_1 phase [300-302]. Movement of glutathione into the nucleus is indicative of S phase and mitosis initiation [300]. T cell proliferation is also highly dependent upon glutathione in that treatment with a gamma-glutamylcysteine synthase inhibitor, L-buthionine-(S,R)-sulfoximine (BSO), inhibited DNA synthesis and thus T cell clonal expansion [303].

Work by our laboratory and others have suggested the beneficial effects of a manganese metalloporphyrin as a means of modulating immune responses in T1D, due to its highly oxidative nature [199, 201, 292] These studies have demonstrated that modulating the redox

balance of T cells results in reduced proliferation and cell cycle progression [168, 304]. As T cell effector function (IFNγ production) is tightly coupled to proliferation [305], inhibition of ROS also resulted in reduced cytokine production.

1.12 ROS AND T REGULATORY CELLS

As their name suggests, the role of T regulatory cells (T_{regs}) is to temper active immune responses and protect against unwanted responses, like those demonstrated in autoimmunity. T_{regs} are capable of inhibiting both innate and adaptive responses by modulating APCs or T cells through numerous mechanisms, including: immunosuppressive cytokine secretion (i.e. TGFβ and IL-10), inhibitory receptor engagement (i.e. PD-L1 and CTLA-4), and amino acid scavenging (i.e. tryptophan and cysteine) [177, 306]. The importance of T_{regs} in suppressing autoimmune responses was highlighted by studies demonstrating that reconstitution of athymic mice with T_{eff} resulted in systemic autoimmunity [307]. This ensuing autoimmunity was only suppressed upon adoptive transfer of $CD4^{+}CD25^{+}$ T regulatory cells [307]. While T_{regs} have been shown to be derived from both $CD4^{+}$ and $CD8^{+}$ T cell lineages, the most well-studied are the naturally occurring $CD4^{+}$ T_{regs} that demonstrate surface expression of the high affinity IL-2 receptor, CD25 [306, 308]. Other surface markers have been used to delineate different subsets of T_{regs} including, CTLA-4, LAG-3, and PD-L1 [177] Most importantly of all Treg markers, expression of the transcription factor Forkhead box P3 (FoxP3), is essential for driving necessary genes for T_{reg} function and development [309].

As in T_{eff}, expression of a functional NOX in T_{regs} has been shown to have a dramatic effect on their development and suppressive capabilities. In models of inducible T_{regs}, studies

demonstrated that macrophage-derived ROS via NOX were necessary for supporting optimal T_{reg} differentiation [310]. *In vitro* co-cultures of naturally occurring T_{regs} and T_{eff} indicated that T_{regs} deficient in NOX (Ncf-1$^{-/-}$) were less capable of suppressing T_{eff} proliferation via TGF-β production, as compared to WT T_{regs} [311]. TGF-β production in other cell types has been shown to be redox sensitive, as antioxidant treatment with N-acetylcysteine (NAC) resulted in reduced expression of the cytokine [312]. Additionally, TGF-β can induce NOX expression and activity [313, 314]. Taken together, these studies highlight the necessity for T_{regs} to produce ROS to maintain suppressive functionality. While suppression was measured by inhibition of T_{eff} proliferation, the effector cytokine response was not examined. As the presence and absence of ROS during T_{eff} activation have been shown to skew towards distinct T helper subsets, characterization of the cytokine profile may have elucidated if NOX expression by T_{regs} influences this as well. Also of note, not all T_{regs} mediate suppression via TGF-β; therefore, the requirement for NOX may differ, depending upon the mode of suppression.

T_{regs} demonstrate an enhanced ability to circumvent oxidative environments. Polarization of $CD4^+$ T cells to T_{regs} under hypoxic conditions increased T_{reg} yields *in vitro* [315]. Not only were more T_{regs} generated, but they also demonstrated enhanced FoxP3 expression as compared to those cultured under normoxic conditions. Although, the enhanced FoxP3 expression had no apparent effect on suppressive capacity [315]. T_{regs} also demonstrate high levels of cell surface thiols, which aid in counteracting oxidative stress [316]. Studies using human T_{regs} have shown they possess increased expression of the antioxidant enzyme thioredoxin, compared to conventional T cells, also enabling improved ROS scavenging [317]. These reducing mechanisms protect T_{regs} from oxidative stress-induced cell death, while maintaining their immunosuppressive function [316, 317].

Based on these studies, presumably T_{regs} would be better equipped to thrive in the highly oxidative environment of the pancreatic islet, resulting in greater immune regulation. T_{regs} are present in the pancreas preceding diabetes onset; however, they are accompanied by activated effector T cells as well [318]. Adoptive transfer studies of T_{eff} with T_{regs} from non-diabetic NOD donors revealed a significant delay in T1D progression as compared to transfer of these subtypes isolated from diabetic animals [196]. Interestingly, T_{regs} from diabetic animals successfully suppressed effectors from non-diabetic donors, indicating that T_{reg} functionality is maintained during overt disease. However, T_{regs} from non-diabetic donors were unable to suppress T_{eff} from diabetic donors *in vivo* [196]. This underscores that while T_{reg} functionality may be intact in T1D, T_{eff} surpass a pathogenic threshold that enables them to escape suppression.

In work by *Padgett et al.,* NOD.BDC.2.5.Ncf-1^{m1J} mice were generated to study the influence of NOX-derived ROS on an antigen-specific model of T1D. Results demonstrated that these NOX-deficient $CD4^+$ T cells were more reactive to antigen as compared to their wildtype counterparts [319], and adoptive transfer experiments further elucidated that this enhanced diabetogenicity was due to defective regulatory T cell suppression. While there have been human studies that also showed defective regulatory T cell responses in T1D patients and at-risk relatives [320-322], there is a lack of evidence linking this to redox-related mechanisms or specifically, NOX machinery. Alternatively, effector T cells from T1D patients have also shown enhanced resistance to T_{reg}-mediated suppression [323]. Therefore, aiming to amplify T_{reg} suppressive capacity may prove ineffective. A more suitable approach could be to make T_{eff} more vulnerable to mechanisms of T_{reg} suppression; thereby, allowing the immune system to remedy the situation itself.

1.13 ROS AS A THERAPEUTIC TARGET IN T1D

As mentioned previously, the NOD mouse model is used to recapitulate T1D as these mice develop spontaneous autoimmune diabetes. In addition, the adoptive transfer of BDC-2.5.TCR transgenic splenocytes into NOD.*scid* mice leads to rapid immune-mediated β cell destruction and diabetes onset within 7-14 days. To understand the role that ROS play in the NOD spontaneous T1D model, the NOD.NCF-1^{m1J} model was developed [202]. These mice contain a point mutation that results in truncation of the p47^{phox} subunit and prevents functional NOX assembly and ROS production [200, 202]. NOD.NCF-1^{m1J} mice experienced a delay in spontaneous diabetes progression, in large part due to suboptimal immune activation [200, 202, 240]. These results further supported targeting NOX and ROS as a potential therapeutic in T1D.

To combat the chronic inflammation and oxidative stress displayed in diabetes, manganese metalloporphyrin antioxidants (MnTE-2-PyP^{5+}, MnP) are seemingly a viable therapeutic. Not only can these agents scavenge free radicals, but they also possess a high bioavailability and are catalytic, displaying oxidoreductase properties [292, 324]. These characteristics allow for preservation of β cell integrity [325-327] and dampening of anti-islet immune responses in T1D [168, 199, 201].

1.13.1 MnP treatment delays Type 1 Diabetes onset in murine models

Early studies investigating redox modulation via MnP treatment in T1D were performed using an adoptive transfer model of the diabetogenic BDC2.5 T cell clone into NOD.*scid* animals [199]. MnP intraperitoneal injections were continued several days during the experiment, and then

ceased. Of the mice treated with MnP, 50% developed diabetes by 28 days post-transfer, while 100% of the untreated animals progressed to fulminant disease [199]. Most notably, histology results showed decreased pancreatic immune cell infiltrate with MnP treatment. Additional *in vitro* studies indicated that MnP treatment reduced IFNγ production by BDC-2.5 T cells, and inhibited respiratory burst of peritoneal macrophages upon LPS stimulation [199]. Together these results demonstrated the immunomodulatory capacity of MnP treatment in T1D and its potential therapeutic use in autoimmunity.

More recent studies in T1D with MnP have included the use of 14-day sustain release MnP pellets implanted subcutaneously at the nape of the neck in NOD mice [168]. This administration allows for systemic MnP treatment, and drastically decreases the number of administrations. In this study, all untreated NOD mice developed T1D by 20 weeks of age [168]. By 30 weeks of age, only 50% of animals treated with MnP developed diabetes. These data, together with the data from the adoptive transfer models, further support targeting ROS for preventing T1D. Nevertheless, the protective effects of MnP cease when treatment ends, as 25% more animals progressed to diabetes following MnP treatment conclusion [168]. Overall, MnP treatment does delay diabetes onset; yet, it does not provide durable tolerance. Therefore, the need for other therapies in conjunction with MnP treatment is apparent.

1.13.2 Manganese Metalloporphyrins and T cell inhibition

As both $CD8^+$ and $CD4^+$ T cells play important roles in mediating T1D, our laboratory has performed extensive *in vitro* and *in vivo* work, including various T1D and transplantation models, in order to understand how MnP treatment modulates responses by both T cell subsets

[168, 199, 304, 328]. Each of these models serves as a potential mimic for delaying T1D onset and preserving transplanted allogenic β cell grafts in human T1D patients. Specifically in $CD8^+$ T cells, *Sklavos et al.* showed that proliferation and IFN-γ and TNF-α production were significantly inhibited by MnP treatment in a mixed lymphocyte reaction (MLR) [304]. Cytotoxicity of MnP-treated and untreated $CD8^+$ T cells was tested in an antigen-specific cytotoxicity assay, where MnP treatment resulted in a 50% reduction in targeted killing by CTLs [304]. MnP treated OT-I $CD8^+$ T cells during *in vitro* stimulation with SIINFEKL peptide also exhibited similar reductions in IFN-γ and TNF-α production, proliferation, and targeted killing. In both models, the reduced cytotoxicity by MnP treatment was in part due to reduced expression of both perforin and granzyme B [304]. Taken together, the results from both the MLR and OT-I models indicated that MnP's ability to inhibit $CD8^+$ T cell activation and effector function was not specific to any one model, supporting its applicability to suppress diabetogenic and anti-graft T cell responses. Here, MnP's effects are relevant to islet transplantation in autoimmune prone individuals, as it would suppress both anti-graft and autoimmune responses.

Delmastro et al. investigated the effects of MnP on activation and effector function of $CD4^+$ diabetogenic T cells. These studies used the NOD.BDC-2.5.TCR.Tg mouse expressing the rearranged TCR of the diabetogenic BDC-2.5 T cell clone that respond to the β cell antigen, chromogranin [329]. Splenocytes isolated from these mice were stimulated *in vitro* with their cognate peptide, mimotope, with or without MnP treatment. *In vitro* results indicated that MnP treatment inhibited $CD4^+$ T cell activation and effector function by reducing IFNγ production, proliferation, and CD69 (marker of activation) and Tbet expression [168]. These results correlated with MnP treatment inhibiting the cleavage of LAG-3. As described previously, in order for optimal T cell activation, LAG-3 must be cleaved from the T cell surface by the

metalloproteases ADAM10 and ADAM17 [159]. ADAM17, like many metalloproteases, is a redox-dependent enzyme in that oxidation of specific cysteine residues must occur to change its confirmation from its latent to its activated form [330-332]. As MnP has been shown to scavenge ROS during immune cell activation, *Delmastro et al.* determined that MnP treatment inhibited ADAM17 activation, thereby maintaining LAG-3 expression on the T cell surface, leading to $CD4^+$ T cell inhibition and delayed T1D onset [168].

In vivo studies by *Delmastro et al.* further showed that MnP treatment of diabetogenic splenocytes diminished their potential for inducing T1D in a murine adoptive transfer model. Severe combined immunodeficiency mice on the NOD background (NOD.*scid)*, lacking endogenous adaptive immunity, received diabetogenic BDC-2.5.TCR.Tg splenocytes. Throughout the course of the study, serum was taken from the MnP treated and control animals, to measure levels of sLAG-3. Results indicated that elevated levels of sLAG-3 (suggesting $CD4^+$ T cell activation) correlated with progression to diabetes in untreated animals [168]. Alternatively, sLAG-3 levels in MnP treated mice remained steady and all animals were euglycemic. Collectively, these results not only demonstrate that MnP treatment *in vivo* inhibits T1D onset, but also that sLAG-3 may serve as an early biomarker of T1D progression.

Recent work from our laboratory has demonstrated that MnP treatment can modulate the metabolic profile of diabetogenic T cells *in vivo* [328]. During *in vivo* studies, NOD.BDC-2.5.TCR.Tg mice were either treated with or without MnP for 7 days, and then various metabolic pathways of bulk splenocytes were assessed. Not only did MnP treatment inhibit diabetogenic potential of splenocytes in an adoptive transfer model, but it also resulted in reduced utilization of glucose via aerobic glycolysis [328]. While aerobic glycolysis was decreased, indicated by reduced lactate production, splenocytes demonstrated more efficient glucose oxidation. These

effects were attributed to enhanced activation of the tricarboxylic acid cycle (TCA) enzyme, aconitase, which is responsible for catalyzing the reaction of citrate to isocitrate in the mitochondria.

1.14 SUMMARY

The goal of this body of work was to more fully elucidate mechanisms that govern the bioenergetics of CD4+ T cells, as a means of understanding potential therapeutic targets for ameliorating autoimmunity. The focus on T cell metabolism was driven by the more recent insight that T cell differentiation and bioenergetics are greatly intertwined – T cell phenotype can dictate the metabolic pathways required for function and metabolism reinforces characteristics attributed to specific T cell subsets. Moreover, studies in diseases like RA, SLE, and MS have indicated that autoreactive T cells are bioenergetically distinct from non-pathogenic T cells, suggesting these alterations contribute to disease. Here, we utilized murine models of T cell homeostasis and T1D to elucidate how LAG-3 (Chapter 2) and ROS (Chapter 3) modulate T cell metabolism during these processes, respectively.

2.0 LYMPHOCYTE ACTIVATION GENE-3 MAINTAINS METABOLIC AND MITOCHONDRIAL QUIESCENCE IN NAÏVE CD4$^+$ T CELLS

"Lymphocyte activation gene-3 maintains metabolic and mitochondrial quiescence in naïve CD4$^+$ T cells"

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This work is currently in preparation for submission.

2.1 SUMMARY

Lymphocyte Activation Gene-3 (LAG-3) is an inhibitory receptor expressed on the surface of various immune cells, including $CD4^+$ T cells. While LAG-3 is more frequently associated with exhausted effector and regulatory T cells, it is also a negative regulator of T cell homeostatic expansion. Recent studies exploring how cellular metabolism dictates immune cell function and fate have elucidated that $CD4^+$ T cell function is tightly coupled to bioenergetics. Therefore, given that LAG-3 is a regulator of $CD4^+$ T cell homeostatic expansion, and the ability to proliferate is highly dependent upon metabolism, we hypothesized that LAG-3 regulates the metabolic profile of naïve $CD4^+$ T cells. Naive OT.II T cells were isolated from wildtype and LAG-$3^{-/-}$ animals, and analyzed by Seahorse Flux Analyzer. LAG-$3^{-/-}$ OT.II T cells demonstrated significantly increased basal respiration, spare respiratory capacity, and aerobic glycolysis, as compared to wildtype OT.II T cells, indicating an enhanced bioenergetic profile. Further analysis indicated that LAG-3-deficient OT.II T cells displayed increased mitochondrial biogenesis, via increased signaling through the AMPK/Sirt-1 pathway. In an adoptive transfer model of homeostatic expansion, LAG-$3^{-/-}$ OT.II cells expanded to a greater extent and continued to demonstrate an enhanced oxygen consumption and glycolysis as compared to WT OT.IIs. This proved to be extrinsically regulated, as LAG-3 blockade in WT recipients recapitulated similar results to LAG-$3^{-/-}$ T cells. These results demonstrate that LAG-3 is critical for actively maintaining metabolic quiescence in naïve $CD4^+$ T cells, and future directions include investigating this mechanism and its implications in autoimmunity.

2.2 INTRODUCTION

CD4$^+$ T cells are a critical component of the adaptive immune system, thereby regulating their development, activation, and survival is of the utmost importance. Homeostasis is an essential period of the T cell lifespan, during which T cells require low-level TCR stimulation and gamma chain cytokines (i.e. IL-7 and IL-15) to ensure their survival [59, 333]. In immunocompetent environments, naïve T cells actively maintain quiescence as a means of avoiding over population and loss due to neglect [52]. Alternatively, in lymphopenic hosts, naïve T cells exhibit increased proliferative capacity as a means of reconstituting the host's immune system. At various times during homeostatic expansion, naïve T cells can adopt a more memory-like phenotype, including increased CD44 and CD25 expression and faster transition to effector function upon antigen encounter [99, 100, 334]. Regulatory mechanisms are in place to maintain naïve T cell homeostasis and quiescence to protect against aberrant T cell activation and hyperproliferation, like that demonstrated in autoimmunity and cancer.

In particular, lymphocyte activation gene-3 (LAG-3; CD223) has gained prominence as a key inhibitory receptor in various disease models [168, 173, 335]. Structurally, LAG-3 and CD4 are considered homologs, with the former demonstrating a higher affinity for their shared ligand, MHC class II [139, 146, 148]. While the various inhibitory effects mediated by LAG-3, including dampening proliferation and inhibiting IFNγ production [336, 337], have been characterized, its downstream signaling remains to be fully defined. Naïve CD4$^+$ T cells express low levels of LAG-3 in comparison to other T cell subsets [141]; nevertheless, LAG-3

engagement can still negatively regulate these cells. Studies examining global knockout [157] and antibody blockade [338] indicated that homeostatic expansion is tightly regulated by LAG-3 in immunocompetent and lymphopenic animals. In addition, these studies suggested that LAG-3 mediated this effect by tempering STAT5 signaling [338].

It is becoming more evident that cellular metabolic programs are essential for driving the identity and functionality of T cells during their various life stages. Unlike activated T cells, which demonstrate robust aerobic glycolysis to maximize macromolecule synthesis and energy, naïve $CD4^+$ T cells are considered metabolically quiescent [48, 49]. Naïve T cells rely predominantly on oxidative phosphorylation (OXPHOS) via the mitochondria for generating ATP [53, 339]. Signaling via the cytokine IL-7 in naïve T cells is essential for maintaining not only their survival via Bcl-2 expression, but also their bioenergetic profile [68, 91]. IL-7 has been shown to increase glucose uptake via increased glucose transporter expression, in an Akt/STAT5-dependent manner [68, 75, 91]. Alternatively, loss of CD127 (IL-7Rα) expression or antibody blockade disrupts naïve T cell homeostasis, resulting in decreased proliferation and viability [72, 340].

Considering that loss of LAG-3 expression results in increased $CD4^+$ T cell homeostatic expansion, and that the ability of a cell to proliferate is tightly coupled to its metabolic profile, we hypothesized that LAG-3 expression regulates naïve $CD4^+$ T cell metabolism. Our results indicate that indeed, LAG-3-deficient naïve T cells exhibited enhanced oxidative and glycolytic metabolisms, with this being attributed to increased mitochondrial content. Also, this heightened bioenergetic profile allowed for greater homeostatic proliferation upon adoptive transfer. Moreover, LAG-3 antibody blockade of wildtype OT.IIs enhanced their metabolic capacity to levels similar to LAG-3 knockout T cells, suggesting that LAG-3 mediates regulation through

extrinsic signaling. LAG-3-deficient T cells were also more resistant to IL-7 deprivation as compared to their wildtype counterparts, which was attributed to increased STAT5 activation concomitant with reduced expression of the inhibitory phosphatase PTEN. Upon activation, LAG-3$^{-/-}$ T cells outperformed wildtype cells by demonstrating enhanced metabolic reprogramming and effector function. These findings suggest that LAG-3 expression on naïve T cells acts as a metabolic regulator to protect against hyperproliferation and maintain naïve T cell homeostasis to ensure controlled T cell activation.

2.3 MATERIALS AND METHODS

2.3.1 Animals

C57BL/6.*Lag3*$^{-/-}$ mice were bred and housed under pathogen-free conditions in the Animal Facility of Rangos Research Center, Children's Hospital of Pittsburgh, University of Pittsburgh Medical Center (UPMC). Female and male mice aged 8-12 weeks were used in all experiments. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Children's Hospital. C57BL/6 WT controls were purchased from Jackson Laboratories and housed with the *Lag3*$^{-/-}$ animals. For *Lag3*$^{-/-}$.OT-II transgenics, B6.*Lag3*$^{-/-}$ animals were crossed with B6.OT-II animals (JAX) to homozygosity for the *Lag3* gene and heterozygosity for the OT-II transgene. C57BL/6.*Rag1*$^{-/-}$ breeder pairs were a gift from the laboratory of Dr. Jay Kolls, MD, and animals were maintained in immunocompromised housing.

2.3.2 CD4$^+$ T cell isolation

Whole spleens from B6.*Lag3*$^{-/-}$, B6.WT, WT.OT-II and *Lag3*$^{-/-}$.OT-II animals were homogenized and red blood cells were lysed using RBC lysis buffer (Sigma). CD4$^+$ T cells were isolated by negative selection from splenocytes using EasySep Mouse CD4$^+$ T cell Isolation kits (StemCell Technologies). Purity was determined by flow cytometry.

2.3.3 Metabolic Seahorse Flux Analyzer assays

T cells were plated in Cell-Tak (Corning) coated Seahorse culture plates (Agilent) at 1 x 10^5 or 3 x 10^5 per well in assay medium (unbuffered DMEM, 1% BSA, 1 mM pyruvate, 25 mM glucose, 2 mM glutamine), and analyzed using the Seahorse XFe96 instrument (Agilent). For mitochondrial stress tests, basal oxygen consumption (OCR) and extracellular acidification rates (ECAR) were measured for 30 minutes, and then cells were stimulated with oligomycin (Oligo; 2 mM), Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP; 0.5 μM), 2-deoxyglucose (2-DG; 100 mM) and rotenone/antimycin A (Rot/AntA; 100 μM each) (all inhibitors from Sigma). Spare respiratory capacity (SRC) was determined by the difference between maximal OCR (following FCCP addition) and basal OCR.

2.3.4 Mitochondrial to nuclear DNA PCR

DNA was isolated from 5.0 x 10^6 isolated $CD4^+$ T cells from indicated animal strains using the DNeasy isolation kit (QIAGEN). DNA was quantified using a Nanodrop 2000c (Thermo Scientific). qPCR was performed on 1 ng total DNA as previously described [341]. ΔΔCt values for the mitochondrial gene cytochrome C oxidase subunit 1 (CO1) were normalized to the nuclear gene NDUFV1. The CO1 primers were 5- TGCTAGCCGCAGGCATTAC-3 (fwd) and 5-GGGTGCCCAAAGAATCAGAAC-3 (rev). The primers for NDUFV1 were 5-CTTCCCCACTGGCCTCAAG-3 (fwd) and 5-CCAAAACCCAGTGATCCAGC-3 (rev).

2.3.5 RNA isolation and qRT-PCR

Isolated $CD4^+$ T cells were lysed for RNA in RLT buffer (Qiagen). RNA was isolated using the RNeasy mini kit (Qiagen) and cDNA was synthesized using the RT^2 First Strand kit (Qiagen). RNA was quantified using a Nanodrop 2000c (Thermo Scientific). qRT-PCR was performed for indicated genes using iQ SYBR Green Supermix and iCycler according to manufacturer's instructions (BioRad). Cycling parameters were as follows: 5 min at 95°C, 30 s at 95°C, 30 s at 60°C, 30 s at 72°C (40 cycles of steps 2-4), 1 min at 95°C, and then samples were held at 4°C. In order to calculate relative expression, ΔΔCt values were normalized to expression of the control gene *Rplo* (FWD 5'-GGCGACCTGGAAGTCCAACT-3'; REV 5'-CCATCAGCACCACAGCCTTC-3') [342]. Values were normalized to wildtype controls. The primers for TFAM were 5-AAGTCTTGGGAAGAGCAGATGGCT-3 (fwd) and 5-AGACCTAACTGGTTTCTTGGGCCT-3 (rev). The primers for mitochondrial complex I were

5-CTTCGGCTTTGTGGCTTTCATGGT-3 (fwd) and 5-AAAGCCCATCAAGCCTCCTCAGAT-3 (rev). The primers for the glycolysis associated genes were taken from *Wang et al.* [103].

2.3.6 Cell lysates and Western blotting

Whole-cell lysates were made by lysing cells in anti-pY lysis buffer (50 mM Tris pH 8.0, 137 mM NaCl, 10% glycerol, 1% NP-40, 1 mM NaF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 2 mM Na_3VO_4, and 1 mM PMSF), followed by sonication. Protein concentration was quantified by Bicinchoninic acid protein assay (Thermo Fisher Scientific). 25 μg of protein lysates were separated by gel electrophoresis on 8% SDS-PAGE gels. Western blots were performed as previously described [168] Blots were probed overnight at 4°C with primary antibodies to the following targets diluted 1:1000 in 5% BSA in Tris-buffered saline with Tween (TBST): pAMPK-α (Thr170) and AMPK (both from Cell Signaling). Secondary anti-rabbit antibody was used for detection of primary antibodies (1:2000; Cell Signaling). Chemiluminescence was detected in real time using ECL plus reagent (Amersham Pharmacia Biotech) and the Fujifilm LAS-3000 imager. Blots were analyzed using Multi Gauge software (Fujifilm Life Science). β-actin expression served as the loading control (Sigma).

2.3.7 Extracellular and intracellular flow cytometry staining

Cells were harvested and first incubated in FACS buffer (1% BSA in PBS) with F_c Block (CD16/CD32; BD Biosciences). Surface staining was performed at 4°C in FACS buffer with the following antibodies: CD4, CD127, CD44, CD62L, LAG-3 (all from BD Bioscience). For experiments examining thymocyte populations the following antibodies were used: CD45, CD4, CD8, CD25 and CD44 (all from BD Bioscience). For intracellular staining of PTEN (clone A2B1; BD Phosflow) and pAkt (Ser473; clone D9E, Cell Signaling), cells were fixed/permeabilized as previously described [343]. For pSTAT5 intracellular staining, cells were fixed using the Foxp3/Transcription Factor Fixation/Permeabilization Kit (eBioscience) and stained using the pSTAT5-PE Cy7 antibody (Tyr694; clone 47/Stat5(pY694); BD Phosflow). Cells were analyzed using a BD LSRII and data were analyzed using FlowJo Software (v10.1).

2.3.8 Adoptive transfers and *in vivo* antibody blockade

$CD4^+$ T cells were enriched from spleens of WT.OT-II or *Lag3*$^{-/-}$.OT-II animals as described above. Cells were labeled with Violet Proliferation Dye 450 (BD Bioscience) according to manufacturer's instructions and 5.0 x 10^6 T cells were transferred i.v. into B6.*Rag1*$^{-/-}$ recipients. Recipient animals were sacrificed 10 days post-transfer and transferred cells were recovered from spleens using $CD4^+$ T cell enrichment kits (Stem Cell) and analyzed by flow cytometry and Seahorse Flux Analyzer. Cohorts of *Rag1*$^{-/-}$ recipients were treated with 200μg of either αLAG-3 (clone C9B7W; BioXCell) or αIL-7Rα (clone A7R34; BioXCell) i.p. every-other day starting

the day prior to adoptive transfer. Control animals received either Rat IgG1 (LAG-3) or Rat IgG2a (IL-7Rα) isotype controls.

2.3.9 IL-7 treatment *in vitro*

Whole splenocytes were plated and treated with 100 ng/mL rmIL-7 (R&D Systems) or media alone for 18 hours as previously described [68]. Glucose uptake was measured as described below. IL-7-induced glucose uptake of $CD4^+$ T cells due to IL-7 treatment was calculated as follows:

recovered glucose uptake = (MFI IL-7 treated) – (MFI Media treated).

Viability was assessed by flow cytometry by staining for CD4 and 7AAD. Recovered viability due to IL-7 treatment was calculated as follows:

recovered viability = (% $7AAD^+$ Media treated) – (% $7AAD^+$ IL-7 treated).

2.3.10 Plate-bound antibody stimulation

Isolated B6.WT and B6.*Lag3*$^{-/-}$ $CD4^+$ T cells were stimulated with plate-bound αCD3 (0.5 ug/mL; BD Pharmingen) and αCD28 (1.0 ug/mL; BD Pharmingen) for indicated periods of time at 37°C in a 5% CO_2 incubator. Cells were cultured in complete splenocyte medium (4.5 mg/dL glucose DMEM (Gibco) supplemented with 10% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate (Gibco), 10 mM HEPES buffer (Gibco), 2× non-essential amino acids (Gibco),

100 μg/mL gentamicin (Invitrogen Life Technologies), 4 mM L-glutamine (Gibco), and 61.5 μM 2-ME).

2.3.11 Lactate and IFNγ measurements

Lactate from *in vitro* culture supernatants was measured using the Accutrend Plus meter and lactate strips (Roche) as previously described [344]. IFNγ was measured by ELISA as previously described [168]. ELISAs were read using a SpectraMax M2 microplate reader (Molecular Devices), and data were analyzed using SoftMax Pro version 5.4.2 software (Molecular Devices).

2.3.12 Glucose uptake assay

$CD4^+$ T cells were stimulated with plate-bound antibodies for 24-72 hours. Prior to harvest, cultures were incubated with the fluorescent glucose analog 2-(*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG; Invitrogen Life Technologies) at 100 μM for 10 minutes, and uptake was quenched with PBS as previously described [136]. Cells were stained for surface markers CD4 and LAG-3 and analyzed live by flow cytometry using a BD LSR II instrument (BD Biosciences). Samples were analyzed using FlowJo software (v10.1; TreeStar).

2.3.13 Statistical analysis

Data are given as mean values ± SEM, with n indicating the number of independent experiments or animals, unless otherwise indicated. Student's t-test or One-way ANOVA with Bonferroni post-hoc analysis was used where appropriate. A p-value of $p<0.05$ was considered significant for all statistical analyses.

2.4 RESULTS

2.4.1 LAG-3 regulates the homeostatic expansion of naïve polyclonal and monoclonal $CD4^+$ T cells

Previous reports have shown that global knockout of LAG-3 results in increased proliferation of naïve $CD4^+$ T cells in both monoclonal (OT.II) and polyclonal (B6) mouse models [157, 338]. Both C57Bl/6 and B6.OT.II TCR transgenic animals that lack LAG-3 expression in our colony similarly demonstrated increased total number of splenocytes and $CD4^+$ T cells in comparison to their wildtype counterparts (Figure 8A and B; $p<0.05$). Closer examination of T cell subsets (naïve, effector, memory) based on CD62L and CD44 expression indicated that absolute numbers of naïve $CD4^+$ T cells differed between knockout and wildtype animals (Figure 8C), suggesting that the naïve compartment is the most dependent upon LAG-3-mediated regulation.

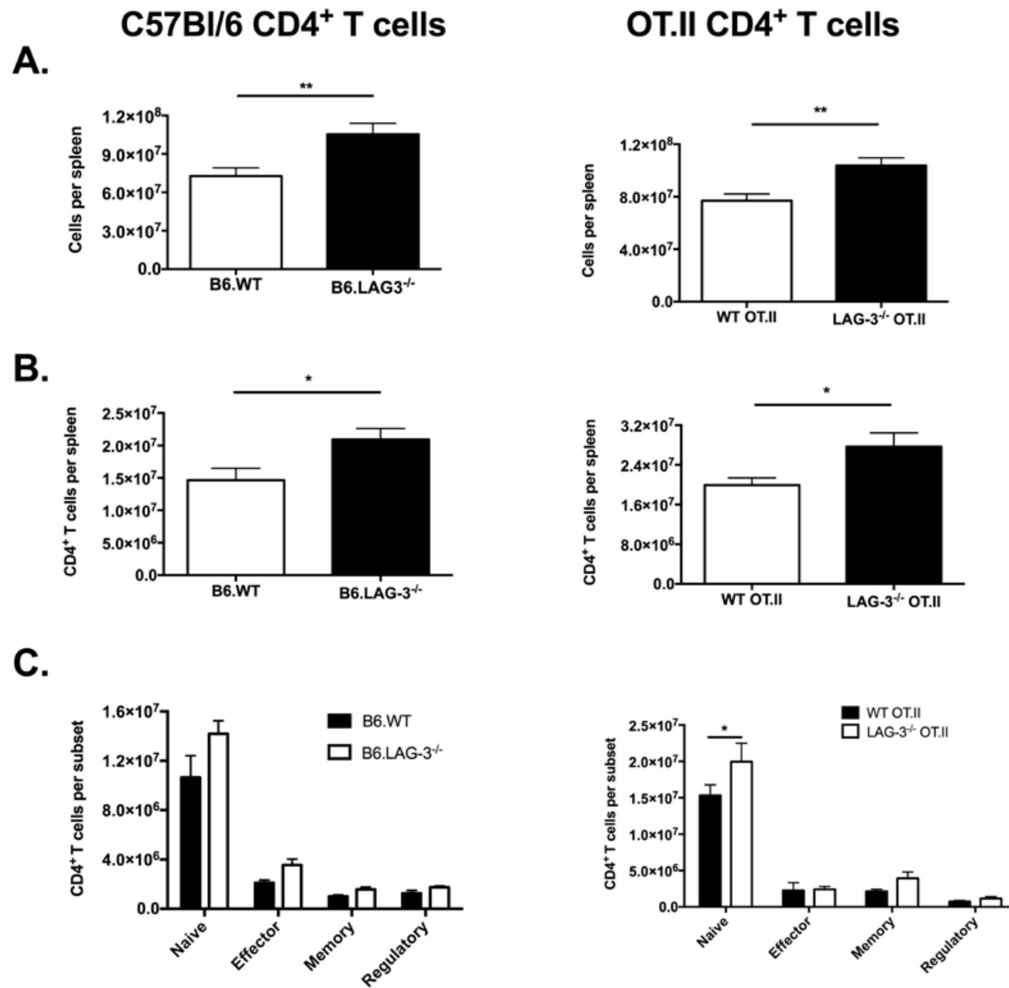

Figure 8. LAG-3 negatively regulates homeostatic expansion of naive CD4+ T cells.
A. Total cells per spleen from C57Bl/6 WT and *Lag3*$^{-/-}$ animals (left panel) and B6. OT.II WT and *Lag3*$^{-/-}$ animals (8-12 weeks of age). **B.** Total CD4+ T cells per spleen. **C.** Absolute numbers of CD4+ T cell subsets from wildtype and *Lag3*$^{-/-}$spleens based on CD62L and CD44 expression. Data are presented as means ± SEM of n = 6-9 animals. *=p<0.05, **=p<0.01.

2.4.2 Naïve CD4$^+$ T cell bioenergetics are regulated by LAG-3

Recent work has revealed that T cell function and proliferation are tightly coupled to cellular metabolic programs. Specifically, naïve CD4$^+$ T cells rely on a more oxidative metabolic program thereby maximizing ATP production for driving basal processes like chemotaxis [49]. As LAG-3-deficient T cells demonstrated increased homeostatic expansion, and the ability to proliferate is highly dependent upon metabolism, we hypothesized that LAG-3 regulates the bioenergetic profile of these cells.

To assess metabolic differences, naïve wildtype and *Lag3*$^{-/-}$ OT.II T cells were isolated from spleens and analyzed using a Seahorse Flux analyzer. Results from a mitochondrial stress test indicated that *Lag3*$^{-/-}$ OT-IIs displayed significantly higher oxidative (measured by oxygen consumption (OCR)) and glycolytic (measured by extracellular acidification rate (ECAR)) metabolisms (Figure 9A-C; $p<0.0001$). Additionally, *Lag3*$^{-/-}$ deficient OT-II T cells presented with an increased spare respiratory capacity (SRC), a measure of mitochondrial reserve and fitness (Figure 9D; $p<0.0001$).

As naïve CD4$^+$ T cells are known to be more oxidative than glycolytic, compared to effector T cells, we calculated the ratio of OCR to ECAR as an indicator of whether these naïve T cells were behaving more like effector T cells. As anticipated, both wildtype and *Lag3*$^{-/-}$ deficient OT.II T cells had OCR to ECAR ratios over one, indicative of a more oxidative profile (Figure 9E); however, this ratio was significantly higher in the knockout OT.IIs ($p<0.01$). These results denote that the loss of LAG-3 leads to in enhanced oxidative and glycolytic metabolisms, but that these T cells still appear "naïve" from a metabolic standpoint.

Similar experiments were performed using isolated naïve T cells from C57Bl/6 animals, and results indicated that indeed, *Lag3*$^{-/-}$ polyclonal T cells demonstrated enhanced OCR, ECAR,

and SRC as compared to those from wildtype animals (Figure 10A-C; $p<0.0001$). OCR to ECAR ratios for both sets of T cells were greater than one, consistent with OT.II T cells, indicating a propensity for OXPHOS (Figure 10D; $p<0.0001$). Together, these findings show that metabolic regulation elicited by LAG-3 expression is not unique to TCR transgenic T cells. Additionally, they suggest that the augmented metabolic phenotype in *Lag3*$^{-/-}$ T cells likely supports their increased homeostatic proliferation (Figure 8).

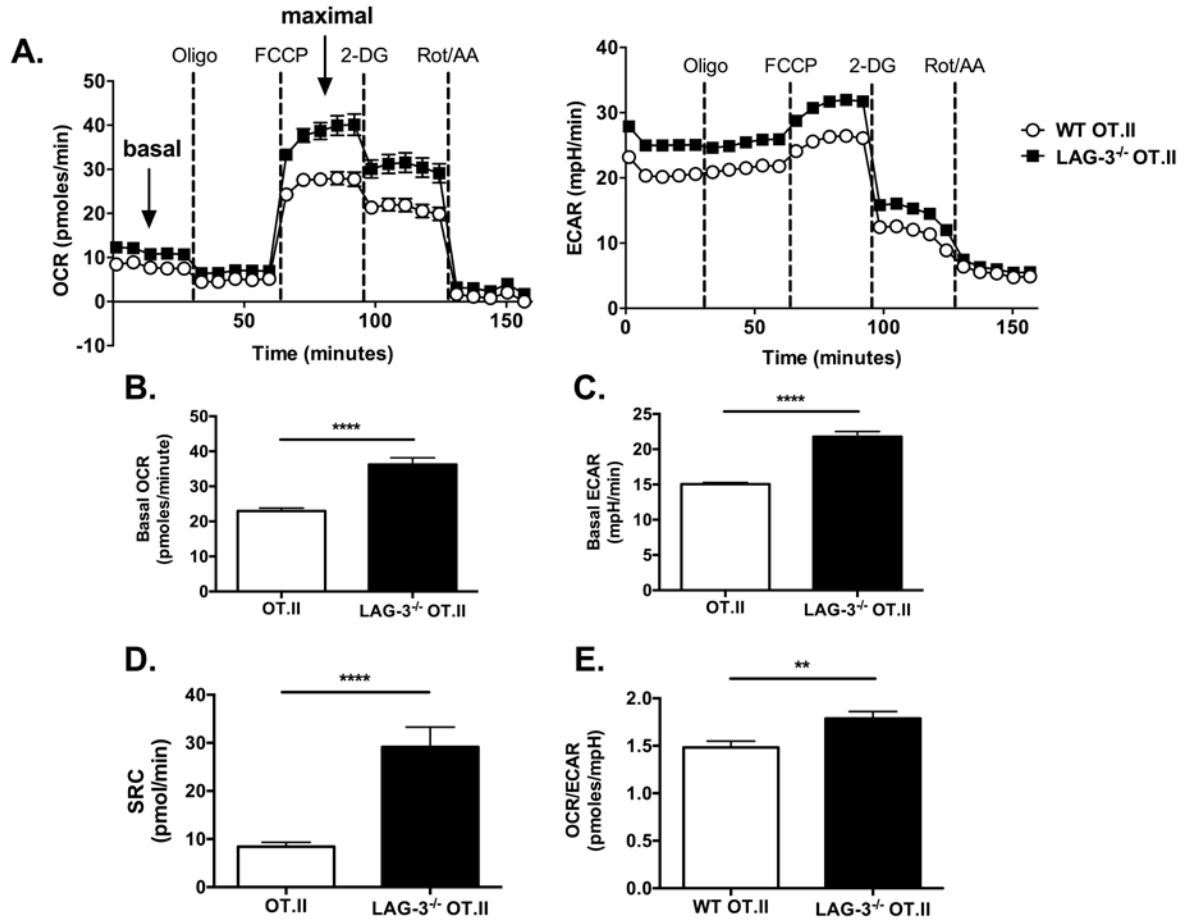

Figure 9. Loss of LAG-3 expression in naïve OT.II cells results in enhanced bioenergetics.
$CD4^+$ T cells were isolated from whole splenocytes, and immediately assayed in a mitochondrial stress test using a Seahorse Flux analyzer. **A.** Representative Seahorse run tracings comparing WT and *Lag3*$^{-/-}$ OT.IIs. Basal oxygen consumption (OCR; **B**) and extracellular acidification rate (ECAR; **C**) were measured prior to oligomycin injection (****$p<0.0001$). **D.** Spare respiratory capacity (SRC) was determined as basal OCR subtracted from maximal OCR (****$p<0.0001$). **E.** Basal OCR to ECAR ratio (**$p<0.01$). Graphs are of n = 5 independent experiments unless otherwise indicated.

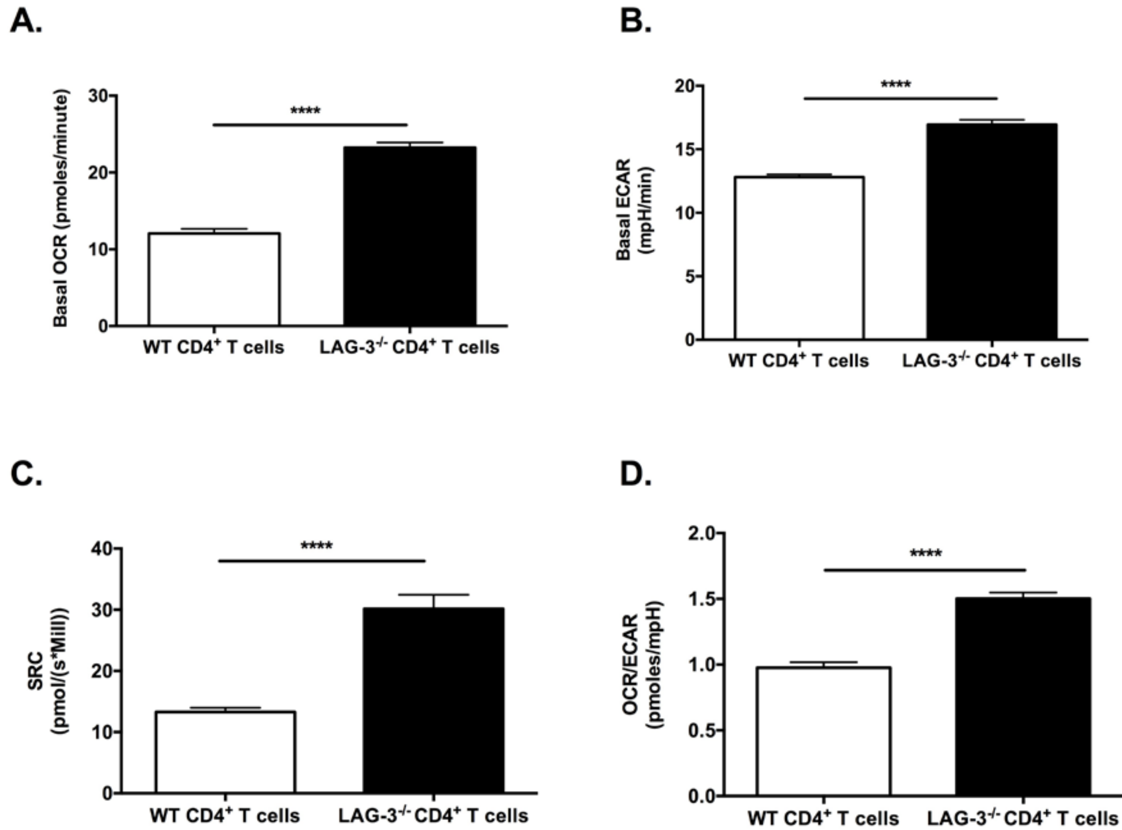

Figure 10. LAG-3 regulates the metabolic profile of naïve polyclonal $CD4^+$ T cells.
$CD4^+$ T cells were isolated from B6.WT and B6.*Lag3*$^{-/-}$ splenocytes, and assayed as described in Figure 4. Basal oxygen consumption (OCR; **A.**) and extracellular acidification rate (ECAR; **B**) were measured prior to oligomycin injection (****$p<0.0001$). **C.** Spare respiratory capacity (SRC) was determined as basal OCR subtracted from maximal OCR (****$p<0.0001$). **D.** Basal OCR to ECAR ratio (****$p<0.0001$). Graphs are of $n>3$.

2.4.3 LAG-3 expression negatively regulates mitochondrial biogenesis in naïve $CD4^+$ T cells

Memory $CD8^+$ T cells demonstrate increased SRC, largely due to their increased mitochondrial mass and biogenesis, compared to effector T cells [128, 129]. As *Lag3*$^{-/-}$ OT-IIs displayed a similar phenotype (Figure 9), we sought to determine if the enhanced mitochondrial oxygen consumption was in part, due to overall increased mitochondrial mass. PCR analysis revealed that *Lag3*$^{-/-}$ OT.IIs exhibited a 1.5-fold increase in their mitochondrial to nuclear DNA ratio, compared to wildtype T cells, indicative of increased mitochondrial mass (Figure 11A; $p<0.01$). qRT-PCR analysis also indicated increased expression of mitochondrial complex I, further corroborating the DNA results (Figure 11B; $p<0.05$). While DNA and RNA analysis revealed increased mitochondrial mass in *Lag3*-deficient T cells, measurement of forward scatter, as a readout of cell size, indicated no difference due to LAG-3 expression (Figure 11C).

Mitochondrial Transcription Factor A, or TFAM, is a key activator of mitochondrial biogenesis, and mRNA analysis revealed a 1.8-fold increase in TFAM expression in *Lag3*$^{-/-}$ OT.IIs compared to wildtype T cells (Figure 11D; $p<0.01$). The adenosine monophosphate activated protein kinase/Sirtuin-1 (AMPK/Sirt-1) pathway drives activation of the transcription factor Peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC-1α), which in turn controls TFAM expression [345, 346]. Therefore, it was important to determine whether if this pathway was mediating the increased mitochondrial mass observed in *Lag3*$^{-/-}$ OT.II T cells. Protein analysis showed increased activated AMPK, as measured by phosphorylation (Thr172) (Figure 11E), concurrent with a two-fold increase in Sirt-1 expression (Figure 11F), suggesting that LAG-3-mediated suppression of this pathway likely dampens mitochondrial biogenesis.

Similar differences in mitochondrial biogenesis and signaling were observed in polyclonal naïve $CD4^+$ T cells (Figure 12A-F). Yet, while there were observed differences due to LAG-3 expression in mitochondrial oxygen consumption (Figure 10) and biogenesis (Figure 12), there was no significant difference in total ATP production between the two cell types (Figure 12G). These studies reveal that LAG-3 expression negatively regulates mitochondrial biogenesis by tempering the AMPK/Sirt-1 pathway, thereby also controlling mitochondrial oxygen consumption, and that these results are independent of TCR specificity.

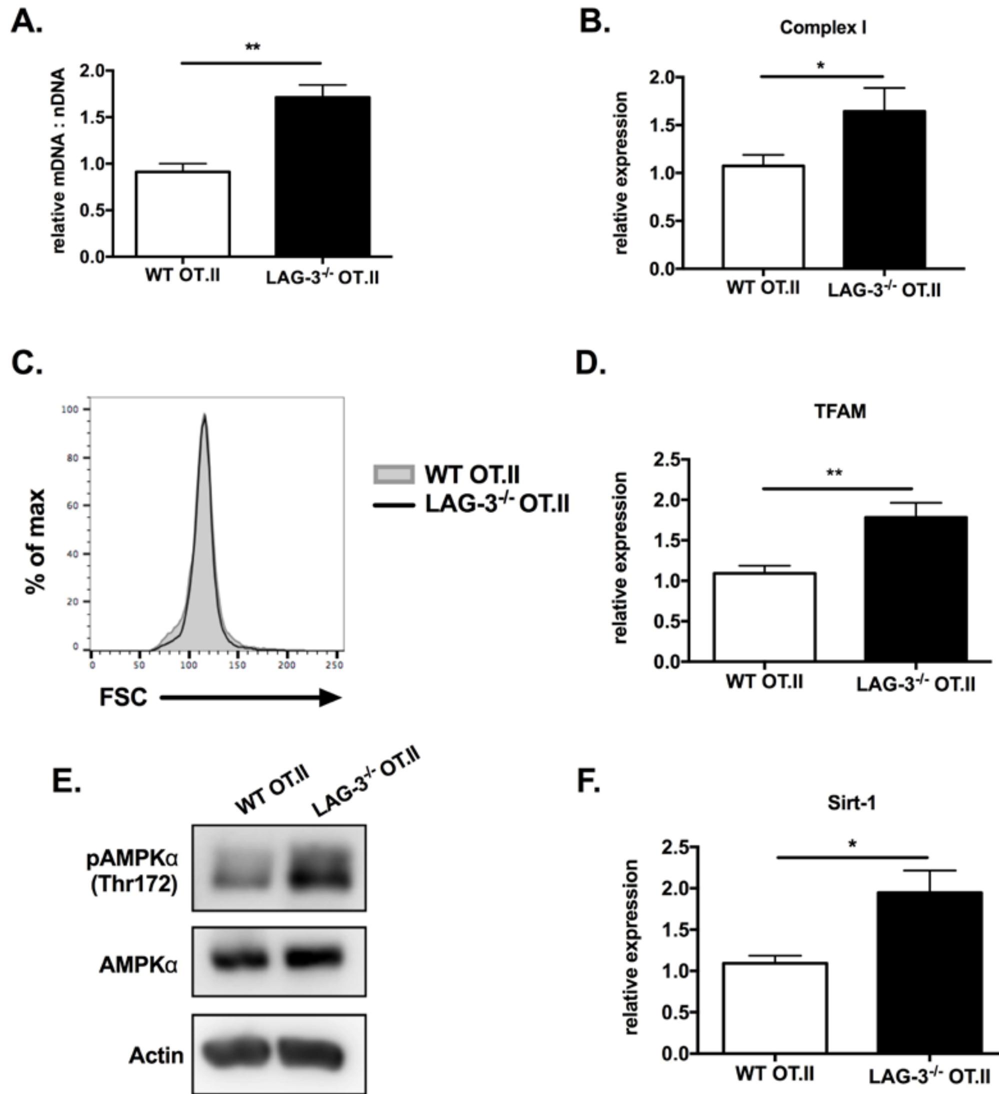

Figure 11. LAG-3 expression negatively regulates mitochondrial biogenesis in naive CD4+ T cells.
Naïve $CD4^+$ T cells were isolated from whole splenocytes and used for downstream analysis. **A.** qPCR for measuring mitochondrial (mDNA) to nuclear (nDNA) DNA ratio. **B.** qRT-PCR analysis of mitochondrial Complex I expression. **C.** Representative histogram measuring forward scatter (cell size). **D.** qRT-PCR analysis of TFAM expression. **E.** Representative Western blots of pAMPK (Thr172), total AMPK, and β actin (loading control). **F.** qRT-PCR analysis of Sirt-1 expression. Data are displayed as means ± SEM of n=5-9 independent experiments. *=$p<0.05$; **=$p<0.01$.

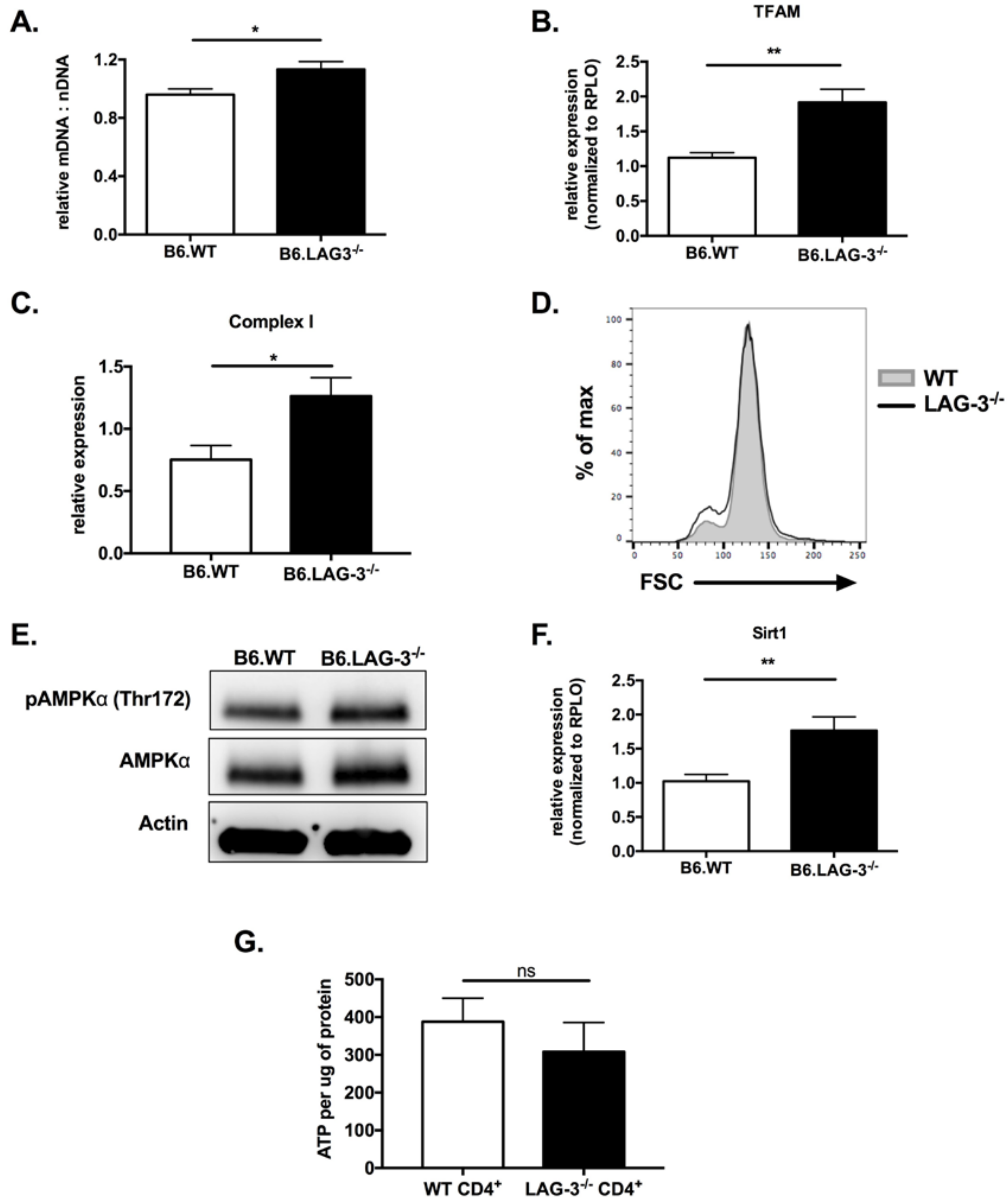

Figure 12. LAG-3 regulates mitochondrial biogenesis in naive polyclonal CD4+ T cells.
Polyclonal CD4$^+$ T cells were isolated from C57Bl/6 wildtype and *Lag3*$^{-/-}$ animals and analyzed for mitochondrial differences. **A.** qPCR for measuring mitochondrial (mDNA) to nuclear (nDNA) DNA ratio. mRNA expression of **B.** TFAM and **C.** mitochondrial complex I. **D.** Measure of cell size by forward scatter (FSC). **E.** Protein analysis of activated (phosphorylated; Thr172) and total AMPK. **F.** mRNA expression of Sirt-1. **G.** Measure of intracellular ATP per ug of protein. Data are displayed as means ± SEM of n=7-9 independent experiments. *=$p<0.05$; **=$p<0.01$.

2.4.4 Loss of LAG-3 expression does not alter the frequency of thymocyte populations

Following the observations that LAG-3 deficiency resulted in enhanced mitochondrial mass (Figures 11-12) and metabolism (Figure 9-10), we wanted to further delineate if this phenotype was due to alterations in thymic development or extrinsic signaling in the periphery. To examine thymic subsets, thymuses were harvested from 4-6-week-old animals, and cells were stained for flow cytometry. Identification of immune cells was based first on CD45 expression and then CD8 versus CD4 expression to distinguish double negative (DN), double positive (DP), and single positive (SP) populations (Figure 13A). Additionally, DN populations were further dissected into DN subsets 1-4 based on CD44 and CD25 expression. Overall, *Lag3*$^{-/-}$ OT.II thymuses had greater cellularity compared to those from wildtype animals (Figure 13B; p=0.082), suggesting increased proliferation in the thymus. However, thymic cellularity remained relatively unchanged in B6 animals (Figure 13C). Following flow cytometric analysis, there were no differences in frequencies of DN, DP, and SP populations between wildtype and LAG-3$^{-/-}$ animals, regardless of strain (Figure 13D). These results held true for DN 1-4 populations as well (Figure 13E). Based on these data, we concluded that it was unlikely LAG-3 was influencing thymocyte development.

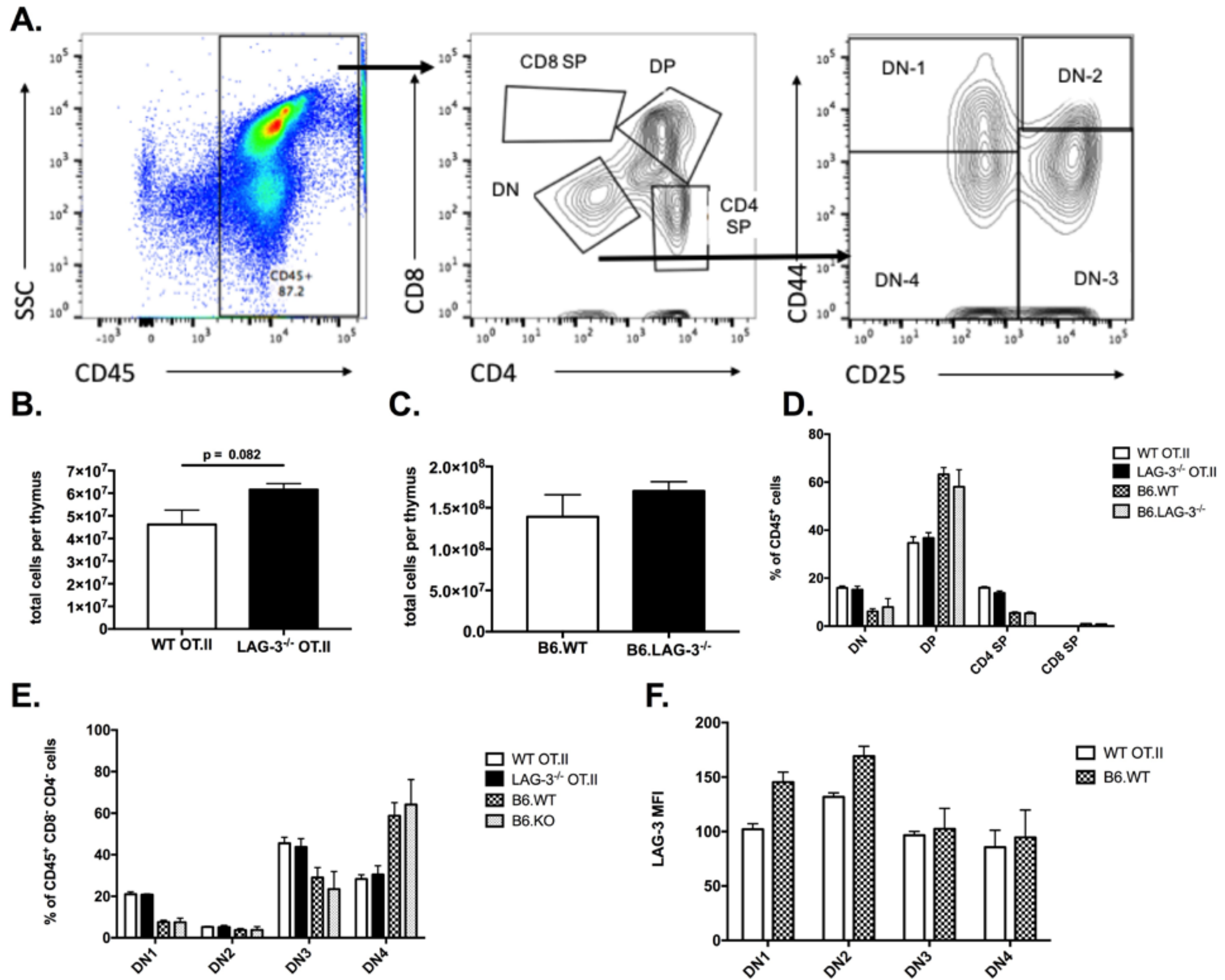

Figure 13. Loss of LAG-3 expression does not alter the frequency of T cell precursors in the thymus.
A. Flow cytometry gating for examining single and double positive thymocytes from OT.II wildtype and LAG-3$^{-/-}$ animals (4-6 weeks old). Total number of cells per thymus comparing **B.** WT and LAG-3$^{-/-}$ OT.II and **C.** B6.WT and LAG-3$^{-/-}$ animals. **D.** Frequencies of double negative (DN), double positive (DP), CD4 single positive (CD4 SP) and CD8 single positive (CD8 SP) populations from indicated strains. **E.** Frequencies of double negative 1-4 populations based on CD44 and CD25 expression. **F.** LAG-3 surface expression of wildtype OT.II and B6 double negative populations. n = 4-5 mice per strain.

2.4.5 LAG-3 signaling is necessary for regulating mitochondrial metabolism in the periphery

As data from thymic populations did not yield significant differences, we next assessed the role of LAG-3 extrinsic signaling in the periphery. To do so, we utilized a model of spontaneous homeostatic proliferation, whereby naïve T cells were adoptively transferred into lymphopenic animals (Figure. 14A). To directly assess if LAG-3 mediated an effect, a cohort of animals that received wildtype OT.II T cells were treated with a LAG-3 blocking antibody. After ten days, splenic $CD4^+$ T cells were analyzed by flow cytometry and results showed a higher percentage of $CD4^+$ T cells per spleen in animals that received *Lag3*$^{-/-}$ OT.II T cells (Figure 14B; $p<0.05$). Additionally, *Lag3*$^{-/-}$ OT.II T cells proliferated to a greater extent, as measured by Cell Trace Violet dilution (Figure 14C; $p<0.05$). Treatment of WT T cells with a LAG-3 blocking antibody fully restored the total percentage and proliferation of transferred T cells to levels demonstrated by LAG-3-deficient cells (Figure 14B-C). Thus, LAG-3 signaling directly regulates homeostatic expansion of naïve $CD4^+$ T cells.

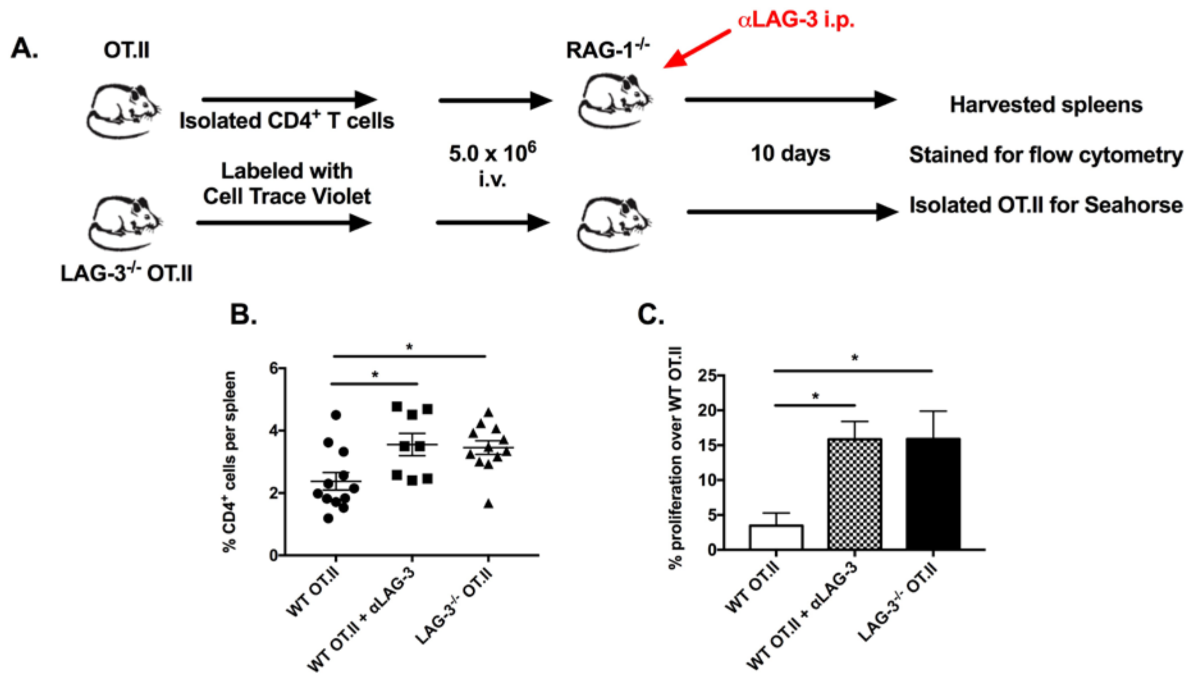

Figure 14. LAG-3 loss and blockade result in increased homeostatic expansion following adoptive transfer. **A.** Schematic of adoptive transfer model to assess homeostatic expansion. A cohort of WT OT.II recipients also received 200μg of αLAG-3 i.p. every-other-day. **B.** Percentage of $CD4^+$ T cells per spleen and **C.** percent proliferation normalized to WT OT.II assessed by flow cytometry. Proliferation was determined by Cell Trace Violet dilution as compared to non-transferred controls. Data are presented as means ± SEM of 3 independent experiments with n=3-5 mice per experiment. *=$p<0.05$

Splenic OT.II T cells from recipient animals were also isolated and analyzed for mitochondrial fitness by Seahorse assay. Similar to what was demonstrated in *ex vivo* analysis (Figure 9-10), OT.II T cells deficient in LAG-3 displayed significantly higher basal OCR and ECAR as compared to their wildtype counterparts (Figure 15A-D). LAG-3 blockade by monoclonal antibody administration augmented both OCR and ECAR of WT cells to levels comparable to *Lag3*$^{-/-}$ $CD4^+$ T cells, demonstrating an extrinsic inhibitory effect of LAG-3 on mitochondrial and glycolytic metabolism (Figure 15A-D). Interestingly, SRC was completely absent in T cells from all three cohorts (Figure 15E) in comparison to values obtained during *ex vivo* analysis (Figure 9D, 10C). When we compared basal OCR and SRC levels pre-transfer

with basal OCR rates post-transfer, we observed an additive effect – the post-transfer basal OCR was equal to the sum of the pre-transfer basal OCR and SRC (Figure 15F). These results suggest that SRC was not lost, but was in fact being utilized to support spontaneous proliferation. These data reveal that LAG-3 engagement and signaling act to limit naïve $CD4^+$ T cell proliferation by regulating mitochondrial and glycolytic metabolism.

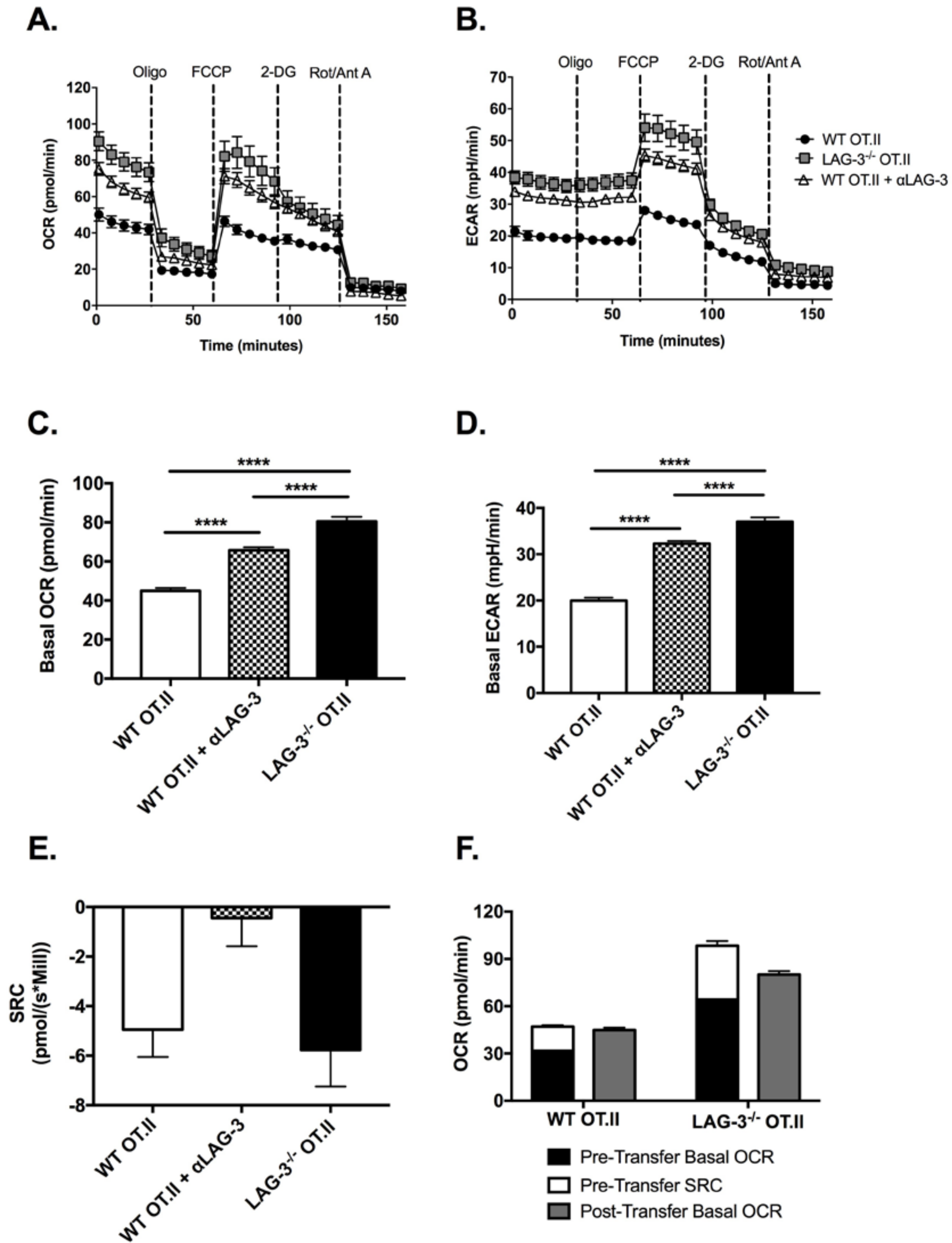

Figure 15. LAG-3 tempers T cell bioenergetic pathways during homeostatic expansion.
OT.II WT and *Lag3*-deficient T cells were adoptively transferred into B6.*Rag1*$^{-/-}$ recipients as a model of homeostatic expansion. A cohort of WT recipients were treated with 200ug of a LAG-3 blocking antibody. **A-B**. Representative Seahorse analysis of isolated OT.II T cells 10 days post-adoptive transfer. Calculated **C.** basal OCR, **D.** ECAR, and **E.** SRC of OT.II T cells post-transfer (****p<0.0001). **F.** Comparison of post-transfer basal OCR to sum of pre-transfer OCR and SRC. Values are indicative of combined n>5 animals per group and displayed as mean ± SEM.

2.4.6 LAG-3 expression increases the dependence of naïve CD4^{+} T cells on Interleukin-7

The common gamma chain (γ_c) family of cytokines (IL-7, IL-15, IL-2, IL-9 and IL-4) play important roles in the homeostasis and proliferation of naïve, effector and memory T cells [74, 79, 347, 348]. In the context of naïve T cell homeostasis and survival, IL-7 is essential for mediating both glucose uptake and oxidation, and survival via Bcl-2 expression [66-68, 91]. Moreover, IL-7 mediates its signaling via Akt and STAT5 phosphorylation. Following the findings that LAG-3 loss resulted in increased mitochondrial oxidation, homeostatic proliferation (Figure 14-15), and STAT5 phosphorylation, all responses that are IL-7 dependent [66-68, 91], we wanted to assess if this enhancement was due to increased IL-7 signaling.

Examination of CD127 (IL-7Rα) expression in adoptive transfer experiments revealed that LAG-3-deficient OT.IIs exhibited an approximate 25% decrease in CD127 surface expression compared to WT T cells both pre- and post-transfer (Figure 16A-B; $p<0.05$). Work by *Park et al.* showed that increased IL-7/IL-7R signaling is indirectly related to IL-7 mRNA and surface expression [85]. Our results, coupled with the studies from *Park et al.*, suggested that LAG-3-deficient T cells experience increased IL-7R signaling, resulting in reduced surface expression. To directly test the influence of IL-7 on naïve T cell responses, wildtype and *Lag3*$^{-/-}$ OT.II splenocytes were treated with either media alone or media containing IL-7 for 18 hours and assessed for glucose uptake and viability. Alternatively, to what was anticipated, wildtype OT.II T cells exhibited a significantly higher change in glucose uptake (Figure 16C-D; $p<0.05$) and viability (Figure 16E-F) due to the presence of IL-7 as compared to *Lag3*$^{-/-}$ T cells, thus suggesting wildtype T cells have a greater dependence on the cytokine. Additionally, the increased CD127 expression by these T cells also suggests an increased IL-7 dependence.

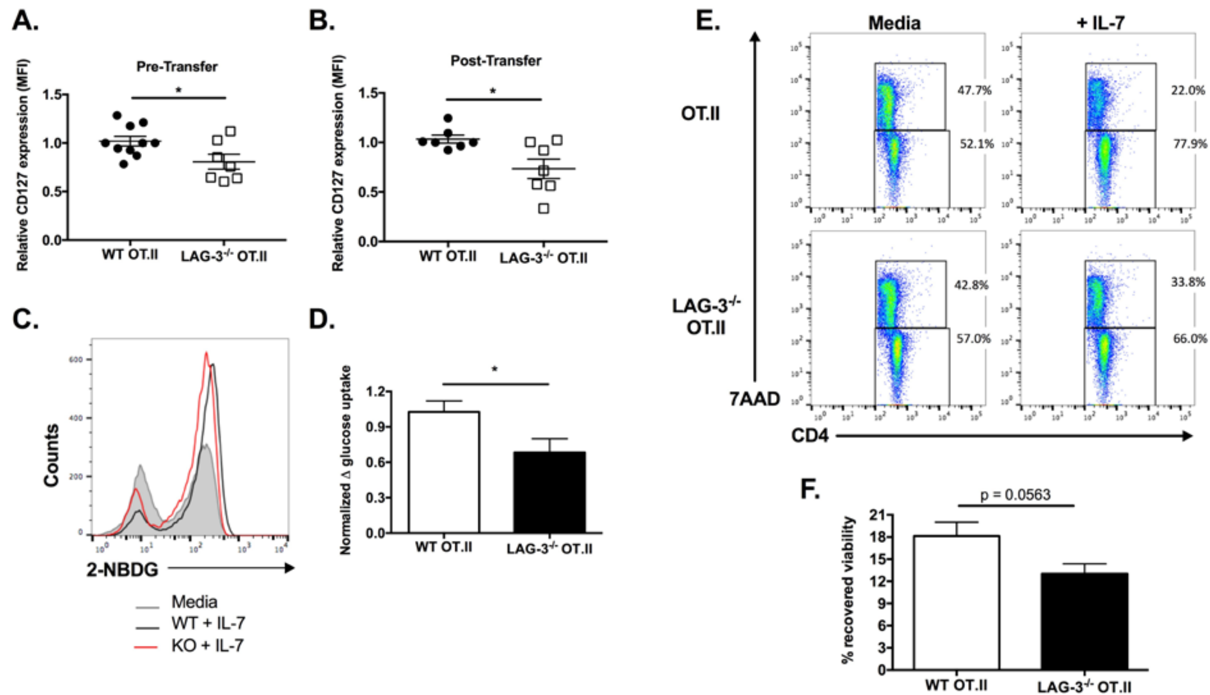

Figure 16. Loss of LAG-3 expression reduces sensitivity to IL-7 in vitro.
Relative IL-7Rα surface expression on naïve $CD4^+$ wildtype and LAG-$3^{-/-}$ T cells **A.** pre- and **B.** post- adoptive transfer. **C-E.** Naïve splenocytes were treated with or without IL-7 *in vitro* for 18 hours. **C.** Representative histograms of glucose uptake (2-NBDG) of naïve $CD4^+$ T cells treated with IL-7 or media alone (control). **D.** Change in glucose uptake due to the presence of IL-7. **E.** Representative dot plots of 7AAD staining of $CD4^+$ T cells to measure viability. **F.** Percent recovered viability due to the presence of IL-7 in cell cultures. Data were normalized to WT values within experiments and are displayed as mean ± SEM of n = 5-6 independent experiments. *=p<0.05.

2.4.7 LAG-3-deficient T cells are refractory to IL-7 deprivation during homeostatic proliferation

Following the *in vitro* studies, we sought to verify our findings in our *in vivo* adoptive transfer model. To do so, *Rag1*$^{-/-}$ recipients were treated i.p. with either a CD127 blocking antibody or an isotype control every other day throughout the course of the experiment. Ten days post-transfer wildtype and *Lag3*$^{-/-}$ OT.II cells were recovered from spleens and analyzed by flow cytometry

and Seahorse as described above. Metabolic analysis revealed that even with IL-7 blockade, *Lag3*$^{-/-}$ OT.II T cells outperformed treated wildtype cells during a mitochondrial stress test (Figure 17A). In comparison to isotype treated controls, IL-7 blockade resulted in a 60% reduction in basal OCR of wildtype cells (Figure 17B; p=0.0544); however, *Lag3*$^{-/-}$ T cells experienced no change in basal OCR (Figure 17C), indicating an IL-7 dependence for OCR in wildtype T cells. Similar to previous experiments, all transferred T cells, regardless of treatment, used the entirety of their SRC to drive homeostatic expansion (Figure 17D). Unlike oxygen consumption, there was no dependence on IL-7 for glycolysis in either cell type, yet there was a trend that IL-7 blockade increased glycolysis in wildtype T cells (Figure 17E,F). This could indicate that without the use of oxidative phosphorylation, wildtype T cells may try to compensate by increasing their dependence on glycolysis. These metabolic differences also directly correlated with overall percentages of T cells per spleen of recipient animals (Figure 17G; $p<0.05$). Lastly, IL-7 is known to regulate naïve T cell size, and cell size analysis by forward scatter indicated that WT T cells demonstrated a greater reduction in cell size between those deprived of IL-7 and those treated with IgG control (Figure 17H-I). Lag-3 deficient T cells exhibited little to no change in forward scatter due to IL-7R blockade (Figure 17H-I).

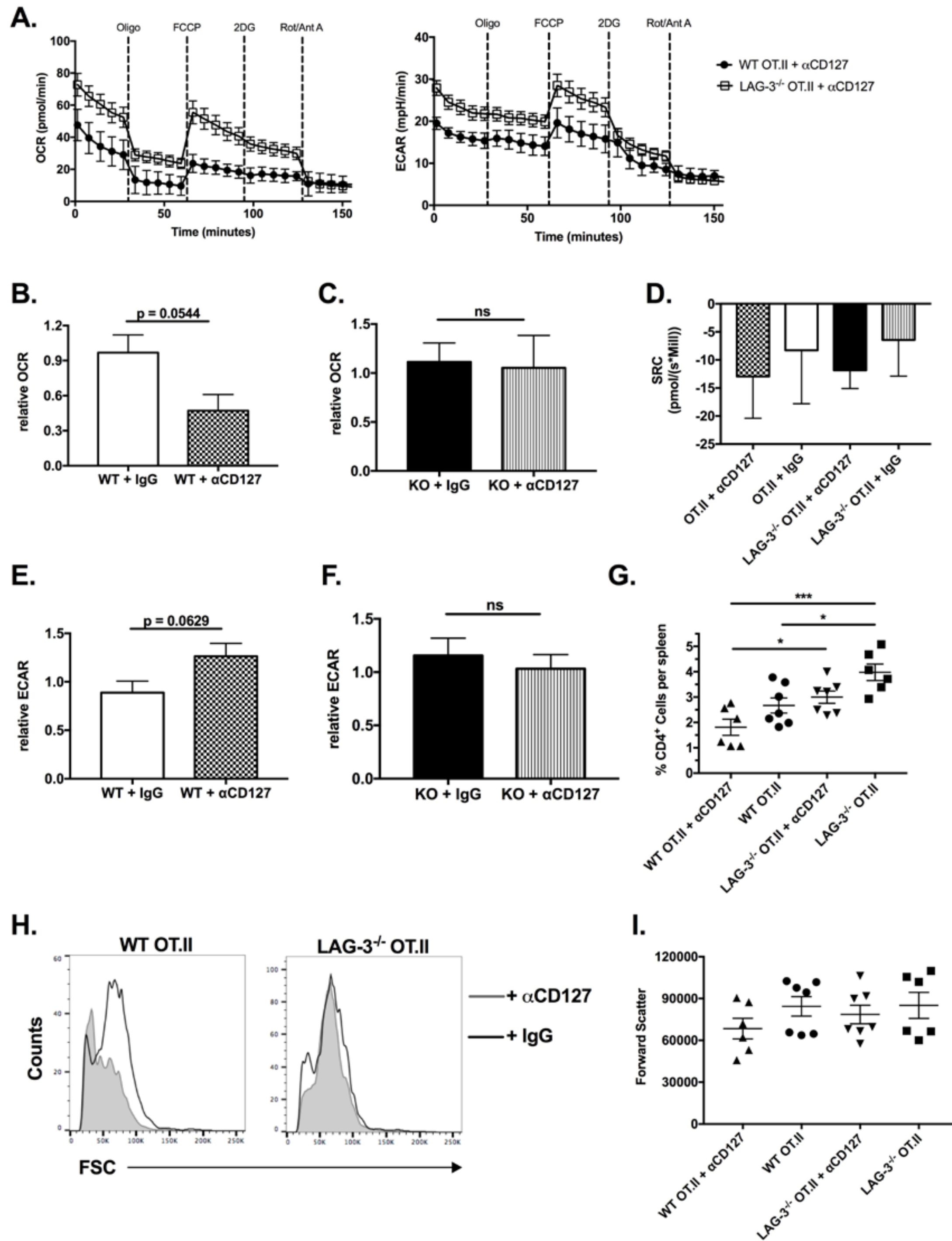

Figure 17. LAG-3-deficient CD4+ T cells are refractory to IL-7 deprivation in vivo.
A. OCR (left panel) and ECAR (right panel) of OT.II T cells following adoptive transfer and treatment with αCD127 or isotype control antibody. Basal relative OCR of **B.** WT T cells and **C.** LAG-3$^{-/-}$ OT.II T cells ± antibody treatment. **D.** Spare respiratory capacity of T cells following adoptive transfer. Relative ECAR of **E.** WT and **F.** *Lag3*$^{-/-}$ OT.II T cells ± antibody treatment. **G.** Percent CD4^{+} T cells per spleen 10 days post adoptive transfer. **H.** Representative histograms of forward scatter (FSC) of adoptively transferred CD4^{+} T cells. **I.** Quantification of forward scatter of CD4+ T cells as calculated by FlowJo software. *=p<0.05; ***=p<0.001.

2.4.8 Inhibition of STAT5 by LAG-3 stabilization of PTEN restrains homeostatic expansion

Previous studies have indicated that LAG-3 engagement results in inhibitory downstream signaling, with implications on proliferation and cytokine production [156, 337, 349, 350]. While the particular domain in the LAG-3 cytoplasmic tail that elicits this inhibitory effect has been identified [145], the exact downstream signaling of the molecule remains elusive. Work by *Durham et al.* indicated that LAG-3 engagement can inhibit phosphorylation of STAT5, resulting in dampened T cell homeostatic expansion [338]. Moreover, activated STAT5 is critical for homeostatic expansion [83, 84], and upon activation, can translocate to the mitochondria and bind mitochondrial DNA, potentially modulating mitochondrial gene transcription [351, 352]. These findings suggest a link between STAT5 and regulation of mitochondrial respiration and biogenesis. Studies in our adoptive transfer model further confirmed that LAG-3 inhibition via knockout or antibody blockade resulted in increased STAT5 phosphorylation in $CD4^+$ T cells (Figure 18A; $p<0.05$).

The PI3/Akt pathway has also been shown to be critical for driving naïve and memory T cell homeostatic proliferation and increased mitochondrial respiration [353, 354]; therefore, we next examined Akt activation in our model. Intracellular staining for phosphorylated Akt in naïve $CD4^+$ T cells showed very modest increases in both WT + anti-LAG-3 Ab. and LAG-$3^{-/-}$ T cells compared to WT OT.IIs; however, these differences were not significant (Figure 18B). These results were not entirely surprising as Akt is critical for mediating homeostatic proliferation, which WT OT.II T cells do exhibit, and could also be activated to serve as a survival signal in these T cells.

Next, we investigated if elevated STAT5 phosphorylation in *Lag3*$^{-/-}$ T cells could be due to decreased expression of inhibitory phosphatases. Specifically, we targeted PTEN, as it is stabilized by another inhibitory receptor, Programmed Cell Death-1 (PD-1), and its stabilization can alter T cell metabolic programming [355, 356]. Additionally, PTEN knockout cells demonstrate hyperproliferation [86, 89], reminiscent of the enhanced proliferation exhibited by LAG-3-deficient CD4^{+} T cells (Figures 8, 14). As anticipated, WT OT.II T cells showed increased PTEN expression as measured by flow cytometry, and LAG-3 knockout resulted in approximately a 20% reduction in PTEN levels (Figure 18C; $p<0.01$). Overall, these results suggest a potential role for LAG-3 in containing STAT5 activation by supporting PTEN expression.

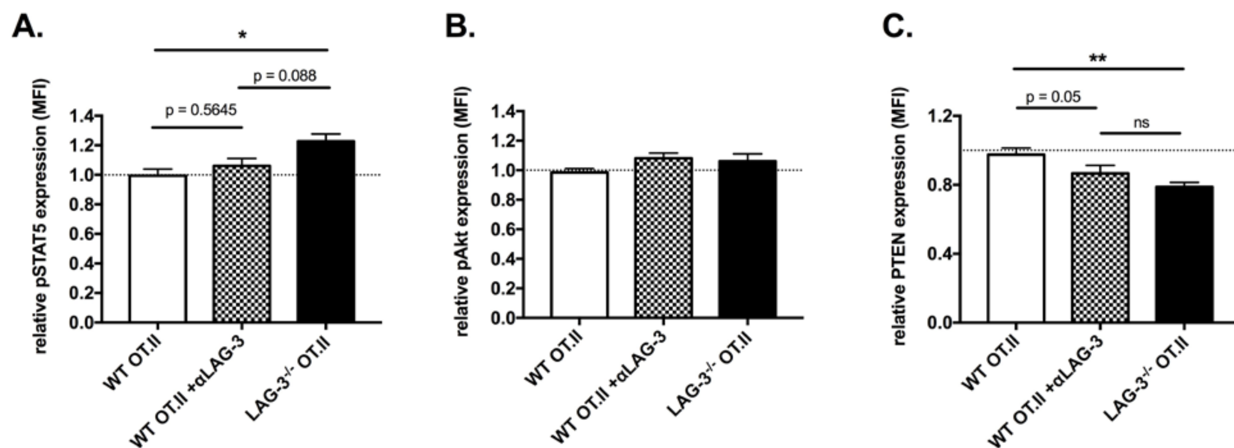

Figure 18. Signaling via LAG-3 stabilizes PTEN expression.
Intracellular Phospho-flow staining of adoptively transferred CD4^{+} T cells for **A.** pSTAT5 (Tyr964), **B.** pAkt (Ser497) and **C.** PTEN. Mean fluorescence intensity (MFI) of CD4^{+} T cells was calculated using FlowJo software, and numbers were normalized to WT values to measure relative expression. Data are presented as means ± SEM of n=5-7 animals. *=$p<0.05$, **=$p<0.01$.

2.4.9 Loss of LAG-3 expression results in enhanced aerobic glycolysis and effector function during activation

One of the key characteristics of memory T cells is their rapid activation upon secondary challenge [137], which is due, in part, to their increased SRC and mitochondrial content [128, 129]. As our data indicated that LAG-$3^{-/-}$ $CD4^{+}$ T cells demonstrated a more memory-like phenotype metabolically (Figures 9 and 11), we hypothesized that this bioenergetic advantage would enable a faster transition to effector function. LAG-3 is known to be upregulated to the T cell surface during activation [149, 150], and ligation by MHC class II, results in diminished proliferation and effector function [337, 349]. Therefore, we utilized an APC-free system to circumvent any regulation in the wildtype cells due to LAG-3 engagement.

During activation, T cells undergo robust metabolic preprogramming, transitioning from OXPHOS (naïve) to aerobic glycolysis (effector) [48]. This transition is critical for supporting increased macromolecule synthesis during both clonal expansion and effector function. Work from *Wang et al.* demonstrated that within 24 hours post-activation, $CD4^{+}$ T cells upregulate the necessary genes required for the metabolic transition to aerobic glycolysis [103]. At 24 hours post-stimulation in our model, *Lag3*$^{-/-}$ $CD4^{+}$ T cells exhibited significantly greater upregulation of the transcription factors *Myc* and *Hif1α* (Figure 19A, $p<0.05$), which orchestrate upregulation of the glycolytic pathway [49]. *Lag3*$^{-/-}$ T cells also more highly upregulated the passive glucose transporter *Glut1*, which is necessary for importing glucose into $CD4^{+}$ T cells (Figure 19A; $p<0.05$) [357]. With increased *Glut1* upregulation by LAG-3-deficient T cells, we next examined glucose uptake by these cells. Indeed, *Lag3*$^{-/-}$ T cell cultures had a higher percentage of glucose^{hi} (2-NBDG^{hi}) T cells at both 48 and 72 hours post-stimulation (Figure 19B-C), indicative of more T cells transitioning to aerobic glycolysis. Lastly, lactate secretion, the

byproduct of aerobic glycolysis was greater in *Lag3*$^{-/-}$ T cell supernatants (Figure 19D; $p<0.01$), signifying enhanced usage of this pathway.

Lag3$^{-/-}$ CD4^{+} T cells also showed greater cell growth during activation, as measured by forward scatter (Figure 19E), a process that is necessary for supporting cell division. IFNγ production is directly regulated by transition to aerobic glycolysis [358], and based on these data we hypothesized that due to their enhanced aerobic glycolysis, *Lag3*$^{-/-}$ T cells would produce more IFNγ as compared to their wildtype counterparts. Indeed, IFNγ ELISA results from culture supernatants revealed that LAG-3-deficient T cells produced three times as much IFNγ as wildtype T cells at both 24 and 48 hours post-stimulation (Figure 19F; $p<0.05$). Lastly, LAG-3 is highly upregulated to the T cell surface upon activation, and can therefore, serve as a marker of activation. Further examination of wildtype cells indicated that LAG-3 expression correlated with glucose uptake, as LAG-3^{hi} T cells demonstrated higher 2-NBDG fluorescence (Figure 19G), suggesting that LAG-3 may serve as a marker for more glycolytic effector T cells.

Overall, these data suggest that the enhanced mitochondrial bioenergetics due to loss of LAG-3 expression support heightened upregulation of aerobic glycolysis during activation, similarly to that observed in memory T cells.

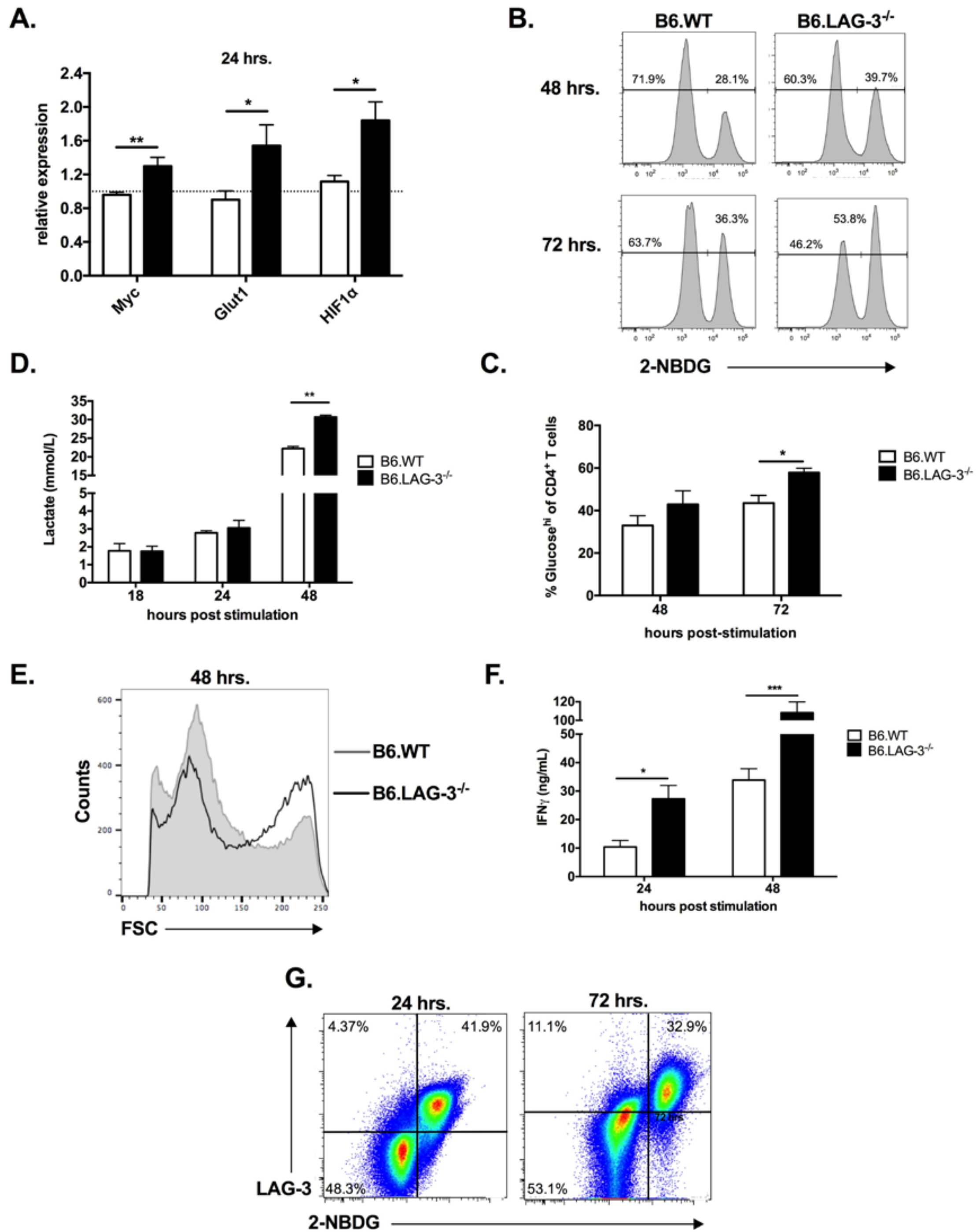

Figure 19. LAG-3-deficient CD4+ T cells demonstrate enhanced aerobic glycolysis and effector function upon activation.

A. qRT-PCR measuring expression of glycolysis-associated genes. **B-C.** Glucose uptake of $CD4^+$ T cells measured by 2-NBDG fluorescence. **D.** Lactate secretion in culture supernatants. **E.** Forward scatter of $CD4^+$ T cells 48 hours post-stimulation. **F.** IFNγ secretion in culture supernatants. **G.** Representative dot plot of LAG-3 by 2-NBDG uptake of $CD4^+$ T cells during activation. Data are presented as means ± SEM of combined n=5-9 independent experiments. *=p<0.05, **=p<0.01, ***=p<0.001.

2.5 DISCUSSION

Our results here indicate that expression of LAG-3 on naïve $CD4^+$ T cells regulates mitochondrial biogenesis and metabolism, as a means of controlling homeostatic expansion and quiescence. While LAG-3 expression on naïve $CD4^+$ T cells is known to be rather low as compared to activated or exhausted T cells, it is clear that the expression levels are sufficient for mediating phenotypic alterations [157, 338]. The enhancement of mitochondrial metabolism and mass due to lack of LAG-3 expression is consistent with the recent study published by *Scharping et al* [343]. Their results showed that increased expression of inhibitory receptors (PD-1, LAG-3 and Tim-3) on exhausted intratumoral $CD8^+$ T cells correlated with reduced mitochondrial mass, further supporting a link between these receptors and the mitochondria.

The fact that antibody blockade of LAG-3 on wildtype $CD4^+$ T cells during adoptive transfer experiments yielded similar proliferation and metabolic responses as LAG-$3^{-/-}$ cells (Figures 10-11), suggests that this phenotype is not solely due to an intrinsic mechanism. Rather, that extrinsic signals, potentially via MHC class II:LAG-3 interactions, drive inhibitory signaling pathways that dampen AMPK-driven mitochondrial biogenesis. This is also supported by finding that there were no differences in frequency of double negative (DN1-4), double positive and single positive populations in the thymus (Figure 13), again suggesting that this phenotype is not a product of thymic developmental alterations, but rather alterations due to T cell interactions in the periphery.

We had initially hypothesized that LAG-3 may inhibit tonic TCR signaling that occurs in the periphery to maintain survival of naïve $CD4^+$ T cells. During tonic signaling, naïve T cells are engaged by MHC-presented self-peptides by antigen presenting cells as a means of initiating low-level TCR signaling. This tonic signal can be sufficient to increase anti-apoptotic Bcl-2

expression and allow T cells to enter the cell cycle to facilitate homeostatic expansion. Fine tuning of this signal is critical for preventing overt T cell activation; therefore, inhibitory mechanisms, like CD5 expression, are in place to modulate TCR signaling [96, 359]. Thus, we hypothesized that LAG-3$^{-/-}$ CD4^{+} T cells would express more CD5, indicative of more tonic TCR signaling; however, our data showed no appreciable differences in CD5 expression between naïve wildtype and LAG-3-deficient T cells, even after adoptive transfer (data not shown). This result does not completely rule out the hypothesis that LAG-3 regulates tonic signaling; however, further experiments would be necessary.

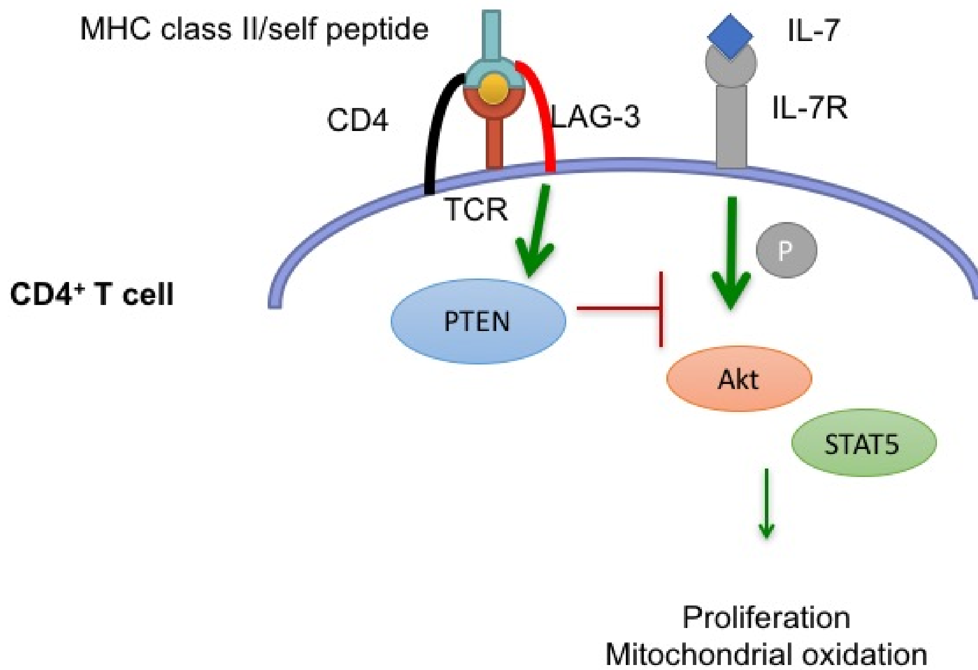

Figure 20. Mechanism for LAG-3's regulation of mitochondrial respiration and homeostatic expansion. We propose that LAG-3 expression on naïve CD4+ T cells stabilizes PTEN expression, thereby resulting in dephosphorylation of STAT5 and Akt, resulting in basal proliferation and mitochondrial respiration. In order to overcome this inhibition, strong IL-7 signaling is required to then drive sufficient Akt and STAT5 activation to mediate homeostatic proliferation and mitochondrial oxidation.

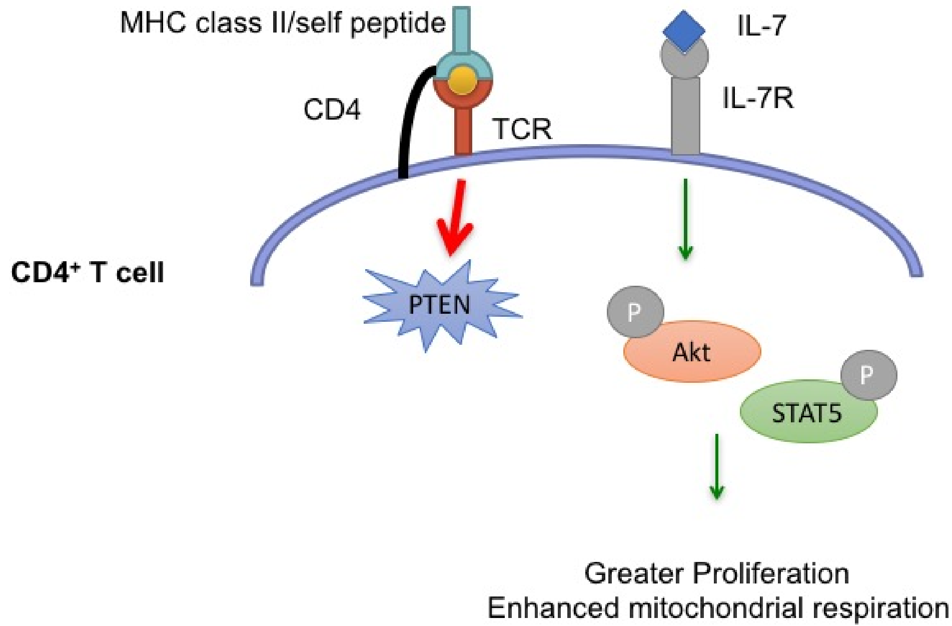

Figure 21. Absence of LAG-3 results in destabilization of PTEN and its inhibitory function.
In the absence of LAG-3, PTEN expression is destabilized thereby allowing greater downstream TCR signaling. This enhancement allows for more robust Akt and STAT5 activation, resulting in greater homeostatic proliferation and enhanced mitochondrial respiration. This increased signaling reduces the requirement for IL-7 signaling to mediate these processes.

Activated *Lag3*$^{-/-}$ CD4^{+} T cells demonstrated enhanced aerobic glycolysis and effector function (Figure 19). Interestingly, memory T cells demonstrate similar enhanced activation in comparison to naïve T cells, in part, due to their enhanced mitochondrial mass and SRC [128, 129]. We propose that the bioenergetic advantage exhibited by *Lag3*$^{-/-}$ CD4^{+} T cells supports their enhanced activation and glycolytic potential, similarly. Following activation, it is necessary for a subset of activated effector T cells to survive as long-lived memory T cells. Metabolically, this transition is dependent upon the ability of the cell to downregulate aerobic glycolysis and transition back to a more oxidative phenotype. Enhancement of OXPHOS via treatment with Metformin, an AMPK activator, resulted in an increased frequency of memory T cells following activation [136]. As LAG-3-deficient T cells are much more glycolytic than wildtype cells, we might suspect that they would have a more difficult time converting back to oxidative

phosphorylation; thus, limiting the potential for generating memory T cells. Studies by *Workman et al.* indicated increased numbers of virus-specific memory $CD4^+$ and $CD8^+$ T cells 30 days post-infection in *Lag3*$^{-/-}$ animals [336]. However, re-challenge experiments to assess memory T cell functionality were not performed; therefore, while numbers may indicate an advantage, there may be functional deficiencies due to LAG-3 loss. Further studies would be necessary to more fully assess memory T cell generation.

In the context of autoimmunity, LAG-3 has been shown to play a critical role in suppressing $CD4^+$ T cell function. Global knockout and antibody blockade of LAG-3 in non-obese diabetic (NOD) animals accelerated autoimmune Type 1 Diabetes progression [155, 158]. Moreover, animals with genetic LAG-3 alterations that resulted in accelerated T1D had equally functional regulatory T cell populations, indicating that this disease progression was not due to dysfunctional peripheral tolerance [158]. Our data imply that the increased T1D progression in NOD.LAG-3$^{-/-}$ animals was due, in part, to metabolic enhancement of naïve $CD4^+$ T cells, conferring greater activation and disease-inducing potential. Human studies have been conducted to identify single nucleotide polymorphisms (SNPs) in genes that are associated with susceptibility to autoimmune diseases. A study by *Zhang et al.* identified SNPs in both the genes encoding LAG-3 and IL-7R that are more highly associated with progression to multiple sclerosis [360]. This work strengthens our conclusion that there is an inherent link between LAG-3 and IL-7R expression and signaling, which could contribute to autoimmunity. Based on these studies and our work, future studies of human autoimmune-associated T cells with LAG-3 mutations may indicate metabolic alterations that could contribute to pathogenicity.

3.0 REACTIVE OXYGEN SPECIES ARE REQUIRED FOR DRIVING EFFICIENT AND SUSTAINED TRANSITION TO AEROBIC GLYCOLYSIS DURING CD4$^+$ T CELL ACTIVATION

This is the author's version of the work.

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3.1 SUMMARY

The immune system is necessary for protecting against various pathogens. However, under certain circumstances, self-reactive immune cells can drive autoimmunity, like that exhibited in type 1 diabetes (T1D). $CD4^+$ T cells are major contributors to the immunopathology in T1D, and in order to drive optimal T cell activation, third signal reactive oxygen species (ROS) must be present. However, the role ROS play in mediating this process remains to be further understood. Recently, cellular metabolic programs have been shown to dictate the function and fate of immune cells, including $CD4^+$ T cells. During activation, $CD4^+$ T cells must transition metabolically from oxidative phosphorylation to aerobic glycolysis to support proliferation and effector function. As ROS are capable of modulating cellular metabolism in other models, we sought to understand if blocking ROS also regulates $CD4^+$ T cell activation and effector function by modulating T cell metabolism. To do so, we utilized an ROS scavenging and potent antioxidant manganese metalloporphyrin (MnP). Our results demonstrate that redox modulation during activation regulates the mTOR/AMPK axis by maintaining AMPK activation, resulting in diminished mTOR activation and reduced transition to aerobic glycolysis in diabetogenic splenocytes. These results correlated with decreased Myc and Glut1 upregulation, reduced glucose uptake, and diminished lactate production. In an adoptive transfer model of T1D, animals treated with MnP demonstrated delayed diabetes progression, concurrent with reduced $CD4^+$ T cell activation. Our results demonstrate that ROS are required for driving and sustaining T cell activation-induced metabolic reprogramming, and further support ROS as a target to minimize aberrant immune responses in autoimmunity.

3.2 INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease where self-reactive T cells escape into the periphery and target pancreatic β cells for destruction. While T1D progression results from the interplay between various immune cell types, $CD4^+$ T cells are considered the principal contributor to disease pathology [12, 361]. We and others have demonstrated that reactive oxygen species (ROS) play an important role in driving the immunopathology exhibited in T1D [16, 206]. Antigen presenting cells (APCs), like macrophages [223], and $CD4^+$ T cells [225] express functional NADPH oxidases (NOX) which generate ROS upon APC-induced T cell activation. Both NOX [202] and mitochondrial-derived ROS from the T cell itself [244] are necessary for optimal $CD4^+$ T cell activation. These ROS, with cytokines, serve as the third signal, during T cell activation. In combination with T cell receptor (TCR; signal 1) and co-stimulatory molecule (signal 2) engagement, these three signals enable cell cycle entry [212] and effector function acquisition [202].

Recently, interest has grown in understanding the role of cellular metabolism in fulfilling the objectives of T cell activation and effector function. Under homeostatic conditions, naïve $CD4^+$ T cells remain relatively quiescent and rely predominantly on oxidative phosphorylation (OXPHOS) to meet basal metabolic needs [362]. Upon antigen (e.g. β cell-derived antigens in T1D) encounter, naïve $CD4^+$ T cells become activated and have two main goals – to clonally expand and to differentiate into effector T cells. To meet these goals during activation, $CD4^+$ T cells undergo dynamic metabolic reprogramming by transitioning to aerobic glycolysis [49, 50,

362], also known as the Warburg Effect, which was first characterized in tumors [29, 50]. The utilization of aerobic glycolysis by activated $CD4^+$ T cells supports increased macromolecule biosynthesis, aiding in daughter cell formation and effector molecule production, along with more rapid production of ATP as compared to OXPHOS [49, 50, 362].

In both tumors and T cells, Myc is a predominant player in coordinating increased glycolysis and cell proliferation [29, 46, 103, 363]. Upstream, activation of mammalian target of rapamycin (mTOR) signaling is critical for Myc expression and thus aerobic glycolysis, as treatment with the mTOR inhibitor rapamycin results in dampened lactate production, proliferation, and cytokine production in $CD4^+$ T cells [364, 365]. In contrast, AMP-activated protein kinase (AMPK) is a known inhibitor of mTOR and is responsible for enhancing oxidative metabolism to restore the ATP to AMP ratio [366, 367]. Overexpression of AMPK in tumors inhibits the Warburg Effect, whereby tumors demonstrate reduced size and lactate production [368]. Similarly, AMPK activation in T cells results in reduced mTOR activation, diminished effector differentiation, and hyporesponsiveness [369]. These results highlight that the interplay between mTOR and AMPK strongly dictates T cell metabolic and functional outcome.

Highly proliferative cells in various models demonstrate enhanced aerobic glycolysis, indicating its requirement for sustaining rapid division. Targeting tumor metabolism via the use of glycolytic inhibitors like 2-deoxyglucose, have proven to be effective in reducing tumor burden and metastasis [370]. The efficacy of metabolic modulation in cancer, and the metabolic similarities between proliferating tumor cells and effector $CD4^+$ T cells, indicate a potential avenue for controlling aberrant T cell responses (like those in autoimmunity) by targeting T cell metabolism. Indeed, others have demonstrated potential for ameliorating autoimmunity by

metabolic manipulation [188]; however, there remains a large gap in understanding the mechanisms by which specifically T cell metabolism is controlled.

Additionally, many metabolic regulators demonstrate redox sensitivity, including the transcription factors HIF-1α [370] and NF-κB [371], and AMPK [372], to name a few, underscoring the potential for redox regulation in modulating metabolism. We and others have shown that a manganese metalloporphyrin, Mn(III) meso tetrakis (N -alkylpyiridinium-2-yl) porphyrin, or MnP, is capable of scavenging ROS (i.e. hydrogen peroxide and superoxide) [373, 374], inhibiting lipid peroxidation [375], and performing redox reactions in cellular systems [324, 376]. As T1D is known to be driven by increased oxidative stress [16, 207], our laboratory has demonstrated that inhibition of ROS during immune activation results in dampened $CD4^+$ T cell responses, thus inhibiting T1D progression [168, 199, 304, 326]. Specifically, work by *Delmastro-Greenwood et al.* showed that treating NOD.BDC.2.5.TCR-Tg mice with MnP *in vivo* for 7 days resulted in increased glucose oxidation and aconitase activity in naïve splenocytes, indicative of enhanced OXPHOS, the predominant pathway used by naïve immune cells [328]. While these studies did demonstrate metabolic alterations due to MnP treatment, they were conducted using naïve immune cells that had no prior exposure to their cognate antigen. As previously stated, T cell metabolic reprogramming occurs only during antigen-mediated activation; therefore, we sought to expand our understanding of the role of ROS and metabolism during such activation events.

Based on these previous studies, we hypothesized that redox modulation by MnP during $CD4^+$ T cell activation would inhibit the transition to aerobic glycolysis, and thus, minimize proliferation and effector function. Our data demonstrate that MnP treatment resulted in reduced Myc upregulation, glycolytic enzyme expression, and lactate production, collectively indicating

inhibition of aerobic glycolysis. These results were in part due to diminished mTOR signaling. Interestingly, redox modulation enhanced activation of the mTOR inhibitor, AMPK, due to MnP's high antioxidant activity. These data show that redox modulation inhibits the metabolic transition of $CD4^+$ T cells by maintaining active AMPK and thus resulting in reduced mTOR signaling and Myc expression. These findings support that ROS are required during the transition from OXPHOS to aerobic glycolysis during T cell activation, and that disruption of ROS may serve as a viable target for modulating immune cell bioenergetics in autoimmune diseases like T1D.

3.3 MATERIALS AND METHODS

3.3.1 Animal models

Non-obese diabetic (NOD) BDC2.5.TCR.Tg and NOD.*scid* mice were maintained in the Rangos Research Center animal facility of the Children's Hospital of Pittsburgh. Animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Children's Hospital of Pittsburgh (Assurance Number A3187-01) and were in compliance with the laws of the United States of America. NOD.BDC2.5.TCR.Tg mice were sacrificed at 8-10 weeks of age for *in vitro* experiments. In this animal, all $CD4^+$ T cells recognize epitopes formed by covalent cross-linking of proinsulin peptides and Chromogranin A (CHgA) in beta cell secretory granules. These T cells can be stimulated with a known peptide mimotope HRPI-RM that has been previously described [377], thus allowing us to examine the effects of MnP on an antigen-

specific immune response physiologically relevant to T1D. NOD.*scid* animals, 6-8 weeks of age, were used for adoptive transfer experiments.

3.3.2 Mn(III) meso tetrakis (N-alkylpyiridinium-2-yl) porphyrin

Mn(III) meso tetrakis (N -alkylpyiridinium-2-yl) porphyrin (MnP) was a generous gift from Dr. James Crapo, MD at National Jewish Health (Denver, CO). MnP was used at a concentration of 68 μM for *in vitro* experiments and a 10 mg/kg dose in all animal experiments.

3.3.3 Splenocyte homogenization

NOD.BDC.2.5.TCR-Tg spleens were harvested and homogenized into single cell suspensions as previously described [168], and red blood cells were lysed using red blood cell lysis buffer (Sigma). $CD4^+$ T cells were stimulated with their cognate peptide, mimotope (EKAHRPIWARMDAKK), at 0.05 uM, with or without MnP in complete splenocyte medium [202]. Splenocytes plated with media alone served as negative controls. Cells were collected for downstream analysis at 24-72 hours post-stimulation. Supernatants were collected for ELISA and lactate measurements.

3.3.4 CD4^{+} T cell isolation and antibody stimulation

CD4^{+} T cells were isolated from whole NOD splenocytes by magnetic bead separation using mouse CD4 MicroBeads (Miltenyi) as per manufacturer's instructions. Purity was assessed by flow cytometric staining pre- and post-isolation. For antibody stimulation, tissue culture plates were coated with αCD3 (0.5 μg/mL) and αCD28 (1.0 μg/mL) in phosphate buffered saline for 2 hours at 37°C, 5% CO_2. The antibody solution was decanted and CD4^{+} T cells were plated at $5.0x10^5$ cells per well of a 96 well, flat-bottom plate, with or without 68 μM MnP. Unstimulated T cells served as negative controls.

3.3.5 ROS and viability assays

NOD or NOD.BDC.2.5 splenocytes were incubated in media alone or media with 68 μM MnP for 2 hours at 37°C. Cells were washed extensively in cold Hank's Balanced Salt Solution (HBSS) and added to flow tubes at $1.0x10^6$ per tube. Dihydroethidium (DHE; Molecular Probes) or MitoSOX Red (Molecular probes) was diluted per manufacturer's instructions, and cells were treated with a final concentration of 50 μM for 20 minutes (DHE) or 5 μM for 15 minutes (MitoSOX) at 37°C. PMA (500 ng/mL) and ionomycin (500 μg/mL) were added to the tubes and incubated at 37°C for indicated periods of time. Cells were read on an LSRII (BD Bioscience). DHE was read in the AmCyan channel using a 585/42 detector and 545LP filter, and MitoSox Red was detected in the PE channel. Mean Fluorescence Intensity (MFI) was determined using FlowJo Software (v10.1). Dye loaded, unstimulated control cells were used to

determine background fluorescence, which was subtracted from stimulated values and graphed as change in MFI due to stimulation (delta MFI). Viability was assessed by 7AAD staining (BD Biosciences) as per manufacturer's instructions. Surface staining for CD4 was performed prior to 7AAD staining. Viability was determined as the percentage of 7AAD negative cells.

3.3.6 Protein lysates and Western blotting

Following stimulation, cells were harvested, washed with phosphate buffered saline (PBS), and sonicated in anti-pY lysis buffer (50 mM Tris pH 8.0, 137 mM NaCl, 10% glycerol, 1% NP-40, 1 mM NaF, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 2 mM Na_3VO_4, and 1 mM PMSF). Protein concentration was determined by Bicinchoninic acid protein assay (Thermo Fisher Scientific). 25 µg of protein per sample were boiled in 6x Lammaeli buffer (BIORAD) for 5 minutes and separated SDS-PAGE gels. Samples were then transferred to PVDF membranes for 1-3 hours in 3% MeOH Tris-Glycine Transfer buffer (BIORAD). Western blots were blocked in 5% non-fat dry milk in Tris-buffered Saline with 1% Tween-20 (TBST). Blots were probed with the following antibodies in 5% BSA/TBST overnight at 4°C: Myc, pmTOR (Ser2448), total mTOR, p4E-BP1 (Thr70), pAMPK-α (Thr170), total AMPK, PFKFB3, p27 Kip1, and Cyclin D3 at 1:1000 (Cell Signaling), and Glut-1 (1:2000; Abcam). Blots were either probed with anti-rabbit secondary antibody (Cell Signaling; 1:2000) or goat anti-rabbit secondary antibody (Jackson Laboratories; 1:10,000) in 5% non-fat dry milk/TBST at RT for 1 hour. β-actin (Sigma) expression was used as a loading control. Protein expression was detected by chemilumenescence using ECL Plus reagent (Amersham Pharmacia Biotech) and the Fujifilm

LAS-3000 Imaging system (FujiFilm Technologies). Multi Gauge software was used to process images (Fujifilm Life Science). Beta-actin expression served as a loading control.

3.3.7 Flow cytometric analysis

Cells were harvested following stimulation and incubated with Fc block (CD16/CD32; BD Biosciences) prior to staining for flow cytometry. Extracellular staining was performed at 4°C using CD4-APC or CD4-FITC (BD Biosciences) in FACS buffer (1% BSA in PBS). For cellular proliferation measurements, splenocytes were stained with 1 μM carboxyfluorescein succinimidyl ester (CFSE; Invitrogen) in PBS at 37°C for 15 minutes and isolated $CD4^+$ T cells were labeled with Cell Proliferation Dye Violet (BD Bioscience) as per manufacturer's instructions. Cells were extensively washed with PBS, plated for stimulation, and surface stained after harvest.

For cell cycle analysis, cells were fixed and permeabilized in 70% cold EtOH for 20 minutes on ice following stimulation and stored at 4°C until staining for flow analysis. Cells were washed with ice cold PBS two times to remove residual EtOH, and surfaced stained for CD4 as described above. After RNase treatment for 1 hour at 37°C, cells were incubated with propidium iodide (0.4 mg/mL; Invitrogen), and analyzed immediately. Media-treated splenocytes served as controls to set gates for no proliferation (CFSE) and cell cycle stages (PI).

To measure glucose uptake, stimulated cells were incubated with 100 μM 2-(*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG; Molecular Probes), a fluorescent glucose analog (Life Technologies), for 10 minutes at 37°C prior to harvest [136]. Uptake was quenched with PBS. Cells were stained for surface CD4 expression and analyzed by

flow cytometry live. Fluorescence was measured using a FACS Calibur or LSR II flow cytometer (BD Biosciences). All data were analyzed using FlowJo software (v10.1) and samples were gated on $CD4^+$ cells.

3.3.8 Cytokine and lactate measurements

Supernatants from cell cultures were analyzed for IFNγ and IL-2 by ELISA according to manufacturer's instructions (BD Biosciences). ELISAs were read on a SpectraMax M2 microplate reader (Molecular Devices), and data were analyzed using SoftMax Pro version 5.4.2 software (Molecular Devices). Lactate, a byproduct of aerobic glycolysis, was measured in culture supernatants using the Accutrend Plus meter and lactate strips (Roche). Samples with high concentrations of lactate were diluted 1:2 in dI H_20 in order to obtain a reading within the meter's range.

3.3.9 Quantitative real-time PCR (qRT-PCR)

At 24 hours post-stimulation *in vitro*, cells were harvested and washed extensively with PBS. 5.0×10^6 cells were lysed using RLT buffer (Qiagen) and 25 gauge needles with 1 mL syringes. mRNA was isolated using the RNeasy kit (Qiagen) and concentration was determined using a NanoDrop 2000c spectrophotometer (Thermo Scientific). cDNA was synthesized from 0.5 µg mRNA using the RT^2 First Strand Kit (Qiagen). Gene expression was quantified by qRT-PCR using the iQ SYBR Green Supermix (BIORAD) and iCycler (BIORAD). Murine glycolytic

primer pair sequences were taken from *Wang et al.* [103]. *Ifnγ* primers were FWD 5'-AGGCCATCAGCAACAACATAAGCG-3' and REV 5'-TGGGTTGTTGACCTCAAACTTGGC-3'. Cycling parameters were as follows: 5 min at 95°C, 30 s at 95°C, 30 s at 60°C, 30 s at 72°C (40 cycles of steps 2-4), 1 min at 95°C, and then samples were held at 4°C. Delta delta Ct values were normalized to expression of the control gene *rplo* (FWD 5'-GGCGACCTGGAAGTCCAACT-3'; REV 5'-CCATCAGCACCACAGCCTTC-3') [342], in order to calculate relative expression. Mimotope and M + MnP expression values were normalized to those of unstimulated, media controls.

3.3.10 Adoptive transfer model of T1D

Spleens from NOD.BDC.2.5.TCR.Tg animals were homogenized and processed as described above. Whole splenocytes were labeled with Cell Proliferation Violet (BD Biosciences) according to manufacturer's instructions, and 1.0×10^7 splenocytes were adoptively transferred into NOD.*scid* recipients i.v. One cohort of recipients was treated with 10 mg/kg MnP i.p. every day or s.c. every other day, starting the day prior to transfer. Serum was collected on days -1, 3, 7, 11, and 15 post-transfer to measure sLAG-3 by ELISA as an indication of T cell activation, as previously described [168]. T1D incidence was monitored by blood glucose post-transfer, and two consecutive readings of > 350 mg/dL was deemed diabetic. At indicated time points, animals were sacrificed and peripheral blood and spleens were taken for downstream analysis by flow cytometry. 1.0×10^6 splenocytes were stained with surface antibodies for CD4, CD25, and LAG-3 following F_c receptor blockade with anti-CD16/CD32 (all from BD Bioscience). For intracellular pS6 staining of peripheral blood, red blood cells were lysed and then lymphocytes

were surface stained. Following fixation and permeabilization using Cytofix/Cytoperm (BD Bioscience), cells were then stained using the pS6 Alexa 488 antibody (Cell Signaling). Cells were then analyzed by flow cytometry using a BD LSRII (BD Bioscience) and FlowJo software (v10.1).

3.3.11 Statistical analysis

Data are given as mean values ± SEM, with n indicating the number of independent experiments or animals, unless otherwise indicated. Student's t-test and Two-way ANOVA with Bonferroni post-hoc analysis were used where appropriate. Kaplan-Meier analysis was used to measure significance of diabetes incidence. A p-value of $p < 0.05$ was considered significant for all statistical analyses.

3.4 RESULTS

3.4.1 Treatment of T cells with MnP effectively scavenges NADPH oxidase and mitochondrial-derived ROS and without toxicity

T cells generate ROS via two sources – a phagocyte-like NADPH oxidase [213, 225] and mitochondrial electron leak [244]. As blockade of each of these sources have differential effects on T cell activation and differentiation, we wanted to further delineate if MnP treatment successfully scavenges ROS from both sources. To do so, the fluorescent indicators

dyhidoethidium (DHE) and MitoSOX were utilized as both dyes only fluoresce upon modification by superoxide. DHE measures total superoxide generation, whereas MitoSOX specifically measures that from the mitochondria. Following pre-treatment with either media alone or media with MnP, splenocytes were stimulated with PMA and ionomycin which are known to induce ROS production by T cells [201, 213, 244]. As anticipated, MnP treatment successfully reduced total superoxide generation as measured by DHE (Figure 22A). Additionally, mitochondrial-derived superoxide generation was also diminished by MnP treatment (Figure 22B), indicating that MnP is capable of entering the mitochondria. Together these data reveal that MnP effectively dissipates ROS from both NADPH oxidase and the mitochondria, resulting in reduced total cellular ROS production.

Viability of splenocyte cultures was also assessed to confirm that effects on T cell activation and metabolism were not simply due to MnP toxicity. 7AAD staining results demonstrated no significant difference in viability of either whole splenocytes (Figure 22C) or $CD4^+$ T cells (Figure 22D), supporting that the subsequent impact of MnP treatment on T cell metabolic reprogramming is not simply due to agent-associated toxicity.

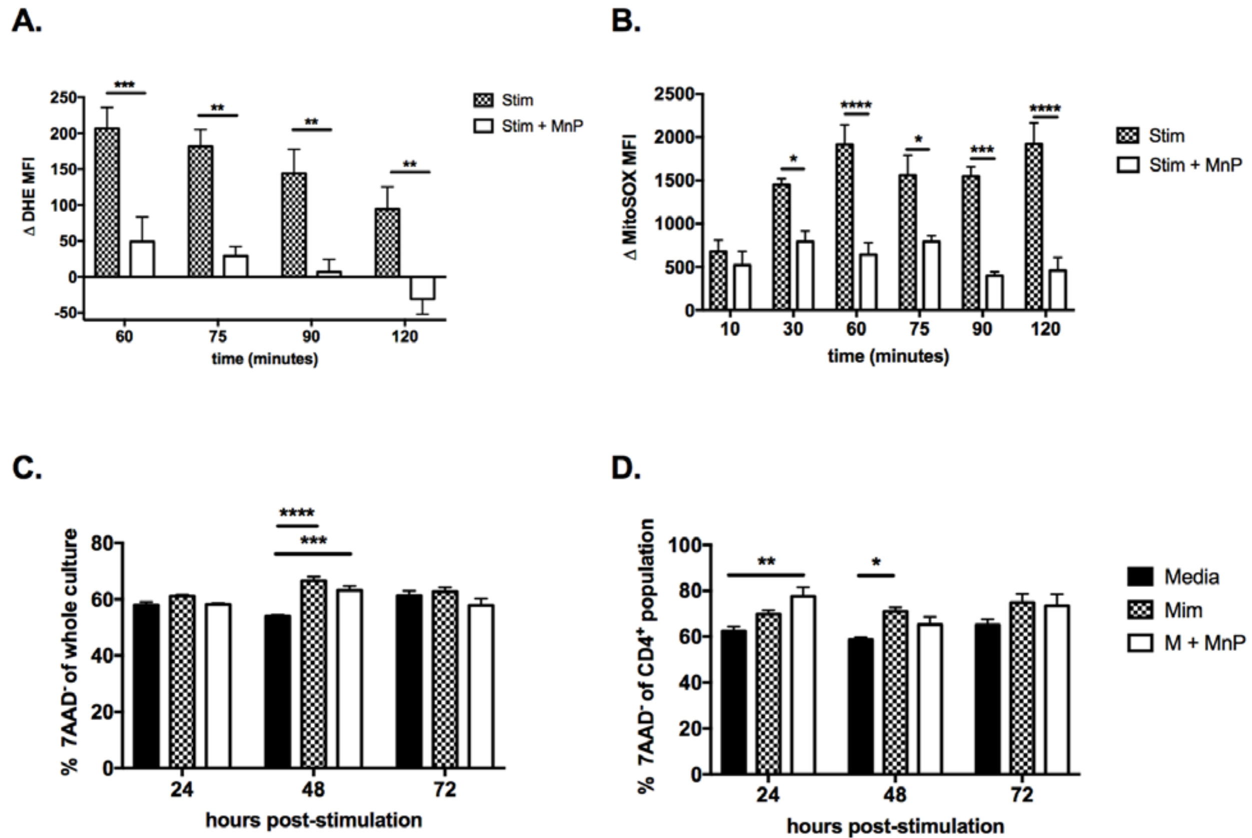

Figure 22. MnP treatment effectively scavenges NADPH oxidase and mitochondrial derived superoxide, while demonstrating no toxicity.

NOD splenocytes were pre-treated with or without MnP and then loaded with either **A.** Dihydroethidium (DHE) or **B.** MitoSOX red. Splenocytes were stimulated with PMA and ionomycin and read for fluorescence by flow cytometry at the indicated time points. Data are displayed as delta mean fluorescence intensity (Δ MFI) ± SEM calculated as $MFI_{stimulated} - MFI_{unstimulated}$. **C-D.** BDC2.5.TCR.Tg splenocyte cultures were stained for 7AAD and CD4 to assess viability of cultures due to MnP treatment. Data are displayed as percent $7AAD^{-}$ of **C.** whole splenocytes and **D.** $CD4^{+}$ T cells. Significance was determined by Two-way ANOVA with Bonferroni post-hoc analysis of a combined n = 3-5 mice (****=p<0.0001; ***=p<0.001; **=p<0.01; *p<0.05).

3.4.2 Scavenging of ROS during activation halts $CD4^+$ T cells at the G_0/G_1 checkpoint and inhibits clonal expansion

Acute doses of ROS are required for cell cycle progression and proliferation of $CD4^+$ T cells [209, 295]. Therefore, we wanted to examine if scavenging of ROS via MnP treatment inhibited this process during activation. Here, we utilized the NOD.BDC.2.5.TCR.Tg (BDC.2.5) mouse model, in which the $CD4^+$ T cells of this animal have been shown to demonstrate diabetogenic potential [7]. $CD4^+$ T cells from BDC.2.5 animals stimulated with their specific antigen mimotope (M), demonstrated high proliferative capacity as demonstrated by CFSE dilution (Figure 23A). ROS inhibition via MnP treatment during antigen-dependent stimulation resulted in $CD4^+$ T cells undergoing fewer rounds of proliferation at both 48 and 72 hrs post-stimulation as compared to stimulation alone (Figure 23A). $CD4^+$ T cells treated with MnP did show low levels of proliferation, which was not surprising as MnP does not fully block all ROS production (Figure 22A and B).

Since overall proliferation was reduced due to MnP treatment, we wanted to examine if $CD4^+$ T cells were being arrested at a specific cell cycle checkpoint. Consequently, propidium iodide staining was used to examine the distribution of $CD4^+$ T cells in the various phases of the cell cycle – G_0/G_1, S, and G_2/M. By 48 hours post-stimulation, significantly fewer $CD4^+$ T cells had progressed to the later stages of the cell cycle (S phase and G_2/M phase) following MnP treatment, as compared to stimulated controls (Figure 23B-C; $p<0.05$). These results correlated with increased expression of the cell cycle inhibitor p27 Kip1 (Figure 23D), which is responsible for restricting progression from G_1 to S phase [378]. Additionally, protein analysis demonstrated reduced expression of Cyclin D3, a promoter of S phase transition, due to MnP treatment, again indicating limited cell cycle progression (Figure 23D). p27 Kip1 is also a potent Cyclin D3

inhibitor [379], and therefore, its maintained expression results in cell cycle arrest. Together, these results indicate that dissipating ROS during activation halts $CD4^+$ T cells at the G_1/S checkpoint in the cell cycle via maintaining p27 Kip1 expression.

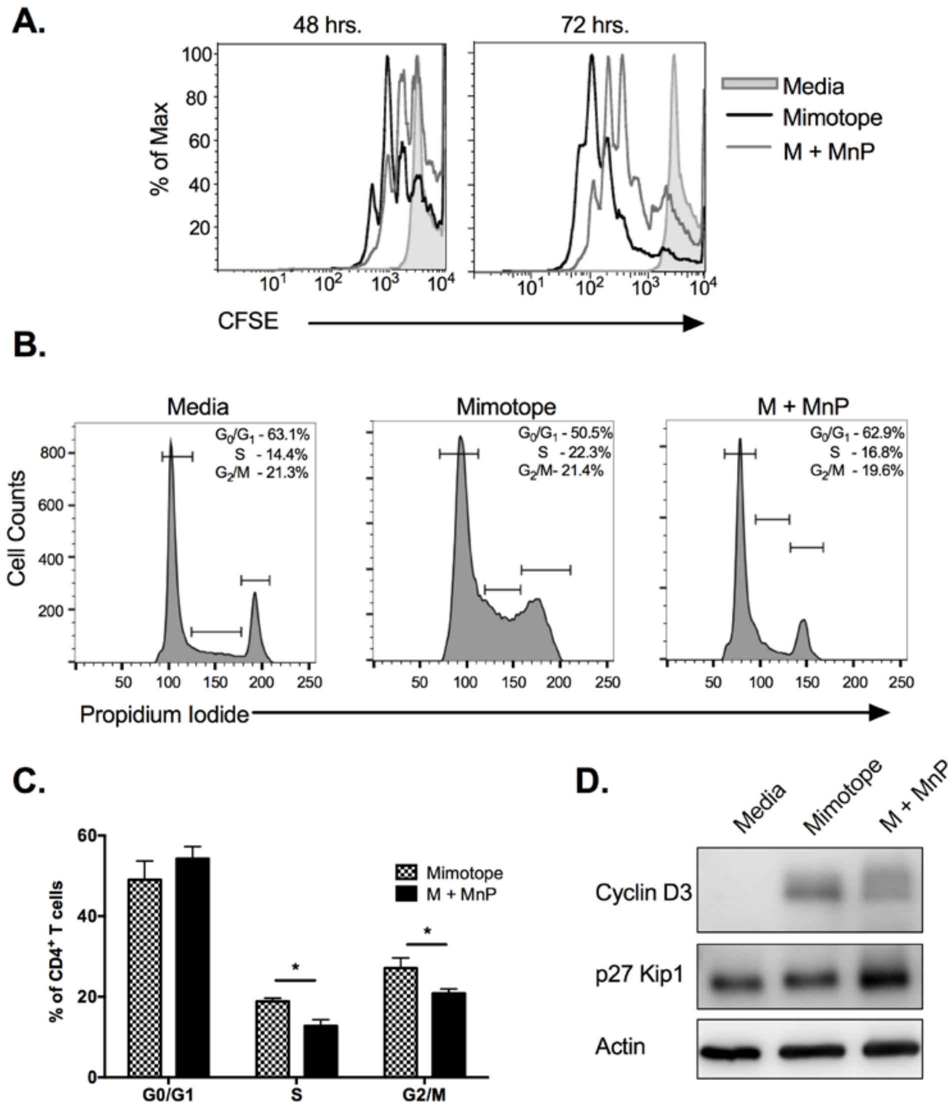

Figure 23. Redox modulation during activation inhibits cell cycle progression of CD4+ T cells. NOD.BDC.2.5.TCR.Tg splenocytes were plated in complete splenocyte media and stimulated with 0.05 μM mimotope with or without 68 μM MnP (M + MnP) for 48-72 hours. **A.** Prior to stimulation cells were loaded with 1 μM CFSE. Cells were stained with CD4 following harvest and analyzed by flow cytometry for CFSE dilution, indicating proliferation. Unstimulated cells (grey shaded curve) served as negative controls to set proliferation gates. CFSE tracings are representative of n > 3 independent experiments. **B.** Cells were fixed, permeabilized, and stained with propidium iodide and CD4-FITC. Cells were analyzed by flow cytometry to determine cell cycle status. $CD4^+$ T cells were gated on and cell cycle phases were set based upon unstimulated controls (left panel). **C.** Percentages of n > 3 experiments were combined and graphed as mean ± SEM (*=p<0.05). **D.** 48 hrs. post-stimulation, cells were harvested and analyzed by Western blot for p27 Kip1 and Cyclin D3 expression. β actin expression served as the loading control.

3.4.3 Transition to aerobic glycolysis during CD4+ T cell activation is dependent upon cellular redox status

CD4+ T cells undergo massive metabolic reprogramming during the transition from naïve to effector states [103, 380], and in order to support this metabolic reprogramming, CD4+ T cells must upregulate various glycolytic enzymes, transcriptional regulators, and glucose transporters [103]. Impeding this process results in reduced clonal expansion [46], effector function [358], and overall T cell responses in disease models [49]. Proliferation and metabolism are two tightly coupled cellular processes, and inhibition of glycolysis has been shown to result in diminished proliferation [362]. Therefore, we hypothesized that as ROS scavenging diminished CD4+ T cell proliferation (Figure 23), it may be doing so by modulating metabolic reprogramming.

The transcription factor Myc is critical for promoting aerobic glycolysis in both CD4+ T cells and tumors alike, by initiating expression of various glycolytic enzymes and glucose transporters [103, 363], along with driving cell cycle progression [363]. Protein and quantitative RT-PCR analysis of *in vitro* stimulated BDC2.5.TCR.Tg splenocytes confirmed increased Myc expression at 24 and 48 hours post-stimulation (Figure 24A,C). In contrast, MnP treatment resulted in contracted Myc upregulation at both time points (Figure 24A,C). These results correlated with reduced mRNA expression of several Myc-dependent genes necessary for aerobic glycolysis including glut1, hexokinase 2 (HK2), lactate dehydrogenase A (LDH A), and pyruvate kinase M2 (PKM2) (Figure 24B,C; $p<0.05$).

Following gene expression analysis, we sought to investigate protein expression of two key Myc-dependent targets – Glut1, the glucose transporter expressed by activated T cells that has the greatest impact on differentiation [357, 381]; and, PFKFB3, a rate limiting enzyme that aids in committing glucose to being metabolized via glycolysis [382]. Also, modulation of

activity and expression of PFKFB3 in autoreactive T cells has been shown to ameliorate disease, suggesting PFKFB3 as a potential therapeutic target for autoimmunity [382, 383]. As anticipated, protein expression of Glut1 was reduced in MnP-treated splenocytes, further corroborating qRT-PCR results (Figure 24C). However, while qRT-PCR results did not show differences in mRNA expression of the enzyme PFKFB3 (Figure 24D), protein analysis did show reduced upregulation due to MnP scavenging ROS (Figure 24D). These results underscore the importance ROS play in enabling the transcriptional alterations necessary for aerobic glycolysis.

As glucose is the primary substrate for aerobic glycolysis, and our data indicated that MnP treatment inhibited Glut1 expression, we wanted to measure the effect of MnP treatment on glucose uptake. To do so, splenocyte cultures were incubated with the fluorescent glucose analog 2-NBDG, and uptake was measured by flow cytometry. As with diminished Glut1 expression, MnP treatment resulted in reduced glucose uptake by $CD4^+$ T cells (Figure 24E). These results correlated with reduced lactate production, the byproduct of aerobic glycolysis, as measured in culture supernatants, further indicating a significant reduction in the utilization of aerobic glycolysis due to redox modulation (Figure 24F; $p<0.01$). Overall, these data reveal that alterations in $CD4^+$ T cell redox status limits upregulation and utilization of the glycolytic pathway necessary for driving effector differentiation and clonal expansion.

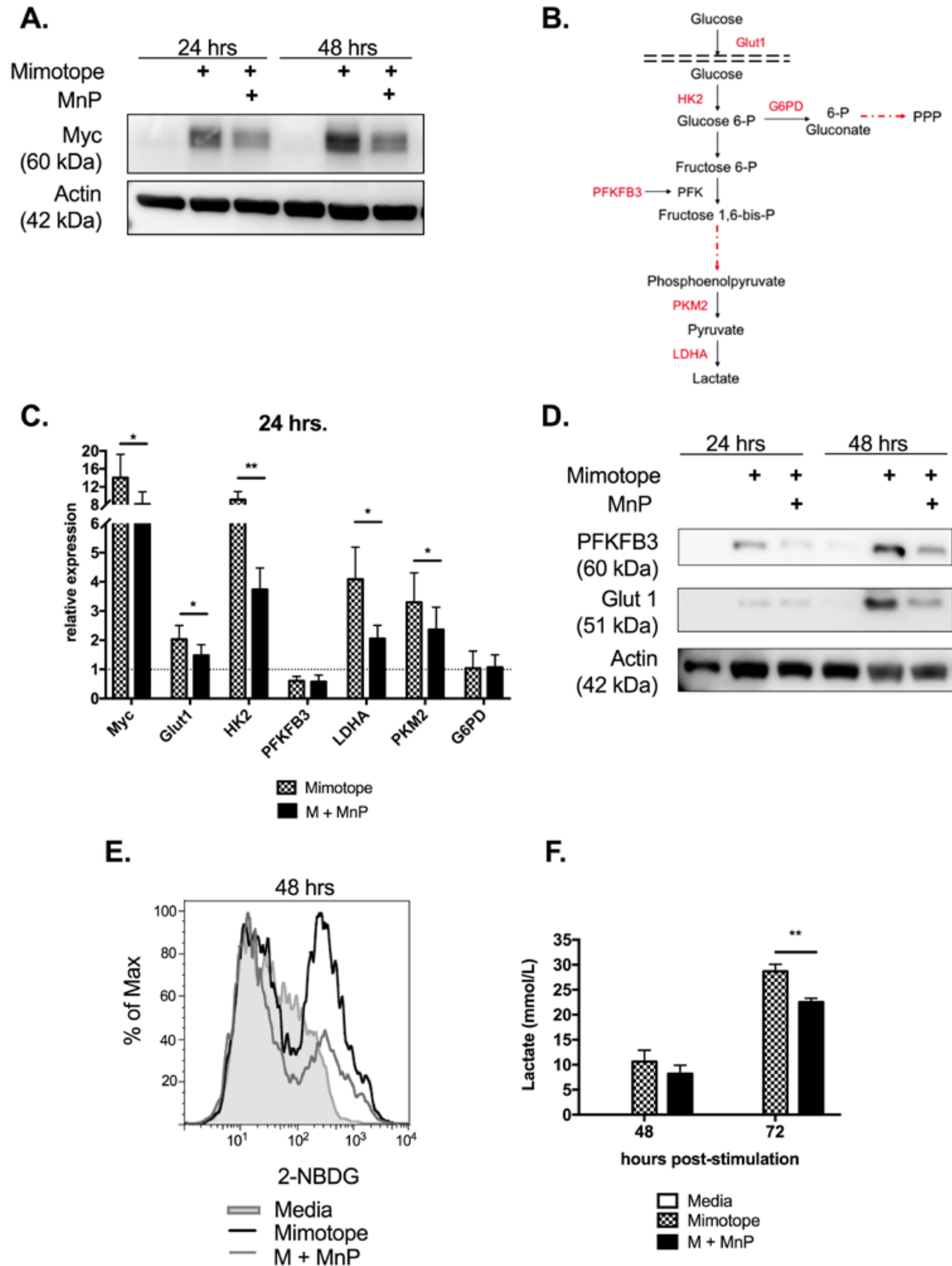

Figure 24. Transition to aerobic glycolysis during CD4+ T cell activation is dependent upon cellular redox status.
A. Protein lysates were probed for Myc expression. β actin expression served as the loading control. **B.** Glycolytic pathway with assayed Myc-dependent genes indicated in red. **C.** 24 hours post-stimulation, mRNA expression of various glycolysis-associated genes was assessed by qRT-PCR. Relative values were normalized to unstimulated, naïve controls (Media). Data displayed are means of n = 5-7 independent experiments ± SEM. **D.** Western blot analysis of specific Myc targets, PFKFB3 and Glut1. **E.** 48 hours post-stimulation, cells were treated with 100 μM 2-NBDG and stained for CD4 following incubation. Glucose uptake was measured as 2-NBDG fluorescence of the $CD4^+$ population. Histograms are representative of n > 3 experiments. **F.** Lactate was measured in culture supernatants using the Accutrend Plus meter (Roche). Data are graphed as means of n > 3 experiments ± SEM. Statistical significance between mimotope and M + MnP groups was calculated using a Student's t test (*=p<0.05, **=p<0.01).

3.4.4 mTOR signaling is reduced upon treatment with MnP

The mammalian target of rapamycin (mTOR) pathway has been shown to be pivotal in driving metabolic changes during T cell activation and regulating $CD4^+$ T cell lineage commitment [384, 385]. Studies examining the effects of rapamycin on T cell metabolism have indicated that inhibition of mTOR activation and signaling during T cell activation resulted in diminished proliferation, effector function, and glycolytic capacity [365]. Given the fact that MnP treatment inhibited cell cycle progression (Figure 23) and aerobic glycolysis (Figure 24), and that the mTOR pathway is critical in mediating both of these processes, we sought to measure mTOR activation and its downstream signaling.

Protein analysis of *in vitro* stimulations indicated that MnP treatment during mimotope stimulation resulted in reduced total mTOR expression and also activation, as measured by phosphorylation at Ser2448 at 48 and 72 hours post-stimulation (Figure 25A). We also examined phosphorylation of mTOR's downstream target 4E-BP1, a translational repressor that is inhibited upon hyperphosphorylation [364]. Reduced phosphorylation of 4E-BP1 (Thr70) was exhibited in MnP treated $CD4^+$ T cells, as compared to stimulated controls (Figure 25A), indicating reduced mTOR signaling.

In addition to driving activation, mTOR signaling is also necessary for mediating the growth phase of mammalian cells in preparation for expansion [386] and driving effector differentiation in T cells [364]. As anticipated, with reduced mTOR signaling, MnP-treated $CD4^+$ T cells demonstrated reduced growth at 48 hours as measured by forward scatter (Figure 25B), and reduced IFNγ secretion (Figure 25C; $p<0.05$). Additionally, we assessed IFNγ mRNA expression by splenocytes 24 hours following stimulation. qRT-PCR results indicated that MnP treatment during stimulation hindered upregulation of IFNγ mRNA as compared to mimotope

stimulation alone (Figure 25D; $p<0.05$), suggesting that the reduced secreted levels due to ROS scavenging was not simply due to reduced proliferation and the presence of fewer T cells. Overall, these results indicate that ROS signaling is required for amplifying mTOR signaling upon activation, which then enables optimal T cell proliferation (Figures 23) and glycolysis (Figures 24).

We also examined Interleukin-2 (IL-2) levels in our *in vitro* cultures, as IL-2 is essential for aiding T cell clonal expansion. Interestingly, we saw no defect in IL-2 production due to MnP treatment as compared to stimulation alone at all time points examined (Figure 25E). Additionally, there was no difference in expression of the high-affinity IL-2 receptor subunit, CD25 (Figure 25F). These results suggest that while redox modulation has no effect on the production of IL-2 or receptor expression, it does alter downstream signaling as mTOR signaling is, in part, IL-2 driven.

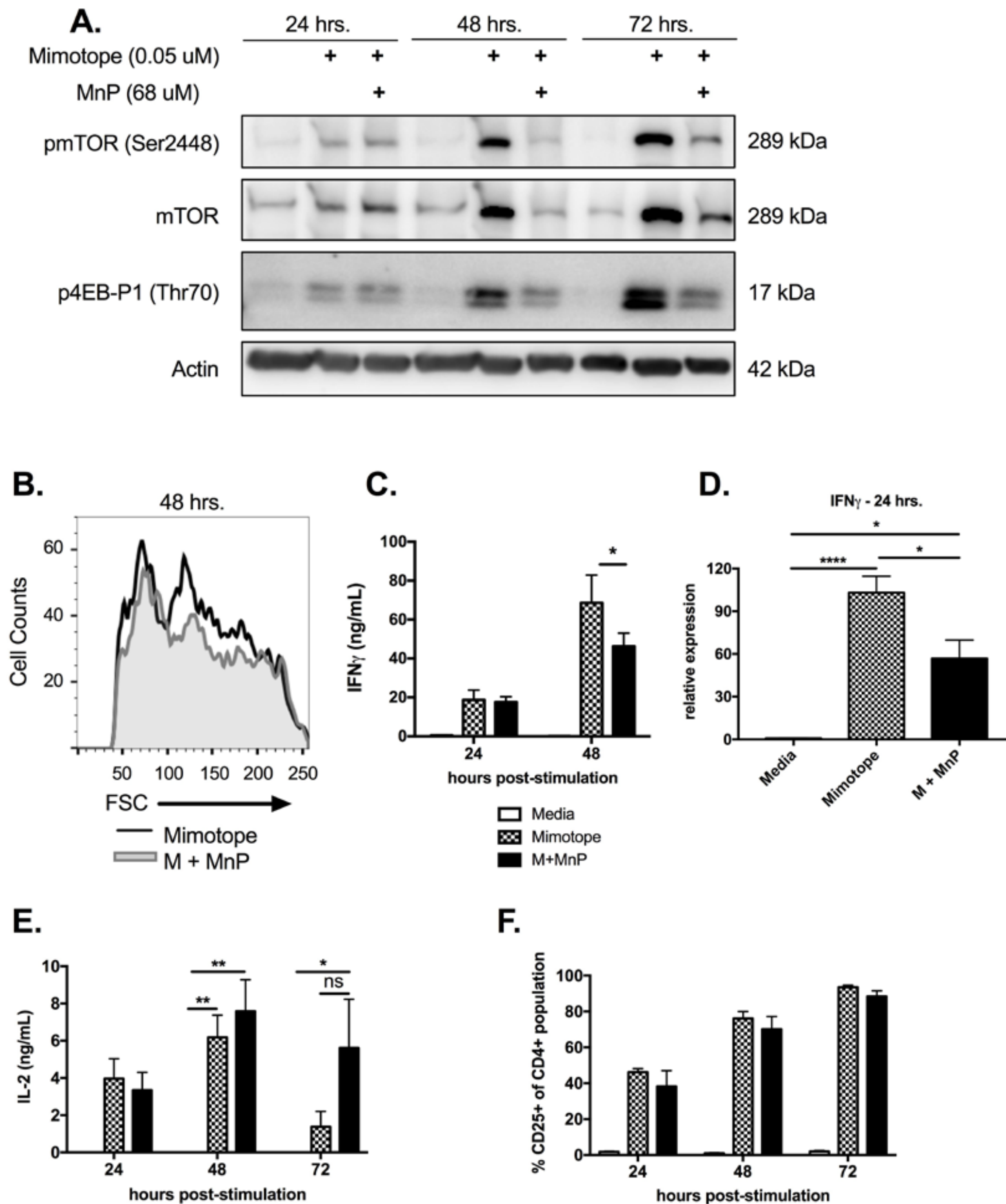

Figure 25. mTOR signaling is inhibited upon MnP treatment.
A. Western blot analysis for phosphorylated mTOR (Ser4998; active), total mTOR, and the downstream mTOR target, phosphorylated 4E-BP1 (Thr70). β actin expression served as the loading control. **B.** Forward scatter (FSC) of $CD4^+$ T cells was measured by flow cytometry as an indication of cell blasting. Histogram is representative of n > 3 experiments. Supernatants from *in vitro* stimulations were analyzed for **C.** IFNγ and **E.** IL-2 production by ELISA. **D.** qRT-PCR to measure IFNγ expression at 24 hrs. post-stimulation. **F.** Frequency of $CD4^+$ T cells expressing CD25 following activation. Data displayed are combined means ± SEM of n > 5 experiments. Statistical significance was calculated using a two-way ANOVA with Bonferroni post-hoc analysis. (*=$p<0.05$). Media alone treated splenocytes served as negative controls.

3.4.5 Inhibition of T cell generated ROS reduces proliferation, growth, and glucose uptake

Third signal ROS are necessary for driving T cell activation, and our data here indicate they are also critical for facilitating metabolic reprogramming (Figure 24-25). Previous work has revealed that both APCs and T cells express functional NOX enzymes that are activated during MHC-TCR engagement, resulting in ROS production from both the APC and T cell [201, 213, 226]. Therefore, we sought to further delineate if T cell-derived ROS were alone sufficient for mediating the transition to aerobic glycolysis during activation, and if MnP treatment would inhibit this process.

To do so, $CD4^+$ T cells were isolated from whole splenocytes and stimulated *in vitro* with plate-bound αCD3/αCD28, with or without MnP. Flow cytometric analysis of stimulated T cells indicated that ROS inhibition by MnP resulted in reduced proliferation at both 48 and 72 hours post-stimulation as compared to activated T cells not treated with MnP (Figure 26A). ROS scavenging also resulted in reduced cell growth during activation as measured by forward scatter (Figure 26B) and glucose uptake at 72 hours post-stimulation (Figure 26C-D; $p<0.01$). These results are in accordance with data presented in Figures 23-25, and further elucidate that the role of ROS in T cell transition to aerobic glycolysis is not specific to APC-derived ROS.

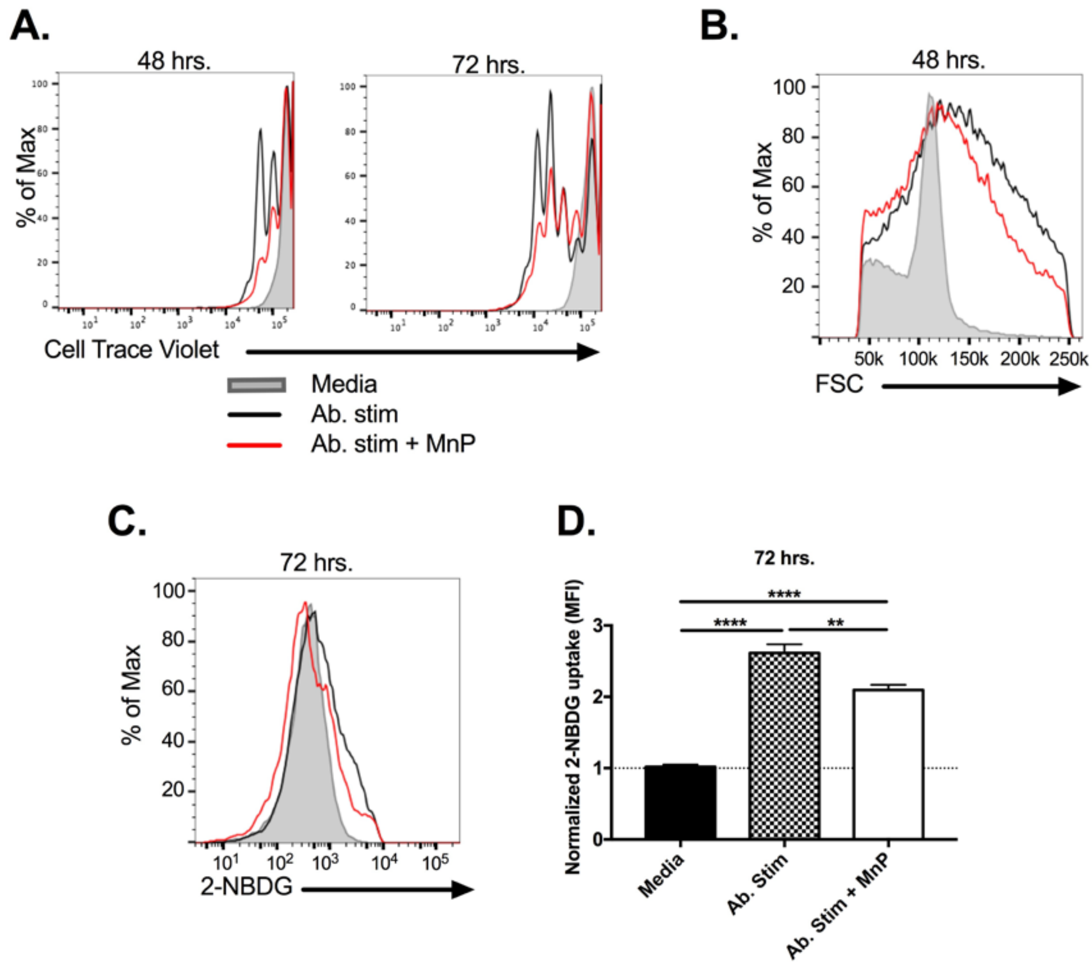

Figure 26. T cell-derived ROS are sufficient for enabling aerobic glycolysis during activation.
Isolated CD4+ T cells were stimulated with plate-bound αCD3/αCD28 with or without MnP treatment. A. Proliferation as measured by dilution of Cell Trace Violet. B. Forward scatter as measure of size following activation. C-D. Glucose (2-NBDG) uptake by CD4+ T cells measured by flow cytometry and normalized to unstimulated control T cells. **=$p<0.01$; ****=$p<0.0001$. Data are combined or representative of n = 5 independent experiments.

3.4.6 MnP treatment enhances activation of AMPK

AMPK, or adenosine monophosphate activated protein kinase, is a nutrient sensor shown to be responsible for driving oxidative metabolism due to ATP depletion, and it becomes active following phosphorylation of its catalytic α subunit [203, 367, 387]. The antioxidant resveratrol has been shown to activate AMPK [366], and AMPK is an established negative regulator of the Warburg effect (aerobic glycolysis) [368] and mTOR signaling [203, 369]. Since our data indicated that MnP treatment inhibits mTOR-driven aerobic glycolysis in $CD4^{+}$ T cells, and MnP is a potent antioxidant [324, 376], we hypothesized that MnP treatment would result in AMPK activation, thereby inhibiting mTOR signaling and aerobic glycolysis.

AMPK is highly activated in naïve T cells, and protein analysis demonstrated that the levels of AMPK phosphorylation (Thr172) due to MnP treatment were comparable to those of media-treated, naïve cells, as indicated by a relative expression value of 1 (Figure 27). Mimotope stimulation alone resulted in reduced AMPK phosphorylation at 48 hours as compared to both media and MnP-treated splenocytes (Figure 27). These results reveal that MnP treatment may not enhance AMPK activation, but rather maintain active levels even in the presence of antigenic stimulation, thus impeding mTOR signaling and aerobic glycolysis.

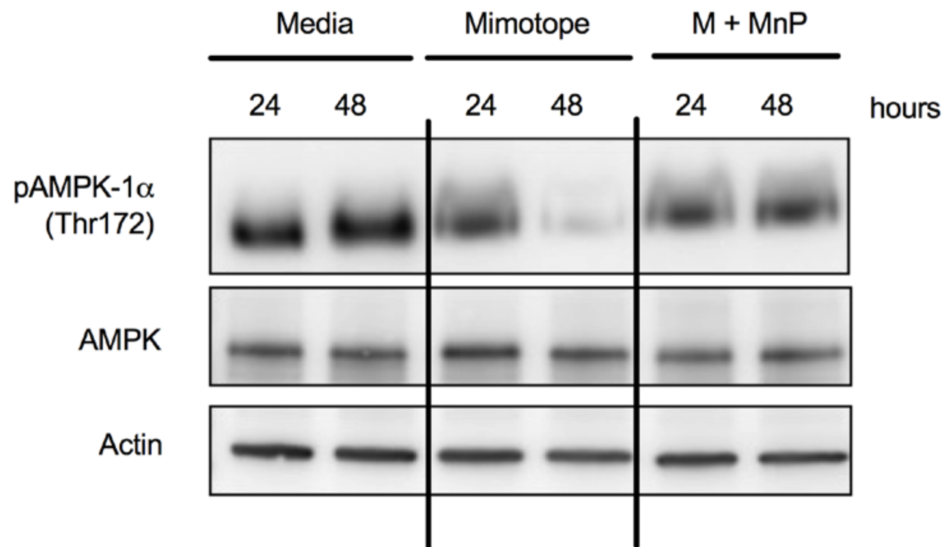

Figure 27. Alteration in redox status of CD4+ T cells maintains AMPK activation.
Protein lysates were probed with antibodies for phosphorylated AMPK-1α (Thr172; activated), total AMPK, and β actin (loading control). Data are a representative of n = 5 independent experiments.

3.4.7 Inhibition of aerobic glycolysis due to redox modulation limits the diabetogenic potential of autoreactive $CD4^+$ T cells

In order to test if inhibiting progression to aerobic glycolysis in $CD4^+$ T cells delayed diabetes incidence, an adoptive transfer model was used (Figure 28A). Here, BDC.2.5.TCR.Tg splenocytes were isolated and transferred i.v. into non-diabetic NOD.*scid* recipients. A cohort of the recipient animals was treated with 10 mg/kg MnP starting the day prior to adoptive transfer as described in the methods. 80% of untreated controls exhibited fulminant diabetes by 12 days post-transfer (Figure 28B); however, MnP treatment inhibited diabetes progression, with only 20% of treated animals becoming diabetic throughout the duration of the experiment (Figure 28B; p=0.0233).

We and others have reported that increased serum levels of the inhibitory receptor Lymphocyte Activation Gene-3 (LAG-3) due to its redox-dependent cleavage from the surface of T cells, serves as a viable marker of T cell activation [169, 170] and conceivable predictor of T1D [168]. Therefore, we measured soluble LAG-3 (sLAG-3) in the serum of recipient animals at various time points post-transfer, as a means of measuring T cell activation. At day 7 post-transfer, while all animals were euglycemic, control animals presented with elevated serum levels of sLAG-3 as compared to those treated with MnP (Figure 28C). Those controls went on to become diabetic by day 11 post-transfer, while animals treated with MnP did not ($p<0.01$). Together, these data demonstrate that sLAG-3 serum levels, as a measure of T cell activation, predicts diabetes onset, and MnP treatment controls this redox-dependent process.

Splenocytes from recipient animals were analyzed by flow cytometry to assess T cell activation *in vivo*. Two markers of activation were measured: CD25, the IL-2 high affinity receptor, and LAG-3 (Figure 28D), as both markers are more highly expressed on the surface of activated $CD4^+$ T cells than on naïve T cells [139, 347]. $CD4^+$ T cells from diabetic controls displayed increased frequency of $CD25^+LAG\text{-}3^+$ double positive T cells, indicative of an activated phenotype, in comparison to $CD4^+$ T cells from MnP treated non-diabetic animals (Figure 28D; $p = 0.0761$).

As *in vitro* results indicated reduced mTOR signaling (Figure 26), we also wanted to confirm these results *in vivo*. Peripheral blood samples were taken at day 4 post-adoptive transfer and levels of phosphorylated S6 ribosomal protein (S6), an mTOR target, were measured by flow cytometry. Results indicated two populations of $CD4^+$ T cells with respect to pS6 expression, $CD4^+pS6^{hi}$ T cells and $CD4^+pS6^{lo}$ T cells (Figure 28E). $CD4^+$ T cells from MnP treated animals exhibited a lower frequency of $pS6^{hi}$ cells (Figure 28E-F), as compared to T cells

from control animals, indicating reduced T cell activation due to MnP treatment. Moreover, pS6 expression (as measured by mean fluorescence intensity) was increased in $CD4^{+}pS6^{hi}$ T cells from control animals compared to the MnP-treated cohort (Figure 28G). These results suggest that those T cells that do receive some activation signal despite the presence of MnP, are still unable to induce mTOR signaling to the extent of stimulated controls. Lastly, studies from *Pollizzi et al.* demonstrated that T cells with high levels of mTOR signaling during activation, were larger than those with lower mTOR activation [126]. In accordance with these and other studies, $CD4^{+}pS6^{hi}$ T cells were larger than the $CD4^{+}pS6^{lo}$ T cells, regardless of treatment (Figure 28H). Yet, as with higher mTOR signaling induction in control T cells (Figure 28G), $pS6^{hi}$ T cells from these animals were also larger than those treated with MnP (Figure 28H), supporting their finding that T cell size is directly related to mTOR activity and activation [124]. These results demonstrate that MnP treatment *in vivo* alters mTOR signaling during T cell activation, resulting in reduced $CD4^{+}$ T cell diabetogenicity.

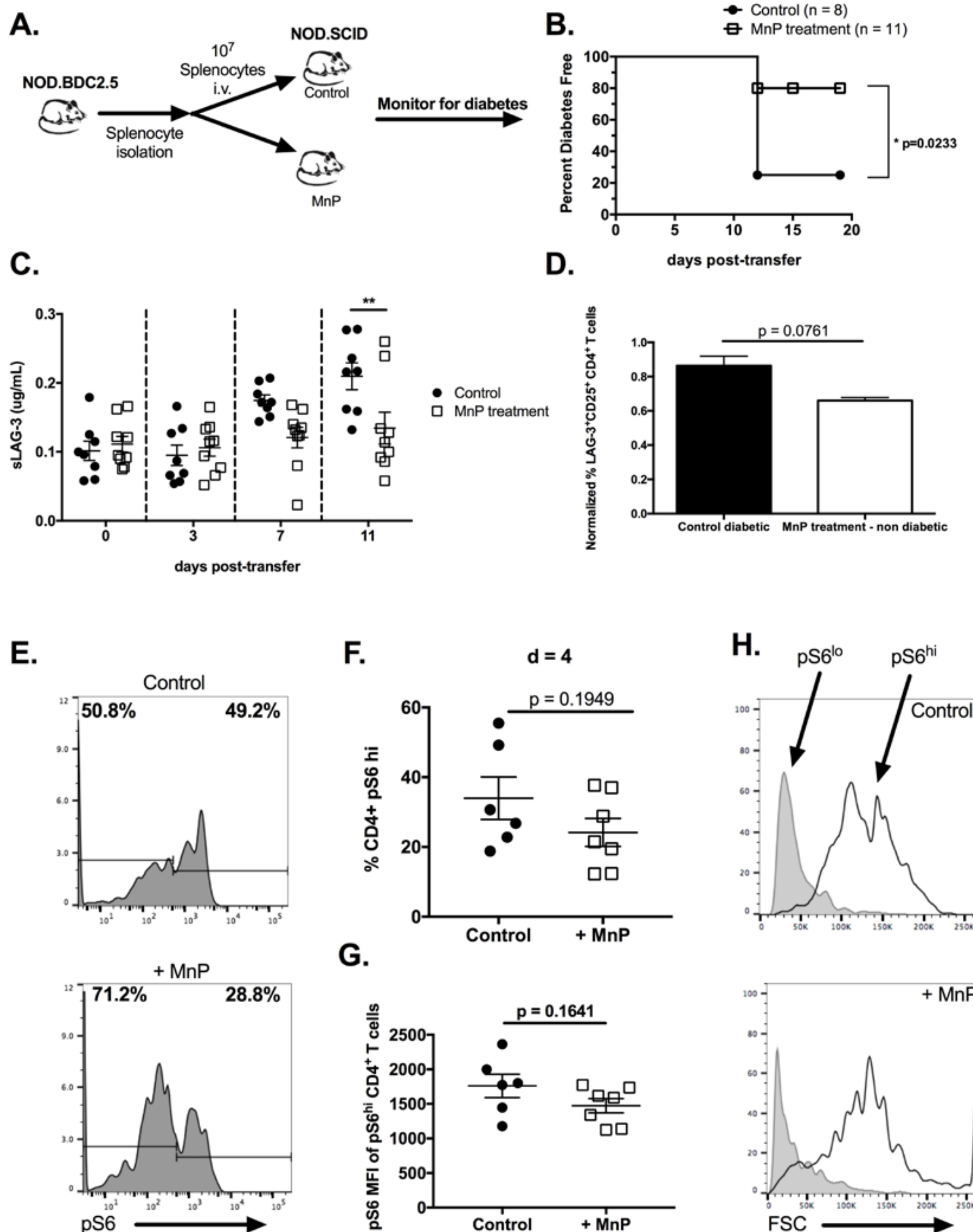

Figure 28. Reducing glycolytic capacity by redox modulation inhibits diabetogenic potential of CD4+ T cells. **A.** Schematic of adoptive transfer model of T1D. **B.** Survival curve of diabetes free animals following adoptive transfer. Animals were deemed diabetic following 2 consecutive blood glucose readings of >350 mg/dL. Statistical significance of disease progression was calculated using a Kaplan-Meier test (*=p<0.05). **C.** T cell activation and prediction of diabetes was measured by serum levels of sLAG-3 by ELISA. Statistical significance was calculated using a Two-way ANOVA with Bonferroni post-hoc analysis. (**=p<0.01). **D.** Normalized percent of LAG-3^{+}CD25^{+}CD4^{+} T cells from spleens of control diabetic and MnP-treated non-diabetic animals (n = 6-8 animals per group; p = 0.0761). **E.** Mean fluorescence intensity of pS6 in peripheral blood CD4^{+} T cells from animals at day 4 post-transfer. **F.** Representative histograms of S6 phosphorylation from peripheral blood CD4^{+} T cells at day 4 post-transfer from control and MnP treated animals. **G-H.** Quantification of frequency of pS6^{hi} and pS6^{lo} CD4^{+} T cells. **I.** Forward scatter analysis of pS6^{lo} and pS6^{hi} CD4^{+} T cells.

3.5 DISCUSSION

Work from our laboratory and others has demonstrated that acute doses of ROS are required for mediating T cell activation, and that ROS inhibition results in dampened T cell responses [168, 202, 225, 244, 304]. Treatment with a manganese metalloporphyrn (MnP) successfully scavenges a significant amount of the ROS produced, with no toxicity to the T cells themselves (Figure 23). Cell cycle entry and proliferation have been shown to be highly dependent upon ROS signaling, as many cyclin-dependent kinases that mediate cell cycle progression, and cell cycle inhibitors are known to be redox sensitive [295]. Indeed, our results indicated that dissipating ROS by MnP resulted in reduced cell cycle entry via maintenance of the cell cycle inhibitor p27 Kip1 (Figure 23). Interestingly, T cell proliferation and effector function were not completed ablated upon MnP treatment, potentially since MnP treatment did not completely dissipate all superoxide generated upon T cell activation (Figure 22A-B). Additionally, completely depleting the cell of all ROS would result in toxicity. These results highlight that fine-tuning of ROS signaling has dramatic effects on T cell outcome.

In order to transition to an effector after activation, naïve $CD4^+$ T cells must undergo massive reprogramming at the metabolic level, transitioning from oxidative phosphorylation to aerobic glycolysis [49, 50, 362]. Aerobic glycolysis, or the Warburg effect, is required for supporting optimal T cell clonal expansion and macromolecule synthesis, yet how ROS affect metabolic reprogramming during T cell activation remains poorly understood. Here, our data indicate that ROS are necessary for driving optimal mTOR signaling (Figure 26A) and upregulation of the transcription factor Myc (Figure 25A), two key players that have roles in coordinating both aerobic glycolysis and cell cycle entry [104, 363, 364]. It has been reported that T cell effector function is tightly regulated by both aerobic glycolysis and cell cycle. In fact,

proliferation and IFNγ production have a direct relationship – as rounds of proliferation increase so does IFNγ production [305] Our results reiterate these findings in that redox modulation inhibits aerobic glycolysis (Figure 24) and proliferation (Figure 24), concomitant with IFNγ secretion (Figure 26C). Also, these findings suggest that redox reactions in fact supersede these pathways.

A well described characteristic of T cells is that they divide asymmetrically, generating two distinctly different daughter cells. These daughter cells exhibit differential mTOR activation, cellular metabolism, and eventual differentiation [123, 126, 127]. Specifically, the larger, $mTOR^{hi}$, glycolytic daughters demonstrate a more T effector phenotype, whereas the smaller, $mTOR^{lo}$, oxidative daughters are more memory-like [124, 126, 127]. Interestingly, redox modulation skewed T cells towards a higher percentage of $mTOR^{lo}$ T cells as compared to untreated controls (Figure 28E-H), which may suggest a role for redox in asymmetric division. It is also plausible that scavenging of ROS is simply inhibiting activation and the $pS6^{lo}$ T cells are those that remain naïve. Also in this report, *Pollizzi et al.* indicated that there was no difference in CD25 surface expression between $mTOR^{hi}$ and $mTOR^{lo}$ T cells [124]. Even with CD25 expression and the presence of IL-2, $mTOR^{lo}$ T cells failed to proliferate as robustly [386], which is in accordance with the *in vitro* results demonstrated (Figure 25D-E), suggesting some additional regulatory mechanism(s) at play. It seems plausible that ROS could be a contributing factor in mediating T cell asymmetry; however, further studies would be necessary.

As mentioned, one of the downstream targets of mTOR is 4E-BP1, a translational repressor [386]. For translation to ensue, hyperphosphorylation of 4E-BP1 by mTOR is critical for inhibition of the repressor. Since our data indicate a decrease in phosphorylation or inhibition of 4E-BP1, it is likely that maintenance of the repressor contributed to the reduced

protein expression of Glut1 and PFKFB3 (Figure 24E). This, coupled with reduced Myc expression, may synergistically impede metabolic reprogramming. With regards to cell cycle progression, inhibition of 4E-BP1 by mTOR is required for promoting expression of Cyclin D3 [365]. Additionally, mTOR signaling has been shown to lead to increased p27 Kip1 degradation as a means of supporting cell cycle progression [365]. Therefore, MnP-mediated decreased 4E-BP1 hyperphosphorylation likely contributed to decreased Cyclin D3 expression and decreased p27 Kip1 degradation (Figure 23D), promoting cell cycle arrest.

$CD4^+$ T cells are a primary mediator of immunopathology in T1D; therefore, we wanted to determine if inhibiting metabolic reprogramming by MnP treatment reduced their diabetogenic potential. As anticipated, modulating the $CD4^+$ T cell glycolytic rate via MnP delayed T1D onset in an adoptive transfer model (Figure 28B). T1D is known to be highly driven by free radicals. Not only does oxidative stress result in islet beta cell death, but it also serves to activate and mobilize inflammatory macrophages and T cells, driving even more immunopathology. Therefore, further outlining the mechanisms in which these molecules influence immune cells is vital. More recent studies in other autoimmune diseases like systemic lupus and rheumatoid arthritis have helped to delineate potential for metabolic-based therapies in ameliorating disease [188, 189, 383], further supporting the need to more fully understand mechanisms governing immune cell metabolism. It is worth noting that in rheumatoid arthritis, pathogenic T cells were shown to produce increased levels of ROS that resulted in glycolysis inhibition and increased apoptosis [388]. Consequently, ROS may play different roles depending on the disease context, making it even more imperative to further understand their influence.

In summary, we have demonstrated that ROS inhibition by a manganese metalloporphyrin during diabetogenic $CD4^+$ T cell activation is capable of impeding the

metabolic transition from oxidative phosphorylation to aerobic glycolysis necessary for optimal T cell responses. We propose a model in which ROS and cellular redox balance is critical for amplification of the mTOR/Myc pathway, and thus aerobic glycolysis (Figure 29). These findings present potential implications in tempering T cell responses in autoimmunity, and also controlling tumor metabolism and cell growth in cancer.

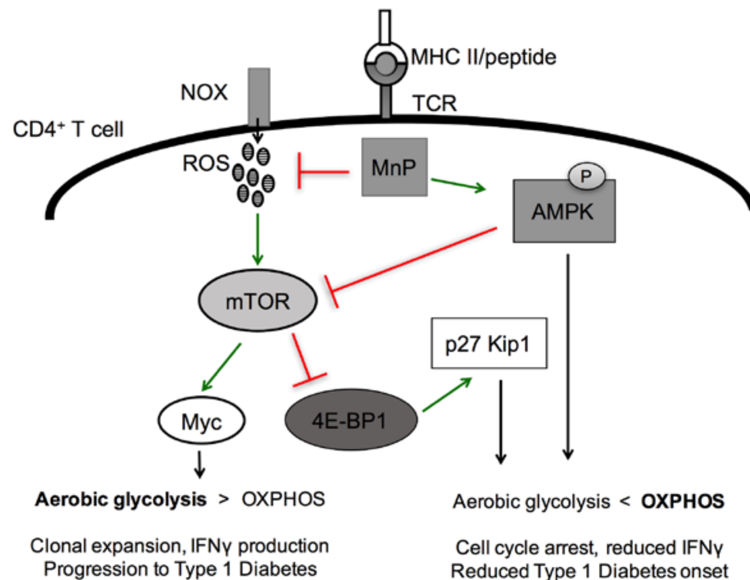

Figure 29. Mechanism for the effect of ROS inhibition on CD4+ T cells during activation-induced metabolic reprogramming.

Upon T cell receptor – MHC interaction, ROS are generated by a functional NOX expressed by T cells. These ROS serve as signaling molecules to help propagate mTOR signaling resulting in Myc upregulation and progression to aerobic glycolysis. Treatment with the ROS-scavenging and potent antioxidant results in inhibition of ROS and maintains potent AMPK activation; thereby, inhibiting mTOR via a two-pronged approach, stabilizing OXPHOS, and limiting T cell proliferation

4.0 FUTURE DIRECTIONS

4.1 LAG-3 AND T CELL METABOLISM

4.1.1 Metabolite oxidation and mitochondrial phenotype

While the data presented in chapter 2 demonstrate that LAG-3 negatively regulates mitochondrial biogenesis and metabolism, one question that remains is which metabolite is responsible for supporting this function. Fatty acids, glucose and glutamine can all be oxidized by the mitochondria, making them each a potential candidate in our model. Seahorse experiments were performed using specific pathway inhibitors during mitochondrial stress tests to see which resulted in reduced basal OCR and/or SRC. However, they proved inconclusive. These experiments are still ongoing and our working hypothesis is that fatty acids will be the critical metabolite. This hypothesis is based upon the fact that our studies concluded LAG-3$^{-/-}$ T cells are metabolically similar to memory T cells, and fatty acid metabolism is necessary for supporting enhanced spare respiratory capacity and rapid recall in memory T cells [129, 135, 389].

4.1.2 T cell metabolism in Type 1 Diabetes

As discussed in Chapter 1, there is mounting evidence that autoreactive T cells are metabolically different from other T cells. These differences include increased mitochondrial membrane potential and mutations, enhanced glycolysis and OXPHOS, and a greater dependence on glutamine metabolism. The majority of the work investigating metabolic differences have been done in diseases like MS, Lupus, and RA, with very little investigating T cell metabolism in T1D. With the knowledge we have gained from studies in these other autoimmune diseases, its highly plausible that there are metabolic abnormalities in diabetogenic T cells. Future experiments comparing glycolytic and oxidative capacities during naïve and effector states of diabetogenic (NOD) and non-autoimmune (B6) T cells may reveal differences, which in turn could suggest the use of metabolic-based therapies in the disease, as these types of therapies have proven efficacious in other autoimmune diseases. For example, in the animal model of Lupus, inhibition of utilization of the chemicals 2-deoxyglucose and metformin reversed metabolic dysfunction, thus ameliorating disease [188]. Additionally, targeting glutamine metabolism in EAE reduced Th17 differentiation and pathogenicity [111]. There is some evidence that effector T cells in T1D are refractory to T regulatory cell mediated suppression [323]. It is highly plausible that underlying metabolic advantages protect effector T cells from being suppressed, and weakening this advantage via targeted therapy could lower this metabolic threshold, thereby leaving effector T cells vulnerable to T regulatory cell-mediated suppression.

As mentioned previously, LAG-3 knockout in the NOD model leads to accelerated T1D progression, due to heightened $CD4^+$ T effector cell responses [155, 158]. Our data would suggest that LAG-3 deficiency in these T cells results in enhanced bioenergetics thus driving greater activation potential, resulting in disease acceleration. These data provide the basis for

future studies examining what effect this metabolic phenotype has in the context of autoimmunity. Based on the data presented in chapter 2, we would hypothesize that increased mitochondrial mass and metabolism in naïve T cells, coupled with an autoreactive TCR, could make for an even more pathogenic T cell. Future studies could examine if metabolic differences exist during activation by comparing mitochondrial characteristics and metabolic profile of NOD.LAG-3 sufficient and deficient T cells during T1D induction.

While these proposed studies would further our understanding of metabolic differences during activation, they would not elucidate what advantage alterations during T cell homeostasis provide for activation. The difficulty lies in that NOD T cells exhibit characteristics of activation even in young animals, since they develop in the presence of their antigen, the β cell. Currently, we are working on experiments utilizing a novel NOD TCR transgenic animal, the NOD.BDC-6.6.9 strain. Here, all $CD4^+$ T cells recognize an autoantigen that is unique to NOD islets [390]. The region of the genome encoding this peptide was mapped and replaced with a Balb/c sequence that does not encode the antigenic peptide [390]. Therefore, these $CD4^+$ T cells, that are capable of inducing T1D following adoptive transfer into NOD.*scid* animals, are educated and develop in an environment devoid of their cognate antigen. Thus, they are autoreactive, yet entirely naïve. We plan to recapitulate adoptive transfer experiments into NOD.*scid* animals with and without LAG-3 antibody blockade (as LAG-3 knockout animals on this background do not exist) to assess metabolic differences as described in chapter 2. These experiments would expand our understanding of the role of LAG-3 in autoreactive T cell homeostatic expansion and metabolism and its relevance in T1D.

One caveat is that we will need to deplete the endogenous β cells in the NOD.*scid* animals via streptozotocin treatment as to eliminate the source of antigen, and therefore, blood

glucose in these animals will need to be maintained by insulin pellets. If similar differences do arise due to LAG-3 blockade, we could then transplant NOD islets back into the recipient animals to assess if the differences in T metabolism augment activation and disease progression.

Translating our studies into humans would also be a future direction for this project. As with studies performed using samples from SLE patients, it would be interesting to receive peripheral blood samples from patients and first degree relatives to investigate the metabolic profile of T cells. Taking this one step further, DNA sequencing could be used to identify SNPs in the LAG-3 gene or any others that may have implications on metabolism. We currently have a collaboration with Dr. Eddie James, PhD., who has isolated autoreactive T cell clones from Type 1 diabetics. Preliminary DNA sequencing studies using these T cells and those from health controls could be performed to then provide a basis for further investigation.

4.1.3 LAG-3 expression and memory T cell formation

One mechanism of memory T cell formation is by the contraction of activated effectors following resolution of the immune attack. With this contraction, glycolytic effectors must transition back to relying on OXPHOS to support memory T cell longevity. Lowering the glycolytic threshold of T effectors has been shown to improve memory T cell formation [135, 136]. The data presented in Figure 15 indicate that LAG-3-deficient T cells exhibit enhanced aerobic glycolysis upon activation. Together, these studies suggest that LAG-3$^{-/-}$ T cells, due to their highly glycolytic nature, may not transition to memory T cells as effectively as wildtype T cells. Moreover, LAG-3 expression may serve as a metabolic regulatory mechanism to dampen glycolytic potential thereby ensuring efficient T effector contraction.

Preliminary *in vivo* studies were performed to address this question, where animals were immunized with ovalbumin and memory T cell function was assessed 30 days later (Appendix A). Results indicated trends in reduced cellularity of draining lymph nodes in LAG-3$^{-/-}$ animals (Figure 30A), along with a lower frequency of memory T cells (CD62L^{hi} CD44^{hi}) as compared to wildtype animals (Figure 30B; $p<0.05$). Upon restimulation *in vitro*, wildtype memory T cells produced greater levels of IFNγ, suggesting a more robust memory response (Figure 30C). Repeating these experiments with animals receiving adoptive transfers of OT.II T cells may prove beneficial in that it would provide a more distinct population that could also be isolated by tetramer staining. Future experiments would be necessary to further understand potential differences, and to also assess metabolic differences. *Van der Windt et al.* has developed an *in vitro* method of generating both effector and memory T cells [128, 129], which could be used with wildtype and LAG-3$^{-/-}$ OT.II cells for Seahorse analysis.

4.1.4 LAG-3 and its effect on T regulatory cell metabolism

Some naturally occurring CD4^{+}CD25^{+} T regulatory cells demonstrate increased LAG-3 expression as compared to naïve, effector, and memory subsets [141]. Moreover, they have been shown to utilize LAG-3 as a mode of suppressing effector T cell responses [141, 177]. Studies examining the metabolic profile of T regulatory cells have indicated that like naïve and memory subsets, they rely predominantly on OXPHOS. Specifically, they utilize fatty acid metabolism, driven by high levels of AMPK expression [391]. Both mTOR inhibition (by knockout or

rapamycin treatment) or AMPK activation (via metformin treatment) enhanced T regulatory cell differentiation as well [384, 392], further supporting a role for this pathway.

Work from *Huang et al.* indicated that LAG-3$^{-/-}$ regulatory T cells proliferated to a greater extent than wildtypes, which is in line with our work and other previous work that LAG-3 expression inhibits T cell proliferation [157]. Our studies would further suggest that LAG-3-deficient T regulatory cells may demonstrate increased mitochondrial mass and metabolism, as a means of supporting increased proliferation. This could prove beneficial in autoimmune settings where T regulatory cell numbers wane. However, it would be important to also verify that bioenergetic differences due to LAG-3 loss do not interfere with suppressive capabilities. In some instances, where LAG-3 is utilized to mediate suppression, LAG-3 blockade would not be advantageous as the ability to restrain T cell responses is likely more important than increasing T_{reg} numbers. Unless this can be done temporally to boost numbers, and then remove treatment to enable suppression. Future studies would be necessary to further elucidate potential metabolic implications on T regulatory cells.

4.2 REDOX MODULATION IN TYPE 1 DIABETES

4.2.1 MnP as a mono-therapy in T1D

Data from our laboratory have demonstrated that use of MnP as a mono-therapy appears insufficient for maintaining durable tolerance, as once treatment ceases, NOD females succumb to T1D [168]. Therefore, understanding its deficiencies is critical for adjunct therapy development. From a metabolic standpoint, the AMPK/mTOR axis is one pathway by which T

cell anergy is achieved. *Zheng et al.* showed that anergic T cells are also metabolically anergic and express high levels of active AMPK which maintain OXPHOS and quiescence [369]. AMPK also results in mTOR inhibition, again inhibiting activation potential. We have shown here that redox modulation via MnP results in dampening mTOR and enhancing AMPK activation (Figures 25,28). It is possible that the degree of these events is insufficient for permanent modification of the T cell phenotype. Since MnP-treated T cells are still capable of full activation following MnP cessation and do not undergo apoptosis, AMPK activation in this context may serve as a survival mechanism for treated $CD4^+$ T cells by maintaining efficient OXPHOS.

Our data here also indicated that MnP-treated T cells still produced sufficient levels of IL-2; but, downstream signaling was interrupted (Figure 25). IL-2 is known to be effective in reversing T cell anergy, and this may provide a mechanism for reversal of hyporesponsiveness during MnP termination. Specifically, IL-2 signaling results in inhibition of the protein deacetylase Sirtuin-1 (Sirt-1), reversing T cell induced anergy [393]. As anergic T cells express higher levels of Sirt-1 as compared to activated effector T cells, we tried to examine Sirt-1 expression in our model, but were unable to see any appreciable differences with and without MnP treatment. It is also interesting in that Sirt-1 is a downstream target of AMPK [394], begging the question that while MnP enhances AMPK activation, it might not result in enhanced Sirt-1 activity; thereby, not inducing T cell anergy. Further experiments involving an IL-2 depletion antibody or further examining Sirt-1 expression and activity may help to clarify the limitations of MnP treatment.

4.2.2 Metformin and Rapamycin as combinatorial therapies with MnP

Combinatorial therapies with MnP and other agents may aid in providing long-term T cell hyporesponsiveness. For example, metformin, an AMPK activator, may be a viable option as it has been shown to maintain AMPK activation in autoreactive T cells, resulting in reduced disease. Additionally, T regulatory cells, which are highly oxidative, rely on AMPK activation for maintenance of the population and enabling their suppressive capabilities. Driving reduced T effector responses while also potentially enhancing T regulatory function, may provide greater protection from disease onset. Metformin has also been shown to protect islet β cells and preserve their glucose sensitivity and insulin secretion, providing even greater therapeutic potential. Rapamycin, an mTOR inhibitor, might also be considered a viable option. While rapamycin has shown potential for modulating T cell responses and metabolism in various models, including T1D, it is well documented that it is toxic to β cells [361]. Unpublished work from our laboratory has indicated that MnP administration in combination with rapamycin, protects β cells from these toxic effects. Consequently, as both MnP and rapamycin provide immune suppressive functions, this could be a viable dual therapy in T1D. Further studies would be necessary for examining this potential.

APPENDIX A

INVESTIGATING THE ROLE OF LAG-3 IN MEMORY T CELL FORMATION

Preliminary studies were performed to investigate if LAG-3 deficient $CD4^+$ T cells capable of forming functional memory responses. Studies were performed using wildtype and $LAG\text{-}3^{-/-}$ B6 animals, where animals were immunized with whole ovalbumin in Complete Freund's Adjuvant (OVA; 100ug per mouse) at the base of the tail. 30 days post-immunization, animals were sacrificed, and draining inguinal lymph nodes (dLN) were harvested and re-stimulated with whole OVA *in vitro* (25 ug). IFNγ production was used to measure activation. Cells were also stained for flow cytometry to distinguish naïve, effector, and memory T cell subsets. Vβ5 expression was used to further gate on OVA-specific T cells. dLN from non-immunized mice served as negative controls for flow cytometry staining.

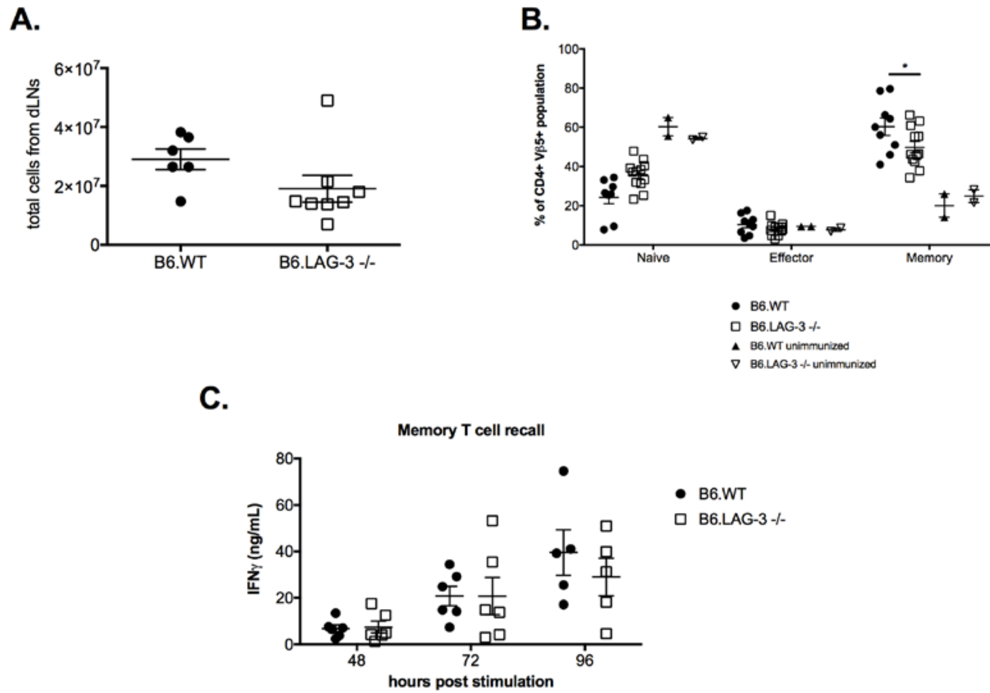

Figure 30. LAG-3 deficient CD4+ T cells demonstrate impaired memory T cell formation and functionality. A. Absolute number of cells from draining inguinal lymph nodes (dLN) 30 days post-immunization with OVA. B. Frequency of naïve, effector, and memory CD4+ T cells following immunization. $CD4^{+}V\beta5^{+}$ T cells were gated on, and expression of CD62L and CD44 were used to delineate naïve, effector, and memory subsets. C. IFNγ ELISA results from *in vitro* recall assay. Data points are indicative of individual animals, and significance was determined using a Student's t test. *=$p<0.05$.

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