# Overcoming Self- Reactivity in Type 1 Diabetes via Modulation of Immune Cell Metabolism

by

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## ABSTRACT

The immune system is responsible for mediating protection of its host from foreign pathogens, and its ability to do so efficiently is vital to the maintenance of health. Recognition of pathogens relies on a keen ability to discriminate self from non- self in order to minimize detrimental immunopathology against host- tissues. However, under certain circumstances, self- reactive T cells become activated and drive autoimmune targeting of host tissues, like that exhibited in Type 1 Diabetes (T1D). T1D occurs when insulin secreting β cells residing in the pancreas are mistakenly recognized as foreign and targeted for destruction. While various immune cells work cooperatively to mediate β cell death, autoreactive $CD4^+$ T cells are considered the primary contributors to disease pathology. Recent interest in the field of immunometabolism has demonstrated the importance of cellular metabolic programs in the activation and differentiation of immune cells, especially T cells. Specifically upon antigen encounter, T cells become activated and rely on the less efficient aerobic glycolysis to support rapid growth, clonal expansion, and effector functions. Based on the importance of glycolysis in promoting T cell activation and effector function, use of glycolytic inhibitors to suppress autoreactive T cell responses in Systemic Lupus Erythematosus (SLE), Multiple Sclerosis (MS), and Rheumatoid Arthritis (RA) have proven beneficial in alleviating autoimmunity. However, the ability to modulate T cell metabolism in the context of T1D has remained unexplored. Here, we assessed the ability of the small molecule PFK15, a competitive inhibitor of a rate- limiting enzyme in the glycolysis pathway PFKFB3, to

suppress autoimmunity during T1D. Our results demonstrated that inhibiting glycolysis reduced diabetogenic T cell responses *in vitro*, and significantly delayed the onset of T1D in adoptive transfer models. The protective benefits associated with PFK15 treatment were due to induction of terminal $CD4^+$ T cell exhaustion. This phenotype was due to a direct effect on autoreactive T cells, as antigen presenting cell (APC) function was unaltered by PFK15. Collectively, our data support modulating cellular metabolism as a novel approach to controlling aberrant T cell responses in T1D; with broad implications in our understanding of chronic infection, cancer, and autoimmunity.

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# PREFACE

"A great experiment takes great determination. The will to do the work and then the wisdom to keep refining, keep tinkering, keep perfecting."

Madam Vice President Kamala D. Harris

Most people will agree that graduate school is a challenging experience, however for me, it has also been a time of immense personal and professional growth that has greatly shaped the person I am today. I would be lying if I said this process has been easy, with the highs and lows of my graduate training often feeling like a turbulent rollercoaster. The struggles associated with graduate school were compounded even further by the COVID-19 pandemic when labs were forced to close, and experiments put on hold. This quote by Vice President Kamala Harris perfectly describes the grad school experience, as science does not happen overnight and requires the determination, hard work, and will to troubleshoot and refine experiments, expand scientific ideas, and an open mind to follow wherever the data may lead you. I cannot count the number of times I wanted to give up, however my passion for moving science forward, learned persistence, scientific curiosity, and the support of some very special people helped me push through the difficult times, and ultimately allowed me to accomplish my goal and earn my PhD.

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today without your advisement, and for that I will forever be thankful. I know that I will do great things in the future thanks to the foundation you helped me build within myself. I would also like to thank my thesis committee members Dr. Robbie Mailliard, Dr. Josh Mattila, and Dr. Steve Little for agreeing to serve on my committee and providing their wisdom, thoughtful critique, and astounding mentorship. Your belief in me as a scientist, and commitment to my training were invaluable, and I appreciate each and every one of you for the time you invested in me.

I am under the personal belief that it takes a village to successfully train a graduate student, and for me, past and present members of the Piganelli lab were instrumental members of my "village." Specifically, I would like to thank Dr. Gina Coudriet, Dr. Meghan Marre, Dr. Dana Previte, as well as our lab technician LeeAnn New for all playing pivotal roles in my journey. Gina and Meghan- you were there for me during my formative years in the lab, when I didn't know anything. From helping me master splenocyte isolations and ELISAs, to guiding me as I navigated and prepared for my comprehensive exam, your unwavering patience and commitment to teaching me were fundamental to my training, and for that I will forever be thankful. I would like to extend a very special thank you to Dr. Dana Previte, who is by far one of the best scientists I have had the pleasure of learning from. Dana- I cannot even begin to describe how much your mentorship and friendship these last 6 years has meant to me. As the resident metabolism expert in the lab, you took me under your wing and helped point me in the right direction when I was completely lost and out of my comfort zone. Even after you graduated and left the lab to start your postdoc, you were always ready and willing to meet for coffee to support me during the lows of graduate school, discuss new data, and to provide valuable insight through any scientific and technical challenges I was facing. Thank you for sticking with me through it all, I feel so lucky to have learned from one of the best. LeeAnn- thank you for your help maintaining the animal colonies and ensuring I always

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To my mom- Laura Carrizales, who from the time I was young worked so very hard to provide a wonderful life for me and my sister Brooke. You were the first to inspire me that women can be strong, fierce, independent leaders. Thank you for believing in me even when I didn't believe in myself, and for always reminding me that hard work pays off. I love you so much and appreciate the sacrifices you made to make my life the best it could possibly be. To my dad- Anthony Singe,

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Last, but certainly not least, I would like to thank my beautiful wife, Dr. Kasey Cargill, for without her none of this would've been possible. You have seen me at my very best and worst and have loved and supported me through it all. Two scientists being married to one another can be tough, and often times I'm sure it felt like the only thing we had to talk about was our science and lab experiments. However, it has been nice to be able to go through this process at the same time, supporting one another through the good and the bad. I am sure at this point you have grown tired of hearing about PFK15 and T cells, but your willingness to talk through my crazy ideas, be a shoulder to lean on when I was struggling, and your reminders that I am enough and can do anything I put my mind to made all the difference. Through it all, you ensured that I knew I was smart enough and loved, and your support has instilled in me a confidence and belief in myself that I didn't know was possible. We started this journey together, and I have truly enjoyed seeing

our growth into the independent scientists we are today. You are one of the most intelligent and ambitious individuals I know, and I feel so lucky that I have you in my life. You are the epitome of what it means to be a woman in science, and I hope to be half as good of the scientist you are one day soon. I love you so much, and appreciate all of the time, support, and unconditional love you have provided to me. I hope that I have made you proud!

Thank you again to all those individuals who made up my "village." I couldn't have done this without all of you, and I feel so lucky to have had such a wonderful support system. Ultimately, it was all of you who made accomplishing my dream possible, and for that I will forever be grateful. I hope I continue to make each and every one of you proud.

# 1.0 INTRODUCTION

A portion of this section was adapted from the author's version of a review paper. The definitive version of this work was published in *Immunometabolism*.

**"Targeting T cell Metabolism to Combat Autoimmunity: Implications for the Future of Type 1 Diabetes Therapeutics"**

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## 1.1 IMMUNITY DEPENDS ON AN ABILITY TO DESCRIMINATE BETWEEN SELF AND NON- SELF

The immune system is tasked with the vital role of protecting the host from foreign invaders, and acts as a critical barrier from infectious agents and pathogens [1]. This relies on an ability to distinguish self from non- self, and acts as a safeguard against the onset of autoimmunity (i.e. targeting of self- tissues) [1, 2]. Broadly speaking, the immune system has two arms: the innate and the adaptive immune systems. While the innate immune system relies on non- specific recognition of pathogen- associated molecular patterns (PAMP's) by macrophages, dendritic cells (DCs), and neutrophils; the adaptive immune system is comprised of initiating T and B cell responses to specific antigens and establishing memory phenotypes that mediate lifelong protection [2]. Ultimately, the interplay and cooperation between these two systems is instrumental to the development of an effective immune response that clears the invading pathogen.

Importantly, while a hallmark of immunity is recognition of foreign antigens, this specialized sensing relies on a keen ability for host immune cells to differentiate between self and non- self-tissues. Innate immune cells rely on pattern recognition receptors (PRRs) that sense the presence of microorganisms to provide initial discrimination between self and non-self [1]. PRRs of macrophages and DCs allow for the simple recognition of evolutionarily conserved structures common to many pathogens that are no present on the body's own cells (i.e. peptidoglycans, lipopolysaccharides, mannose- rich oligosaccharides, etc.), leading to non- specific recognition [2]. Pattern recognition and detection of foreign antigens triggers an initial adaptive immune response characterized by engulfment of the pathogen by macrophages and DCs, and the processing and presentation of peptides on the cell surface via MHC-I and MHC-II molecules that are recognized by naïve T cells to activate adaptive immunity [1, 2].

Presentation of antigens by APCs to naïve T cells in secondary lymphoid organs (i.e., draining lymph nodes) leads to the commencement of an adaptive immune response that brings the specificity lacking from the innate arm of the immune system. Activation of $CD4^+$ T cells requires three distinct signals, including 1) recognition and binding of the T cell receptor (TCR) with peptide: MHC complexes, 2) co- stimulation provided by triggering of CD28 on T cells by CD80 and CD86 on APCs, and 3) cytokine micromilieu and reactive oxygen species (ROS) that directs the polarization and differentiation of unique T helper subsets (Figure 1) [1-5]. Activation of T cells results in the clonal expansion of antigen- specific effector T cells that mediate adaptive immunity via secretion of cytokines that promote inflammation via immune cell recruitment, and maturation of other immune cells, like $CD8^+$ T cells, that can mediate direct lysis of infected host cells [2]. Specificity of the T cell response relies on a principle known as clonal selection, which occurs during T cell development in the thymus, leading to a diverse repertoire of T cells bearing diverse T cell receptors and specificities [1]. This selection process is vital for maintaining tolerance to self and ensures potentially self- reactive T cells are deleted prior to thymic egress and maturation.

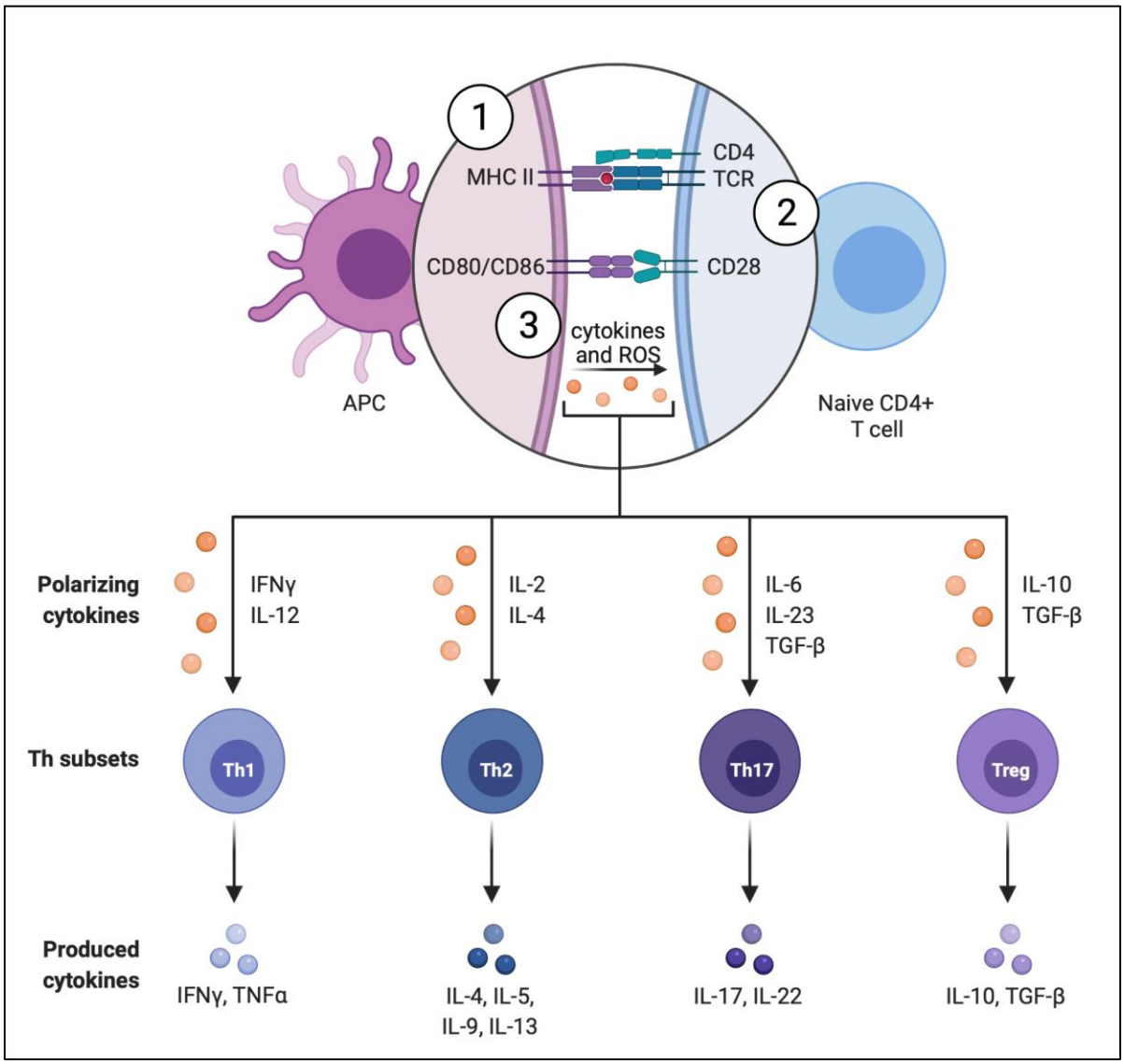


**Figure 1. Three signals are required for the activation and differentiation of CD4$^+$ T cell subsets.**
Naïve CD4$^+$ T cells require three signals for proper T cell activation and function. Signal 1 consists of binding of the TCR to peptides bound to MHC molecules on APCs and subsequent downstream signaling. Signal 2, or costimulation, is provided by the binding of costimulatory molecules CD80/CD86 to CD28 co- receptor on T cells, which amplifies intracellular processes associated with T cell activation. Cytokines and the presence of ROS in the local micromilieu to skew the differentiation of various T helper cell subsets, including Th1, Th2, Th17, and Treg populations. These 3 signals are required to produce a productive immune response against invading pathogens and to maintain tolerance to self- antigens. Diagram created using BioRender.com.

### 1.1.1 Development of the T cell repertoire occurs in the thymus

T cell precursors originating in the bone marrow migrate to the thymus where development into mature T cell populations occurs. Here, immature T cell progenitors undergo a rigorous selection process that ultimately shapes the mature repertoire of T cells [1]. Upon the formation of antigen receptors, T cells are required to undergo testing to select for useful antigen receptors that are able to recognize pathogens and do not react against an individual's host cells and occurs in 2 stages: positive and negative selection [1, 2]. These two processes aim to eliminate T cell clones expressing self- reactive as well as nonfunctional TCRs from the host, and are vital for the proper function of the adaptive immune response.

During positive selection, developing thymocytes whose receptors are able to interact weakly with self- antigen receive survival signals. The ability of thymocytes to interact with self-peptide-MHC complexes is particularly crucial, as it ensures that an individual's T cells can respond to antigens bound to their own MHC molecules to initiate immune responses in the periphery [1, 2]. Successful interaction of developing thymocytes with peptide- MHC complexes triggers a survival signal, while cells unable to bind self- peptide complexes leads to the induction of cell death by neglect [1, 2]. However, why selection on self- peptide:MHC complexes benefits the production of a competent T cell repertoire able to respond to unknown foreign antigens the host will encounter in the future has not been clearly resolved. One study by Mandl et al. demonstrated that positive selection uses the strength of self- peptide:MHC binding to optimize recognition to foreign antigens, where the strength of self- reactivity correlates directly with the strength of TCR binding to foreign peptides [6]. Specifically, it was determined that T cells with receptors that interacted strongly to self-antigens predominate in responses to diverse pathogens, thus selecting for high affinity T cell clones that are effective at clearing potential threats. Ultimately, positive selection

is crucial in ensuring the preferential maturation of T cells with the greatest capacity to respond to foreign antigen [6]. Further, prior to positive selection, thymocytes bear both the CD4 and CD8 co- receptors. Positive selection via MHC-I or MHC-II dictates expression of the corresponding CD8 or CD4 co- receptor respectively, that will be retained by thymocytes upon maturation, and therefore is important for lineage commitment of developing T cells [1].

As previously mentioned, positive selection ensures that TCRs expressed by thymocytes are capable of interacting with host MHC complexes, however the risk of selecting for self- reactive thymocytes remains. To mitigate this risk, developing T cells undergo a process termed negative selection, whereby lymphocytes that bind too strongly to self- reactive TCRs are eliminated from the repertoire [1]. This occurs when self- antigens are displayed by MHC molecules on the surface of thymic epithelial cells or resident DCs and macrophages. The self- antigens presented within the thymus encompass a wide range of both tissue specific (i.e. insulin) and ubiquitously expressed antigens, whose expression is mediated by the autoimmune regulator (AIRE) [1, 2]. Mutations in AIRE in mice and humans can lead to the development of autoimmune disorders [1]. Consequently, the collection of self-antigens expressed in the thymus have a direct impact on ability for the host to delete autoreactive T cell clones, thereby influencing the occurrence of autoimmunity. The deletion of self- reactive T cell clones during this process is vital to the establishment of central tolerance, and prevents their potentially pathogenic activation later should they encounter self- peptides after maturation.

### 1.1.2 Maintenance of self- tolerance relies on specific checkpoints in the periphery

Although developing T cells undergo robust testing and selection in the thymus to 1) produce a diverse T cell repertoire capable of mounting immune responses against a plethora of pathogens

and foreign invaders and 2) protect against the onset of autoimmune diseases, this process is not perfect. In fact, some T cells do escape these processes in healthy individuals, therefore allowing autoreactive T cell clones capable of mediating autoimmunity to migrate into the periphery [7]. Moreover, not all self- proteins, especially those that are tissue- restricted, are expressed in the thymus. Notably, many T cells with some underlying level of autoreactivity are able to mount immune responses to foreign antigens, therefore can play beneficial roles as the deletion of all weakly self- reactive T cells would leave a hole in the diversity of T cells an individual has to mediate life- long protection from disease [1, 7]. Luckily, there are a number of mechanisms in the periphery that exist to prevent the activation of autoreactive T cells as a means to achieve a state of self- tolerance. These checkpoints act synergistically to mediate protection against autoimmunity without inhibiting the ability of the immune system to remain effective at clearing invading pathogens. However, a failure in one or more of these tolerogenic mechanisms can lead to the onset of autoimmunity, like that of which occurs when pancreatic islet β cells are attacked during Type 1 Diabetes (T1D). These checkpoints, and how they fail in T1D will be discussed in more detail in the following sections.

#### 1.1.2.1 Checkpoint 1: Central Tolerance

As discussed in great detail above, central tolerance mechanisms within the thymus are the first checkpoint of self- tolerance. It is here that immature thymocytes undergo rigorous testing via positive and negative selection mechanisms as a means to delete strongly self- reactive T cells [1, 2, 7]. However, as not all potentially autoreactive T cells are removed from the T cell repertoire during their development, other mechanisms in the periphery must be in place as safeguards against the activation and expansion of autoreactive lymphocytes that escape negative selection processes.

#### 1.1.2.2 Checkpoint 2: Peripheral Tolerance

A number of mechanisms exist in the periphery to keep autoreactive T cells in check, which include 1) suppression by regulatory T cells (Tregs), 2) induction of anergy, 3) activation- induced cell death, and 4) immune suppression by immune inhibitory receptors (IRs). These 4 processes will be described in more detail herein.

#### 1.1.2.2.1 Suppression by Regulatory T cells (Tregs)

Regulatory T cells, or Tregs (defined simply as $CD4^+$ $CD25^+$ $FoxP3^+$ T cells), are a subpopulation of T cells that can modulate the immune system by maintaining tolerance to self- antigens, and preventing autoimmune diseases. Tregs have immunosuppressive capabilities that can suppress or downregulate the proliferation and effector functions of effector T cells via a number of mechanisms [1, 2, 8, 9]. There are two types of Tregs: natural Tregs (nTreg) and induced (iTregs). nTregs develop in the thymus. Moderately autoreactive nTregs can escape deletion in the thymus similarly to autoreactive effector T cells, and when activated by self- antigen in the periphery, can inhibit the activation of other self- reactive T cells and prevent their subsequent differentiation and effector functions [1, 2, 8, 9]. iTregs on the other hand develop in peripheral tissues in the presence of TGF-β [1]. Although Tregs exist to mediate suppression of pathogenic T cells responsive to self- tissues, evidence in the literature suggests these cells are defective in a number of autoimmune diseases [8-12]. This is true in Type 1 Diabetes (T1D), where insulin- secreting β cells in the pancreas are targeted and destroyed by autoreactive T cells. Although Treg numbers remain unchanged in T1D, suppressive function is often dysfunctional. Further, in some instances, Tregs can adopt a proinflammatory phenotype characterized by the secretion of IFNγ, further potentiating autoimmune mediated damage [8-12].

The potent suppressive nature of Treg cells have led to the development of Treg based therapies for the treatment of autoimmune diseases. Several approaches aiming to increase Treg numbers or support suppressive function have shown promise, however these approaches often times ultimately fail or lead to non-specific global immunosuppression and off- target effects [13, 14]. Regardless, Tregs are a promising therapeutic target for suppressing autoimmunity, especially in regard to restoring Treg function under circumstances where they become defective. Ultimately,

finding a balance or use of combinatorial therapies to induce antigen- specific Tregs are vital to the success of Treg mediated therapies for the treatment of autoimmune disorders, albeit without off target global immunosuppression [1, 12-16].

#### 1.1.2.2.2 Anergy

Anergy is defined as a state of hyporesponsiveness to antigen, ultimately leading to immunological ignorance. This phenotype occurs when autoreactive T cells are stimulated with antigen along with sub- optimal CD28 co- stimulation (signal 2), or high co-inhibition [1]. This results in low IL-2 production and cell cycle arrest [1]. Ultimately, potent and lasting immunoregulatory signaling via lack of proper co-stimulation or engagement of IRs with their associated ligands (PD-1: PD-L1, LAG-3: MHC-II, CTLA-4: CD80/CD86) can lead to a hyporesponsive state resulting in long-lived T cells with reduced effector functions and tolerance to self-antigens [17]. Notably, anergic T cells maintain expression of the high affinity IL-2 receptor CD25, and therefore can be rescued via IL-2 stimulation, indicating that while anergy is a tolerogenic phenotype, it is not permanent and can lead to re- activation of potentially pathogenic T cells [18]. Regardless, induction of a hyporesponsive, anergic T cell phenotype in NOD mice and human T1D patients is associated with protection from disease and is an important mediator of peripheral tolerance to self- antigens [17-21].

#### 1.1.2.2.3 Activation- Induced Cell Death (AICD)

Deletion of autoreactive T cells not only occurs during negative selection in the thymus, but also can occur in the periphery. In peripheral T cells, those responding to self- antigens can be eliminated through activation- induced cell death (AICD), which is a form of apoptosis induced by repeated TCR stimulation [1, 21]. AICD results from the interaction between Fas and Fas ligand (FasL) on activated T cells. Ultimately, the ligation of Fas with FasL induces a signaling cascade that initiates apoptosis, thereby eliminating self- reactive T cells [21, 22]. However, defects in the AICD pathway exist and contribute to autoimmunity, especially in T1D. Interestingly, activated T cells from the non- obese diabetic (NOD) mouse model of T1D are more resistant to AICD than T cells from non- autoimmune prone control strains. Analysis of T cells from T1D patients and at-risk populations are highly defective in their surface expression of Fas, and are more resistant to cell death [23]. Cumulatively, these data indicate that dysregulated apoptosis pathways may be involved in the pathogenesis of T1D, and highlight the importance of peripheral tolerance mechanisms in maintaining quiescence of autoreactive T cells.

#### 1.1.2.2.4 Signaling via Immune Inhibitory Receptors act as a "brake" for T cell activation

Immune inhibitory receptors (IRs), also termed checkpoint molecules, are important regulators of immunity, especially in the context of mediating protection against the onset of autoimmunity [24-28]. While the interaction of co- stimulatory molecules (i.e. CD28:CD80/CD86) are important for directing T cell activation, differentiation, and expansion; the opposite is true for co- inhibitory receptors, which negatively regulate TCR driven signals and T cell activation [21]. A number of IRs have been identified and include cytotoxic T-lymphocyte- associated protein 4 (CTLA-4), programmed cell death protein- 1 (PD-1) , lymphocyte Activation Gene-3 (LAG-3), and T cell immunoglobulin and mucin- domain containing- 3 (TIM-3), and their expression is most often associated with a type of hyporesponsive phenotype termed T cell exhaustion during cancer and chronic infection [24-26]. However, the role of IRs goes beyond mediating T cell exhaustion, as these signaling molecules play a crucial role in regulation of T cell activation as well as Treg suppressive function. During activation, TCR signaling upregulates IR expression, where signaling with their associated ligands controls and contracts expanded T cell populations as a means to balance the immune response (Figure 2) [24-29]. This regulation is crucial to limiting autoimmunity and detrimental immunopathology. Evidence of this is in T1D specifically has been supported by studies where animals deficient in CTLA-4, PD-1 or LAG-3, or mice treated with antibody blockade demonstrate accelerated disease kinetics, further underscoring the importance of IRs in maintaining quiescence of autoreactive T cells [30-32].

Although the expression of IRs and specifically T cell exhaustion is typically associated with detrimental outcomes in cancer and chronic infection, the opposite is true in autoimmunity. In one study by McKinney et al., it was demonstrated that levels of T cell exhaustion in patients across a variety of autoimmune diseases strongly correlated with their clinical outcomes, with a T cell

exhaustion signature predicting favorable clinical responses [29]. Therefore, based on these correlations, promoting T cell exhaustion could be a beneficial phenotype for inhibiting autoimmunity, especially in those individuals with defects in IR expression and signaling. These concepts will be discussed in more detail below in a later section of this introduction.

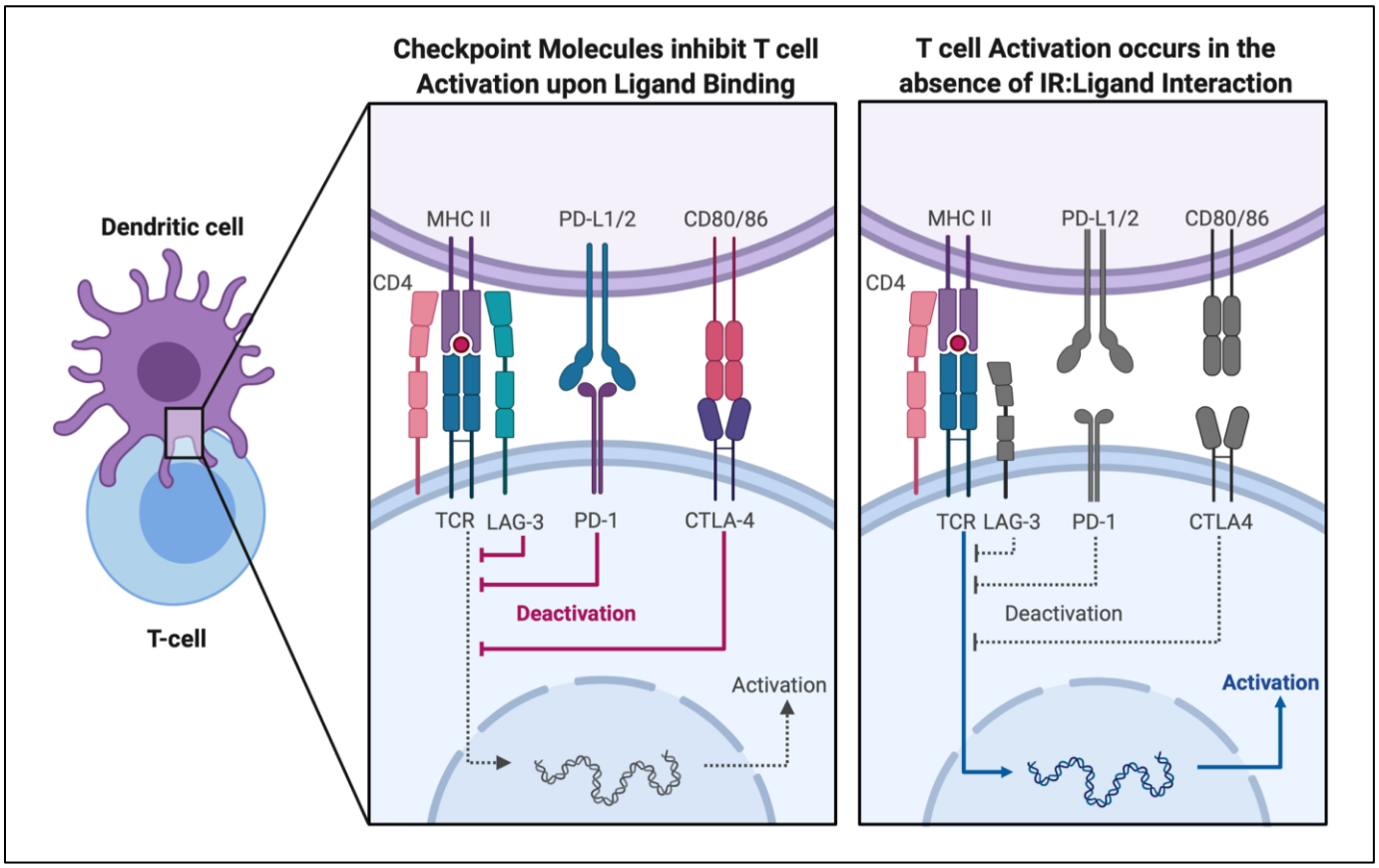


**Figure 2. Mechanisms of Immune Inhibitory Receptor (IR) Signaling**
Signaling of IRs such as CTLA-4, LAG-3, and PD-1, with their associated ligands are important in mediating tolerance to self- antigens in the periphery, and also play a role in tempering T cell responses to foreign antigens as a means to protect against aberrant immunopathology. During T cell activation, CTLA-4, LAG-3, and PD-1 expressed by T cells can interact and bind to their ligands CD80/CD86, MHC-II, and PD-L1/PD-L2, respectively. If ligand binding occurs, downstream signaling leads to a stop signal that inhibits T cell activation, proliferation, and cytokine production (left panel). In contrast, absence of IR:ligand binding results in fulminant T cell activation and associated effector functions (right panel). Diagram created using BioRender.com.

## 1.2 AUTOIMMUNE RESPONSES RESEMBLE NORMAL IMMUNE RESPONSES TO PATHOGENS

Despite the checkpoints in place to prevent the activation of autoreactive T cells, a breakdown in one or more self- tolerance safeguards leads to the activation of autoreactive T cells that mediate the destruction of host tissues and the onset of autoimmune diseases, like Type 1 Diabetes (T1D) [33-35]. Notably, the activation of autoreactive T cells is no different than T cell activation to foreign pathogens. Unfortunately, the inability to effectively delete autoreactive T cells from the repertoire and control their activation in the periphery leads to autoimmunity, where self- reactive T cells mistakenly recognize host tissues as foreign. These concepts will be explored in more detail in the following sub- sections, with a specific focus on the pathogenesis of T1D, and the resulting attack of insulin- secreting β cells that occurs in affected patients.

### 1.2.1 Immunopathology of Type 1 Diabetes

Invasion of the pancreatic islets by immune cells (insulitis) is a hallmark of T1D pathogenesis that leads to progressive destruction of the β cell and precedes disease onset. This along with a break in tolerance to β cell antigens lead to the activation and clonal expansion of autoreactive $CD4^+$ T cells that drive T1D. While the etiology of T1D is not completely understood, we do know that in those with a genetic predisposition to autoimmunity, a triggering event (i.e. chemical exposure, dysglycemia, endoplasmic reticulum (ER) stress, reactive oxygen species (ROS), or viral infection) affecting β cells in the pancreas leads to release of β cell antigens that are phagocytosed by antigen presenting cells (APCs), like macrophages and dendritic cells (DCs), and leads to their subsequent activation [36-41]. Activated APCs traffic to the pancreatic draining lymph node where

they present β cell self-antigens to autoreactive $CD4^+$ T cells that become activated and travel back to the pancreas as effector $CD4^+$ T cells. Effector $CD4^+$ T cells coordinate the resulting autoimmune attack of the β cell via secretion of proinflammatory cytokines like IFNγ and tumor necrosis factor-α (TNFα) that can directly cause β cell death, provide help to activate $CD8^+$ T cells and B cells, and provide an inflammatory milieu that favors continual APC activation [36, 39]. Ultimately, this perpetuates a cycle of autoimmune attack that when left unhindered, leads to the organ- specific autoimmunity and β cell destruction that culminates in hyperglycemia and T1D onset (Figure 3) [36, 39]. Orchestration of autoimmune responses by APCs, T cells, and B cells together leads to β cell death and hyperglycemia. The diversity of immune cells involved in autoimmune attack of the β cell provide a variety of potential therapeutic targets to control aberrant responses in T1D. Further, finding ways to alter the differentiation of immune cell subsets to more anti-inflammatory phenotypes or impeding activation of autoreactive T cells will allow for the design of novel therapies to prevent, delay, or reverse T1D.

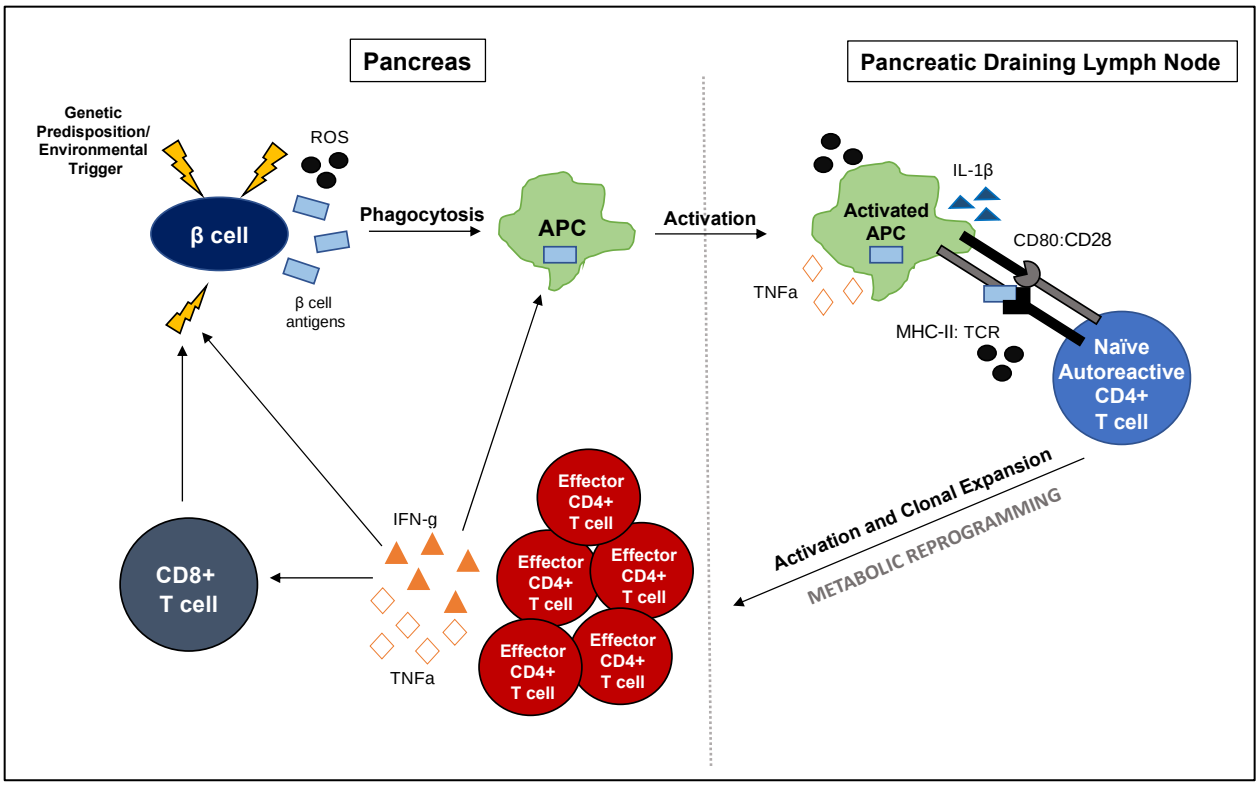


**Figure 3. Immune- mediated β cell killing leads to onset of T1D.**
In genetically predisposed individuals, an initial threat targeting the β cell triggers the release of β cell antigens in the pancreas. These autoantigens are phagocytosed and processed by islet- resident macrophages and DC that become activated and traffic to the pancreatic draining lymph node. It is here that naïve autoreactive $CD4^+$ T cells specific for islet antigens become educated, leading to their subsequent activation and clonal expansion, supported in large part by dramatic changes in cellular metabolism. Once activated, effector $CD4^+$ T cells travel back to the target organ where they are instrumental in orchestrating autoimmune attack of the β cell that leads to β cell death and hyperglycemia.

### 1.2.2 $CD4^+$ T cells Drive T1D Pathogenesis

While a number of immune cells are involved in autoimmune destruction of the β cell, autoreactive $CD4^+$ T cells are largely responsible for mediating and driving disease pathogenesis [33, 35-37, 39-47]. This is also true for other autoimmune diseases like SLE, MS, and RA. $CD4^+$ T cells are fundamental and required for T1D development, as studies in the non- obese diabetic (NOD) mouse have shown that depletion of $CD4^+$ T cells or treatment with non- depleting anti- CD4 antibodies prevents disease onset [42, 48-50]. Further, genetic predisposition to autoimmunity and T1D disease development has been linked to human leukocyte antigen (HLA) class II alleles DR4, DQ8, and DQ2, which confer the highest genetic risk of T1D in human patients. This suggests that HLA class II restricted $CD4^+$ T cells play a key role in T1D [34, 42, 51]. While $CD8^+$ T cell mediated β cell killing is a major mechanism of disease development, $CD4^+$ T cells lead the way in orchestrating the attack against islet β cells [34, 40, 42, 43]. While their role includes providing help to activate $CD8^+$ T cells, B cells, and antigen presenting cells, activation of $CD4^+$ T cells and their subsequent secretion of proinflammatory cytokines, especially IFNγ,TNFα, and interleukin-1β (IL-1β), can directly induce β cell apoptosis [33, 34, 36, 40, 42, 43]. Without intervention, this results in a cycle of β cell killing, resulting in dysregulation of dynamic glucose sensing and subsequent insulin secretion [36]. Therefore, understanding the mechanisms governing $CD4^+$ T cell effector function, and using this knowledge to find therapeutic targets to inhibit autoreactive T cell activation are of the utmost importance, as they are critical for disease prevention and future therapies.

The ability of autoreactive T cell clones to escape deletion during development in the thymus leads to autoimmunity [40, 41, 45, 47, 52]. However, a failure in peripheral tolerance mechanisms to control these rogue T cells also plays a significant role. Tregs (simply defined as $CD4^+$ $CD25^+$

Forkhead box $P3^+$ (FoxP3; master regulator of Treg development and function)), are members of the CD4 compartment and are widely regarded as the primary mediators of peripheral tolerance [35, 41, 47]. However, in T1D and other autoimmune disorders, Tregs are known to be functionally defective. In T1D specifically, Tregs have an altered phenotype, most notably in terms of their stability, survival, and functionality [10, 12, 16, 40-42, 45, 47, 52]. While there appears to be no change in levels of circulating Tregs in mice or human patients with T1D, previous studies have shown a decline in intra- islet Treg frequencies that correlated with disease development in the NOD mouse [10, 12]. Moreover, a number of investigations have shown that $FoxP3^+$ Treg function and suppressive capacity is defective in those with T1D, as Tregs from affected individuals were less able to control the proliferation of autologous T effectors when compared to healthy controls [10, 12, 16, 41] Further, Tregs in autoimmunity suffer from lineage instability, as emerging data suggests that functional Tregs can either convert to effector T cells or become Th1- like Treg cells associated with reduced or loss of FoxP3 expression and suppressive function, expression of the Th1 specific transcription factor T-box protein expressed in T cells (T-bet), and enhanced proinflammatory cytokine secretion, especially IFNγ [11, 53]. Further insight is needed to discern the causative reason behind impaired Treg stability and function in T1D, as reinvigorating Treg function may promote tolerance in those predisposed to autoimmunity.

## 1.3 FUELING IMMUNITY: METABOLIC PROGRAMS DICTATE IMMUNE CELL FUNCTION

### 1.3.1 Cellular Metabolism Governs $CD4^+$ T cell Function

Cellular metabolism dictates T cell fate and function. Consequently, cellular metabolism has the

ability to influence the final outcome of the innate and adaptive immune responses, further impacting health and disease outcome [54]. T cells undergo substantial metabolic reprogramming from oxidative phosphorylation (OXPHOS) to aerobic glycolysis during activation upon encounter with cognate antigen [54-62]. This section will briefly review the major metabolic pathways utilized by T cells during activation and quiescence, however this topic has been reviewed in more detail in reports cited here [58-61].

As T cells move through the various stages of their life cycle, their metabolic demands vary. Naïve $CD4^+$ T cells are metabolically quiescent, as they utilize OXPHOS via the mitochondria to support basic cellular processes, maintain homeostasis, and to circulate the periphery surveying the body for foreign invaders [58, 59, 62]. In fact, 96% of the adenosine triphosphate (ATP) generated by naïve T cells comes from oxidative metabolism, with roughly 4% coming from glycolytic metabolism [61]. Upon recognition of foreign antigen, along with the necessary costimulatory signals, naïve T cells become activated and undergo metabolic reprogramming to support this change in function. During activation, T cells switch to a program of anabolic growth and biomass accumulation to generate daughter cells, requiring an increased demand for ATP and metabolic resources [54, 58-62]. This developmental program is characterized by an increase in rapid growth, proliferation, and acquisition of specialized effector functions [57-60, 62]. Activated T cells are more metabolically active, and engage primarily in aerobic glycolysis, marked by the conversion of glucose to lactate despite the presence or availability of oxygen [59, 60]. This phenomenon, known as the Warburg Effect, was characterized by Dr. Otto Warburg in the 1920's, and is a hallmark of cancer metabolism [63, 64]. Although far less efficient than OXPHOS in terms of energy production, aerobic glycolysis allows for proliferating T cells to generate ATP quickly, and also allows for the generation of metabolic intermediates important for clonal expansion and

effector capabilities [54, 59]. After clearance of the initial threat, most effector T cells die, however a small population of long- lived antigen- specific memory T cells remain that mediate protection against re-challenge [65]. Memory T cells, much like naïve T cells, are quiescent and rely primarily on OXPHOS and fatty acid oxidation (FAO) for their development and long-term survival [58-60] (Figure 4).

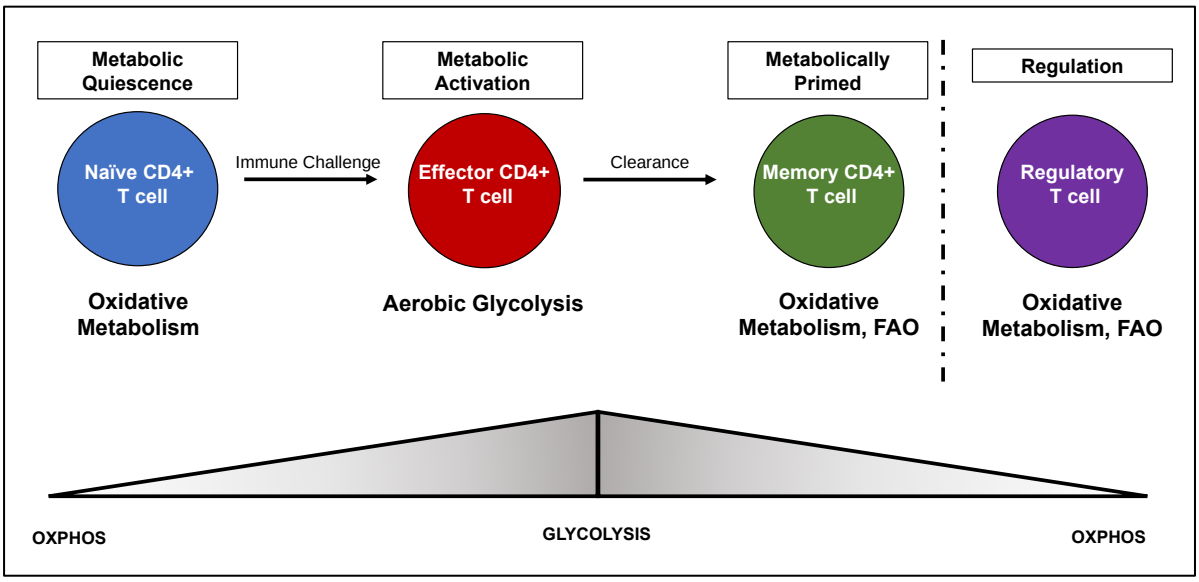


**Figure 4. Distinct metabolic profiles dictate T cell fate and function.**
The metabolic needs of $CD4^+$ T cells drastically change over the course of an immune response. Consequently, immunometabolism has the ability to dictate whether a pro- or anti- inflammatory response will ensue. While there is some layover in the metabolic programs of different T cell subsets, clear distinctions exist. Naïve T cells are quiescent and utilize OXPHOS to generate ATP to support antigenic surveillance. Upon encounter with antigen, naïve T cells become activated and undergo a metabolic to utilize aerobic glycolysis, which is vital for T cell proliferation and acquisition of effector functions. After clearance, a small subset of T cells become memory T cells and revert back to a program of FAO and OXPHOS, which promotes longevity and survival. Like memory T cells, Tregs too are thought to utilize FAO. This pathway has been implicated in supporting Treg stability and suppressive capabilities.

Tregs too have their own metabolic preferences that dictate their function and stability, however there have been conflicting reports in both mouse and human studies, as it appears Tregs utilize a number of metabolic programs depending on their environment and specific needs [11, 57-61, 66-71]. Overall, Tregs are thought to preferentially rely on oxidative metabolism [57, 67] (Figure 4). This idea was supported in a paper by Angelin et al., where it was determined that FoxP3 induced OXPHOS and shut down glycolysis via binding to the myelocytomatosis oncogene (Myc) (transcription factor that drives glycolytic metabolism and metabolic reprogramming) gene promoter and suppressing Myc gene expression [72]. More specifically, Tregs rely on FAO, which feeds into the mitochondria to supply OXPHOS, and readily take up externally derived fatty acids to support high rates of FAO. Interestingly, treatment with etomoxir, an inhibitor of the enzyme Carnitine palmitoyltransferase Iα (CPT1α), which converts acyl- CoAs to acylcarnitines allowing for entry of fatty acids into the mitochondria and FAO, abrogates Treg development and suppressive function suggesting that Tregs depend on the FAO pathway to differentiate and perform their effector functions [12, 57, 58, 69, 70]. On the other hand, Tanimine et al. and others have indicated that glycolysis controls the induction of functional FoxP3 during early activation and proliferation in Tregs isolated from human subjects [73]. Another study of human Tregs found that the glycolytic enzyme enolase-1 was required for Treg suppressive capacity via control of FoxP3 splice variants [74]. While the exact metabolic preferences of Tregs remain elusive, it's clear that these cells display a mixed metabolism depending on nutrient availability and environmental cues. Further studies are desperately needed to fully understand the metabolic requirements of Tregs in order to have a better understanding of mechanisms driving Treg differentiation, stability, and functional capacity. All in all, while each phase of the T cell life cycle has distinct metabolic requirements, each program allows for T cells to function and respond to

various situations effectively during their life span. Moreover, as cellular metabolism is responsible for shaping immune responses, it's clear that changes in immunometabolism drive autoimmunity, specifically T1D pathogenesis (Figure 5).

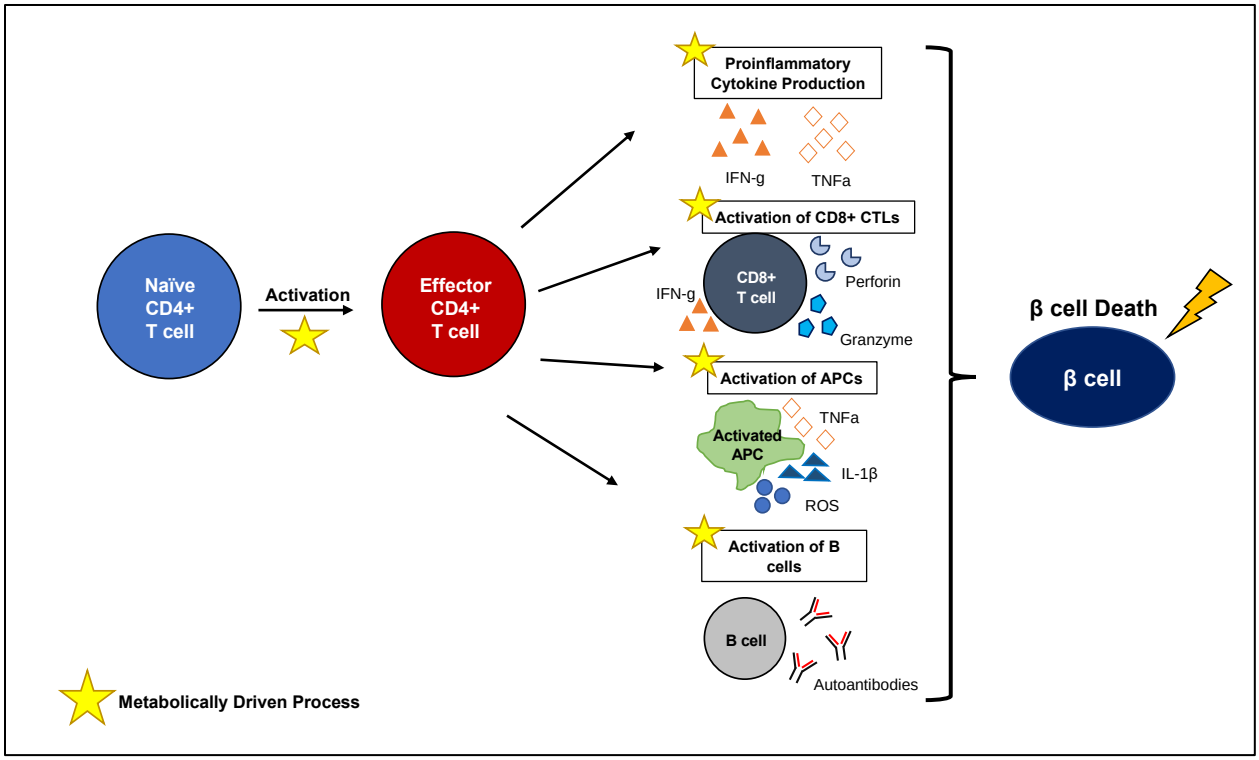


**Figure 5. Changes in Immunometabolism drive β cell death in T1D.**
T1D pathogenesis is largely mediated by the activation of autoreactive $CD4^+$ T cells that is fueled by metabolic reprogramming. Activation of $CD4^+$ T cells is a metabolically demanding process accompanied by a metabolic switch to aerobic glycolysis, that although less efficient, is required for T cell clonal expansion and effector capabilities. Like $CD4^+$ T cells, other immune cells undergo metabolic reprogramming to support immune function. $CD4^+$ T cells are critical mediators of orchestrating β cell destruction, where proinflammatory cytokine production can lead to direct β cell death and support the downstream activation of other immune cells like $CD8^+$ CTLs, APCs, and B cells. The importance of metabolic reprogramming and immune cell activation therefore provide an opportunity to modulate immune cell activation and differentiation by exploiting specific bioenergetic programs to limit autoimmunity.

### 1.3.2 Immunometabolism of macrophages and dendritic cells

Professional APCs, including macrophages and dendritic cells (DCs), are important in mediating innate and adaptive immune responses to both self and foreign antigens. As is the case with all immune cells, a number of metabolic transitions occur over the course of the APC lifecycle, as reviewed in more detail in chapter 4 and in the following reviews [59, 75]. Similarly to the immunometabolism of $CD4^+$ T cell lineages, metabolic differences exist between pro- and anti-inflammatory subsets of APCs [75-78]. Proinflammatory DCs and classically activated M1 macrophages switch from an oxidative metabolic profile to aerobic glycolysis upon activation, where glucose is preferentially fermented to lactate despite the availability of oxygen [59, 75, 76, 78]. In contrast, tolerogenic DCs and alternatively activated M2 macrophages involved in wound healing and the resolution of inflammation rely on oxidative phosphorylation and the breakdown of fatty acids via β- oxidation [59, 75, 76, 78]. As T1D is associated with an aberrant proinflammatory phenotype of autoreactive T cells and APCs, this section will focus on this subset of APC metabolism.

Reliance on aerobic glycolysis to fuel the energy needs of proinflammatory APCs have been demonstrated in a number of studies [59, 75, 76, 78]. Specifically, use of inhibitors of the glycolysis pathway, especially 2-DG, have demonstrated an ability to reduce APC activation, upregulation of costimulatory molecules required for antigen presentation, and effector cytokine production (i.e., TNFα, IL-1β, IL-6) [59, 75, 76, 78]. There is also evidence to support the idea that APCs are metabolically flexible, and can rely on other metabolic substrates to fuel energy needs during times of nutrient restriction [79, 80]. Due to their importance in the pathogenesis of T1D, further exploration of the metabolic programs used by islet infiltrating macrophages and DCs

warrant investigation to allow for the generation of combinatorial therapies that modulate both APC and T cell functions to control autoimmunity.

## 1.4 METABOLIC DYSREGULATION AS A CONSEQUENCE AND TARGET IN AUTOIMMUNITY

Studies investigating the mechanisms that dictate T cell activation and function have shed light on the importance of cellular metabolism in guiding these processes. While there is some layover in terms of the pathways that are vital for immune cell function, metabolic signatures exist between T cell subsets and are important in controlling T cell differentiation [56-60]. Further, different metabolic processes can influence the final outcome of an immune response and can govern whether a pro- or anti- inflammatory response will ensue. In the case of autoimmunity, recent work has highlighted that metabolic differences exist between immune cells in healthy individuals versus in patients with autoimmune disorders [55, 81, 82]. Due to the importance of metabolism in controlling cell fate and function, as well as the importance of $CD4^+$ T cells that coordinate autoimmune attack of the β cell in T1D, understanding metabolic signatures of autoreactive $CD4^+$ T cell subsets in autoimmunity are vital to providing new therapeutic targets to control these responses. The following section will review immunometabolism as a therapeutic target to control autoreactive T cell responses in SLE, MS, and RA. This will afford insight on the metabolic requirements of autoreactive T cells and provide evidence to support metabolic regulation as the future of immunotherapies in autoimmunity, especially in T1D.

### 1.4.1 Systemic Lupus Erythematosus

Studies focused specifically on the role of immunometabolism in autoimmunity have indicated that autoreactive T cells display altered metabolic dependencies and programs [55, 81, 82]. Interestingly, self- reactive T cells from different autoimmune diseases studied thus far display distinct metabolic programs with little overlap, further indicating a role for the specific immune microenvironment in driving T cell differentiation and effector function. Due to the importance of effector T cells in orchestrating attack of the various tissues targeted in autoimmunity, inhibiting the activation of these $CD4^+$ T cells is an ideal therapeutic target [61, 71, 83]. Generally speaking, autoreactive T cells are thought to have enhanced bioenergetics compared to healthy controls, although the metabolic demands of autoreactive T cells in T1D specifically remain largely unexplored. This was demonstrated in a lupus model by Yin et al., where it was determined that both glycolysis and mitochondrial oxidative metabolism, as well as mammalian target of rapamycin complex 1 (mTORC1) activity, are elevated in $CD4^+$ T cells from lupus prone mice compared to non-autoimmune control animals [84]. The authors concluded that this metabolic dysregulation was a major contributor to the immunological abnormalities associated with lupus pathogenesis including T cell hyperactivation and increased IFNγ production [84]. The enhanced cellular metabolism of $CD4^+$ T cells from lupus prone animals preceded onset of disease and increased as T cells became more activated throughout disease progression, indicating that metabolic differences may be able to be specifically manipulated to prevent onset of autoimmunity prior to $CD4^+$ T cell hyperactivation [84]. In an effort to normalize autoreactive $CD4^+$ T cell functions *in vitro* and *in vivo*, the authors utilized a combination treatment of the glycolysis inhibitor 2-DG and metformin, an inhibitor of mitochondrial metabolism (specifically Complex 1 of the electron transport chain (ETC). *In vitro*, this treatment combination inhibited excessive IFNγ

production and promoted interleukin-2 (IL-2) production which is otherwise defective during lupus [84]. *In vivo*, targeting glucose and mitochondrial metabolism reversed disease and simultaneously led to significant decreases in glycolysis and OXPHOS in autoreactive T cells to levels similar to that of non- autoimmune prone controls, decreased the expression of activation and effector function markers, decreased autoantibody production, and improved production of IL-2 by autoreactive $CD4^+$ T cells; ultimately leading to significant reductions in disease pathology [84]. This enhanced bioenergetic profile of lupus- prone $CD4^+$ T cells was also seen in T cells from human SLE patients, and could be normalized via treatment with metformin [85]. While treatment with either 2-DG or metformin alone were sufficient to prevent disease, combination therapy was required for disease reversal. Moreover, a continuous treatment was required to maintain tolerance, as cessation of treatment lead to disease flare- ups [85].

Recent work by Choi et al. confirmed that $CD4^+$ T cells from lupus prone animals were glycolytic, and found that Inhibiting glucose metabolism selectively targeted autoreactive follicular helper T cells (Tfh), which are expanded in SLE and are required for production of high affinity autoantibodies [86]. This treatment was capable of preventing onset of disease in various mouse models of SLE. Use of CG-5, a glucose transporter inhibitor, was also capable of ameliorating autoimmune activation in lupus [87]. Tfh cells from lupus prone animals displayed increased mTORC1 activation compared to B6 controls, consistent with the findings by Yin et al., and had a pre- activation status indicated by higher mTORC1 activation in naïve CD44- negative $CD4^+$ T cells in lupus- prone animals [84-86]. Moreover, $CD4^+$ T cells from lupus animals displayed increased expression of the pro- survival factor B-cell lymphoma 2 (Bcl2), indicating a resistance of autoreactive T cells to apoptosis. Preventative treatment (animals were autoantibody positive without clinical disease) with 2-DG reduced autoantibody production as well as the

frequency and number of Tfh and germinal center B cells and reduced the number of total splenic $CD4^+$ T cells in four lupus- prone mouse strains, but not in control B6 mice [86]. Interestingly, treatment with 2-DG had variable effects on $CD4^+$ T cell activation, as activation and $CD44^+CD4^+$ T cell frequency were only decreased in a few of the lupus models tested, indicating that spontaneous lupus Tfh cells are uniformly sensitive to glycolysis inhibition compared to other T cell subsets that showed a variable response depending on strain. Finally, while proliferation and signal transducer and activator of transcription 3 (STAT3) signaling were similar between lupus-prone and B6 Tfh cells, these were increased in naïve TC $CD4^+$ T cells in which glycolysis is also higher than in naïve B6 $CD4^+$ T cells. This finding is interesting, in that it indicates that autoreactive $CD4^+$ T cells display heightened bioenergetics that may contribute to the ability for autoreactive T cells to escape deletion and persist in the periphery. This heightened activation status of naïve $CD4^+$ T cells in autoimmune prone animals also gives precedence for selectively targeting $CD4^+$ T cell metabolism as a means to control autoimmunity [84-86].

As was demonstrated in mouse models of SLE, $CD4^+$ T cells from human patients also exhibit elevated cellular metabolism compared to $CD4^+$ T cells from healthy control patients [84]. Enhanced bioenergetics correlated with increased production of IFNγ that could be normalized *in vitro* via treatment with Metformin [84]. These data corroborate earlier studies that demonstrated lupus T cells from human patients have increased mTOR activity, mitochondrial dysfunction, increased oxidative stress, and glutathione depletion [88-93]. Aberrant oxidative stress and ROS in SLE T cells was attributed to increased oxygen consumption and mitochondrial ETC complex I activity [89]. As ROS are important mediators in rewiring $CD4^+$ T cell metabolism during activation, the phenotype exhibited by SLE T cells is indicative of enhanced bioenergetics, aberrant T cell activation, and overall T cell dysfunction that contributes to SLE immunopathology

[91]. Further, higher mTOR activity leads to expansion of pathogenic Th17 cells and reduction of functional Tregs, further contributing to autoimmunity [90]. Interestingly, treatment with rapamycin or the antioxidant N-acetylcysteine (NAC) were capable of reducing SLE disease activity by decreasing mTOR activity in effector T cells, expanding Tregs, and enhancing Treg suppressive functions [88-90, 92-94]. All in all, these studies in murine and human models of SLE disease indicate metabolic aberrations exist in the $CD4^+$ T cell compartment compared to healthy counterparts, and that targeting metabolic pathways can reverse T cell dysfunction to correct known defects that contribute to SLE pathogenesis.

### 1.4.2 Multiple Sclerosis

The metabolic profile of $CD4^+$ T cells from patients with multiple sclerosis (MS) also display altered bioenergetics compared to those of healthy patients, however the literature has had conflicting reports. As is the case in other autoimmune disorders, Th17 cells contribute to autoimmune disease pathogenesis and progression, where a metabolic switch to glycolysis accompanies T cell activation and drives effector functions [57, 59, 60, 95]. This finding that Th17s relied on glycolysis was confirmed in a study by Shi et al., where it was demonstrated that Th17 differentiation was dependent on hypoxia- inducible factor 1α (HIF1α) dependent up-regulation of glycolytic activity, as $CD4^+$ T cells deficient in HIF1α had diminished Th17 differentiation due to a failure to upregulate the glycolytic enzymes required for metabolic reprogramming during activation [71]. Further, blocking glycolysis via 2-DG or the mammalian target of rapamycin (mTOR) inhibitor Rapamycin reduced Th17 differentiation of naïve T cells *in vitro* and reduced the ability of Th17 to cause experimental autoimmune encephalomyelitis (EAE), a murine model of MS, *in vivo* [71]. This was associated with reduced lymphocyte infiltration and

spinal cord inflammation [71]. Other work in EAE has demonstrated the importance of glutamine in driving autoreactive $CD4^+$ T cell activation, differentiation, and effector functions, and is dependent on the amino acid glutamine transporter alanine-serine-cysteine transporter 2 (ASCT2) [96]. Indeed, genetic knockout of ASCT2 resulted in reduced Th1 and Th17 differentiation, cytokine production, reduced leukocyte infiltration into the central nervous system, and reduced EAE disease severity compared to wild type (WT) controls [96]. Interestingly, the importance of glutamine in mouse models of MS was also true in patients, where increased concentrations of glutamine and glutamate has been found in the cerebrospinal fluid and brain biopsies from affected individuals. Increases in these metabolites were also associated with disease severity and have been suggested to be potential biomarkers for MS [97, 98].

In human patients, altered energy metabolism has been a common finding, however the exact metabolic phenotype of autoreactive $CD4^+$ T cells remain elusive. Studies analyzing the gene-expression profile in brains from post- mortem MS subjects have showed evidence of an upregulation of genes involved in cellular metabolism like HIF1$\alpha$ and the protein kinase B (AKT) signaling pathways, which are involved in upregulating glycolysis [99]. Further, MS patients have significant changes in serum levels of different metabolites, including an increase in serum nicotinamide adenine dinucleotide (NADH) compared to healthy patients. Interestingly, increased concentrations of glutamine and glutamate has been found in the cerebrospinal fluid and brain biopsies from affected individuals. Increases in these metabolites were also associated with disease severity and have been suggested to be potential biomarkers for MS [97-99]. In MS lesions, mitochondrial changes have also been reported, including a loss of complex I and IV activity in the mitochondrial ETC, implicating a role for mitochondrial damage and oxidative stress in MS disease pathogenesis [100-105]. While a number of metabolic differences exist between MS

patients and healthy controls based on what has been reported in the current literature, these studies have not focused on the metabolic characteristics of $CD4^+$ T, which similar to T1D and lupus, are the main effectors in driving autoimmunity in MS. In 2015, De Riccardis et al. set out to determine the metabolic phenotype of immune cells in the MS autoimmune response using $CD4^+$ T cells isolated from peripheral blood mononuclear cells (PBMCs) of relapsing remitting MS patients [101]. They found that $CD4^+$ T cells from these patients had impaired OXPHOS activity and decreased activity of mitochondrial complex 1 and IV compared to healthy subjects, confirming the findings from earlier studies [101, 102]. When analyzing glucose metabolism in $CD4^+$ T cells from these patients, MS $CD4^+$ T cells displayed increased glycolytic flux as measured by increased activities of two major rate limiting enzymes in the glycolysis pathway, hexokinase and phosphofructokinase [101]. During activation, $CD4^+$ T cells switch to a program dominated by aerobic glycolysis, which is the conversion of glucose to lactate despite the presence or availability of oxygen. Because of this phenomenon, lactate secretion is a useful output to measure reliance on glycolytic metabolism in T cells. As expected, extracellular lactate release was enhanced in T cells from MS patients compared to control subjects [101]. This, along with the findings that $CD4^+$ T cells from MS patients had reduced ratios of ATP from mitochondrial metabolism over glycolytic ATP, as well as increased glucose transporter 1 (Glut1) expression (the most important glucose transporter for T cells) compared to healthy controls indicates an increased reliance on glycolysis by autoreactive T cells in MS [101]. Interestingly, the enhanced reliance on glycolysis by circulating $CD4^+$ T cells from MS patients supplies further evidence that autoreactive T cells have enhanced bioenergetics compared to T cells from healthy controls. However, there are limitations to this study, which included total $CD4^+$ T cells. It would be interesting to note whether the enhanced glycolytic metabolic phenotype described was due to an increase in Teffectors in the

periphery in those with MS compared to healthy individuals, or whether metabolism was truly dysregulated in naïve, effector, and memory T cell populations between MS patients and healthy subjects, as performed in work described earlier in lupus [86, 101].

Contrary to the findings by De Riccardis et al., another study found that T cells from MS patients were in fact less glycolytic and had impaired engagement of mitochondrial respiration than their healthy counterparts, as indicated by a lower extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) measured by Seahorse Extracellular Flux analysis [106]. These measurements are indicators of glycolysis and OXPHOS, respectively. This metabolic defect was confirmed by measuring the expression of key enzymes involved in both glycolysis and mitochondrial respiration. Reduced protein levels of key enzymes such as Glut1 and hexokinase, correlated with the seahorse analysis performed, further confirming a metabolic defect in $CD4^+$ T cells from MS patients [106]. This finding is in conflict with the enhanced bioenergetic profile of T cells observed in MS patients from the study outlined above, and could be due to differences in the patient cohort, as the T cells in the De Riccardis study were from patients that had been treated in the past, albeit these patients had not been treated for at least 3 months [101]. On the contrary, samples from the patients in the La Rocca study were isolated from affected individuals who had not underwent treatment for their condition. This difference in treatment regimens between the two studies could account for these metabolic differences, as immunomodulatory agents have been shown to modulate cellular metabolism [106]. Moreover, the methods used to assess metabolism in both studies were vastly different, as the De Riccardis study relied on the evaluation of enzymatic activity and the La Rocca study utilized an approach that took the whole cell into consideration using a functional analysis like Seahorse extracellular flux. Regardless, the impaired metabolic profile of MS $CD4^+$ T cells in the La Rocca study is an interesting finding, as glycolysis

is required not only for T cell differentiation but also for acquisition of specialized effector functions, like IFNγ secretion [107]. Further, this is different than the metabolic phenotype of autoreactive T cells from other autoimmune diseases in that instead of having enhanced bioenergetics, T cells from these patients had reduced glycolysis and OXPHOS. How these T cells are still able to become activated and perpetuate disease under a reduced bioenergetic state warrants investigation so as to obtain a full picture of the metabolic choices T cells have at their disposal. Further, the activation status of these T cells would also be important to investigate. It has been reported in the cancer literature and other autoimmune disorders that a chronic exposure to antigen and metabolic competition in the tumor microenvironment (TME) can lead to T cell exhaustion or anergy [108]. T cell exhaustion in particular is associated with increased expression of checkpoint molecules like programmed cell death protein 1 (PD-1) and lymphocyte activation gene-3 (LAG-3), which are capable of reprograming T cell metabolism themselves, and will be discussed in more detail below [109, 110]. Further characterization of the expression of key surface proteins and delineation of the molecules that regulate metabolism and cell fate decisions should be further explored, in addition to understanding the activation status. Finally, it is vital to our understanding of autoimmune disease pathogenesis to study metabolic profiles of T cells over the course of autoimmune destruction. These studies are important to better understand the metabolic dependencies of autoreactive T cells over the course of disease pathogenesis and will aid in the discovery of novel biomarkers and pathways that can be solicited to specifically target these pathogenic T cells therapeutically to prevent or reverse disease in affected individuals.

### 1.4.3 Rheumatoid Arthritis

In RA, altered immune tolerance and metabolic defects contribute to chronic synovial inflammation and joint destruction, mediated in large part by $CD4^+$ T cells [111, 112]. The first evidence that altered bioenergetics played a role in RA pathogenesis was found in early studies analyzing serum samples from RA patients, where aldolase A and α-enolase, two enzymes of the glycolytic pathway, were identified as autoantigens [55, 113, 114]. Similarly to the results described above, $CD4^+$ T cells from a spontaneous mouse model of RA were more metabolically active compared to controls. Further, glycolysis inhibition with 2-DG significantly reduced joint inflammation and activation of adaptive and innate immune cells, as well as autoantibody production [115].

Contrary to the findings in animal studies, naïve $CD4^+$ T cells isolated from the PBMCs of RA patients were found to be energy deprived and were unable to fully engage aerobic glycolysis [111]. This was indicated by reduced glucose utilization, lactate production, and generation of intracellular ATP 72 hours post *in vitro* stimulation, and correlated with increased apoptosis, which was 50% higher in RA T cells compared to controls [111]. To understand whether RA T cells were anergic thus leading to reduced glucose utilization, parameters of T cell responsiveness were monitored post stimulation. Interestingly, RA T cells responded as vigorously as their control counterparts, proliferated faster, and were able to produce IL-2 to the same extent as control T cells, indicating that naïve RA T cells were able to respond to environmental stimuli and undergo activation at a rate comparable or better than control T cells, however metabolically speaking RA T cells displayed abnormalities in the utilization of glucose, ATP production, and were more susceptible to undergo cell death in response to stimulation [111]. This metabolic phenotype was

found to be due to a defect in up regulating the rate limiting enzyme 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3), a key regulator of glycolytic flux [111].

The immune microenvironment plays a significant role in T cell differentiation and function, as evidenced by studies researching the impact the TME has on tumor- infiltrating lymphocyte (TIL) function. In order to determine whether the failure to induce PFKFB3 expression in RA T cells was a consequence of the inflammation associated with RA disease progression, PFKFB3 transcript levels in $CD4^+$ T cells were correlated with a disease activity score, indicating disease severity. Contrary to what you would expect, patients with lower disease activity had no advantage over patients with more severe disease [111]. Further, a failure to induce PFKFB3 expression did correlate with diagnosis of RA, but not with the inflammatory milieu, indicating that reduced PFKFB3 expression by RA T cells is a defect in all RA patients regardless of disease clinical score or severity [111]. Nonetheless, it's important to note that autoimmunity precedes the onset of clinical symptoms, and that exposure of $CD4^+$ T cells to chronic stimulation in an inflammatory environment likely plays a key role in altering immune function and lymphocyte stability in the local micromilieu. For example, in the TME, a competition for nutrients, especially glucose between tumor cells and tumor infiltrating lymphocytes, is thought to drive T cell dysfunction and exhaustion thus leading to cancer persistence and tumorigenesis [108]. This too could be the case in RA, as the fibroblast- like synoviocytes (FLS) that play a major role in the initiation and development of synovial inflammation and joint destruction in patients display an activated phenotype characterized by enhanced glycolytic metabolism similarly to cancer cells [112, 116]. This enhanced metabolic profile allows RA FLSs to exhibit an abnormal capacity for migration, invasion, and proinflammatory cytokine production that contribute to disease pathology [112]. The proinflammatory nature of FLSs in RA most likely promotes a hostile immunosuppressive

environment that over time, renders autoreactive infiltrating T cells dysfunctional due to high lactate output and a lack of nutrients available to fuel proper T cell activation [72, 108]. Therefore, its plausible that the metabolic defects in $CD4^+$ T cells from RA patients does not cause disease, rather it occurs as a consequence of the environment. Indeed, treatment of RA FLSs with the anti-glycolytic PFK15, a competitive inhibitor of the enzyme PFKFB3, lead to decreased expression of proinflammatory cytokines, migration, and proliferation [112]. Whether metabolic regulation of RA FLS lead to normalization of T cell metabolism and function remains unknown and should be investigated more thoroughly. Moreover, more studies are needed to fully dissect the metabolic features of $CD4^+$ T cells in RA from disease onset through progression. This would allow for thorough examination of the metabolic defects plaguing autoreactive T cells in RA, and may provide new details that explain differences seen in mouse models and patient samples.

## 1.5 EVIDENCE OF METABOLIC DYSREUGLATION IN T1D: OPPORTUNITIES FOR INTERVENTION?

While metabolism as a therapeutic target has been exploited in many autoimmune diseases, such as the ones detailed in this review, it has been largely unexplored in T1D research. However, like $CD4^+$ T cells in lupus, MS, and RA, there is growing evidence from our laboratory and others that β cell reactive T cells demonstrate enhanced bioenergetic demands and metabolic defects that drive autoimmune targeting of the pancreatic β cell. While most of this review has focused on utilization of glycolysis to fuel T cell activation, mitochondrial OXPHOS and ROS are also enhanced during activation, as mitochondrial derived ROS not only act as a third signal for efficient T cell activation, but also are essential for IL-2 production and proliferation upon antigenic stimulation

[4, 117]. T cell mitochondrial dysfunction is seen often in autoimmunity, and is attributed to increased mitochondrial inner- membrane hyperpolarization (MHP) [117]. Recently it has been identified that the production of ROS and synthesis of ATP are tightly regulated by maintenance of mitochondrial membrane potential. Further, mitochondrial dynamics and function are thought to drive T cell differentiation [118, 119]. More specifically, it has been demonstrated that increased mitochondrial membrane potential leads to an effector T cell phenotype characterized by increased glycolysis and ROS production [120]. Interestingly, T cells from patients with T1D display MHP and increased mitochondrial membrane potential, indicating a more activated phenotype compared to healthy control T cells [117]. This phenotype was seen in T cells in the CD4 and CD8 compartment regardless of activation status, indicating that MHP is a general characteristic of T cells in patients with T1D. Moreover, enhanced MHP was linked to increased ROS production and IFNγ secretion, indicating that mitochondrial dysfunction promotes effector functions of autoreactive T cells due to an altered proinflammatory T cell effector response [117]. This finding that ROS drive T cell activation and effector capabilities correlate with data from our laboratory, where use of a manganaese metalloporphyrin (MnP) with ROS scavenging capabilities during T cell activation inhibited diabetogenic $CD4^+$ T cell responses *in vitro* and delayed diabetes progression in an adoptive transfer model of T1D *in vivo* [37, 39, 121-123]. Work by Previte et al. demonstrated that redox modulation via MnP treatment during activation maintained AMP-activated protein kinase (AMPK) activation that resulted in decreased mTOR activation and a reduced ability to undergo metabolic reprogramming to aerobic glycolysis [123]. These data demonstrated that ROS are required for driving and sustaining T cell activation and promoting the metabolic switch to glycolysis [123]. Altogether, this data suggests that self- reactive $CD4^+$ T cells have enhanced bioenergetic demands fueled by glycolysis, and that this increased reliance on the

glycolytic pathway long- term induces MHP, increases ROS production, and makes these T cells super- effectors that drive Th1 mediated β cell destruction. Moreover, the fact that T1D T cells secrete more IFNγ after activation is in itself indicative of an enhanced glycolytic profile, as aerobic glycolysis controls preferential translation of IFNγ mRNA and is required for acquisition of T cell effector functions [107]. Finally, data presented by Chen et al. indicating that naïve T cells had higher MHP supports the idea that autoreactive T cells are in a heightened activation state. This data, along with the potential for poor peptide binding due to dysfunctional antigen presentation, may provide further insight as to how and why autoreactive T cells are able to escape deletion in the thymus and go unabated in the periphery [117, 124]. It may also be a reason as to why effector T cells become resistant to Treg mediated control or explain why Treg function is defective in T1D, as enhanced glycolysis may lead to FoxP3 destabilization and subsequent loss of suppressive capabilities.

### 1.5.1 Hyperglycemia and the immune microenvironment's role in shaping β cell reactive T cell responses in T1D

A common theme discussed thus far has been understanding the role of the immune microenvironment on T cell differentiation and function, and how that contributes to autoimmune disease progression and pathology. As glucose is the substrate of choice for T cells undergoing activation and is required for metabolic reprogramming to occur, it would be interesting to know whether hyperglycemia contributes to the heightened bioenergetic profile and metabolic defects of β cell reactive $CD4^+$ T cells in T1D. One could postulate that autoreactive T cells in T1D, which appear to be more glycolytic compared to T cells from healthy controls, are able to utilize glucose more freely due to its abundant availability in the blood, thereby enforcing a proinflammatory

program that leads to a predominate effector T cell phenotype. This phenomenon has been previously described for $CD8^+$ T cells, where *in vitro* simulated hyperglycemia increased IFNγ secretion [125, 126]. In human studies, Chen et al analyzed the impact of hyperglycemia on T cell mitochondrial membrane potential by measuring membrane potential in T cells from a cohort of patients with Type 2 Diabetes (T2D) and comparing it to the membrane potential of T cells from patients with T1D [117]. This comparison allowed for delineating whether the high glucose environment or immune abnormalities associated with autoimmune conditions lead to the metabolic defects in T cells from T1D patients. Intriguingly, it was found that T cells from T2D had membrane potential levels that were indistinguishable from controls. However, this was significantly lower than the membrane potential of T cells isolated from T1D patients. These data indicate that the mitochondrial defects seen in T cells from individuals affected by T1D is a consequence of autoimmunity, and not a consequence of the high blood glucose levels and loss of glucose control in these patients [117]. Whether T cells between T1D and T2D were more glycolytic in hyperglycemic environments remains unknown. It would be intriguing to follow metabolic and mitochondrial dysfunction in autoreactive T cells starting during development in the thymus through early initiation of autoimmunity, all the way to onset and progression of clinical symptoms and disease. While this would be difficult to do using patient samples, a large-scale study following the course of disease could be more easily done using the NOD mouse, a spontaneous model of T1D that closely mimics key characteristics of human disease. While not a perfect system, it would allow for a better understanding of how T cells respond to various environmental stimuli encountered during development and in the periphery that could contribute to metabolic and immunologic dysfunctions.

Furthermore, defective Treg function is a common feature of autoimmune diseases [8, 12, 41]. Metabolism plays a key role in regulating the balance between Teffectors and Tregs [10, 11, 53, 57, 66, 67, 71]. While Treg suppressive capabilities and FoxP3 stability are thought to be supported by use of FAO, glycolysis has been demonstrated to be required for Treg proliferation and circulation [57, 74]. Glycolysis, which is associated with Teffector activation, drives proinflammatory responses while OXPHOS and FAO are associated with Tregs, and drive an anti-inflammatory response. Modulating the glycolysis pathway has been shown to inhibit effector T cell responses while also promoting the induction of $FoxP3^+$ Tregs [57, 59, 71, 72, 84-86, 115]. In SLE, Treg cells exhibit increased mTOR activity that correlated to their dysfunction and diminished suppressive capacities, which could be corrected with rapamycin treatment [94]. This provides evidence that Tregs in autoimmune conditions are more glycolytic, and could indicate why they fail to control autoreactive T cells in SLE, MS, RA, and T1D [94]. Notably, while rapamycin treatment is capable of expanding SLE Tregs and correcting functional defects, mixed results have been obtained with Treg expansion in T1D patients [88-90, 92-94, 127]. One study by Monti et al. investigating the effect of rapamycin on human Tregs *in vivo* demonstrated that treatment did not alter the frequency, proliferation, or cytokine production of circulating Tregs, but it enhanced their capability to suppress proliferation of effector T cells in T1D [127]. The varying effects of rapamycin on Treg expansion seen in SLE and T1D could be attributed to the type of pathogenic T cells that mediate autoimmune damage in both conditions. In SLE, patients have enhanced Th17 cell responses that correlate with disease activity [128]. On the other hand, T1D is a primarily Th1 mediated disease [37, 39, 40, 42, 121]. This difference in Th cell differentiation could indicate why Treg expansion is more easily accomplished in SLE patients. In particular, recent literature has indicated that T cell metabolism, namely the glycolysis pathway,

is an important checkpoint controlling the differentiation of Th17 and Treg cells, as blocking glycolysis has the ability to inhibit Th17 differentiation while promoting Treg cell generation [57, 71]. Further research is needed to dissect the metabolic features of effector T cells and Tregs in autoimmune conditions, with particular attention to how T cells in various disease pathologies differ from one another. Specifically in T1D, it would be of interest to know whether high blood glucose levels and enhanced glycolysis leads to destabilization of the Treg lineage, and to further characterize whether Treg dysfunction in autoimmunity is a result of metabolic alterations, as appears to be the case in SLE [94].

### 1.5.2 Enforcing T cell exhaustion: A foe in cancer, a friend in autoimmunity?

Immune dysfunction in cancer is thought to occur as a result of T cell exhaustion and increased Tregs in the tumor microenvironment that limit anti-tumor immunity. T cell exhaustion, which is typically associated with $CD8^+$ T cells but can occur in the CD4 compartment, is characterized by loss of effector functions, altered cytokine responses, overexpression of checkpoint inhibitory receptors (i.e. PD-1, CTLA-4, LAG-3, and TIM-3 and altered metabolic programs leading to T cell hyporesponsiveness and cancer progression [129]. Checkpoint blockade of inhibitory receptors, especially PD-1 have revolutionized cancer immunotherapy, as they have proven to be effective in reinvigorating exhausted T cells in the TME. Unfortunately, immunotherapy is known for a multitude of immune- related adverse effects, including onset of autoimmune disorders like PD-1 inhibitor induced T1D [130]. Specific work in T1D has revealed that PD-1/Programmed death- ligand 1 (PD-L1) deficiency and blockade or deletion of LAG-3 in NOD animals accelerates the onset of autoimmune diabetes, indicating an important role for checkpoint inhibitory receptors in regulating T cell responses in T1D [30, 131, 132].

Studies using mouse models and samples from human patients have revealed that the PD-1/PD-L1 pathway is dysregulated in T1D [133-135]. Indeed, activated T cells from new- onset T1D patients fail to upregulate PD-1 compared to healthy subjects [134]. This was true too for $CD4^+$ T cells in adults with long- standing T1D [136]. Interestingly, this phenotype was seen only in $CD4^+$ T cells from T1D patients, whereas PD-1 expression was similar between T cells from SLE patients and healthy controls , indicating PD-1 dysregulation is specific to T1D onset and pathogenesis [136].

LAG-3 is another checkpoint molecule that has been tied to immune cell dysfunction in T1D. This was confirmed in work by Bettini et al. where deletion of LAG-3 in autoimmune prone NOD animals resulted in accelerated diabetes with 100% incidence that corresponded to increased T cell islet infiltration, and not due to Treg dysfunction [30, 31]. This phenotype was exclusive to autoimmunity, as LAG-3 deletion in non- autoimmune prone C57BL/6 mice resulted in a minor phenotype with no development of spontaneous disease, similarly to the minimal immunopathologies associated with PD-1 knockout in non- autoimmune prone mice [137, 138]. Together, these data indicate a critical role for LAG-3 in controlling early expansion of autoreactive T cell clones. It would be important to study LAG-3 expression on T cells from healthy individuals and T1D patients to better understand whether LAG-3, like PD-1, is dysregulated during autoimmunity. Due to the synergistic nature of PD-1 and LAG-3, we would hypothesize that LAG-3 expression is decreased or signaling is dysfunctional in T cells from T1D patients. Moreover, it’s clear that checkpoint inhibitory receptors, including both PD-1 and LAG-3, are important for maintaining self- tolerance and may be critical mediators that drive autoimmunity.

As one would expect, the state of T cell exhaustion is accompanied by distinct metabolic features [139]. Moreover, both PD-1 and LAG-3 have been implicated in reprogramming lymphocyte metabolism. For example, PD-1 signaling specifically promotes lipolysis and FAO and leaves T cells unable to engage in aerobic glycolysis via stabilization of phosphatase and tensin homolog (PTEN) [109]. As glycolysis is required for acquisition of T cell effector functions, this finding makes sense as exhausted T cells expressing PD-1 are dysfunctional in their ability to secrete proinflammatory cytokines and have limited effector capabilities [129]. This reliance on FAO is associated with T cell longevity and enhanced survival while exhausted and unable to utilize other key nutrients [109, 140]. A paper published this past year by our laboratory investigated the role of LAG-3 in maintaining metabolic quiescence in naïve $CD4^+$ T cells [110]. Interestingly, it was determined that LAG-3 -/- $CD4^+$ T cells demonstrated greater glycolytic capacity and enhanced proliferation and effector functions (i.e. IFNγ secretion) during activation, indicating a role for LAG-3 in regulating T cell activation and clonal expansion. This is an interesting finding and may provide evidence for enhanced metabolic phenotypes of T cells in autoimmune conditions, as inhibitory receptor expression is impaired in diabetogenic autoreactive T cells [134]. Impaired expression of PD-1 and LAG-3 could explain the hyperactivated phenotype and enhanced effector capabilities of autoreactive $CD4^+$ T cells that are responsible for disease-inducing potential of these cells. Finally, altered bioenergetics and enhanced glycolysis in diabetogenic $CD4^+$ T cells may confer resistance to Treg mediated suppression or lead to Treg dysfunction that is known to occur during T1D pathogenesis [10, 12, 40, 41, 45]. Based on these data, it would be interesting to know whether recapitulating an environment like the TME in cases of autoimmunity could prevent or reverse disease. As T cells in autoimmune prone individuals fail to upregulate these inhibitory receptors, enforcing the checkpoints and modulating T cell

metabolism to promote T cell exhaustion may lead to tolerance in affected individuals. In fact, a study by Tilstra et al. demonstrated that kidney- infiltrating T cells in a mouse model of lupus nephritis become metabolically and functionally exhausted after organ infiltration as a means to limit damage to self [141]. Therefore, enforcing this phenotype prior to disease onset via metabolic regulation may provide a new way to limit autoimmune disease associated pathologies [141]. Further investigation is needed to determine whether glycolysis inhibition or promotion of FAO leads to an exhausted state.

## 1.6 METABOLIC INTERVENTION: A NEW OPPORTUNITY FOR T1D PREVENTION AND THE FUTURE OF IMMUNOTHERAPY

Current therapeutic strategies for T1D have focused on 2 different approaches including 1) restoring or preserving endogenous β cell mass or 2) combatting autoreactivity and autoimmunity. While efforts to restore and preserve β cell mass have been interesting and shown promise in preclinical studies, these strategies ultimately fail due to a recurrence of autoimmunity [142, 143]. The first proof of principle study showing that immunotherapy is effective in human patients with T1D was published in 1984, and investigated the use of Cyclosporine in recent onset T1D patients [144, 145]. This has given precedence for finding ways to limit the autoimmune destruction of β cells by the immune system. Two clinical trials have been published this year that investigated the use of low dose anti- thymocyte globulin (ATG) in new onset T1D patients and the anti- CD3 antibody Teplizumab in relatives at risk for developing disease and found that both low- dose ATG preserved β cell mass while Teplizumab delayed disease onset [146, 147]. While disease was successfully delayed and β cell mass was preserved for a period of time, both treatments

consequently lead to lymphopenia in treated patients [146, 147]. Moreover, these new studies, while effective, have not addressed the broad immunosuppression associated with the treatment. This is a major hurdle to overcome especially in T1D, which affects juvenile patients, as wiping out the immune system could have broad implications for the ability to overcome potential infections and pathogens. Instead, they have used newer therapeutics that have the same lymphopenic affect that was demonstrated 35 years ago with Cyclosporine. Importantly though, these studies, along with the ability to predict onset of T1D based on the presence of one or more autoantibodies, has given precedence for manipulating T cell responses to prevent or delay disease onset in those predisposed to autoimmunity.

As discussed in detail in this review, modulating $CD4^+$ T cell metabolism has the ability to both prevent and reverse autoimmune conditions [71, 84-87, 115]. While not yet explored in T1D, the potential of metabolic intervention to be successful to control islet- antigen specific T cell responses exists and deserves further examination. At the time of diagnoses, patients with T1D have already experienced longstanding autoimmune onslaught to pancreatic β cells, and thus only have approximately 10-20% β cell mass still present [33-35, 40]. As in most autoimmune conditions, autoimmunity (specifically activation of autoreactive $CD4^+$ T cells) precedes downstream damage, and is an important consideration to make when thinking about where and when metabolic intervention is best suited. Studies in T1D have shown that immunotherapy is successful in delaying and/ or preventing onset [144-147]. Based on the immunopathology of T1D, and due to the importance of $CD4^+$ T cells in disease initiation and progression, we would hypothesize that use of metabolic inhibitors would be best suited as a prophylactic strategy, prior to diagnoses. Genetic predisposition and the presence of multiple autoantibodies can predict who will progress to have T1D [33, 35, 40, 146, 147]. Although not currently used in the clinic, testing

for the presence of autoantibodies to β cell antigens in children with a family history of T1D or in those at risk to developing autoimmunity could be used to determine whether metabolic intervention should be initiated. Moreover, the natural history of T1D takes place over a number of years, giving ample time for intervention prior to complete loss of dynamic glucose sensing and insulin secretion due to β cell loss [33]. This idea of disease prevention was outlined above. Studies in SLE have demonstrated the success of metabolic modulators in preventing autoimmunity (52-54). Also, while it is possible that chronic administration may be necessary, inhibiting glycolysis has been shown to promote Treg differentiation while inhibiting effector T cell expansion (47, 52-58,61-62). If metabolic intervention can restore the balance between effector T cells and Tregs, and improve suppressive function of dysfunctional Tregs in autoimmune diseases, a shorter treatment regimen may be successful in establishing durable tolerance to self- antigens. However, more work is needed to fully understand the ability of metabolic inhibitors to prevent or reverse disease, especially T1D, in order to determine length of treatment needed to establish tolerance.

On the contrary to immune- targeted therapies that result in repression of global immunity, metabolic therapies have demonstrated great success in selective targeting of self- reactive T cells while leaving humoral immunity unscathed and intact [61, 84-87, 115]. This specific targeting of autoreactive T cells via use of anti- metabolites even when administered systemically is a phenomenon termed “cellular selectivity based on demand,” and was first described by Lee et al, in a study where targeting glucose and glutamine metabolism specifically prevented allograft rejection [148-150]. The idea behind this theory is that use of generic inhibitors of universal metabolic processes selectively affect cells with the greatest metabolic demand, without altering normal cellular homeostatic function [148-150]. While effector T cells alone have increased metabolic demands to support activation, proliferation, and effector functions, research in

autoimmunity has provided evidence that autoreactive T cells have altered bioenergetic programs that allow for their increased effector capabilities. These metabolic abnormalities could contribute to their persistence in the periphery and disease- promoting capabilities compared to T cells from healthy individuals. This makes metabolism an ideal target for autoimmunity, where enhanced metabolically demanding programs that self- reactive T cells rely on can be specifically modulated to promote tolerance. Moreover, as immune cells utilize distinct metabolic programs, inhibition of glycolysis for example should in theory have minimal effects on Tregs or established memory T cells to vaccines. When using metabolic inhibitors, like 2-DG, to alleviate autoimmune targeting by $CD4^+$ T cells as described in detail above, the remainder of the immune system is otherwise unharmed and functionally able to respond appropriately to non- self-antigens (i.e. to immunization). However, it's important to consider that metabolic intervention may also modulate immune cells other than $CD4^+$ T cells. All immune cells, including macrophages, dendritic cells, B cells, and $CD8^+$ T cells, undergo metabolic reprogramming to aerobic glycolysis upon activation [59, 75, 78]. Therefore, its plausible that use of inhibitors like 2-DG, metformin, or PFK15 could also alter APC, B cell, or $CD8^+$ T cell function. In fact, treatment with glycolysis inhibitors like 2-DG in mouse models of SLE and T1D resulted in reduced $CD8^+$ T cell expansion and effector function and also lead to reduced autoantibody production by B cells [84, 86, 151]. All in all, current immunotherapeutic strategies to combat T1D have had harsh repercussions on the entire immune system. Moreover, there is evidence that autoreactive T cells do not play by the same rules metabolically and have altered metabolic demands and requirements compared to their non-autoimmune counterparts. Whether abnormal metabolism is a feature of other immune cell compartments in autoimmunity warrants further investigation. Moreover, further work is needed to determine the impact of metabolic modulators on $CD8^+$ T cell, B cell, and APC function, as

most of the studies done thus far have focused on modulating $CD4^+$ T cell metabolism. The importance of $CD4^+$ T cells in autoimmunity and their distinct metabolic features provide a unique opportunity to move immunotherapy towards targeting metabolism, which has a proven track record of specifically controlling rogue self- reactive T cell responses without interfering with basal cellular metabolic programs of other cells in the body or serious side effects.

# 2.0 INVESTIGATING SYSTEMIC AND CONTROLLED RELEASE MICROPARTICLE ADMINISTRATION OF THE ANTI- GLYCOLYTIC PFK15 TO CONTROL ABERRANT T CELL ACTIVATION IN TYPE 1 DIABETES

This chapter is an ongoing study and more experimentation is required before it can be considered for publication.

## "Controlled Release of the Anti- Glycolytic PFK15 reduces T cell Activation and Effector Functions in Type 1 Diabetes"

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## 2.1 SUMMARY

Although safeguards are in place to protect against the onset of autoimmunity, a breakdown in tolerance mechanisms can lead to the activation of autoreactive T cells that mediate immunopathology against self-tissues, like that which occurs during T1D. T1D is an autoimmune disease in which insulin-producing islet β cells are targeted and destroyed by the immune system, with $CD4^+$ T cells largely mediating pathogenesis. Importantly, distinct metabolic programs dictate T cell differentiation and function over the course of the T cell lifecycle. Notably, $CD4^+$ T cells undergo metabolic reprogramming to the less efficient aerobic glycolysis during activation to support clonal expansion and effector cytokine secretion. This is true too for diabetogenic $CD4^+$ T cells, who upregulate the glycolysis pathway, including the rate- limiting enzyme PFKFB3, upon encounter with β cell antigen. Targeting the glycolysis pathway as a means to maintain tolerance has been investigated in a number of autoimmune disease models, including Lupus, Multiple Sclerosis, and Rheumatoid Arthritis, but remains a largely understudied therapeutic approach to control self- reactivity against islet β cells T1D. However, while other studies using immunomodulatory agents have been used successfully in the clinic to delay disease onset, their systemic delivery regimens often lead to global immune suppression, lymphopenia and/or a recurrence of autoimmunity. In this study, we utilized the small molecule PFK15, a competitive inhibitor of PFKFB3 that has shown efficacy in FDA clinical trials for advanced malignancies [152]. As a means to circumvent systemic administration of an anti- glycolytic, we formulated PFK15 into a microparticle (MP) in order to provide a sustained and constrained release of the drug to a targeted area to limit systemic exposure, thus allowing for selective control of β cell reactive T cells while keeping global immune function intact. Our results demonstrated that metabolic regulation of $CD4^+$ T cell activation via PFK15 soluble and MP formulations inhibits

effector function *in vitro* and delays disease onset *in vivo*. These data support our hypothesis that the glycolysis pathway is a novel therapeutic target to combat rogue autoimmune responses in T1D without altering global immune function, as other studies have failed to address this consequence of current immunotherapeutic regimens. The work presented here are crucial in expanding our knowledge regarding the design of more effective and selective therapies for patients at risk for T1D.

## 2.2 INTRODUCTION

The immune system is tasked with mediating protection of its host against various pathogens, where it relies on the ability to distinguish self from non- self to avoid detrimental immunopathology [1]. Under certain circumstances, a breakdown in tolerance leads to the activation of self- reactive T cells that can initiate autoimmunity, like that exhibited in T1D [33, 34, 43]. A number of immune cells contribute to autoimmune destruction of insulin- secreting β cells [33, 34, 43, 45]. However, $CD4^+$ T cells are considered the primary mediators of disease pathology, and are crucial in perpetuating attack of insulin- secreting pancreatic islet β cells [33, 34, 43, 45].

Interest in the mechanisms governing the activation and effector capabilities of T cells have grown immensely, with a specific interest in understanding the role cellular metabolic programs play in mediating proper T cell function [15, 54-62, 71, 83, 95, 125, 148, 153-155]. Under homeostatic conditions, naïve $CD4^+$ T cells are metabolically quiescent and rely on oxidative phosphorylation (OXPHOS) via the mitochondria to support basal processes [15, 58-60, 62, 71, 95, 154]. Upon TCR mediated recognition of antigen, activated T cells adopt an anabolic metabolism characterized by a metabolic transition from OXPHOS to glycolysis. Costimulation and the presence of growth factors like IL-2 promotes metabolic reprogramming via induction of phosphoinositol 3- kinase (PI3K) – dependent activation of AKT [58-60, 95, 154]. Active AKT then acts as a positive regulator of the mammalian target of rapamycin (mTOR), thereby stimulating glycolysis and cellular metabolism [60, 95, 154].

Activated T cells preferentially ferment glucose into lactate, a process that occurs despite sufficient oxygen levels to support mitochondrial respiration [60, 95, 154]. This phenotype, known

as the Warburg effect, is a hallmark of highly proliferative cancer cells. Utilization of the glycolysis pathway is far less efficient than OXPHOS, with a net gain of 2 versus 36 molecules of ATP generated per one molecule of glucose, respectively [60, 95, 154]. However, lack of efficiency is secondary to the processes and intermediates glucose and the glycolysis pathway provide to highly proliferative cells. Not only is glycolysis required for proliferation and clonal expansion, but it's also necessary to support the specialized effector functions of both $CD4^+$ and $CD8^+$ T cells [60, 95, 154]. Most notably, use of aerobic glycolysis is required for effector cytokine production (i.e. IFNγ synthesis), nucleotide and reducing equivalent generation via the pentose phosphate pathway (PPP) for producing biosynthetic precursors for cellular components necessary for proliferation, and for the production of the cytolytic effector molecules granzyme and perforin by $CD8^+$ T cells [60, 95, 107, 154]. Evidence supporting the necessity of glycolysis for T cells comes from studies demonstrating that glucose deprivation inhibits IFNγ gene expression and cytolytic activity of $CD8^+$ T cells [60, 95, 107, 154].

An often-overlooked consequence of immunotherapy is the broad non- specific targeting of either non- autoreactive T cells or other cell types within the body, which ultimately may lead to global immunosuppression. Controlled drug delivery systems through use of micro (MP)- or nano-particle (NP) technologies have provided an effective way at delivering therapeutics locally to specific tissues to mitigate systemic delivery- induced toxic effects [156]. These approaches have proven beneficial in a number of disease settings, including a recent paper by our laboratory demonstrating use of MP technologies in preclinical models of RA [13].

As T cells are critical contributors to the immunopathology of T1D, and due to the importance of a metabolic switch to glycolysis in fueling effector T cell functions, we hypothesized that inhibiting glycolysis would dampen $CD4^+$ T cell activation and effector functions, that would

prevent autoimmune attack of pancreatic beta cells in a mouse model of T1D. Here we demonstrate the ability for systemic and local MP delivery of the glycolysis inhibitor PFK15 to dampen T cell responses *in vitro*, and delay T1D *in vivo*.

## 2.3 MATERIALS AND METHODS

### 2.3.1 Animals

Non-obese diabetic (NOD), NOD.BDC2.5.TCR.Tg (BDC2.5) and NOD.*scid* mice were maintained under specific pathogen free conditions in the animal facility located at the Rangos Research Center within UPMC Children's Hospital of Pittsburgh. All animal experiments were approved by the University of Pittsburgh's Institutional Animal Care and Use Committee (IACUC; Assurance Number: D16-00118). Male and female mice aged 6-12 weeks old were used in all experiments.

### 2.3.2 Splenocyte isolation and *in vitro* stimulation

NOD animals were sacrificed, and spleens harvested and homogenized into single cell suspensions as described [123]. Red blood cells were lysed using RBC lysis buffer (Sigma Aldrich). For *in vitro* experiments, 2.5 x $10^6$ splenocytes were plated per well in 24 well plates and stimulated with 2.5 μg/mL of ConA ± 2.5- 10 μM PFK15 soluble drug (Selleck) for 48-72 hours. In some studies, PFK15 was administered via porous MP formulations, and were a generous gift formulated and provided by Dr Abhinav Acharya and Dr. Steven Little (University of Pittsburgh). The Little laboratory performed release kinetic studies and MP characterization and scanning electron microscopy as previously described [13, 157, 158]. MP experiments were performed in 24 well

Transwell plates (Corning). Cells and culture supernatants were collected for downstream flow cytometry, ELISA, and lactate measurement analyses.

### 2.3.3 Flow Cytometry

$5x10^5 – 1x10^6$ cells were harvested at indicated timepoints and surface stained for flow cytometric analysis as described [123]. Briefly, cells were incubated with Fc block (CD16/CD32; BD Biosciences) for 15 minutes prior to staining for flow cytometry. Surface staining was performed at 4°C using antibodies against CD4, CD69, CD25, PD-1, CTLA-4, Vβ4, LAG-3 (BD Biosciences), or hen egg lysozyme (HEL) tetramers (NIH Tetramer Core Facility) in FACS buffer (1% BSA in PBS). For proliferation measurements, splenocytes were labeled with Cell Proliferation Dye Violet (BD Bioscience) per manufacturer's instructions prior to stimulation. After indicated timepoints, cells were harvested, and surface stained as described above. In some instances, cells were fixed in 2% PFA for 15 minutes at 4°C (Thermo Fisher Scientific). Cells were stored at 4°C until time of analysis.

To measure glucose uptake, splenocytes were incubated with 100 μM of the fluorescent glucose analog 2-(*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) Amino)-2-Deoxyglucose (2-NBDG; Cayman Chemical) for 10 minutes at 37°C prior to harvest as described [110, 123]. Cells were washed with PBS and surface stained for CD4 expression and analyzed live by flow cytometry. To measure apoptosis, cells were stained with Annexin V and Propidium Iodide (PI) apoptosis detection kit per the manufacturer's instructions (BD Biosciences), For all flow cytometry studies, fluorescence was measured using a FACS Aria II flow cytometer (BD Biosciences). All data were analyzed using FlowJo software (v10.5.3) and samples were gated on $CD4^+$ cells.

### 2.3.4 Lactate and Cytokine Measurements

Cell culture supernatants from *in vitro* experiments were harvested and used to measure IFNγ by ELISA. Antibody pairs for IFNγ were purchased from BD Biosciences. ELISAs were read on a SpectraMax M2 microplate reader (Molecular Devices) and data analyzed using SoftMax Pro version 7.0.2 software (Molecular Devices). Lactate, a byproduct of aerobic glycolysis, was measured using the Lactate Plus meter and test strips per manufacturer's instructions (Novus Biologics).

### 2.3.5 Nominal Antigen Immunization

For immunization experiments, non- diabetic NOD mice were immunized with 100 µg HEL (Sigma Aldrich) emulsified in Complete Freund's Adjuvant (CFA; Sigma Aldrich) at the base of the tail. Animals were treated with 2 mg of blank or PFK15 MPs as described above on each side near the inguinal lymph nodes. After 7 days, draining lymph nodes and spleens were harvested for downstream analyses. Specifically, cells from the dLNs and spleens were plated and stimulated with 25 µg HEL in an *in vitro* recall assay for 72 hours to measure activation and effector cytokine (IFNγ) production.

### 2.3.6 Adoptive Transfer Model of T1D

1 x $10^7$ splenocytes from BDC2.5 mice were injected i.v. into NOD.*scid* recipients as described by our laboratory [123]. Recipient animals were split into three groups. One cohort of recipients received 25 mg/kg PFK15 (Selleck) dissolved in DMSO prepared fresh as described and used in previous literature [152, 159, 160]; the second cohort received blank MPs, and the third cohort received PFK15 MPs (5 mg/kg PFK15). The dose of PFK15 MPs administered was based on

preliminary *in vitro* studies. Animals were treated every third day for two weeks. Body weights and blood glucose (BG) levels were monitored over the course of the experiments. Animals were deemed diabetic after two consecutive BG readings ≥ 350 mg/dL. Diabetic animals were sacrificed at indicated timepoints and peripheral blood, pancreata, and spleens were harvested for downstream analyses.

### 2.3.7 Tissue Collection and Histological Assessment

Pancreatic tissue was collected and fixed in 4% paraformaldehyde (PFA; Thermo Fisher Scientific) overnight at 4° C. Fixed tissue was processed and embedded in paraffin by the Histology Core Laboratory located at UPMC Children's Hospital of Pittsburgh's Rangos Research Center [161]. Embedded tissue was sectioned at 4 μm thickness and stained with hematoxylin and eosin (H&E) for histological examination of immune infiltration in pancreatic islets. Samples were imaged using a Nikon Eclipse E800 microscope (Nikon) and associated software.

### 2.3.8 Immunofluorescent Staining

Immunofluorescent staining was performed on paraffin embedded samples prepared as described above. Antigen retrieval was performed in sodium citrate buffers followed by overnight incubation with primary antibodies against insulin (1:100; Santa Cruz), FoxP3 (1:100; Abcam), and TUNEL staining as previously described [162]; all co-stained with DAPI (1:3000; Thermo Fisher Scientific). The following day, slides were incubated with Alexa Fluor 488 or 594 conjugated donkey anti- rabbit or rat secondary antibodies (Invitrogen). Samples were imaged using a Leica DMi8 inverted microscope (Leica) and LAS X Navigator software (Leica).

### 2.3.9 Statistical Analyses

All data are presented as mean values ± standard error of the mean (SEM), with n indicating the number of independent experiments or animals. Student's t-test or One- way ANOVA were used where appropriate. For survival studies, 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. Histology and immunofluorescent images were generated using Photoshop. All statistics and graphs were generated using GraphPad Prism software.

## 2.4 RESULTS

### 2.4.1 Soluble PFK15 treatment reduces T cell activation induced glucose uptake and lactate secretion in a dose dependent manner *in vitro*

T cell activation is accompanied by a metabolic switch to glycolysis, marked by increased glucose uptake and the subsequent fermentation of glucose to lactate [155]. We first confirmed whether the glycolysis inhibitor PFK15, a competitive inhibitor of a rate limiting enzyme in the glycolysis pathway PFKFB3, inhibited T cell metabolic reprogramming during activation [155]. Briefly, splenocytes from non- diabetic NOD animals were stimulated with the lectin mitogen concanavalin A (ConA), a global T cell activator dependent on T cell:APC interaction ± 2.5 – 10 µM PFK15 *in vitro* [163, 164]. After 48- 72 hours, glucose uptake was measured via staining with the fluorescent glucose analog 2-NBDG, and lactate secretion was measured in cell culture supernatants as previously described [110, 123, 155]. As expected, we observed increased glucose uptake in ConA activated NOD splenocytes, compared to cells left in media alone 48 hours post stimulation. Further, PFK15 treatment reduced glucose uptake in a dose dependent manner, with

a significant reduction observed with 10 μM PFK15 treatment (Figure 6A-B). Coinciding with this, ConA stimulation led to significant increases in lactate secretion, with PFK15 treatment significantly reducing ConA induced lactate production (Figure 6C).

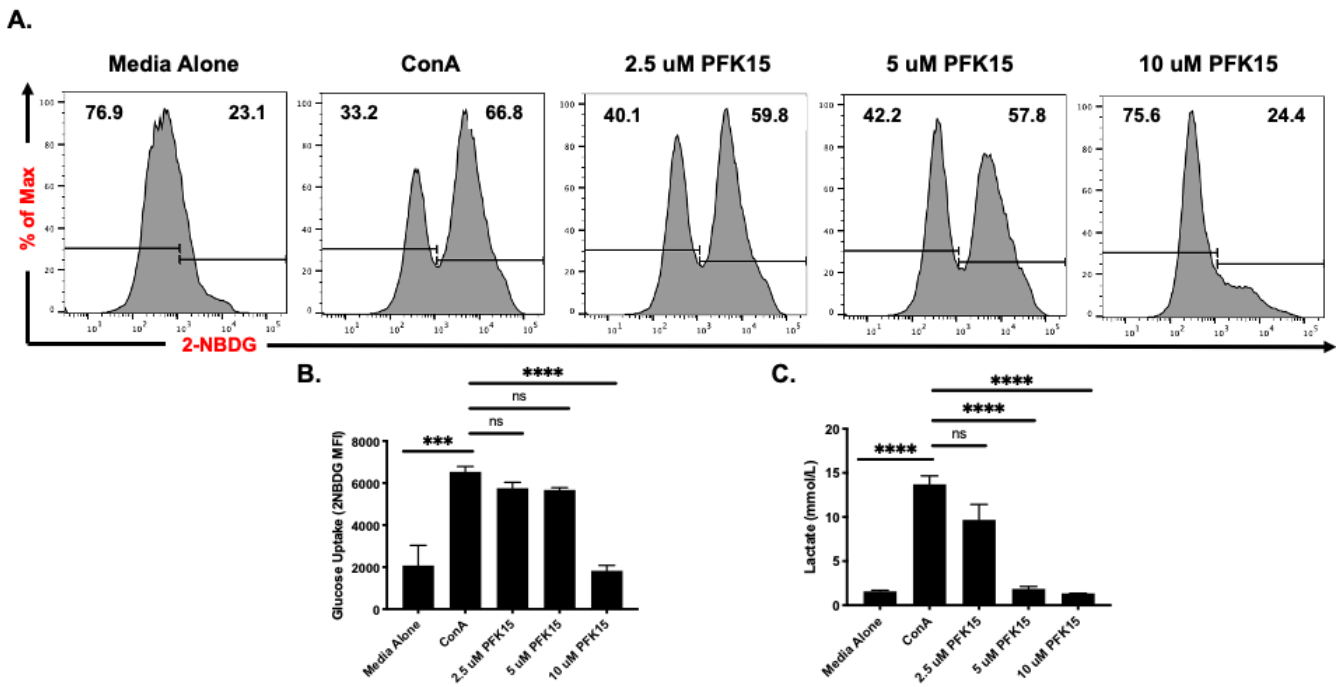


**Figure 6. PFK15 treatment reduces parameters of glycolysis in activated CD4+ T cells.**
NOD splenocytes were stimulated 2.5 ug/mL ConA ± PFK15 for 24-72 hrs. **A, B.** Representative histogram and statistical analysis showing 2-NBDG fluorescence and MFI of $CD4^+$ T cells, indicative of glucose uptake 48 hrs post stimulation (n = 3). **C.** Lactate measurements in cell culture supernatants (n = 3). All data are presented as the mean ± SEM. ( *** = $p < 0.005$, **** = $p < 0.0001$).

### 2.4.2 Inhibiting glycolysis reduces CD4$^+$ T cell responses to ConA stimulation *in vitro*, with minimal toxicity

Glucose restriction, as well as glycolysis inhibition, have been shown to reduce CD4$^+$ T cell growth and IFNγ secretion during activation [57, 107, 165, 166], however whether this occurs in T cells from the autoimmune prone NOD mouse remains unknown [57, 59, 60, 95, 107, 165, 166]. To assess this, we measured functional parameters of CD4$^+$ T cell activation using the *in vitro* PFK15 ± ConA stimulation described in the previous section. After 72 hours in culture, we assessed early (CD69) and late (CD25) activation marker expression on CD4$^+$ T cells, as well as IFNγ secretion and CD4$^+$ T cell proliferation (Figure 7). Consistent with the literature, inhibiting glycolysis with PFK15 led to reduced expression of CD69 (Figure 7A) and CD25 (Figure 7B), diminished effector cytokine (IFNγ secretion) production (Figure 7C), and greatly inhibited CD4$^+$ T cell proliferation (Figure 7D), with 5-10 μM of PFK15 having the most optimal effect. These data indicate that CD4$^+$ T cells from the NOD mouse are susceptible to metabolic modulation, and provide evidence to support a therapeutic benefit of PFK15 in suppressing T cell mediated destruction of β cells in T1D.

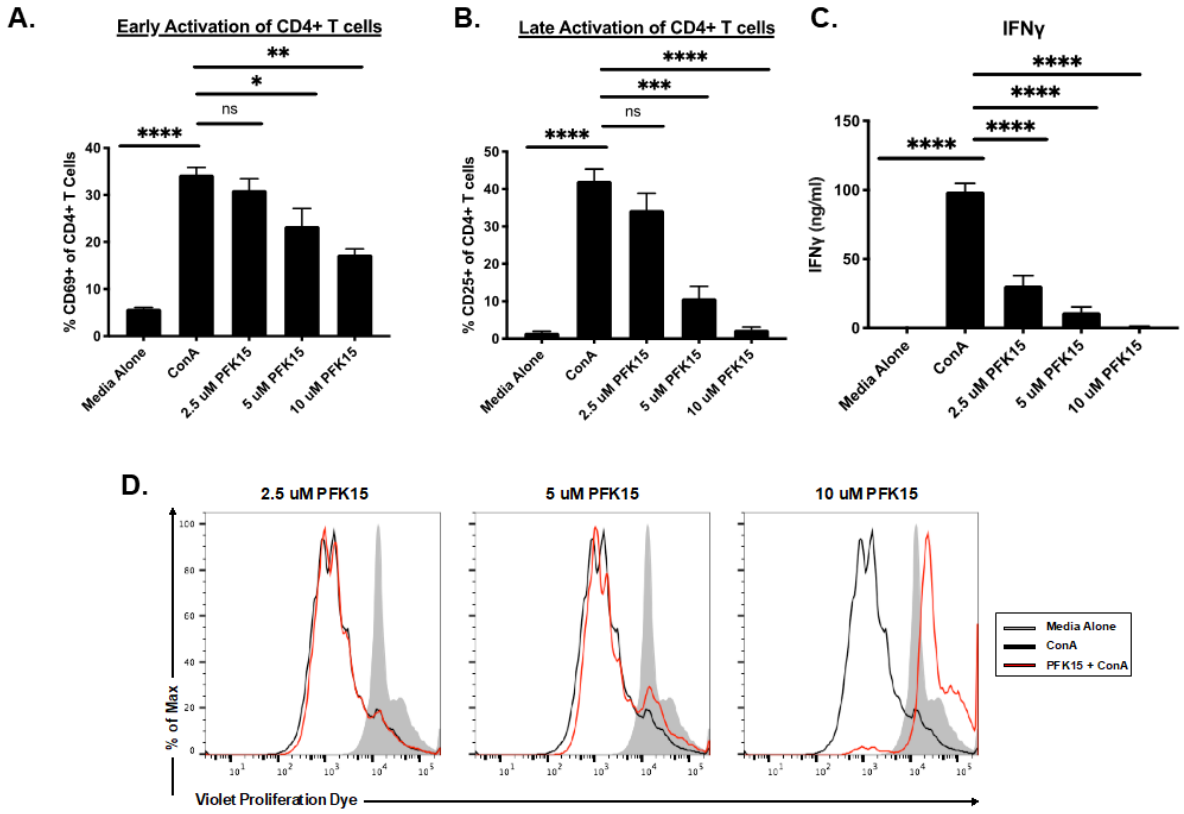


**Figure 7. Inhibition of glycolysis suppresses CD4$^+$ T cell activation and effector functions *in vitro*.**
Assessing the impact of glycolysis inhibition on diabetogenic CD4$^+$ T cell responses *in vitro*. **A.** Frequency of CD4$^+$ CD69$^+$ T cells (n = 3). **B.** Frequency of CD4$^+$ CD25$^+$ T cells (n = 3). **C.** ELISA analysis of IFNγ in cell culture supernatants 72 hrs post stimulation (n = 3). **D.** Representative histogram of CD4$^+$ T cell proliferation assessed by cell proliferation dye violet (CPDV) dilution (n = 3). All data are presented as the mean ± SEM. (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.005$, **** = $p < 0.0001$).

Previous literature has demonstrated an ability for PFK15 treatment to induce apoptosis in transformed cells [112, 152]. To determine whether PFK15 reduced $CD4^+$ T cell responses to ConA stimulation due to induction of apoptosis, and to better define a therapeutic dose, we assessed cell death in 48-hour cultures via Annexin V/propidium iodide (PI) staining by flow cytometry. We observed a dose dependent reduction in viability of $CD4^+$ T cells with PFK15 treatment, with 10 µM demonstrating overt toxicity with a majority of cells not- viable at the indicated timepoint (Figure 8). Further, 5 µM PFK15 treatment reduced T cell viability compared to ConA stimulated cells only, however cell viability remained similar to cells in media alone, indicating an optimal therapeutic dose. Based on these data, 5 µM of PFK15 was used for the remainder of our studies with PFK15, as it demonstrated an ability to dampen $CD4^+$ T cell responses independent of apparent cell death.

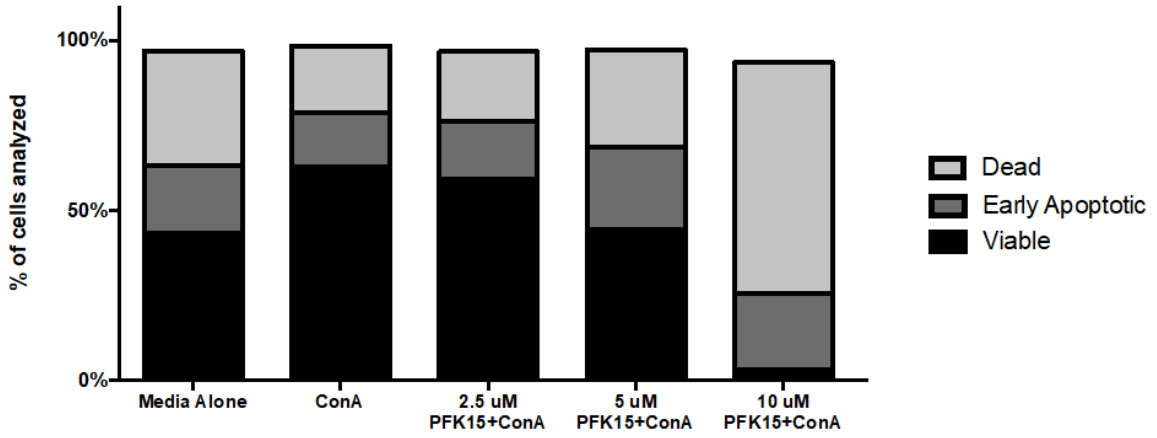


**Figure 8 PFK15 induces cell death in a dose dependent manner**
NOD splenocytes were stimulated 2.5 ug/mL ConA ± a dose titration of PFK15 for 48 hrs. Apoptosis was measured by staining for Annexin V/ PI. Data are presented as the mean ± SEM (n = 2).

### 2.4.3 PFK15 MPs recapitulate soluble drug treatment *in vitro*, and dampen CD4$^+$ T cell responses to ConA stimulation

Systemic delivery of immunotherapies can have detrimental consequences, either through non-specific targeting, depletion of lymphocytes, and/ or suppression of global immunity; thereby altering treated patients' ability to fend off possible infections [14, 156]. As glycolysis is an evolutionarily conserved pathway utilized not only by immune cells, but also by other cell types in the body, we aimed to determine whether controlled release of PFK15 near the pancreas would limit the activation of autoreactive CD4$^+$ T cells, while limiting any associated systemic toxicities associated with PFK15 treatment. We developed porous PFK15 PLGA MPs to promote fast and sustained release of PFK15 in a specific microenvironment. To test the ability of these MPs to suppress T cell responses, we employed the same ConA stimulation protocol utilized in our soluble drug experiments using transwell plates, to allow for MPs to sit in an upper chamber, and release PFK15 into the bottom chamber where splenocytes were plated and stimulated (Figure 9A). Blank MPs loaded without drug were used as a vehicle control for PLGA MPs formulations. PFK15 MPs demonstrated fast release kinetics, with a majority of PFK15 released within 4 days of culture (Figure 9B). Scanning electron microscopy (SEM) of PFK15 MPs confirmed the porous nature of MP formulations (Figure 9C). For all *in vitro* studies, PFK15 MPs were administered to release 5 μM of PFK15, the optimal dose determined from our dose titration studies (Figures 6-8).

To confirm glycolysis inhibition, we measured lactate secretion in cell culture supernatants, and observed significant knockdown with PFK15 MP treatment, while blank MP treated cells were left unaffected (Figure 9D). In our assessment of T cell activation, PFK15 MPs had no impact on early activation (CD69 expression), but led to a significant reduction in the ability for CD4$^+$ T cells to transition to late activation status (CD25 expression) (Figure 9E-F). Further, as with soluble drug, PFK15 MPs led to significant reductions in IFNγ secretion and T cell proliferation (Figure

9G-H). Blank MPs had no impact on T cell function, indicating the phenotypes we observed with PFK15 MP administration were due to release of PFK15 and not due to any effect of PLGA MPs in general. All in all, these data confirm that PFK15 MP treatment recapitulates our experiments with PFK15, and are able to inhibit T cell responses to ConA stimulation *in vitro* (Figure 9).

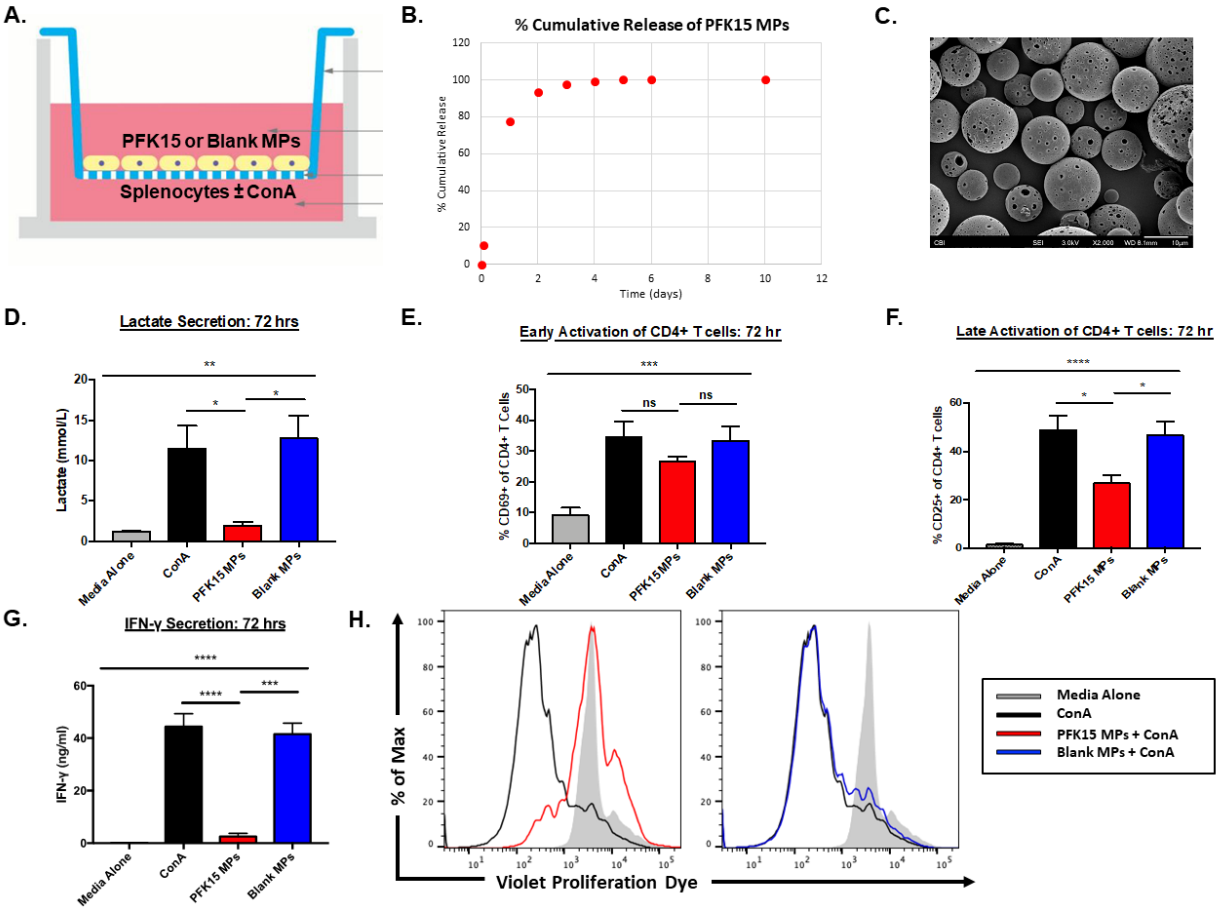


**Figure 9. PFK15 MPs display quick kinetic release, and reduces T cell responses to ConA stimulation similarly to soluble drug.**

Assessing the impact of PFK15 MPs on diabetogenic $CD4^+$ T cell responses *in vitro*. **A.** Schematic diagram detailing our experimental design. **B.** Release kinetics of PFK15 MPs**. C.** Scanning electron micrograph of PFK15 MPs. **D.** Lactate measurements in cell culture supernatants (n = 3). **E**. Frequency of $CD4^+$ $CD69^+$ T cells (n = 3). **F.** Frequency of $CD4^+$ $CD25^+$ T cells (n = 3). **G.** ELISA analysis of IFNγ in cell culture supernatants 72 hrs post stimulation (n = 3). **H.** Representative histogram of $CD4^+$ T cell proliferation assessed by cell proliferation dye violet (CPDV) dilution (n = 3). All data are presented as the mean ± SEM. (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.005$, **** = $p < 0.0001$).

### 2.4.4 PFK15 MPs inhibit antigen- specific T cell responses *in vivo,* with no impact on global immune function

Administration of PFK15 MPs to *in vitro* ConA stimulated NOD splenocytes decreased the activation, proliferation, and effector function of $CD4^+$ T cells, however whether they are able to dampen responses to nominal antigens *in vivo* remained unknown (Figure 9). To test the use of PFK15 MPs in an *in vivo* model of T cell activation, non- diabetic NOD animals were immunized with hen egg lysozyme (HEL) emulsified in Complete Freund's Adjuvant (CFA). On the same day, PFK15 or blank MPs were injected subcutaneously at sites near each of the inguinal draining lymph nodes (dLN). HEL- specific T cell responses were measured 1 week post immunization, when dLNs and spleens were harvested from blank MP and PFK15 MP treated animals. Cells from the dLNs and spleens were plated and stimulated with HEL in an *in vitro* recall assay for 72 hours to measure activation and effector cytokine (IFNγ) production (Figures 10-11).

In the dLN, we observed no differences in the number of cells recovered from blank or PFK15 MP treated animals, indicating that PFK15 treatment had no impact on T cell numbers and did not cause lymphopenia (Figure 10A). We also assessed expansion of HEL-specific $CD4^+$ T cells by tetramer staining, and observed reduced frequencies of antigen specific T cells in response to stimulation with HEL from PFK15 treated animals, indicating decreased expansion (Figure 10B). Consistent with *in vitro* studies (Figure 7 and 9), HEL specific T cells failed to upregulate CD69 upon encounter with their antigen, as evidenced by reduced activation marker expression compared to T cells from control blank MP treated animals (Figure 10C). Finally, T cells from the dLN of PFK15 MP treated animals secreted less IFNγ than dLN cells from blank MP treated animals, confirming that glycolysis inhibition reduces T cell responses to HEL immunization *in vivo* (Figure 10D).

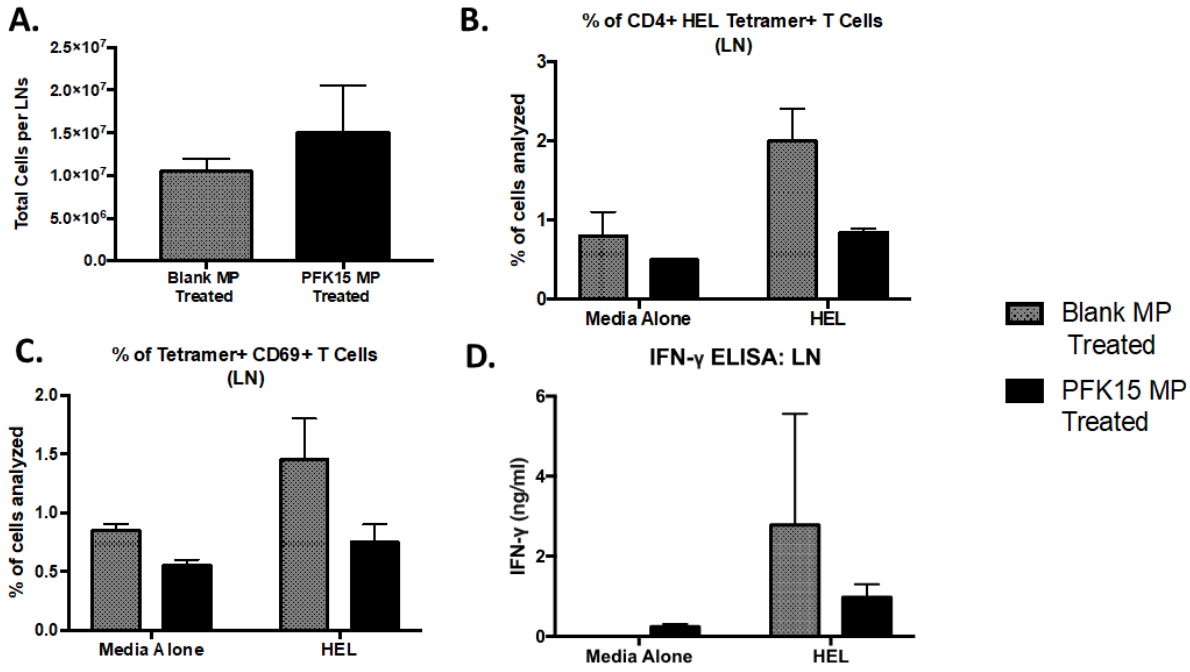


**Figure 10. PFK15 MPs inhibit antigen- specific T cell responses in the dLN.**
NOD animals were immunized with HEL emulsified in CFA, with animals receiving either blank or PFK15 MP treatment. 1 week post immunization, dLN cells were stimulated with HEL in an *in vitro* recall assay for 72 hours. **A**. Total number of cells isolated from the inguinal dLNs. **B.** Frequency of HEL specific T cells in the dLN. **C**. Frequency of HEL specific T cells expressing CD69. **D**. ELISA analysis of IFNγ in cell culture supernatants 72 hrs post stimulation. (n = 2 animals/ group); All data are presented as the mean ± SEM.

We also assessed HEL- specific T cell responses from the spleens of blank and PFK15 MP treated animals, as a measurement of the impact MP delivery has on T cells at a site that's not in close proximity to the local micromilieu where MPs were subcutaneously implanted. Similar to results obtained from the dLN, PFK15 MPs failed to cause a lymphopenia, as there were equal numbers of cells recovered from the spleens of blank and PFK15 MP treated animals (Figure 11A). In contrast to the reduced T cell responses caused by glycolysis inhibition in the dLN with PFK15 MP treatment, we saw no differences in the frequency of HEL- specific T cells (Figure 11B), CD69 expression on HEL- specific T cells (Figure 11C), or in the ability of splenocytes from PFK15 MP treated animals to secrete IFNγ in recall responses to HEL antigen *in vitro* compared to splenocytes from blank MP treated animals (Figure 11D). Together, these data, along with the data presented in Figure 10, demonstrate an ability to specifically reduce T cell responses to nominal antigen immunization *in vivo*, and more importantly provide evidence that delivery of PFK15 MPs have no impact on global immunity, as T cell responses in the spleen, at a site away from where PFK15 MPs were administered, remained unaffected compared to T cell responses in the dLN.

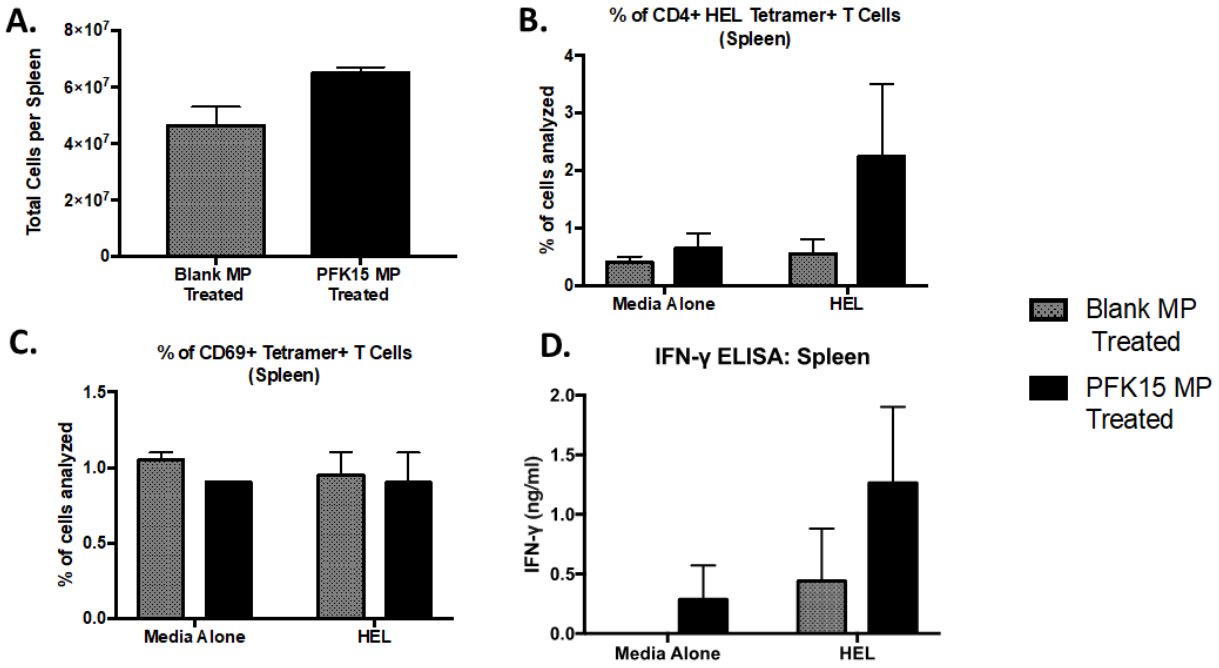


**Figure 11. PFK15 MPs do not affect T cell responses to HEL immunization in the spleen.**
NOD animals were immunized with HEL emulsified in CFA, with animals receiving either blank or PFK15 MP treatment. 1 week post immunization, splenocytes were stimulated with HEL in an *in vitro* recall assay for 72 hours. **A**. Total number of cells isolated from the spleens. **B.** Frequency of HEL specific T cells. **C**. Frequency of HEL specific T cells expressing CD69. **D**. ELISA analysis of IFNγ in cell culture supernatants 72 hrs post stimulation. (n = 2 animals/ group); All data are presented as the mean ± SEM.

### 2.4.5 Soluble PFK15 prevents, while PFK15 MP formulations delay T1D onset *in vivo*

Glycolysis inhibition via PFK15 treatment reduced T cell responses to ConA stimulation *in vitro*, and to HEL immunization *in vivo*, indicating that PFK15 treatment may have therapeutic benefit in preclinical models of T1D, an autoimmune disorder whereby autoreactive T cells are aberrantly activated and target β cells for destruction. To test the preventative capabilities of PFK15 in a mouse model of T1D, we utilized an adoptive transfer model. Briefly, splenocytes from NOD.BDC2.5.TCR.Tg animals, a mouse model derived from the diabetogenic $CD4^+$ specific BDC2.5 T cell clone, were adoptively transferred into immunodeficient NOD.*scid* recipients as previously described [121, 123]. This model leads to fulminant $CD4^+$ T cell mediated diabetes in approximately 14 days. Recipient animals were divided into three cohorts: 1) control animals treated with blank MPs; 2) PFK15 MP treatment; 3) Soluble PFK15 treatment. MPs were administered subcutaneously near the spleen, as the pancreas in an effort to release PFK15 near the pancreas (Figure 12A). As expected, a majority (80%) of animals in the control group were hyperglycemic by the end of the study period. While 100% of animals remained diabetes free in the soluble PFK15 treatment group, 50% remained diabetes free in the PFK15 MP group (Figure 12B). Although a portion of animals in the PFK15 MP treated group succumbed to diabetes, onset of disease was delayed compared to blank MP treated animals. Further, it's not entirely surprising that soluble PFK15 treatment led to more robust protection from disease in comparison to PFK15 MPs, as animals receiving MPs were administered $1/4^{th}$ the total dose of animals receiving systemic delivery of soluble drug. Interestingly, PFK15 treatment in any form had no impact on body weights and did not cause lymphopenia, indicating that even systemic delivery did not cause any overt toxicity (Figure 12C-D).

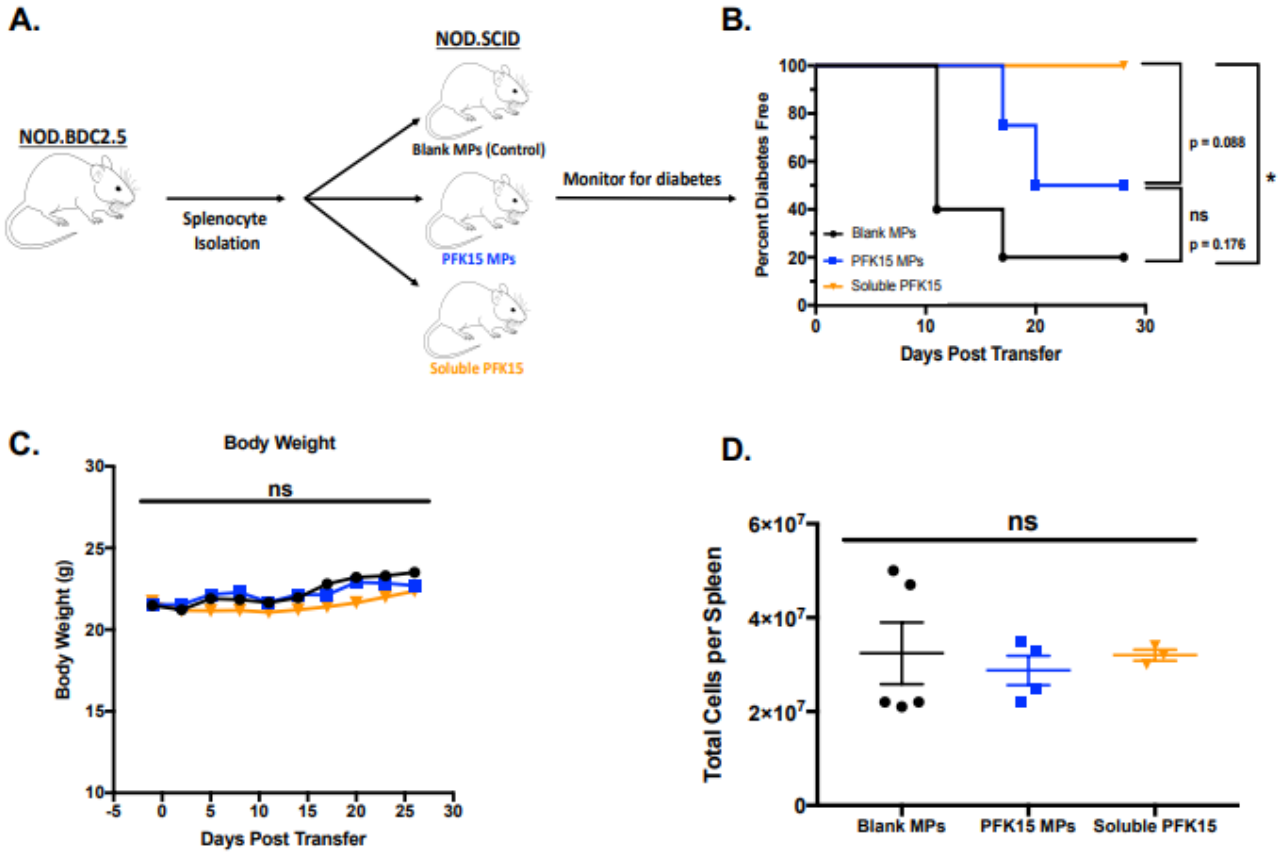


**Figure 12. PFK15 MPs delay T1D onset in an adoptive transfer model.**
The effect glycolysis inhibition has on Type 1 Diabetes onset was assessed using an adoptive transfer model. **A.** Schematic diagram of experimental design for adoptive transfer studies. **B.** Survival analysis of diabetes incidence in blank MP, PFK15 MP, and PFK15 treated groups. Kaplan-Meier survival analysis test was performed for statistical significance. **C.** Body weight measurements. **D.** Total cells isolated from spleens in mice from all three treatment groups**.** All data are presented as the mean ± SEM. (n = 3-5 animals/ group; * = $p < 0.05$).

### 2.4.6 Investigating the mechanisms leading to tolerance in PFK15 treated animals

To determine the mechanisms leading to tolerance due to glycolysis inhibition in PFK15 treated animals, we performed a global histological assessment to assess changes in islet infiltration by H&E staining for gross histology, as well as immunofluorescent staining for insulin, TUNEL (indicator of apoptosis), and FoxP3 (Treg marker). Control animals displayed invasive insulitis and a loss in insulin staining, correlating with disease status. In contrast PFK15 soluble drug and MP treated animals had reduced islet infiltration with peri- islet insulitis observed, indicating an ability for immune cells to make it to pancreatic islets, albeit without the ability to completely penetrate and cause disease (Figure 13A-F). Further, PFK15 treatment was not associated with induction of apoptosis, as indicated by no appreciable differences observed by TUNEL staining of pancreatic tissue sections (Figure 12G-I). Finally, glucose restriction in the TME, and glycolysis inhibition during $CD4^+$ T cell differentiation *in vitro*, have been shown to drive T cell exhaustion and/ or induce Treg generation, respectively [155]. To assess this, we measured FoxP3 expression in pancreatic tissue sections, and assessed T cell exhaustion markers CTLA-4, PD-1, and LAG-3 on $CD4^+$ T cells from the peripheral blood of a subset of protected animals who received PFK15 soluble drug, and donor BDC2.5 animals to compare baseline levels. Interestingly, we observed increased FoxP3 expression in pancreatic islets of PFK15 treated animals compared to controls (Figure 13J-L), and an increased frequency of $CD4^+$ T cells expressing immune inhibitory receptors CTLA-4, PD-1, and LAG-3 (Figure 13M). Together, these data demonstrate that the protective benefits associated with PFK15 treatment are due to induction of Tregs that reside in the islets and mediate protection against effector T cells. Moreover, increased IR expression could indicate the induction of T cell exhaustion, however, further studies are required to confirm these data and are explored in more detail in chapter 3.

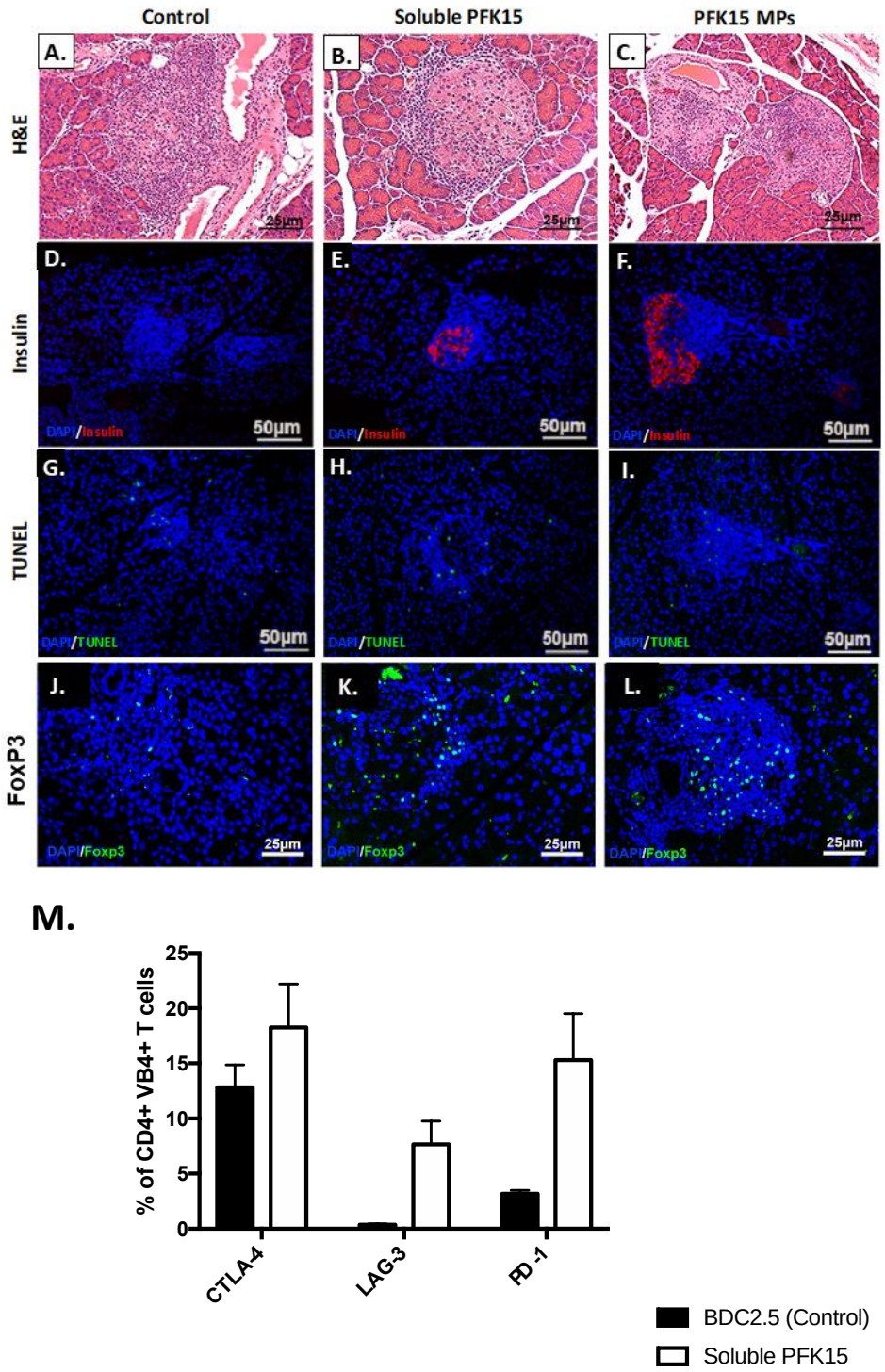


**Figure 13. Interrogating the mechanisms of PFK15 mediated protection.**

**A-C.** Representative H&E staining to assess islet infiltration in pancreatic sections. **D-F.** Representative pancreatic tissue immunostaining for insulin co-stained with DAPI. **G-I.** Representative pancreatic tissue TUNEL staining for apoptosis co-stained with DAPI. **J-L.** Representative pancreatic tissue immunostaining for FoxP3 co-stained with DAPI. M. Frequency of $CD4^+$ T cells expressing CTLA-4, LAG-3, and PD-1 in the peripheral blood compared to control BDC2.5 animals at baseline. All data are presented as the mean ± SEM. (n = 10 BDC2.5 control animals and n = 2 PFK15 soluble drug treated animals).

## 2.5 DISCUSSION

In this study, we provide evidence that T cells from the autoimmune prone NOD mouse are susceptible to metabolic modulation with the glycolysis inhibitor PFK15 when stimulated *in vitro* with global T cell activator ConA and immunized with nominal antigen HEL *in vivo*. Moreover, PFK15 treatment had beneficial therapeutic capabilities, as we have demonstrated an ability to restrain autoreactive $CD4^+$ T cell mediated damage of pancreatic β cells in an adoptive transfer model of T1D. Although the benefits associated with glycolysis inhibition of T cell proliferation and IFNγ secretion are not entirely novel, this is the first study testing the use of the inhibitor PFK15 to suppress T cell mediated autoimmunity in T1D [155]. Further, use of MPs delivering glycolysis inhibitors have never been reported or used in settings of autoimmunity, and underlies another novel aspect of this study.

A limitation of the current study lies in the lack of an antigen- specific stimulus for T cell activation. Instead, we used the global T cell activator ConA, as it is more realistic to the other non- antigen specific alternative αCD3/αCD28 due to its reliance on APC and T cell interaction [163, 164]. However, these data provided proof of principle evidence suggesting that use of PFK15 could have a therapeutic benefit in modulating aberrant T cell activation that occurs during the pathogenesis of T1D. Further, we performed an *in vivo* prevention study where PFK15 was able to prevent adoptive transfer of diabetes, indicating the results we obtained in our *in vitro* studies translated well to *in vivo* experiments. In light of this limitation and to expand on this study, we investigated the ability to modulate T cell glycolysis during activation with specific antigen in chapter 3, where we stimulated diabetogenic T cells from the NOD.BDC2.5 mouse with their peptide mimotope; thus allowing for an investigation into how glycolysis inhibition alters T cells responses to diabetes relevant antigens.

Use of PFK15 MPs were able to recapitulate soluble drug administration *in vitro*, and prevent diabetes onset in 50% of animals in adoptive transfer studies. These finding are important as treatment with $1/4^{th}$ the total dose of PFK15 administered via MPs compared to the dose delivered systemically had therapeutic benefits. Although beneficial, we decided to focus the remainder of our studies for this work using soluble drug. However, MP therapies have shown great promise in the treatment of T1D in mouse models, and we plan to further optimize the dose and delivery of PFK15 to reduce systemic delivery and allow for a more targeted approach to use of glycolysis inhibitors [167].

In an adoptive transfer study, PFK15 soluble drug and MP formulations were capable of delaying and in some cases completely prevent the onset of T1D, suggesting an ability to modulate autoreactive T cell responses. Specifically, we observed increased expression of FoxP3 within pancreatic islets of treated animals, indicating increased Tregs. Further, T cells in the peripheral blood of treated animals had increased expression of the checkpoint molecules CTLA-4, PD-1, and LAG-3, potentially indicative of possible T cell exhaustion [17, 29, 154, 155, 168]. Regarding Tregs, glycolysis inhibition has been shown to promote the differentiation of Tregs [71]. However, it remains unknown whether we actually induced the differentiation of Tregs, or whether reduced effector T cell responses due to PFK15 treatment selected for Tregs, allowing them to maintain their stability and mediate their suppressive function. Further studies are needed to confirm whether PFK15 mediated tolerance is dependent on Tregs, or whether an increased frequency of Tregs was observed due to selective targeting of effector T cells, thus leaving Treg frequency and function intact.

In the TME, nutrient competition between tumors and T cells drives T cell exhaustion, indicating that reduced glycolytic flux may contribute to T cell hyporesponsiveness [108, 155].

We observed increased expression of checkpoint molecules on T cells in the peripheral blood of PFK15 treated animals. This, along with the hyporesponsive phenotype of transferred T cells based on their inability to cause disease as efficiently as T cells in control animals indicates the induction of possible T cell exhaustion. However, the expression of IRs alone is not enough to mark exhausted T cell subsets, effector T cells transiently upregulate these molecules upon activation [155, 169]. Further experiments are required to fully understand the extent to which PFK15 treatment induces T cell exhaustion. These studies were performed and are a part of the studies detailed in chapter 3.

In conclusion, we demonstrated for the first time an ability to modulate autoreactive T cell effector function by specifically targeting the glycolysis pathway in T1D. Although the therapeutic benefits associated with glycolysis inhibition have been extensively reported in the literature, and have proven effective at controlling T cell responses during autoimmunity, targeting this pathway in T1D have remained largely unexplored. Moreover, our findings indicating an ability to prematurely exhaust $CD4^+$ T cells by modulating their ability to utilize glycolysis is novel, and has never been reported in the literature. Although future experiments are required to further our understanding of the impact PFK15 has on T cell responses in T1D, these data suggest that use of metabolic inhibitors may be efficient in controlling autoreactive T cell responses and preventing disease onset in those genetically predisposed to autoimmunity.

# 3.0 GLYCOLYSIS INHIBITION INDUCES FUNCTIONAL AND METABOLIC EXHAUSTION OF CD4$^+$ T CELLS IN TYPE 1 DIABETES

This is an adaptation of the author's version of the work. The definitive version is published in *Frontiers in Immunology*.

**"Glycolysis Inhibition induces Functional and Metabolic Exhaustion of CD4$^+$ T cells in Type 1 Diabetes"**

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## 3.1 SUMMARY

In Type 1 Diabetes (T1D), $CD4^+$ T cells initiate autoimmune attack of pancreatic islet β cells. Importantly, bioenergetic programs dictate T cell function, with specific pathways required for progression through the T cell lifecycle. During activation, $CD4^+$ T cells undergo metabolic reprogramming to the less efficient aerobic glycolysis, similarly to highly proliferative cancer cells. In an effort to limit tumor growth in cancer, use of glycolytic inhibitors have been successfully employed in preclinical and clinical studies. This strategy has also been utilized to suppress T cell responses in autoimmune diseases like Systemic Lupus Erythematosus (SLE), Multiple Sclerosis (MS), and Rheumatoid Arthritis (RA). However, modulating T cell metabolism in the context of T1D has remained an understudied therapeutic opportunity. In this study, we utilized the small molecule PFK15, a competitive inhibitor of the rate limiting glycolysis enzyme 6-phosphofructo-2-kinase/fructose-2,6- biphosphatase 3 (PFKFB3). Our results confirmed PFK15 inhibited glycolysis utilization by diabetogenic $CD4^+$ T cells and reduced T cell responses to β cell antigen *in vitro*. In an adoptive transfer model of T1D, PFK15 treatment delayed diabetes onset, with 57% of animals remaining euglycemic at the end of the study period. Protection was due to induction of a hyporesponsive T cell phenotype, characterized by increased and sustained expression of the checkpoint molecules PD-1 and LAG-3 and downstream functional and metabolic exhaustion. Glycolysis inhibition terminally exhausted diabetogenic $CD4^+$ T cells, which was irreversible through restimulation or checkpoint blockade *in vitro* and *in vivo*. In sum, our results demonstrate a novel therapeutic strategy to control aberrant T cell responses by exploiting the metabolic reprogramming of these cells during T1D. Moreover, the data presented here highlight a key role for nutrient availability in fueling T cell function and has implications in our understanding of T cell biology in chronic infection, cancer, and autoimmunity.

## 3.2 INTRODUCTION

Invasion of pancreatic islets by immune cells is a hallmark of Type 1 Diabetes (T1D), where the innate and adaptive immune systems work cooperatively to mediate damage of insulin- secreting β cells [33, 45]. This attack is largely orchestrated by self-reactive $CD4^+$ T cells, which are fundamental drivers of disease pathology [45]. During their life cycle, $CD4^+$ T cells rely on specific metabolic pathways to generate energy in the form of adenosine triphosphate (ATP) [59, 60]. However, it has become abundantly clear that these programs are not merely for energy production, but rather are necessary for the ability of T cells to carry out specialized effector capabilities, including interferon gamma (IFNγ) secretion [107]. During homeostasis, naïve $CD4^+$ T cells utilize oxidative phosphorylation (OXPHOS) to support surveillance efforts and migration in the periphery. Upon encounter with antigen (i.e., islet β cells in T1D), activated T cells undergo robust metabolic reprogramming marked by a transition to the less efficient aerobic glycolysis [59, 60]. Although net energy obtained through the glycolysis pathway is far less than what is generated via OXPHOS (net gain of 2 versus approximately 36 ATP molecules, respectively), glycolysis is required to generate ATP quickly to support T cell activation, clonal expansion, and effector cytokine production [107].

While distinct metabolic programs dictate T cell differentiation and function, a number of studies have also implicated nutrient availability as an important determinant of T cell fitness. This is especially evident in the tumor microenvironment (TME), where reports have demonstrated that cancer cells outcompete T cells for key nutrients like glucose and amino acids, which are required for acquisition of specialized effector functions [108]. This battle for metabolic substrates, along with persistent antigen exposure, has been implicated in suppressing immune responses and driving T cell exhaustion. Exhaustion is defined as a state of dysfunction characterized by

increased expression of immune inhibitory receptors (IRs) like programmed cell death protein- 1 (PD-1) and lymphocyte Activation Gene-3 (LAG-3), and a marked decrease in T cell effector functions that allow tumors to go unabated by the immune system [108, 169]. Although metabolic insufficiencies demonstrate a major hurdle in reinvigorating tumor-specific T cell responses, the opposite is plausible in settings of autoimmunity, where enforcing T cell exhaustion by targeting metabolism may be a novel mechanism by which tolerance to self-antigens is restored [129].

Efforts to target the glycolysis pathway have been successfully employed to limit tumor growth and metastasis in cancer, with a number of these inhibitors undergoing FDA clinical trials [15, 152, 159, 170]. One such inhibitor, PFK15, is a small molecule inhibitor of 6-phosphofructo-2-kinase/fructose-2,6- biphosphatase 3 (PFKFB3), an enzyme involved in a cells commitment to metabolize glucose via glycolysis [152, 159, 160]. Regarding autoimmunity, researchers have successfully targeted metabolism as a means to control T cell responses in models of Systemic Lupus Erythematosus (SLE), Multiple Sclerosis (MS), and Rheumatoid Arthritis (RA), however the ability to target metabolic pathways to control T1D have been largely unstudied [84, 86, 87, 115] . Previously, our laboratory demonstrated that redox modulation via disruption of third signal reactive oxygen species (ROS) impeded T cell metabolic reprogramming to glycolysis, and altered the diabetogenic potential of autoreactive $CD4^+$ T cells [123]. However, whether specifically modulating the glycolysis pathway could be used to limit the activation of autoreactive $CD4^+$ T cells and prevent attack of pancreatic β cells deserves further exploration. Based on these previous studies, we hypothesized that use of the glycolysis inhibitor PFK15 would inhibit the activation, proliferation, and effector capabilities of autoreactive $CD4^+$ T cells, thereby delaying the onset of T1D. Herein, we demonstrate that PFK15 treatment interrupts metabolic reprogramming to glycolysis upon activation with β cell antigen, and reduces T cell responses *in vitro*, while delaying

the onset of T1D *in vivo*. These results were, in part, due to increased and sustained expression of checkpoint molecules PD-1 and LAG-3 and downstream functional and metabolic exhaustion of diabetogenic T cell clones. These findings support that inhibition of glycolysis drives T cell exhaustion, and that metabolic modulation may serve as a novel therapeutic target to control T cell metabolism and restore tolerance in autoimmunity.

## 3.3 MATERIALS AND METHODS

### 3.3.1 Animals

Non-obese diabetic (NOD), NOD.BDC2.5.TCR.Tg (BDC2.5) and NOD.*scid* mice were maintained under specific pathogen free conditions in the animal facility located at the Rangos Research Center within UPMC Children's Hospital of Pittsburgh. All animal experiments were approved by the University of Pittsburgh's Institutional Animal Care and Use Committee (IACUC; Assurance Number: D16-00118). Male and female mice aged 6-12 weeks old were used in all experiments.

### 3.3.2 Splenocyte isolation and *in vitro* stimulation

BDC2.5 animals were sacrificed, and spleens harvested and homogenized into single cell suspensions as described [123]. Red blood cells were lysed using RBC lysis buffer (Sigma Aldrich). For *in vitro* experiments, 2.5 x $10^6$ splenocytes were plated per well in 24 well plates and stimulated with 0.05 μM of their peptide mimotope ± 5 μM PFK15 soluble drug (Selleck), 0.2-1 mM 2-DG (Sigma Aldrich), 25-50 μM YN1 (Millipore Sigma), or 2.5-5 μM PFK158 (Selleck) for

24-72 hours. Cells and culture supernatants were collected for downstream flow cytometry, western blotting, ELISA, and lactate measurement analyses.

### 3.3.3 Flow Cytometry

$5x10^5 – 1x10^6$ cells were harvested at indicated timepoints and surface stained for flow cytometric analysis as described [123]. Briefly, cells were incubated with Fc block (CD16/CD32; BD Biosciences) for 15 minutes prior to staining for flow cytometry. Surface staining was performed at 4°C using CD4-PE, PerCP-Cy5.5, or APC (Clone RM4-5), CD69-PeCy7 (Clone H1.2F3), CD25-APC (Clone PC61), CD223-PE (LAG-3; Clone C9B7W), CD279-BV480 (PD-1; Clone J43) antibodies (BD Biosciences) in FACS buffer (1% BSA in PBS). For proliferation measurements, splenocytes were labeled with Cell Proliferation Dye Violet (BD Bioscience) per manufacturer's instructions prior to stimulation. After indicated timepoints, cells were harvested, and surface stained as described above. In some instances, cells were fixed in 2% PFA for 15 minutes at 4°C (Thermo Fisher Scientific). Cells were stored at 4°C until time of analysis.

To measure glucose uptake, splenocytes were incubated with 100 μM of the fluorescent glucose analog 2-(*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) Amino)-2-Deoxyglucose (2-NBDG; Cayman Chemical) for 10 minutes at 37°C prior to harvest as described [110, 123]. Cells were washed with PBS and surface stained for CD4 expression and analyzed live by flow cytometry. To measure fatty acid uptake, cells were harvested, surface stained for CD4 expression, and incubated with the fluorescent fatty acid BODIPY FL C16 (4,4-Difluoro-5,7-Dimethyl-4-Bora-3a,4a-Diaza-*s*-Indacene-3-Hexadecanoic Acid; Invitrogen) in serum-free warm PBS for 30 minutes at 37°C. Cells were washed with PBS and analyzed live by flow cytometry.

To assess mitochondrial function, Day 14 BDC2.5 T cell clones were harvested, surface stained for CD4 expression, and incubated with MitoTracker green, MitoSOX red, or TMRE (tetramethylrhodamine, ethyl ester, perchlorate; Invitrogen) in warm PBS at 37°C for 15-30 minutes. Cells were washed with PBS and analyzed live by flow cytometry as described previously [110]. For all flow cytometry studies, fluorescence was measured using a FACS Aria II flow cytometer (BD Biosciences). All data were analyzed using FlowJo software (v10.5.3) and samples were gated on $CD4^+$ cells. Forward scatter of $CD4^+$ T cells was also determined by flow cytometry.

### 3.3.4 Preparation of Protein Lysates and Western Blotting

Cells were lysed by sonication 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 (BCA) assay (Thermo Fisher Scientific). 25 μg of protein per sample were boiled in 6x Lammaeli buffer (BIORAD) for 5 minutes and separated on 4-20% gradient SDS-PAGE gels (BIORAD). Samples were then transferred to PVDF membranes for 2 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: Glut-1, CPT1α (1:1000; Abcam), HK2, PFKFB3, LDHA (1:1000; Cell Signaling), and β-actin as a loading control (1: 10,000; Sigma- Aldrich). Membranes were washed with TBST and incubated with HRP-conjugated goat anti-rabbit or rabbit anti-mouse (Jackson ImmunoResearch 1:10,000) secondary antibodies for 2 hrs in 5% non- fat milk/TBST at room temperature. Chemiluminescence was detected using SuperSignal West Pico PLUS Chemiluminescence solution (Thermo Fisher Scientific) and the iBright FL1500 imaging system (Invitrogen).

### 3.3.5 Lactate and Cytokine Measurements

Cell culture supernatants from *in vitro* experiments were harvested and used to measure IL-2, IFNγ, and TNFα by ELISA. Antibody pairs for IFNγ and IL-2 ELISAs were purchased from BD Biosciences, and TNFα ELISA kits were purchased from R&D according to the manufacturer's instructions. ELISAs were read on a SpectraMax M2 microplate reader (Molecular Devices) and data analyzed using SoftMax Pro version 7.0.2 software (Molecular Devices). Lactate, a byproduct of aerobic glycolysis, was measured using the Lactate Plus meter and test strips per manufacturer's instructions (Novus Biologics).

### 3.3.6 $CD4^+$ T cell Isolation and *ex vivo* Activation and Expansion

Spleens from BDC2.5 animals were harvested and homogenized into single cell suspensions as described above. $CD4^+$ T cells were isolated by magnetic bead separation using the EasySep $CD4^+$ T cell Negative Selection isolation kit (StemCell) per the manufacturer's instructions. For *ex vivo* activation, 6 well plates were coated with plate bound αCD3 (BD Biosciences; 1 ug/mL) in PBS for at least 3 hours in a cell culture incubator (37° C, 5% $CO_2$). The antibody solution was decanted, and $5x10^6$ isolated cells were plated with 100 U/mL IL-2 and 1 ug/mL soluble αCD28 (BD Biosciences) for 3 days in a cell culture incubator. After 3 days in culture, cells were harvested and transferred to T75 flasks with an additional 100 U/mL IL-2 for expansion. At the end of the 3-day expansion in IL-2, cells were isolated, counted, and washed with sterile PBS for adoptive transfer experiments.

### 3.3.7 Adoptive Transfer Model of T1D

1 x $10^7$ *ex vivo* activated $CD4^+$ T cells from BDC2.5 mice (described above) were injected i.p. into NOD.*scid* recipients. For initial prevention studies, recipient animals were split into two groups. One cohort of recipients received 25 mg/kg PFK15 (Selleck) dissolved in 5% DMSO $^+$ 45% PEG300 $^+$ 1% Tween80 $^+$ 49% dd$H_2O$ prepared fresh; the other cohort received vehicle control every other day for 2 weeks. In reversibility studies, recipient animals were placed into one of the following treatment groups: 1) Vehicle Control, 2) PFK15 $^+$ IgG (Isotype controls for αPD-1 and αLAG-3 blocking antibodies; 200 μg each per treatment), or 3) PFK15 + 200 μg αPD-1 (clone J43; BioXCell), + 200 μg αLAG-3 (clone C9B7W; BioXCell) as previously described [30, 110]. Animals were treated every other day for two weeks, with checkpoint blockade or IgG treatment initiated during the second week. Body weights and blood glucose (BG) levels were monitored over the course of the experiments. Animals were deemed diabetic after two consecutive BG readings ≥ 350 mg/dL. Diabetic animals were sacrificed at indicated timepoints and peripheral blood, pancreata, and spleens were harvested for downstream analyses.

### 3.3.8 Tissue Collection and Histological Assessment

Pancreatic tissue was collected and fixed in 4% paraformaldehyde (PFA; Thermo Fisher Scientific) overnight at 4° C. Fixed tissue was processed and embedded in paraffin by the Histology Core Laboratory located at UPMC Children's Hospital of Pittsburgh's Rangos Research Center [161]. Embedded tissue was sectioned at 4 μm thickness and stained with hematoxylin and eosin (H&E) for histological examination of immune infiltration in pancreatic islets. Samples were imaged using a Nikon Eclipse E800 microscope (Nikon) and associated software.

### 3.3.9 Immunofluorescent Staining

Immunofluorescent staining was performed on paraffin embedded samples prepared as described above. Antigen retrieval was performed in either sodium citrate or Tris-EDTA buffers followed by overnight incubation with primary antibodies against insulin (1:100; Santa Cruz), CD3 (1:100; Abcam), and PD-1 (1:50; Abcam) all co-stained with DAPI (1:3000; Thermo Fisher Scientific). The following day, slides were incubated with Alexa Fluor 488 conjugated donkey anti- rabbit secondary antibody (Invitrogen). Samples were imaged using a Leica DMi8 inverted microscope (Leica) and LAS X Navigator software (Leica).

### 3.3.10 Maintenance of BDC2.5 T cell Clones

$CD4^+$ MHC-II restricted BDC2.5 T cells, a generous gift from Dr. Kathryn Haskins (University of Colorado), were maintained in supplemented DMEM as previously described [38, 122, 171, 172]. Briefly, BDC2.5 T cells were cultured in T-25 flasks with β membrane (antigen), irradiated NOD splenocytes (antigen presenting cells: APC), and EL-4 supernatant (source of IL-2) for 2 weeks in a cell culture incubator. For mechanistic studies, a subset of flasks were treated with **5** μM PFK15 every third day over the course of the restimulation period. Day 8 and 14 T cells and culture supernatants were harvested for downstream analyses. Similarly, for reinvigoration studies, untreated and PFK15 treated T cells were put into restimulation flasks **± 5**μg/mL αPD-1 (clone J43; BioXCell), αLAG-3 (clone C9B7W; BioXCell), or αPD-1 $^+$ αLAG-3. Cells were treated every third day for 2 weeks.

### 3.3.11 ADP/ATP Ratio Measurements

Day 14 control and PFK15 treated T cell clones were harvested and assayed for the ADP/ATP ratio per the manufacturer's instructions (Millipore Sigma).

### 3.3.12 Statistical Analyses

All data are presented as mean values ± standard error of the mean (SEM), with n indicating the number of independent experiments or animals. Student's t-test, One- way ANOVA, or Two-way ANOVA were used where appropriate. For survival studies, 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. Histology and immunofluorescent images were generated using Photoshop. All statistics and graphs were generated using GraphPad Prism software.

## 3.4 RESULTS

### 3.4.1 PFK15 interrupts metabolic reprogramming to glycolysis and reduces T cell effector functions during activation

To determine the effect of glycolysis inhibition on the activation and subsequent metabolic reprogramming of autoreactive $CD4^+$ T cells in T1D, we stimulated splenocytes from NOD.BDC2.5.TCR.Tg animals *in vitro* with their cognate peptide mimotope (MM) ± PFK15 (a PFKFB3 inhibitor; Figure 14A), as previously described [123]. PFK15 treated BDC2.5 splenocytes failed to upregulate glycolysis-associated proteins glucose transporter-1 (Glut-1), hexokinase 2 (HK2), PFKFB3, and lactate dehydrogenase A (LDHA) in response to MM stimulation compared to T cells stimulated without treatment (Figure 14B). Additionally, PFK15

treatment decreased glucose (2-NBDG) uptake by $CD4^+$ T cells upon stimulation to levels similar to T cells in media alone (Figure 14C-D). A significant reduction in 2-NBDG fluorescence from treated $CD4^+$ T cells was most likely due to reduced expression of Glut-1 (Figure 14B), indicating decreased capacity to engage in aerobic glycolysis [37, 123]. Utilization of the glycolysis pathway by $CD4^+$ T cells is accompanied by increased secretion of the by-product lactate [59, 60, 121, 123]. Splenocytes stimulated with MM alone displayed a significant increase in lactate secretion in cell culture supernatants as expected, indicating increased glycolytic flux (Figure 14E). In comparison, PFK15 treated splenocytes secreted less lactate compared to MM stimulated T cells, further confirming an inability to metabolically transition to glycolysis upon encounter with β cell antigen (Figure 14E).

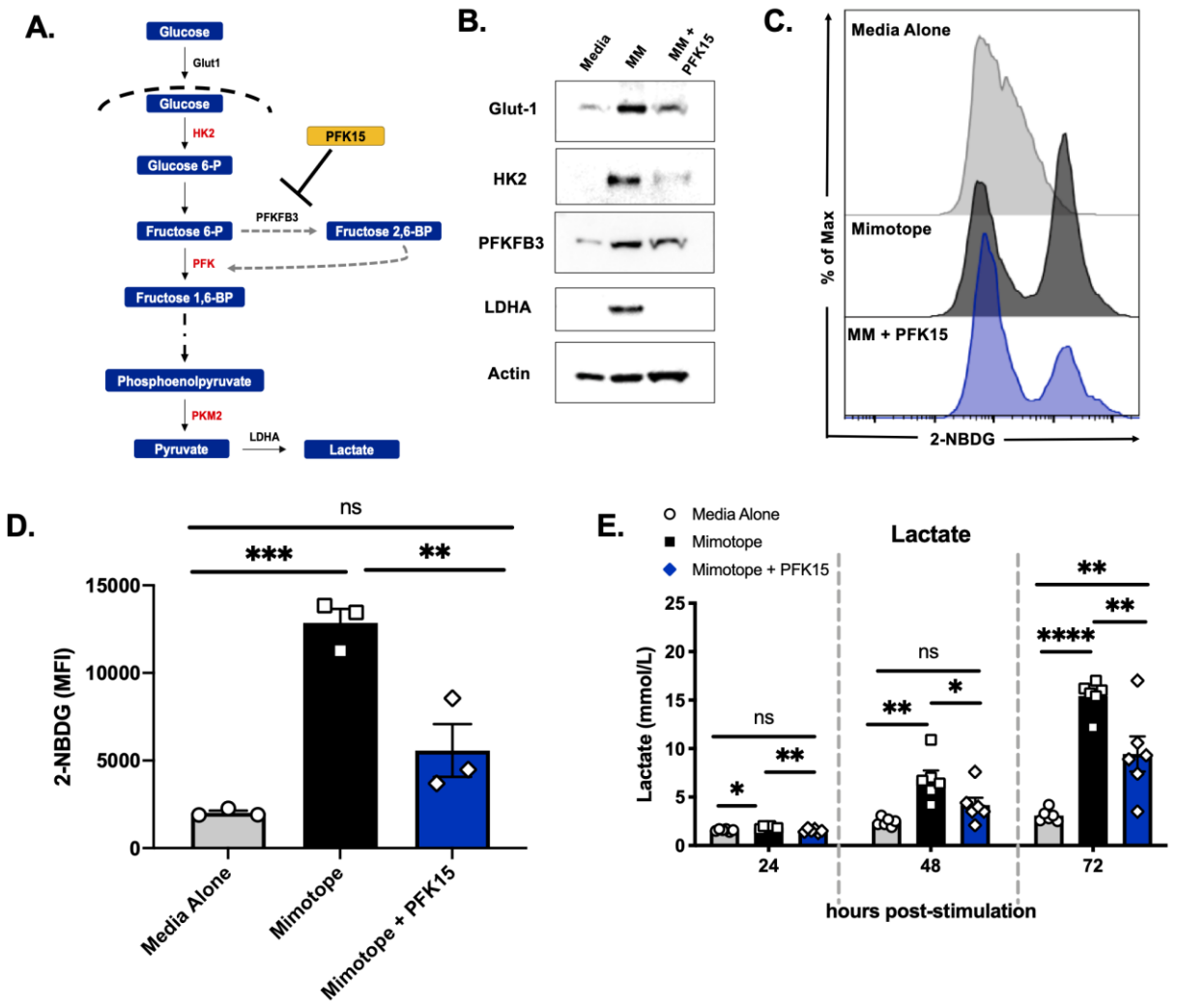


**Figure 14. PFK15 treatment inhibits metabolic reprogramming to glycolysis during T cell activation.** NOD.BDC2.5 splenocytes were stimulated with their cognate peptide MM ± PFK15 for 24-72 hrs. **A.** Schematic diagram displaying the mechanism of action of PFK15. **B.** Representative western blot analysis of glycolysis proteins in untreated, MM stimulated, and MM$^{+}$ PFK15 treated splenocytes after 48 hrs in culture. **C, D.** Representative histogram and statistical analysis showing 2-NBDG fluorescence and MFI of CD4$^{+}$ T cells, indicative of glucose uptake 48 hrs post stimulation (n = 3). **E.** Lactate measurements in cell culture supernatants (n = 6). All data are presented as the mean ± SEM. (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.005$, **** = $p < 0.0001$).

As bioenergetics and T cell function are intricately linked, we next determined the impact of glycolysis inhibition on the activation, proliferation, and effector capabilities of BDC2.5 T cells. While activated $CD4^+$ T cells displayed increased forward scatter (FSC) compared to unstimulated controls, indicative of increased cell growth, PFK15 treatment resulted in reduced FSC compared to stimulated T cells, indicating inhibited growth (Figure 15A). We then assessed proliferative capacity by measuring cell proliferation dye violet (CPDV) dilution by $CD4^+$ T cells in all three treatment groups. MM stimulated T cells displayed robust proliferation in response to antigen; however, PFK15 treatment significantly reduced this response (Figure 15B-C).

To interrogate the activation status of PFK15 treated T cells, expression of the early activation marker CD69, and the late activation marker and high- affinity interleukin-2 (IL-2) receptor CD25, was measured 48-72 hours post stimulation on $CD4^+$ T cells by flow cytometry (Figure 15D-E). There were no appreciable differences in the expression of CD69 in stimulated or PFK15 treated groups indicating that PFK15 treatment does not interfere with early activation (Figure 15D). Conversely, CD25 expression was significantly decreased with PFK15 treatment, suggesting an inability to fully transition to late activation status when glycolysis is inhibited (Figure 15E). Since glycolysis is required for acquisition of effector functions [107], we interrogated this ability by kinetically measuring IL-2, tumor necrosis factor alpha (TNFα), and IFNγ in cell culture supernatants (15F-H). ELISA analysis revealed a reduced ability to secrete all three cytokines. The phenotype we observed was due to specific targeting of PFKFB3, as treatment of BDC2.5 splenocytes with the prototypical glycolysis inhibitor 2-Deoxy-D-glucose (2-DG), a non-metabolizable glucose analog, was only capable of dampening IFNγ secretion, but not IL-2 or TNFα (Figure 26; Appendix A). However, activation of BDC2.5 splenocytes with two other known PFKFB3 inhibitors YN1 and PFK158 recapitulated our data with PFK15 (Figure 26;

Appendix A), further implicating glycolysis, and more specifically PFKFB3, as a vital metabolic pathway required for optimal activation and cytokine secretion by autoreactive effector T cells (Figure 15F-H).

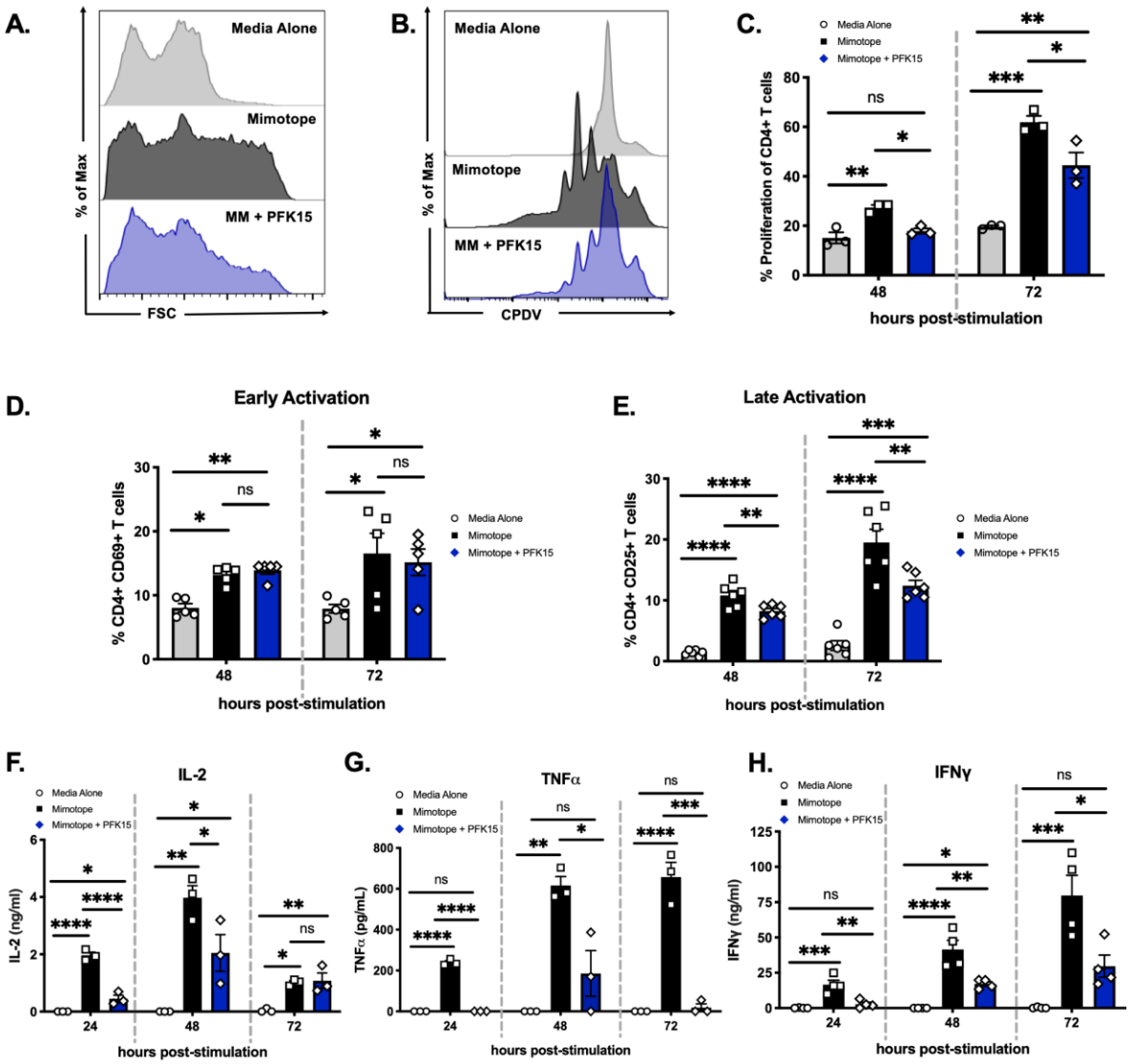


**Figure 15. Inhibiting glycolysis suppresses CD4$^+$ T cell responses to β cell antigen *in vitro*.**
Assessing the impact of glycolysis inhibition on diabetogenic CD4$^+$ T cell responses *in vitro*. **A.** Representative histogram measuring forward scatter (FSC). **B, C.** Representative histogram and statistical analysis of CD4$^+$ T cell proliferation assessed by cell proliferation dye violet (CPDV) dilution (n = 3). **D.** Frequency of CD4$^+$ CD69$^+$ T cells (n = 5). **E**. Frequency of CD4$^+$ CD25$^+$ T cells (n = 6). **F, G, H.** ELISA analysis of IL-2, TNFα, and IFNγ in cell culture supernatants 24-72 hrs post stimulation (n = 3-4). All data are presented as the mean ± SEM. (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.005$, **** = $p < 0.0001$).

### 3.4.2 Targeting glycolysis delays the onset of T1D in an adoptive transfer model

Based on the ability of PFK15 treatment to reduce BDC2.5 T cell responses, we examined the impact of glycolysis inhibition on the onset of diabetes using an adoptive transfer model (Figure 16A). Here, isolated $CD4^+$ T cells from the spleens of NOD.BDC2.5.TCR.Tg animals were activated and expanded *ex vivo* with plate- bound αCD3/αCD28 and EL-4 supernatant as a source of IL-2 as previously described [173]. We confirmed activation by measuring proinflammatory cytokine secretion in culture supernatants 3 days post activation, and observed significant levels of TNFα and IFNγ in BDC2.5 T cell cultures prior to adoptive transfer (Figure 27). Activated T cells were transferred via intraperitoneal (i.p.) injection into immunodeficient NOD.*scid* recipients. A cohort of animals received 25 mg/kg of PFK15 treatment beginning on the day of the adoptive transfer. Animals were treated every other day for two weeks, and monitored for drug related toxicity (body weight) and diabetes onset (BG ≥ 350 mg/dl). 100% of control animals exhibited diabetes 7 days post-transfer, however PFK15 treatment delayed disease onset, with 57% of animals remaining diabetes free for the duration of the study, with no appreciable weight loss observed (Figure 16B-C). Pancreases from control animals exhibited invasive insulitis, while protected animals treated with PFK15 displayed peri- islet insulitis as observed via H&E staining (Figure 16D-E). In agreement with this, immunofluorescent (IF) staining for the T cell marker CD3 revealed reduced T cell infiltration into the islets of PFK15 treated animals corresponding to an inability for T cells to completely penetrate the islets (Figure 16F-G). As expected, loss of insulin staining was observed in diabetic controls, with retention of insulin staining observed in PFK15 treated animals, correlating with disease status (Figure 16H-I). Lastly, PFK15 treatment had no impact on circulating $CD4^+$ T cell frequencies in the peripheral blood, however reduced $CD4^+$ T cell percentages were observed in the spleens of treated animals, indicating reduced

expansion of PFK15 treated T cells (Figure 16J). Analysis of CD25 on $CD4^+$ T cells in control and PFK15 treated animals revealed a significant decrease in the percentage of $CD4^+$ $CD25^+$ T cells in the periphery, however a higher percentage of $CD4^+$ T cells expressed CD25 in the spleens of treated animals compared to controls (Figure 16K). Although a larger percentage of $CD4^+$ T cells in the spleens of treated animals expressed CD25, treated animals had less $CD4^+$ T cell percentages in the spleens, indicating possible sequestration of effector- like T cells in the spleen compared to control animals, as glycolysis is required for proper T cell migration to sites of inflammation [174]. Together, these data indicate that metabolic modulation by PFK15 treatment alters the diabetogenic potential of activated $CD4^+$ T cells, thereby reducing the immunopathological parameters associated with disease onset.

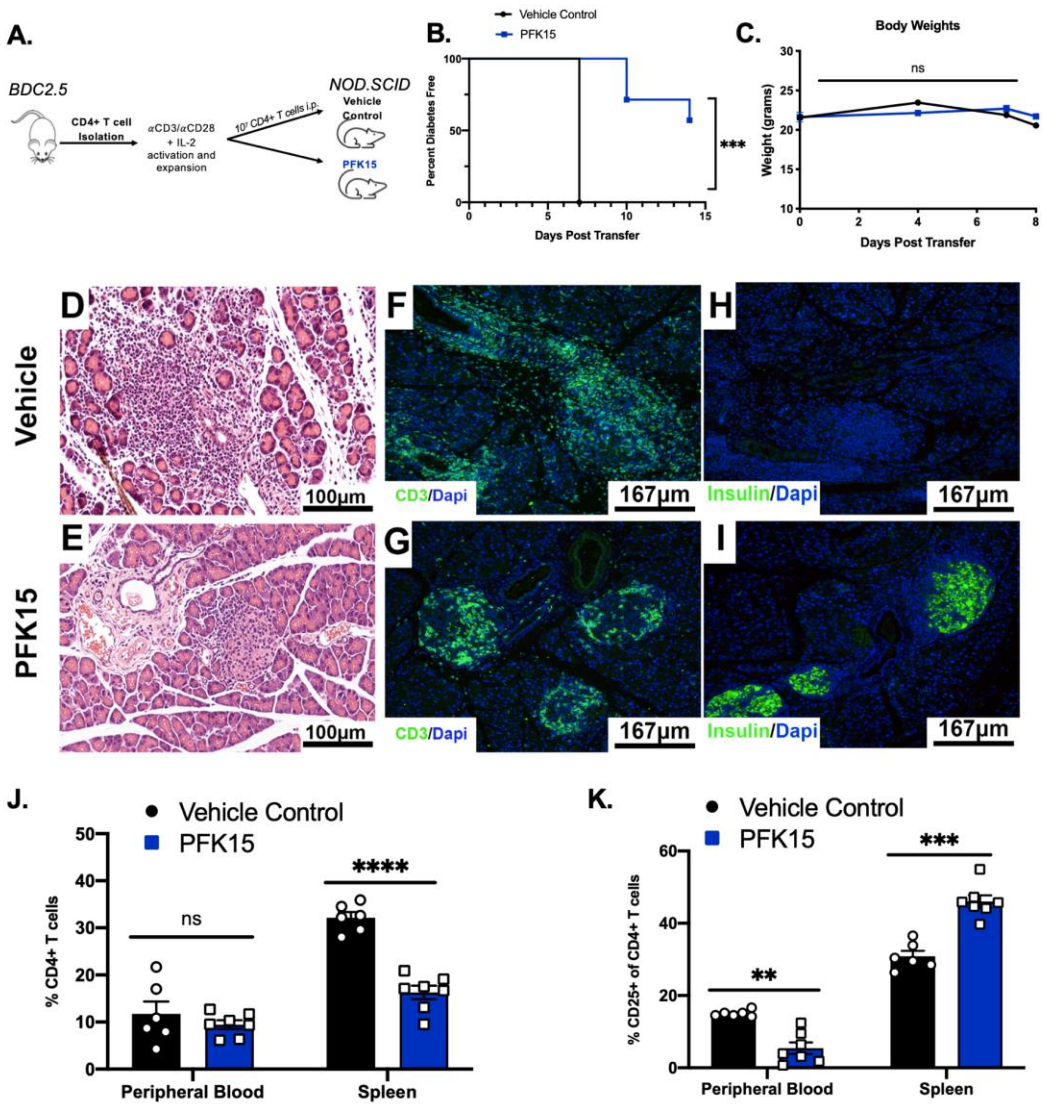


**Figure 16. PFK15 treatment alters the diabetogenic potential of autoreactive CD4$^+$ T cells and delays adoptive transfer of Type 1 Diabetes.**

The effect glycolysis inhibition has on Type 1 Diabetes onset was assessed using an adoptive transfer model. **A.** Schematic diagram of experimental design for adoptive transfer studies. **B.** Survival analysis of diabetes incidence in vehicle control and PFK15 treated groups. Kaplan-Meier survival analysis test was performed for statistical significance. **C.** Body weight measurements. **D, E.** Representative H&E staining to assess islet infiltration in pancreatic sections. **F, G.** Representative pancreatic tissue immunostaining for the T cell marker CD3 co-stained with DAPI. **H, I.** Representative pancreatic tissue immunostaining for insulin co-stained with DAPI. **J.** Frequency of CD4$^+$ T cells in the peripheral blood and spleens of PFK15 treated and control animals. **K.** Frequency of CD4$^+$ CD25$^+$ T cells in the peripheral blood and spleens. All data are presented as the mean ± SEM. (n = 6-7 animals/ group; ** = $p < 0.01$, *** = $p < 0.005$, **** = $p < 0.0001$).

### 3.4.3 PFK15 Treatment increases Expression of Checkpoint Molecules PD-1 and LAG-3 on $CD4^+$ T cells

In the tumor microenvironment (TME), metabolic restriction leads to increased IR expression and subsequent T cell exhaustion [108]. To investigate the mechanisms leading to T cell hyporesponsiveness and protection in PFK15 treated animals, we measured known markers of exhaustion, PD-1 and LAG-3, in the peripheral blood and spleens from control and PFK15 treated animals. Consistent with a hyporesponsive phenotype, PFK15 treatment led to significantly increased frequencies and expression of PD-$1^+$ (Figure 17A-C) and LAG-$3^+$ $CD4^+$ T cells (Figure 17D-F) in the peripheral blood and spleens. Since T cell exhaustion is associated with increased expression of multiple immune inhibitory receptors (IRs), we assessed co-expression of PD-1 and LAG-3 and found that T cells from PFK15 treated animals retained high expression of both PD-1 and LAG-3 on circulating $CD4^+$ T cells and T cells in the spleens (Figure 17G-H). Effector T cells transiently upregulate checkpoint molecules early during activation to temper their initial response and clonal burst [169, 175]. To phenotypically characterize differences between PD-$1^+$ LAG-$3^+$ T cells in control and PFK15 treated animals, we measured the percentage of PD-$1^+$ LAG-$3^+$ T cells expressing the activation marker, CD25. Strikingly, we observed a higher percentage of PD-$1^+$ LAG-$3^+$ T cells in the peripheral blood of control animals expressing CD25, indicative of an effector phenotype, while PD-$1^+$ LAG-$3^+$ T cells in PFK15 treated animals had decreased CD25 expression, consistent with an exhaustion phenotype (Figure 17I). Similar to our analysis of CD25 on $CD4^+$ T cells in Figure 16K, no differences in CD25 expression were observed in the spleen of control and PFK15 treated animals, providing further evidence for sequestration of effector like T cells due to the importance of both glycolysis and PD-1 signaling in T cell trafficking [167, 174]. Finally, to determine the impact glycolysis inhibition had on T cell responses in the pancreas, we stained pancreatic sections for PD-1. Indeed, PFK15 treated animals displayed increased PD-1

staining in the pancreatic islets compared to diabetic control animals (Figure 17J-K). These results demonstrate that PFK15 treatment results in increased frequency of $CD4^+$ T cells expressing checkpoint molecules PD-1 and LAG-3 *in vivo*, suggesting that inhibition of glycolysis induces potential T cell exhaustion thereby contributing to delayed T1D onset.

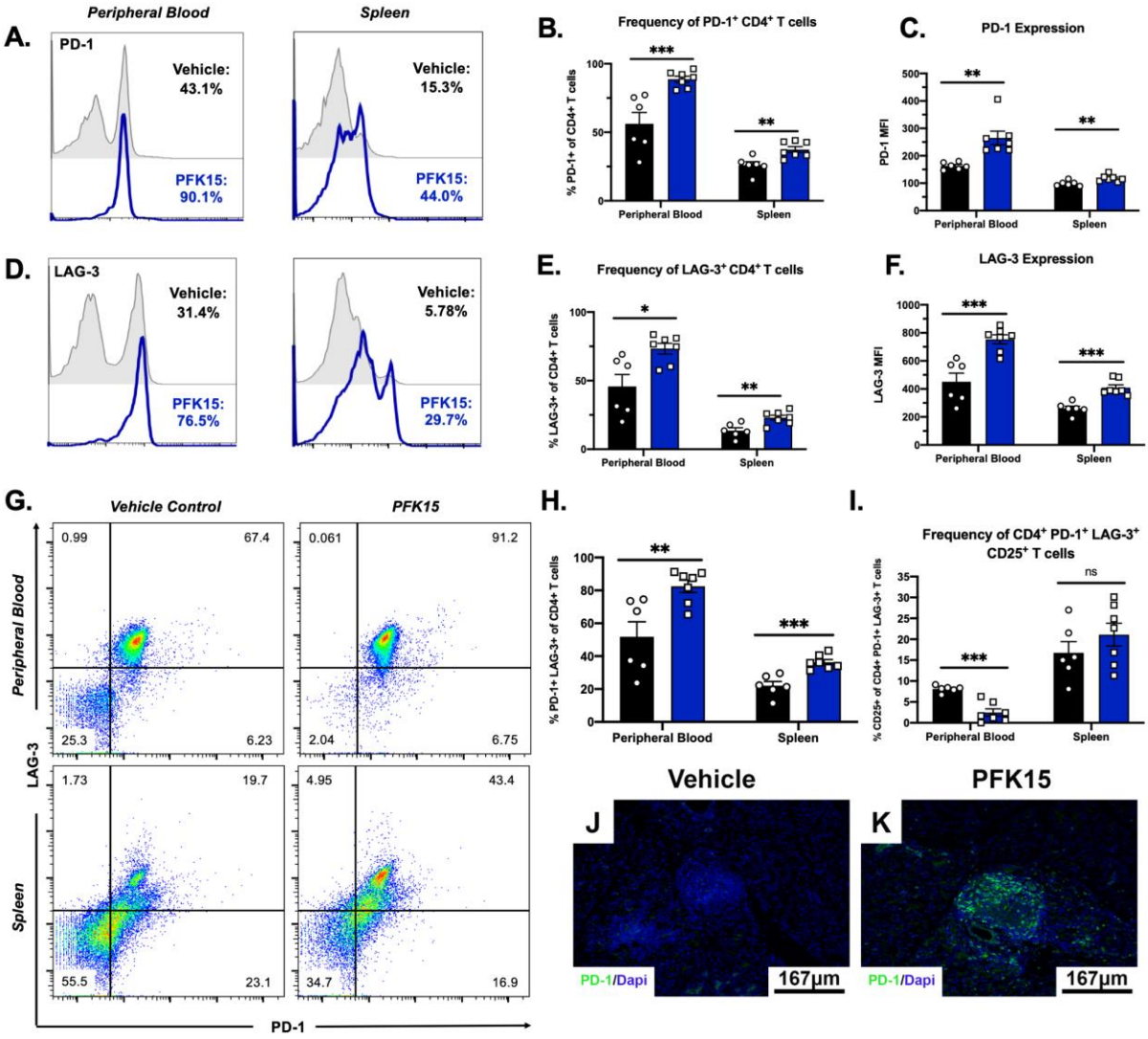


**Figure 17. Inhibition of glycolysis results in increased expression of PD-1 and LAG-3 on CD4$^+$ T cells.**
PD-1 and LAG-3 expression were assessed on CD4$^+$ T cells in PFK15 treated and control animals. **A, B.** Representative histogram and statistical analysis measuring the frequency of PD-1$^+$ CD4$^+$ T cells in the peripheral blood and spleen of PFK15 treated and control animals. **C.** Statistical analysis of PD-1 expression (MFI) on CD4$^+$ T cells in the peripheral blood and spleens. **D-E.** Representative histogram and statistical analysis measuring the frequency of LAG-3$^+$ CD4$^+$ T cells in the peripheral blood and spleen of PFK15 treated and control animals. **F.** Statistical analysis of LAG-3 expression (MFI) on CD4$^+$ T cells in the peripheral blood and spleens of PFK15 and control animals. **G, H.** Representative flow plots and statistical analysis measuring PD-1 and LAG-3 co-expression on CD4$^+$ T cells in the peripheral blood and spleen of PFK15 treated and control animals. **I.** Statistical analysis of the frequency of PD-1$^+$ LAG-3$^+$ CD4$^+$ T cells expressing CD25. **J, K.** Representative pancreatic tissue immunostaining for PD-1 co-stained with DAPI. All data are presented as the mean ± SEM. (n = 6-7 animals/ group; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.005$).

### 3.4.4 Modulating glycolysis leads to functional and metabolic exhaustion of diabetogenic $CD4^+$ T cell Clones

To further substantiate evidence of T cell exhaustion induction by PFK15 treatment, we performed mechanistic studies *in vitro* using the BDC2.5 T cell clone maintained on a 2-week restimulation schedule [38, 122, 171, 172]. This 2- week period allows for the treatment of T cells with PFK15 on a similar regimen to our *in vivo* study. Consistent with our *in vivo* data, sustained PFK15 treatment of BDC2.5 T cell clones significantly increased the expression of PD-1 and LAG-3 alone on $CD4^+$ T cells (Figure 18A-C). However, while a hallmark of T cell exhaustion is the expression of checkpoint molecules, expression of these proteins alone is not indicative of exhaustion, as these molecules are upregulated transiently on the surface of newly activated T cells [169]. We confirmed this by kinetically measuring PD-1 and LAG-3 co-expression on days 4, 8, and 14 post stimulation on $CD4^+$ T cells from untreated and PFK15 treated flasks (Figure 18D), and quantified the percentage of $CD4^+$ T cells co-expressing both PD-1 and LAG-3 on day 14 (Figure 18E). BDC2.5 T cells upregulated both PD-1 and LAG-3 early (day 4) after activation, however by Day 8 most untreated $CD4^+$ T cells began to downregulate expression of the checkpoint molecules as predicted, with an even further downregulation evident by Day 14 (Figure 18D-E) [175]. Interestingly, PFK15 treated T cells displayed increased and sustained expression of PD-1 and LAG-3 over the course of the 14-day restimulation, with a majority of T cells expressing both PD-1 and LAG-3 on Day 14 (Figure 18D-E). Concomitant with this, PFK15 treated T cell clones had decreased expression of CD25 compared to control flasks, confirming that PD-1 and LAG-3 expression on PFK15 treated T cells occurred independently of late T cell activation (Figure 18F-G).

During exhaustion, progressive loss of function occurs in a hierarchical manner, with high proliferative capacity and IL-2 production lost first, followed by a reduced ability to produce TNFα and IFNγ [169, 175, 176]. We assessed these parameters in order to link PD-1 and LAG-3 expression with functional measures of T cell fitness. While control BDC2.5 T cells expanded 9-fold, treated T cells proliferated significantly less, with a mean 5-fold expansion (Figure 18H). Coinciding with this, we measured IL-2 production in culture supernatants on days 8 and 14 post stimulation by ELISA and found significantly more IL-2 accumulated in cultures treated with PFK15 (Figure 18I), indicating a reduced ability to consume IL-2 as a growth factor compared to control T cell cultures. Reduced consumption of IL-2 was likely due to the significantly decreased expression of the high affinity IL-2 receptor CD25 observed in PFK15 treated T cell cultures (Figure 18F-G). Further, the levels of IL-2 in PFK15 treated BDC2.5 cultures were similar to the amount of IL-2 supplemented into restimulation cultures from EL-4 supernatant (gray dotted line on the graph). We also measured the effector cytokines TNFα and IFNγ in cell culture supernatants on days 8 and 14 post stimulation, both of which were significantly reduced upon treatment with PFK15 (Figure 18J-K). In sum, these data indicate that targeting glycolysis leads to severe exhaustion of diabetogenic $CD4^+$ T cell clones, and suggest this as the mechanism by which PFK15 treatment delays T1D onset *in vivo* (Figure 16).

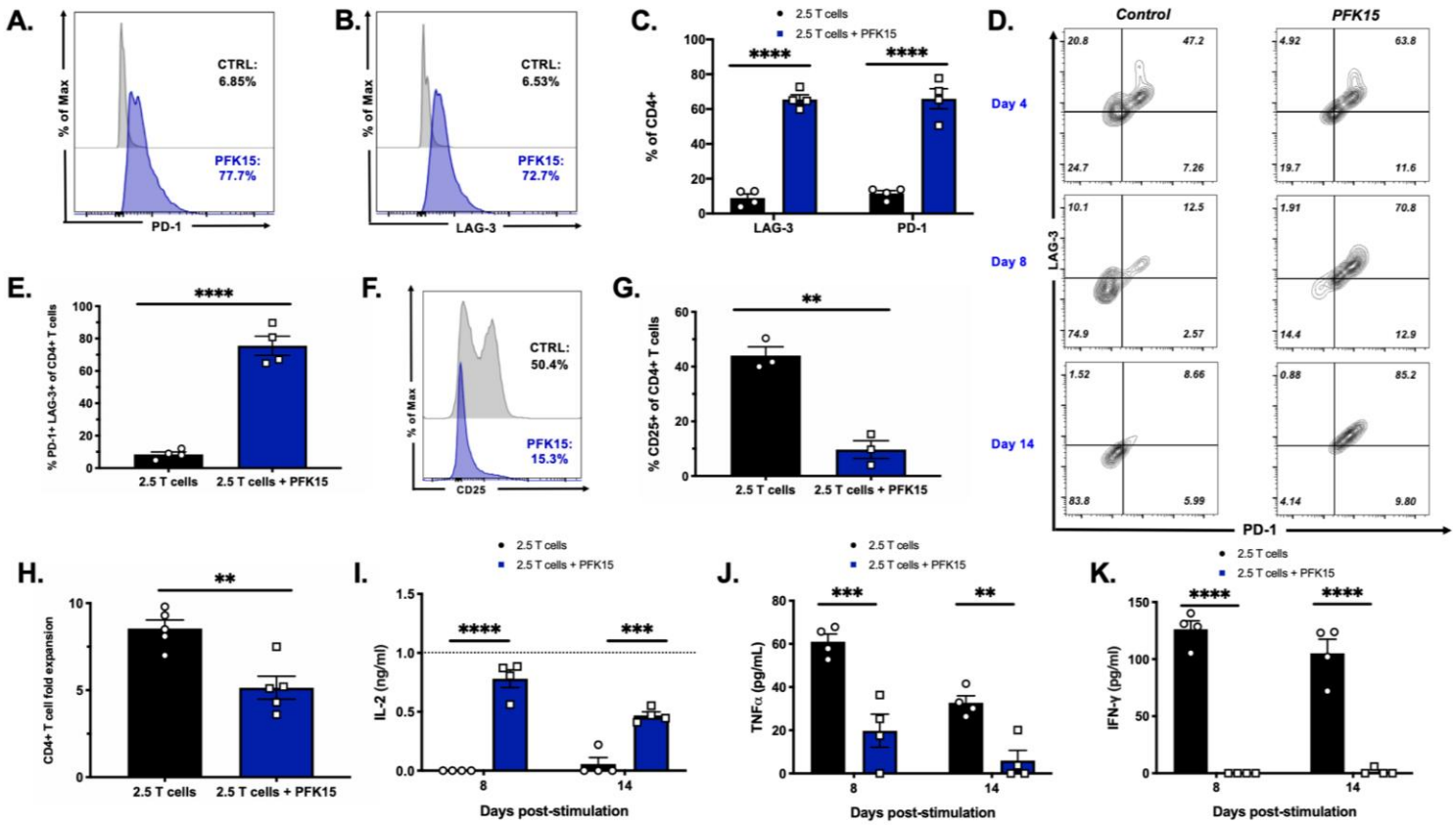


**Figure 18. PFK15 treated T cell clones are functionally exhausted.**
BDC2.5 T cell clones were treated with PFK15 over the course of a 2- week restimulation period to perform mechanistic studies investigating whether glycolysis inhibition induces functional exhaustion of $CD4^+$ T cells. **A, B, C.** Representative histograms and statistical analysis assessing PD-1 and LAG-3 expression on control and PFK15 treated BDC2.5 T cell clones (n = 4). Cells were gated on $CD4^+$ T cells. **D.** Representative flow plots measuring PD-1 and LAG-3 co-expression on PFK15 treated and control BDC2.5 T cell clones on days 4, 8, and 14 post stimulation. Cells were gated on $CD4^+$ T cells. **E.** Statistical analysis of the percentage of BDC2.5 T cells co-expressing PD-1 and LAG-3 in control and treated flasks on day 14 (n = 4). **F, G.** Representative histogram and statistical analysis of the frequency of control and PFK15 treated BDC2.5 T cells expressing CD25 (n = 3). Cells were gated on $CD4^+$ T cells. **H.** Fold expansion of treated and control BDC2.5 T cells (n = 5). **I, J, K.** ELISA analysis of IL-2, TNFα, and IFNγ in culture supernatants on days 8 and 14 post stimulation (n = 4). All data are presented as the mean ± SEM. (** = $p < 0.01$, *** = $p < 0.005$, **** = $p < 0.0001$).

Functional exhaustion of T cells is associated with downstream metabolic consequences. Generally speaking, exhausted T cells are thought to be metabolically deficient, with a majority of metabolic flux supporting cell survival, and limited reserve for fueling effector functions [154]. As PFK15 treated T cell clones were functionally exhausted, we wanted to determine whether this phenotype correlated with decreased metabolic fitness as has been reported in the literature [141, 154, 177]. To begin our investigation, we measured relative adenosine diphosphate (ADP) and ATP levels, and calculated the ADP/ATP ratio from Day 14 control and PFK15 treated T cell clones. While there was no difference in the relative levels of ADP, relative ATP levels were significantly reduced upon PFK15 treatment, indicating decreased metabolic flux (Figure 19A-B). While control T cells had a low ADP/ATP ratio, indicative of cell proliferation, PFK15 treated T cell clones had a significantly higher ADP/ATP ratio, indicating an inability to generate ATP efficiently and metabolic insufficiency (Figure 19C). To characterize the reduced metabolic capacity of PFK15 treated T cells, we measured indicators of glycolysis, fatty acid oxidation (FAO), and mitochondrial metabolism. As expected, PFK15 treated T cells had downregulated levels of the key glycolysis proteins Glut-1, HK2, PFKFB3, and LDHA and reduced supernatant lactate levels as compared to control BDC2.5 T cell clones, confirming an inability to engage in glycolysis upon encounter with β cell antigen (Figure 19D-E).

PD-1 signaling has been reported to promote FAO, with early exhausted T cells having increased carnitine palmitoyltransferase 1α (CPT1α) expression, a rate limiting enzyme that regulates mitochondrial fatty acid transport [109]. To investigate FAO, we first measured fatty acid uptake utilizing a BODIPY C16 fluorescent analog, and found no significant difference in uptake of fatty acids in Day 14 control or PFK15 treated T cells (Figure 19F). CPT1α expression was measured by western blot analysis, and revealed increased expression of CPT1α in Day 8

PFK15 treated T cells, but decreased levels in treated Day 14 clones compared to untreated control T cells (Figure 19G). Together, these results indicate that by Day 8 control T cell clones are engaging in glycolysis in response to presentation of β cell antigen (Figure 19D) while PFK15 treated T cells are utilizing FAO. Although fatty acid uptake was unaltered, reduced CPT1α expression was observed in PFK15 treated clones compared to controls on Day 14, demonstrating a reduced ability to efficiently transport fatty acids into the mitochondria (Figure 19F-G), and likely contributing to the inefficient ATP generation we observed (Figure 19B).

A number of reports demonstrate that exhausted T cells have reduced mitochondrial fitness [141, 168, 177-180]. To investigate the mitochondrial health of PFK15 treated T cells, we measured mitochondrial mass, reactive oxygen species (ROS), and mitochondrial membrane potential. Indeed, PFK15 treated T cell clones exhibited mitochondrial dysfunction as demonstrated by reduced mitochondrial mass (Figure 19H), decreased mitochondrial membrane potential (Figure 19I), and increased generation of mitochondrial ROS when compared to control T cells (Figure 19J). The mitochondrial dysfunction supports the reduced ATP levels measured in PFK15 treated T cells when uptake of fatty acids was unaffected (Figure 19F-G), further pointing to inefficient utilization of nutrients and overall metabolic insufficiency. All in all, these data indicate that inhibition of glycolysis during the activation of autoreactive $CD4^+$ T cell clones enforces an exhausted phenotype that mediates protection from T1D onset *in vivo*

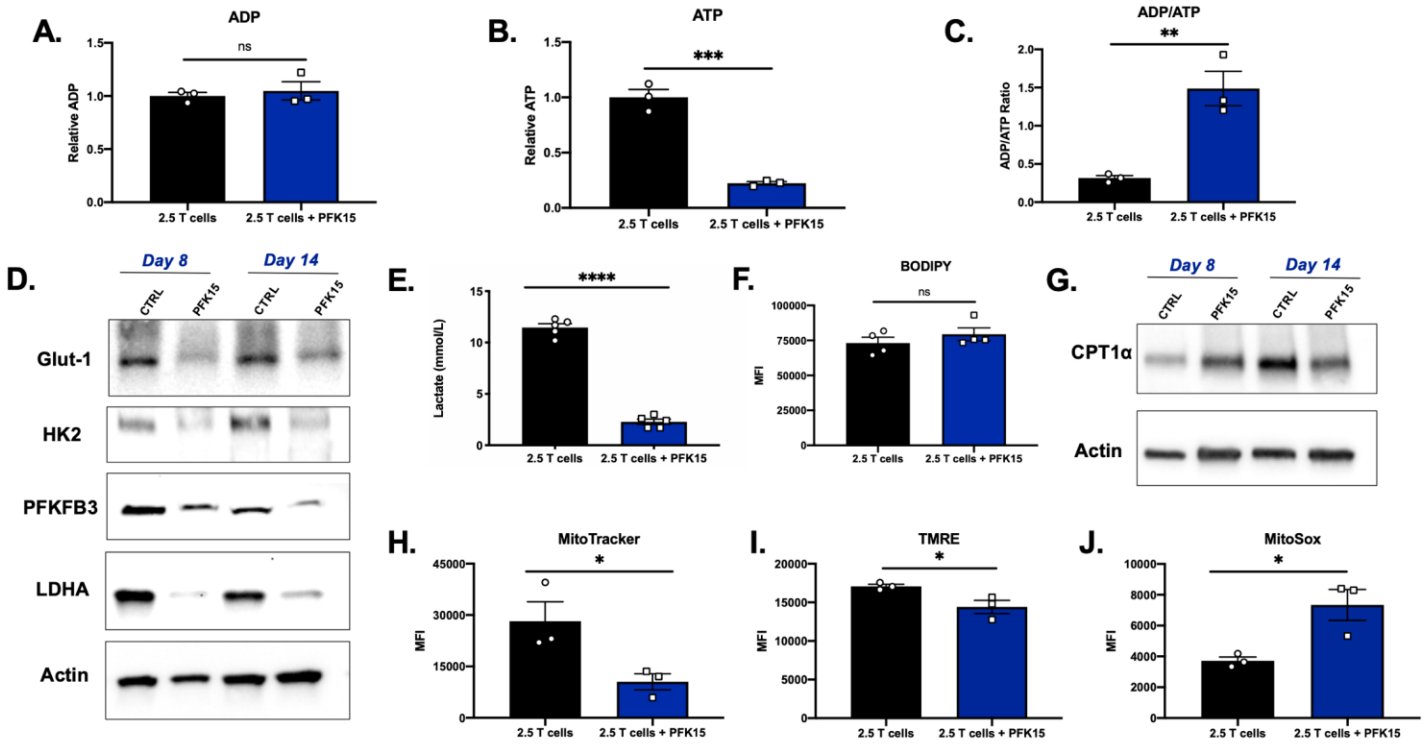


**Figure 19. Glycolysis inhibition during activation renders CD4$^+$ T cells metabolically insufficient.**
Assessing metabolic consequences of PFK15 treatment on BDC2.5 T cell clones. All analyses were performed 14 days post stimulation (Day 14), unless otherwise noted. **A, B, C.** Relative ADP, ATP levels, and the ADP/ATP ratio were measured from Day 14 control and treated BDC2.5 T cells (n = 3). **D.** Representative western blots for glycolysis proteins in control and PFK15 treated BDC2.5 T cell clones on days 8 and 14 post stimulation. **E.** Lactate measurements in cell culture supernatants (n = 5). **F.** Statistical significance of BODIPY fatty acid uptake of Day 14 control and PFK15 treated BDC2.5 T cells (n = 4). Cells were gated on CD4$^+$ T cells. **G.** Representative western blot analysis of CPT1α expression in control and PFK15 treated BDC2.5 T cell clones on days 8 and 14 post stimulation. Cells were gated on CD4$^+$ T cells. **H.** Statistical analysis of mitochondrial mass by MitoTracker green staining on day 14 control and PFK15 treated T cell clones (n = 3). Cells were gated on CD4$^+$ T cells. **I.** Statistical analysis of mitochondrial membrane potential by TMRE staining on day 14 control and PFK15 treated T cell clones (n = 3). Cells were gated on CD4$^+$ T cells. **J.** Statistical analysis of mitochondrial ROS by MitoSOX staining on day 14 control and PFK15 treated T cell clones (n = 3). Cells were gated on CD4$^+$ T cells. All data are presented as the mean ± SEM. (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.005$, **** = $p < 0.0001$).

### 3.4.5 PFK15 treated CD4$^+$ T cells are terminally exhausted

Exhausted T cell lineages display heterogeneity amongst subsets with unique characteristics and varying abilities to become reinvigorated [181, 182]. To determine the state of exhaustion observed in PFK15 treated T cells, we performed reinvigoration experiments where a subset of BDC2.5 T cell clones were treated with PFK15 every third day for two weeks to induce exhaustion. Then, T cells from control or PFK15 treated flasks (PFK15 treated T cells put into restimulation cultures termed PFK15 $T_{EX}$) were restimulated for another two weeks without further PFK15 treatment (Figure 20A). We first measured PD-1 and LAG-3 expression on PFK15 $T_{EX}$ cells after restimulation and found that PFK15 $T_{EX}$ sustained high expression of both PD-1 and LAG-3 compared to control cultures (Figure 20B), consistent with retention of an exhausted phenotype. Notably, PFK15 $T_{EX}$ were unresponsive to IL-2 present in restimulation cultures, further confirming exhaustion and ruling out anergy [154]. We also measured lactate and IFNγ in cell culture supernatants on days 8 and 14 post restimulation as indicators of re-engagement in the glycolysis pathway upon activation and effector function. We observed reduced lactate production and little secretion of IFNγ by PFK15 $T_{EX}$ in response to restimulation, suggesting that treated T cell clones are terminally exhausted (Figure 20C-D).

Reports have demonstrated that terminally exhausted T cells are refractory to checkpoint blockade [154, 168, 177]. To determine whether PFK15 $T_{EX}$ cells were responsive to checkpoint blockade, a subset of restimulation cultures were treated with αPD-1, αLAG-3, or a combination of both αPD-1/αLAG-3 blocking antibodies as described in the methods. We measured lactate and IFNγ in cell culture supernatants to determine whether checkpoint blockade treatment would rescue PFK15 $T_{EX}$ cell's ability to utilize glycolysis and exert their effector function (Figure 20C-

D). Neither αPD-1, αLAG-3, nor a combination of αPD-1 and αLAG-3 blocking antibody treatments were capable of rescuing PFK15 $T_{EX}$ ability to respond to self- antigen, evidenced by significantly less lactate and IFNγ secretion measured in culture supernatants of PFK15 $T_{EX}$ ± PD-1 and/or LAG-3 blockade compared to control T cells (Figure 20C-D). To confirm the induction of terminal exhaustion *in vivo*, we performed reversibility experiments using our adoptive transfer model, described in Figure 16A. Briefly, ex vivo activated BDC2.5 T cells were adoptively transferred into NOD.*scid* recipients, with recipient animals being separated into one of three treatment cohorts: 1) vehicle control, 2) PFK15 $^{+}$ IgG, and 3) PFK15 $^{+}$ αPD-1 $^{+}$ αLAG-3 (Figure 20E). Animals in the PFK15 treatment groups were treated every other day for two weeks, with IgG or checkpoint blockade treatment initiated in the second week. Notably, the dose of αPD-1 and αLAG-3 blocking antibodies administered have been previously shown to accelerate diabetes onset in NOD animals, therefore any delay or protection achieved with PFK15 treatment would be considered durable if irreversible with checkpoint blockade [30]. As expected, 100% of control animals displayed fulminant diabetes by day 7 post- transfer, with significant delays associated with PFK15 treatment (Figure 20F). While 70% of animals receiving PFK15 $^{+}$ IgG remained diabetes free through the end of the study period, 42% receiving PFK15 $^{+}$ αPD-1 $^{+}$ αLAG-3 treatment were protected from disease (Figure 20F). Although more animals receiving checkpoint blockade succumbed to diabetes than PFK15 $^{+}$ IgG treated animals, diabetes incidence between these two groups were not statistically significant ($p = 0.348$), indicating an inability to reverse PFK15 induced T cell exhaustion. Finally, regardless of αPD-1 and αLAG-3 blockade, $CD4^{+}$ T cells in PFK15 treated animals retained high expression of PD-1 and LAG-3 in the periphery and spleens compared to control animals, consistent with an exhausted phenotype (Figure 20G). Together, these data support the findings that inhibition of glycolysis in diabetogenic $CD4^{+}$ T cells

leads to terminal exhaustion, characterized by functional and metabolic dysfunction that is irreversible by restimulation or checkpoint blockade therapy *in vitro* and *in vivo*. This work demonstrates that the rate- limiting glycolysis enzyme PFKFB3 is a novel target for controlling autoreactive T cell activation as a means to protect against the onset of T1D by enforcing exhaustion of pathogenic T cells.

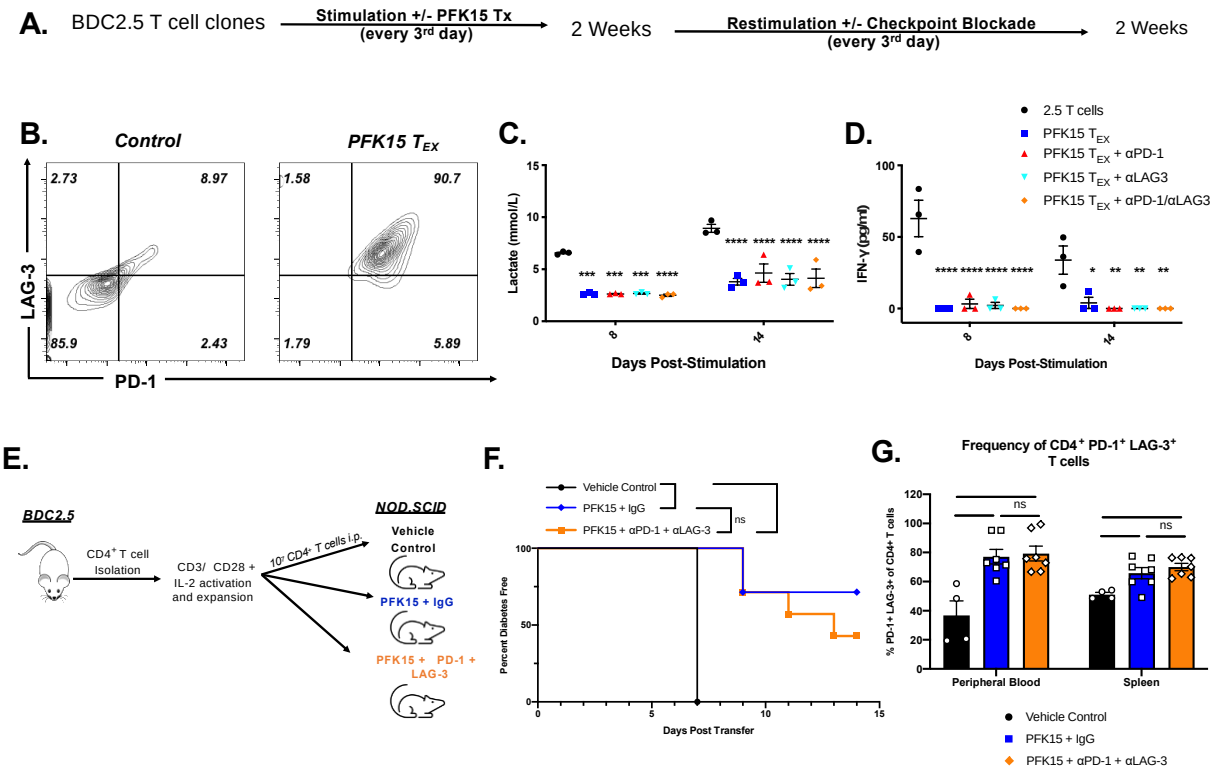


**Figure 20. Inhibition of glycolysis leads to terminal exhaustion of CD4$^+$ T cells that are refractory to checkpoint blockade**

To determine the degree of exhaustion induced by PFK15 treatment, PFK15 treated T cell clones were restimulated ± checkpoint blockade. **A.** Schematic diagram of experimental design for reinvigoration studies. **B.** Representative flow plots of PD-1 and LAG-3 expression on CD4$^+$ T cells on control and PFK15 $T_{EX}$. **C.** Statistical analysis of lactate secretion in Day 8 and 14 restimulation culture supernatants (n = 3). **D.** ELISA analysis of IFNγ in culture supernatants on days 8 and 14 post stimulation (n = 3). **E.** Schematic diagram of experimental design for *in vivo* reversibility studies. **F.** Survival analysis of diabetes incidence in vehicle control and PFK15 treated groups. Kaplan-Meier survival analysis test was performed for statistical significance. **G.** Statistical analysis measuring PD-1 and LAG-3 co-expression on CD4$^+$ T cells in the peripheral blood and spleen of vehicle control (n = 4), PFK15 $^+$ IgG (n = 7), and PFK15 $^+$ αPD-1 $^+$ αLAG-3 (n = 7) treatment. All data are presented as the mean ± SEM. (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.005$, **** = $p < 0.0001$).

## 3.5 DISCUSSION

In the present study, we evaluated the ability of the anti- glycolytic PFK15 to control the activation of diabetogenic $CD4^+$ T cells in T1D. To our knowledge, this is the first study testing the ability of a PFKFB3 inhibitor to prevent the onset of autoimmune diabetes. Our findings confirmed PFK15 inhibited glycolysis upon activation of $CD4^+$ T cells (Figure 14), dampened autoreactive $CD4^+$ T cell responses *in vitro* (Figure 15), and delayed disease onset *in vivo* (Figure 16-17). The protective benefits associated with PFK15 treatment are not entirely surprising as glycolysis is required for $CD4^+$ T cell activation and IFNγ secretion. In fact, targeting T cell metabolism has been successfully used to prevent and reverse disease in other autoimmune diseases, albeit without mediating durable tolerance [84-87, 115]. However, our findings demonstrate an induction of terminal exhaustion by glycolysis inhibition, which to our knowledge, has not been previously reported in the literature.

Previously, use of the prototypical glycolysis inhibitor 2-DG in SLE, RA, and MS failed to generate a long- lasting benefit, as cessation of treatment was associated with disease flare-ups [84, 86, 87, 115]. Dissimilarities in the observed outcomes between our study and others is likely due to differences in the mechanisms of inhibition. 2-DG is a glucose analog that indirectly targets the action of HK2 through competition with endogenous glucose levels [183]. For this reason, an effective reduction in glycolytic flux requires high dose treatments, which are associated with adverse effects and non- specific targeting [183]. In comparison, PFK15 is highly selective for a defined intracellular enzyme, thus requiring a much lower concentration for effective inhibition [183]. These key differences appear to have drastically different outcomes on the T cell response, and the data included in Figure 26 confirm this as treatment of BDC2.5 splenocytes with 2-DG *in vitro* was only able to reduce IFNγ secretion, and failed to recapitulate PFK15's ability to dampen

IL-2 and TNFα (Figure 26). Strikingly, our data demonstrate an ability for PFK15 treated T cells to become early activated (Figure 15D). 2-DG treatment, however, leads to reduced CD69 expression upon activation, indicating maintenance of a quiescent phenotype that is reversible when treatment is stopped [84]. The early activation observed in our model supports the need for T cells to lineage commit to effector subsets in order to induce a terminal phenotype. Finally, PD-1 and LAG-3 expression are induced upon TCR signaling, therefore early activation is required to upregulate IRs that ultimately render PFK15 treated T cells exhausted (Figure 17-18) [169, 175].

Although often overlooked, availability of nutrients is vital to maintaining T cell fitness. As described herein, T cells and cancer cells have a shared reliance on aerobic glycolysis, which becomes problematic in the TME when tumor cells metabolically restrict T cells, thus eliciting poor anti- tumor immunity [108, 177]. Analogously, our data supports the idea that reduced glycolytic flux promotes T cell exhaustion, as PFK15 treatment induced defective effector responses upon activation (Figure 15 and 18). Glycolysis restriction, however, is not the only metabolic pathway dysregulated by TILs. Notably, TILs demonstrate a progressive loss of mitochondrial mass and function [141, 168, 177, 180]. This, along with a low glucose environment, promote a state of metabolic insufficiency due to an inability to meet nutrient requirements, leading to a permanent hyporesponsive state [108, 141, 168, 177, 180]. Unexpectedly, we too observed mitochondrial dysfunction when glycolysis was inhibited (Figure 19). Although other factors in the TME contribute to repressed mitochondrial function, particularly hypoxia, our data strengthens the link between nutrient restriction and T cell exhaustion since glycolysis inhibition led to the development of metabolic insufficiency [184].

While the development of T cell exhaustion is detrimental in cancer and chronic infection, the opposite is true in autoimmunity, where induction of a hyporesponsive phenotype protects the host

from attack [129]. The onset of autoimmunity in T1D occurs due in large part to defective central and peripheral tolerance mechanisms that fail to control pathogenic T cells. This defect is due to dysregulated IR expression in T1D [31, 134, 185]. In healthy individuals, binding of IR proteins to their associated ligand and subsequent downstream signaling act as a metaphorical "brake" that impedes T cell activation and protects against autoimmunity [129, 169, 175]. Evidence of the importance of IRs is underscored by the accelerated diabetes observed in the absence or blockade of PD-1 or LAG-3 in NOD mice [30-32]. Clinically speaking, polymorphisms in the PD-1 gene have been identified and associated with disease susceptibility [185]. Concomitantly, T1D patients fail to upregulate PD-1 on T cells compared to control subjects, correlating to aberrant T cell activation and effector function [134, 136]. In our study, PFK15 treatment led to increased PD-1 and LAG-3 expression on $CD4^+$ T cells that correlated with protection from diabetes onset (Figure 16). Importantly, expression of IRs alone is not sufficient to induce T cell exhaustion, since activated T cells transiently upregulate both PD-1 and LAG-3 upon early activation [169, 175]. In fact, exhaustion can occur even in the absence of checkpoint molecules, further complicating the role these molecules play in enforcing and maintaining functional exhaustion [177]. While IRs may play a lesser role in driving exhaustion in other disease settings, our data reveal a pivotal role for PD-1 and LAG-3 in maintaining tolerance against autoimmune responses. In conclusion, we demonstrated a unique ability to correct defects in peripheral tolerance mechanisms in T1D by inducing PD-1 and LAG-3 expression on $CD4^+$ T cells. Increased IR expression and glycolysis restriction led to the functional and metabolic exhaustion of diabetogenic T cells independent of PD-1 and LAG-3 signaling, as checkpoint blockade failed to reverse this phenotype (Figure 20).

Therapeutic strategies for T1D have focused on two specific areas: 1) β cell replacement via regeneration of endogenous β cell mass or 2) immunomodulation [143, 146]. Although innovative

efforts have been made to restore β cell mass, these strategies ultimately fail due to reemergence of the autoimmune response [143]. Moreover, although immunomodulation has yielded positive results in preclinical studies, success in the clinic has remained limited. Unfortunately, present clinical studies have only administered immunotherapies to patients with diagnosed T1D. By diagnosis, T1D patients have endured longstanding autoimmunity, with significant β cell loss, thus highlighting a need to intervene prior to symptomatic disease [33]. Interestingly, the presence of autoantibodies is known to be a strong predictor for disease onset [186, 187]. In fact, the first in man prevention study was published recently, where the αCD3 antibody teplizumab was utilized in patients at risk for diabetes development [146]. Teplizumab delayed T1D onset by 2 years, and similarly to our study, protection was associated with increased expression of IRs and induction of a T cell hyporesponsive state [146]. However broad immunosuppression was observed in teplizumab treated patients, which is an adverse effect of most immunotherapies due to non-specific targeting. A potential benefit of modulating glycolysis is the ability to specifically target activated T cells, while leaving established memory T cells and regulatory T cell populations unaffected based on their reliance on alternative metabolic pathways [57, 59, 71, 95]. Clinically speaking, we would anticipate glycolysis inhibition to delay or prevent disease onset, while use of PFK15 in conjunction with methods to restore β cell mass may be a novel way to reverse T1D.

In summary, these findings demonstrate an ability to induce terminal exhaustion of autoreactive T cells in T1D by modulating the glycolysis pathway via targeting of PFKFB3 (Figure 21). On a broader note, these data support a key role for glucose utilization in T cell activation and function, since an inability to efficiently metabolize glucose enforces a hyporesponsive phenotype. In our study, this phenotype was associated with expression of PD-1 and LAG-3, which are known to be dysregulated in T1D patients. This study remains focused on the ability to restrain

diabetogenic $CD4^+$ T cells due to their importance in the initiation of autoimmunity. While we would anticipate an overall benefit to other mediators of autoimmunity in T1D, like $CD8^+$ T cells, further investigations are required to fully understand the impact glycolysis inhibition would have on other immune cell subsets. However, with the ability to restore tolerance in preclinical studies, we anticipate the use of metabolic modulators, like PFK15, may have a beneficial impact in both the clinical prevention and reversal of disease.

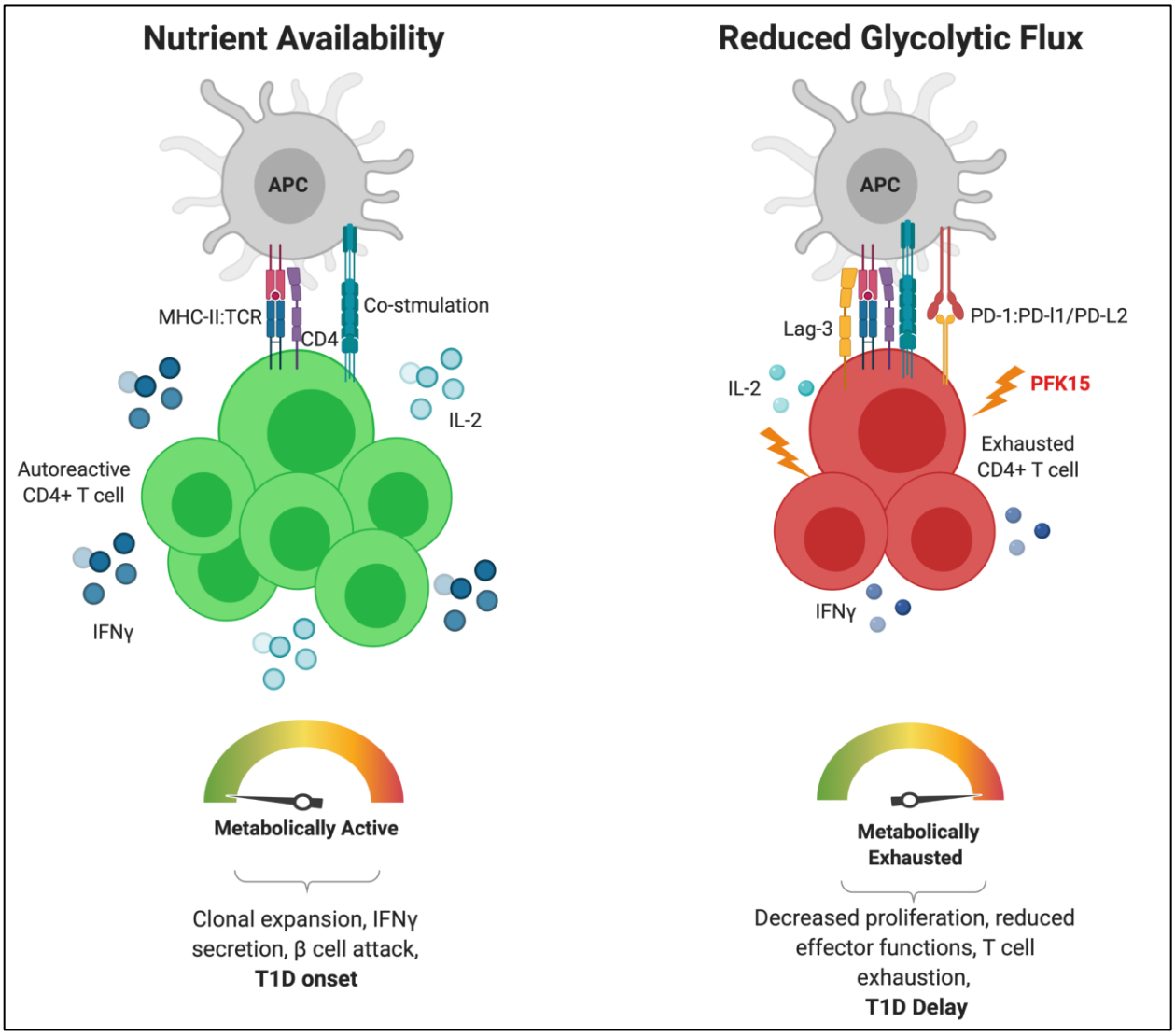


**Figure 21. Mechanism for the effect of glycolysis inhibition on CD4$^+$ T cell activation and Type 1 Diabetes Pathogenesis.**

Upon encounter with antigen via TCR: MHC-II interaction, CD4$^+$ T cells become activated. Under conditions of sufficient nutrient availability, effector CD4$^+$ T cells are metabolically active and transition to aerobic glycolysis to fuel their clonal expansion and effector function (i.e. IFNγ secretion). In the case of T1D, the activation of autoreactive CD4$^+$ T cells ultimately leads to β cell attack, hyperglycemia, and T1D onset. Treatment with PFK15, an inhibitor of a rate- limiting enzyme of the glycolysis pathway, reduces glycolytic flux, and results in the induction of terminal exhaustion, characterized by high PD-1 and LAG-3 expression, decreased proliferative capacity, reduced effector function, metabolic insufficiency, and a delay in T1D onset in an animal model of autoimmune diabetes.

# 4.0 INVESTIGATING THE IMPACT GLYCOLYSIS INHIBITION HAS ON ANTIGEN PRESENTING CELL FUNCTION

This chapter is an ongoing study and more experimentation is required before it can be considered for publication.

## "Therapeutic Targeting of the Glycolysis Enzyme PFKFB3 does not Alter Antigen Presenting Cell Function"

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## 4.1 SUMMARY

The activation of autoreactive T cells that target β cells in the pancreas during T1D is dependent on the presentation of self- antigen by professional antigen presenting cells (APCs), like macrophages and dendritic cells (DCs). Further, their role in the immunopathology of T1D supersedes their ability to present antigen, as macrophages and DCs are the first immune cells to invade the islets, and also mediate β cell death over the course of the autoimmune response. Similar to what has been described for T cells, cellular metabolic programs dictate APC activation and function, where a metabolic transition to aerobic glycolysis accompanies activation. In chapters 2 and 3, we have demonstrated an ability to the glycolysis inhibitor PFK15 to control autoreactive T cells responses in T1D by inducing T cell exhaustion, however due to the shared reliance of T cells and APCs on the glycolysis pathway, we wanted to determine the impact glycolysis inhibition had on APC function. Our data presented herein demonstrate that the therapeutic benefits associated with PFK15 administration are due to direct modulation of $CD4^+$ T cells, as both macrophage and DC function remained intact with PFK15 treatment. The observed outcomes on APC function were independent of the defects associated with macrophages and DCs in the NOD mouse, as APCs from healthy control C57BL/6J animals were also unaffected by PFK15 treatment. In sum, these data confirm that the protective benefits of PFK15 treatment occur independently of any effects on APCs. These data highlight a need to better characterize the metabolic phenotype and flexibility of APCs in T1D, as understanding these pathways are vital to expanding the ability to modulate immune cell metabolism to control self- reactivity.

## 4.2 INTRODUCTION

Professional APCs, such as macrophages and DCs, are important mediators of inflammation. Not only can APCs mount innate immune responses against invading pathogens, they also play a pivotal role in initiating an adaptive immune response, via engagement of peptide-MHC complexes with the TCR [1]. Upon antigen recognition via pattern recognition receptors (PRRs), APCs become activated, increase their expression of MHC-peptide complexes and costimulatory molecules (i.e. CD80/CD86, CD40), and secrete proinflammatory cytokines such as IL-6, TNFα, IL-1β, IL-12, and type 1 IFNs [1]. Synthesis of proinflammatory cytokines act to promote inflammation and initiate the differentiation and effector function of naïve T cells that mediate antigen- specific responses, and are vital for the maturation of adaptive immunity and IFNγ synthesis by effector T cells.

Similarly to T cells, macrophages and DCs too, undergo metabolic transitions over the course of their lifespan with specific pathways utilized to promote anti- inflammatory or immunogenic processes. Generally speaking, tolerogenic DCs or anti- inflammatory M2 macrophages that promote the resolution of inflammation are oxidative while proinflammatory immunogenic dendritic cells and classically activated M1 macrophages are considered more glycolytic, however these cells display plasticity and adaptability in regard to the nutrients required for their function and survival [59, 75, 76, 78, 80, 188]. Upon activation to pro- inflammatory phenotypes, macrophages and DCs increase their glucose consumption and lactate production as a result of a rapid increase in glycolytic flux [59, 75, 76, 78, 80, 188]. While T cells rely on glycolysis to produce biosynthetic intermediates to fuel their high proliferative capacity and clonal expansion,

macrophages and DCs do not proliferate and instead use this pathway both as a survival mechanism and to exert their effector function [189].

Regarding Type 1 Diabetes (T1D), macrophages and DCs have an often-unappreciated role in perpetuating autoimmunity against islet β cells. T -cell mediated destruction of pancreatic β cells during T1D is secondary to islet- infiltrating macrophages and DCs, which are some of the first cells to invade the islets [3, 4]. Notably, resident macrophages are present within the pancreas at all times, however it is not until acquisition of antigen that tissue- resident APCs become activated and secrete inflammatory cytokines. As described in the introduction, environmental triggers like viral infection or chemical exposure in genetically predisposed individuals leads to β cell destruction and the release of β cell antigens within the pancreas. Resident macrophages and DCs within the pancreatic microenvironment become activated, phagocytose β cell antigens and dying β cells, and traffic to the pancreatic draining lymph node to present processed antigenic peptides to autoreactive T cells. Importantly, TNFα, IL-1β, IL-6, and reactive oxygen species (ROS) secreted by activated APCs can damage β cells, with IL-1β specifically able to cause direct β cell lysis and cell death [36, 121, 189]. Therefore, APCs are instrumental in both the initial insult against islet β cells, as well as in the maturation of the adaptive immune response that ultimately leads to complete destruction of β cells, and the onset of hyperglycemia in afflicted individuals.

Our previous work described in chapters 2 and 3 demonstrated an ability to inhibit T cell responses to nominal and β cell antigens *in vitro* and delay T1D onset *in vivo* via administration of the glycolysis inhibitor PFK15. Previous literature has demonstrated that treatment of bone marrow derived macrophages (BMDMs) or dendritic cells (BMDCs) activated with the TLR4 agonist lipopolysaccharide (LPS) in the presence of the glycolysis inhibitor 2-DG reduced costimulatory molecule expression and proinflammatory cytokine production [75, 76, 78, 188,

190, 191]. However, whether specific targeting of PFKFB3 via PFK15 treatment has not been studied, especially in the context of T1D. Due to the importance of APCs in T1D pathogenesis and the reliance of macrophages and DCs on the glycolysis pathway, we sought to determine whether PFK15 treatment would also affect APC function. In contrast to use of other metabolic modulators of the glycolysis pathway, treatment of LPS- activated BMDMs or BMDCs from T1D prone NOD animals with PFK15 *in vitro* failed to reduce their activation and effector functions, as costimulatory molecules and proinflammatory cytokine production remained intact despite PFK15 treatment. Ultimately, these data are interesting and demonstrate that use of other immunomodulatory agents in conjunction with PFK15 may be required to suppress both APC and T cell mediated autoimmunity against islet β cells in T1D.

## 4.3 MATERIALS AND METHODS

### 4.3.1 Mouse Strains

Non- Obese Diabetic (NOD), NOD.BDC2.5.TCR.Tg (BDC2.5), NOD.*scid*, and C57BL/6J animals were bred and maintained under specific pathogen free conditions in the animal facility located at the Rangos Research Center within UPMC Children's Hospital of Pittsburgh. All animal experiments were approved by the University of Pittsburgh's Institutional Animal Care and Use Committee (IACUC; Assurance Number: D16-00118). Male and female mice aged 6-12 weeks old were used in all experiments.

### 4.3.2 Differentiation of Bone Marrow Derived Dendritic Cells and Macrophages

Bone marrow derived macrophages and dendritic cells were differentiated as previously described [3, 192]. Bone marrow cells were flushed from the bones using a 26 g needle. Bone marrow cells were then centrifuged and filtered through a cell strainer. For differentiation of BMDMs to the classical M1 proinflammatory phenotype, $2\times10^6$ cells/well cells were plated on 24-well tissue culture plates. Cells were cultured in macrophage colony- stimulating factor 1 (mCSF1) conditioned RPMI 1640 media, with a media change every two days. After 7 days of differentiation, BMDMs were stimulated with 100 ng/mL or 1 ug/mL lipopolysaccharide (LPS) from *Escherichia coli* (055:B5) (Sigma Aldrich) ± 2.5- 10 μM PFK15. Cell culture supernatants were collected 24-72 hours post- stimulation to assess effector cytokine secretion. For differentiation of BMDCs, $2\times10^6$ cells/well cells were plated on 24-well tissue culture plates. Cells were cultured in IL-4 and GM-CSF (8 ng/mL) conditioned RPMI-1640 media, with a media change after 2 days, followed by an additional 3-day incubation. After 5 days of differentiation, BMDCs were stimulated with 100 ng/mL LPS ± 5 μM PFK15. Cells and cell culture supernatants were collected 24-48 hours post- stimulation to assess the expression of costimulatory molecules by flow cytometry and effector cytokine secretion by ELISA.

### 4.3.3 Flow Cytometry

$1x10^6$ BMDCs cells were harvested at indicated timepoints and surface stained for flow cytometric analysis as described [123]. Briefly, cells were incubated with Fc block (CD16/CD32; BD Biosciences) for 15 minutes prior to staining for flow cytometry. Surface staining was performed at 4°C using CD11b, CD11c, MHC-II, CD80, and CD86 antibodies (BD Biosciences) in FACS

buffer (1% BSA in PBS). Cells were then fixed in 2% PFA for 15 minutes at 4°C (Thermo Fisher Scientific), and stored at 4°C until time of analysis.

### 4.3.4 ELISA and Lactate Measurements

Supernatants were collected from LPS ± PFK15 stimulated BMDMs or BMDCs after 24-48 hours in culture. IL-6 and TNFα cytokines secreted by BMDMs or BMDCs were measured by ELISA using purified IL-6 capture and biotinylated antibody pairs (BD Biosciences) or TNFα ELISA kits (R&D) according to the manufacturer's instructions. ELISAs were read on a SpectraMax M2 microplate reader (Molecular Devices) and data analyzed using SoftMax Pro version 7.0.2 software (Molecular Devices). Lactate, a byproduct of aerobic glycolysis, was measured using the Lactate Plus meter and test strips per manufacturer's instructions (Novus Biologics).

### 4.3.5 Adoptive Transfer Model of T1D

1 x $10^7$ *ex vivo* activated $CD4^+$ T cells from BDC2.5 mice were injected i.p. into NOD.*scid* recipients as described previously by our laboratory [155]. One cohort of recipients received 25 mg/kg PFK15 (Selleck) dissolved in 5% DMSO $^+$ 45% PEG300 $^+$ 1% Tween80 $^+$ 49% dd$H_2O$ prepared fresh; the other cohort received vehicle control every other day for 2 weeks. Body weights and blood glucose (BG) levels were monitored over the course of the experiments. Animals were deemed diabetic after two consecutive BG readings ≥ 350 mg/dL. Diabetic animals were sacrificed at indicated timepoints and Pancreata were harvested for downstream analyses.

### 4.3.6 Tissue Collection and Histological Assessment

Pancreatic tissue was collected and fixed in 4% paraformaldehyde (PFA; Thermo Fisher Scientific) overnight at 4° C. Fixed tissue was processed and embedded in paraffin by the Histology Core Laboratory located at UPMC Children's Hospital of Pittsburgh's Rangos Research Center [161]. Embedded tissue was sectioned at 4 μm. Samples were imaged using a Nikon Eclipse E800 microscope (Nikon) and associated software.

### 4.3.7 Immunofluorescent Staining

Immunofluorescent staining was performed on paraffin embedded samples prepared as described above. Antigen retrieval was performed in sodium citrate buffer followed by overnight incubation with primary antibodies against F4/80 (1:100; BD Biosciences). The following day, slides were incubated with Alexa Fluor 594 conjugated donkey anti- rat secondary antibody (Invitrogen). Samples were imaged using a Leica DMi8 inverted microscope (Leica) and LAS X Navigator software (Leica).

### 4.3.8 Statistical Analysis

All data are presented as mean values ± standard error of the mean (SEM), with n indicating the number of independent experiments or animals. Some data were collected from preliminary experiments, and therefore only one representative experimental data point is shown. Student's t-test or One- way ANOVA were used where appropriate. A p-value of $p < 0.05$ was considered significant for all statistical analyses. All statistics and graphs were generated using GraphPad Prism software.

## 4.4 RESULTS

### 4.4.1 BMDCs and BMDMs fail to secrete aerobic glycolysis byproduct, lactate, in response to LPS stimulation

Macrophages and DCs transition from a resting state to an activated state in response to TLR agonists [59]. Activation is an energetically demanding process, and therefore leads to metabolic transitions that ultimately increase the glycolytic rate [59, 76, 188, 193-195]. Similarly to both tumor cells and T cells, this phenotype is reminiscent of the Warburg effect, where cells preferentially metabolize glucose to lactate despite the presence or availability of oxygen. Further, glucose restriction and treatment of APCs derived from bone marrow progenitors with inhibitors of the glycolysis pathway, such as 2-DG, severely inhibits the lifespan and activation of both macrophages and DCs [59, 76, 188, 195]. To determine the effect PFK15 treatment had on LPS induced metabolic reprogramming of BMDMs and BMDCs, we measured extracellular lactate in cell culture supernatants from BMDC (Figure 22A) and BMDMs (Figure 22B-C) stimulated with LPS ± PFK15 as previously described [110, 123, 155]. In contrast to published literature, we did not observe a significant increase in lactate secretion by LPS stimulated BMDMs or BMDCs (Figure 22A-B). Interestingly, BMDCs secreted more lactate than BMDM cultures, even when unstimulated. This could be due to intrinsic effects of GM-CSF, which has been reported to increase glycolytic capacity [77, 191]. Further, unlike the data obtained in chapters 2 and 3, PFK15 treatment had no impact on lactate secretion and did not reduce lactate levels in 24-hour culture supernatants, indicating 1) that NOD BMDCs and BMDMs do not utilize aerobic glycolysis upon LPS stimulation, and 2) treatment with PFK15 has no impact on APC lactate secretion. Further experimentation is required to fully assess the metabolic changes that occur during APC activation and confirm these data.

It has been well established that APCs from the NOD mouse, and in T1D patients, are defective in their maturation and function [124, 196-200]. To determine whether the phenotypes observed were due to inherent dysfunctionality of NOD APCs, we differentiated bone marrow cells from control C57BL/6J (B6) animals into BMDMs and stimulated them with LPS ± PFK15 (Figure 22C). Similarly to NOD BMDCs and BMDMs, mCSF1 derived BMDMs from B6 animals failed to upregulate aerobic glycolysis upon stimulation with LPS, and were unaffected by PFK15 treatment (Figure 22C).

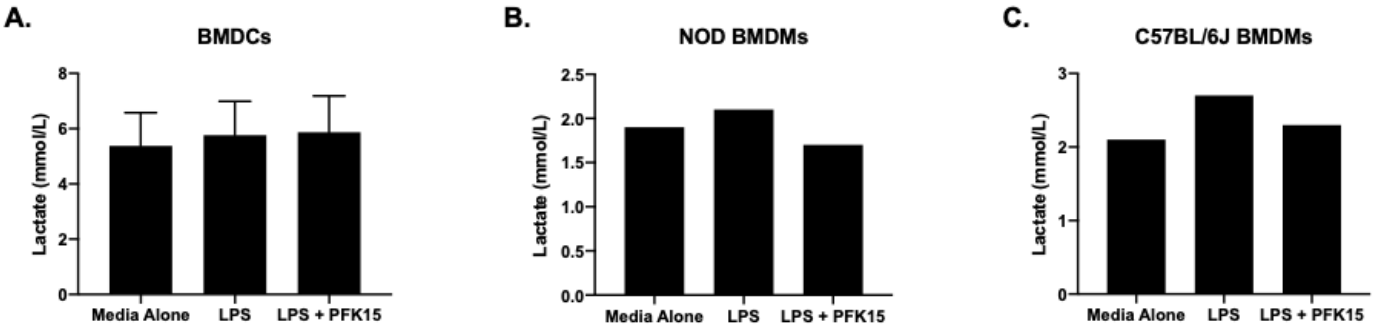


**Figure 22. LPS stimulated BMDCs and BMDMs fail to secrete lactate, the byproduct of aerobic glycolysis and are non-responsive to PFK15 treatment.**

**A-C.** BMDCs and BMDMs were differentiated from bone marrow cells isolated from NOD and C57BL/6J animals. After differentiation, cells were stimulated with 100 ng/mL (BMDMs; n = 1) or 1 µg/mL (BMDCs; n = 3) LPS ± 5 µM PFK15. After 24 hours in culture, lactate in cell culture supernatants was measured.

### 4.4.2 PFK15 treatment does not alter BMDC function

LPS stimulation of BMDCs leads to increased expression of MHC-II and costimulatory molecules, and subsequent synthesis of proinflammatory cytokines, especially IL-6 and TNFα; all of which are required for efficient priming of T cell responses [60, 76, 188]. To determine whether targeting the glycolysis pathway alters BMDC function upon TLR4 ligation with LPS, we first measured expression of MHC-II, and the costimulatory molecules CD80 and CD86 (Figure 23A-C) 24-48 hours post stimulation with LPS ± PFK15. Again, we saw no appreciable increases in MHC-II (Figure 23A), CD80 (Figure 23B), or CD86 (Figure 23C) expression upon LPS stimulation compared to unstimulated controls, although CD80 appeared to be slightly increased (Figure 23B). Moreover, PFK15 did not reduce the expression of either MHC-II, CD80, or CD86 (Figure 23A-C) compared to LPS stimulated only BMDCs. Next, we measured IL-6 and TNFα production as indicators of BMDC effector function (Figure 23D-E) by ELISA. While LPS stimulation led to increased IL-6 and TNFα production 24 – 48 hours post- stimulation compared to unstimulated control BMDCs, PFK15 treatment did not reduce the production of either cytokine, providing further evidence that targeting PFKFB3 in BMDCs does not alter their function (Figure 23D-E).

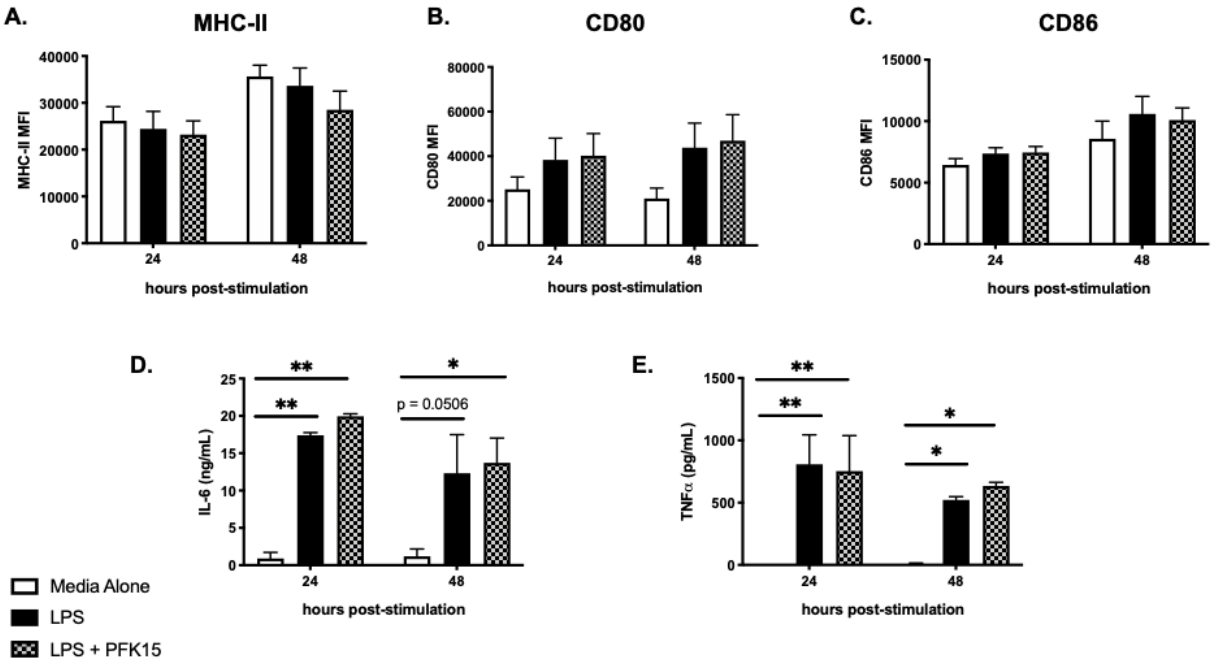


**Figure 23. Glycolysis inhibition via PFK15 treatment does not alter BMDC function.**
BMDCs were differentiated from bone marrow cells isolated from NOD mice. After differentiation, cells were stimulated with 1 µg/mL (BMDCs; n = 3) LPS ± 5 µM PFK15. After 24-48 hours in culture, **A.** MHC-II, **B.** CD80, **C.** CD86 expression were analyzed by flow cytometry. **D-E.** 24-48 hour supernatants were harvested and utilized to measure **D.** IL-6 and **E.** TNFα secretion by LPS ± PFK15 BMDCs. All data are presented as the mean ± SEM. (* = $p < 0.05$, ** = $p < 0.01$).

### 4.4.3 TNFα secretion by C57BL/6J and NOD BMDMs remain intact with PFK15 treatment

PFK15 treatment of LPS stimulated BMDCs from NOD animals did not reduce their effector functions (Figure 23). To determine whether this was specific for BMDCs, or whether PFK15 would fail to regulate other APC subsets, we performed similar experiments using BMDMs derived from NOD animals. We also differentiated BMDMs from C57BL/6J mice as a control, to ensure the phenotypes we observed were not due to inherent defects associated with APC function and maturity that have been extensively reported in the literature for NOD mice [124, 196-200]. After 24 hours in culture, we measured TNFα secretion in cell culture supernatants from BMDMs treated with LPS ± PFK15. While our BMDC studies were performed using 5 μM PFK15, which we found to be the optimal dose in our T cell studies in Chapters 2 and 3, we performed a PFK15 dose titration for our BMDM studies to ensure we were not seeing inefficient downregulation of BMDM function due to insufficient dosing. Similarly to the results obtained with PFK15 treated BMDCs, PFK15 treatment failed to reduce LPS stimulated BMDM secretion of TNFα, a key mediator in the pathogenesis of T1D (Figure 24). Further, an inability to therapeutically target PFKFB3 in NOD BMDMs was not due to defective function associated with the NOD mouse, as PFK15 treatment failed to reduce TNFα secretion by both non- autoimmune prone C57BL/6J (Figure 24A) and autoimmune prone NOD BMDMs (Figure 24B). Cumulatively, these data indicate that PFK15 treatment does not alter BMDM function.

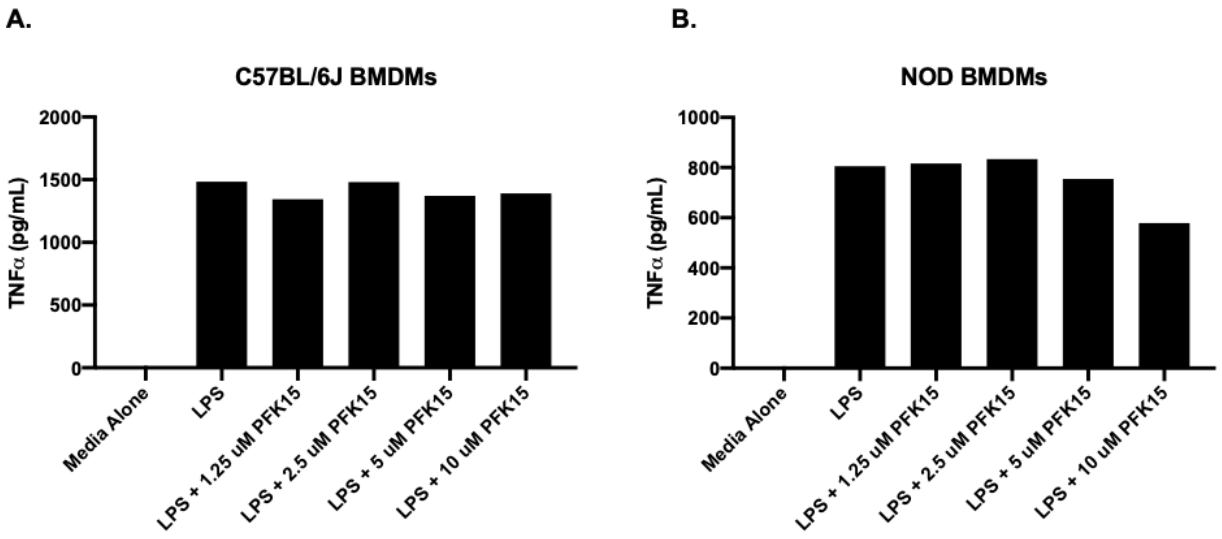


**Figure 24. PFK15 treated BMDMs retain their ability to secrete the effector cytokine TNFα.**
BMDMs were differentiated from bone marrow cells isolated from C57BL/6J or NOD mice. After differentiation, cells were stimulated with 100 ng/mL LPS ± 1.25 - 10 μM PFK15. After 24 hours in culture, supernatants were harvested and utilized to measure TNFα secretion from **A.** C57BL/6J BMDMs or **B.** NOD BMDMs. Data are preliminary and representative of a n = 1.

### 4.4.4 PFK15 treatment *in vivo* does not alter macrophage migration and infiltration into pancreatic islets in an adoptive transfer model of $CD4^+$ T cell mediated T1D

Induction of autoimmune diabetes by diabetogenic BDC2.5 T cells is dependent on macrophages, as they are the final effectors that mediate β cell killing in adoptive transfer models using $CD4^+$ T cells only [201]. Since PFK15 treatment delayed diabetes onset in *in vivo* adoptive transfer studies and reduced $CD4^+$ T cell invasion in pancreatic islets, we wanted to assess the ability of macrophage populations to migrate and infiltrate the islets [155]. To assess this, we stained pancreatic sections from control and PFK15 treated animals from previous adoptive transfer studies (Chapter 3) with the macrophage marker F4/80. No significant differences in islet infiltration of macrophages were observed between vehicle control and PFK15 treated animals, indicating that protection from disease was due to specific targeting and inhibition of autoreactive T cell populations (Figure 25).

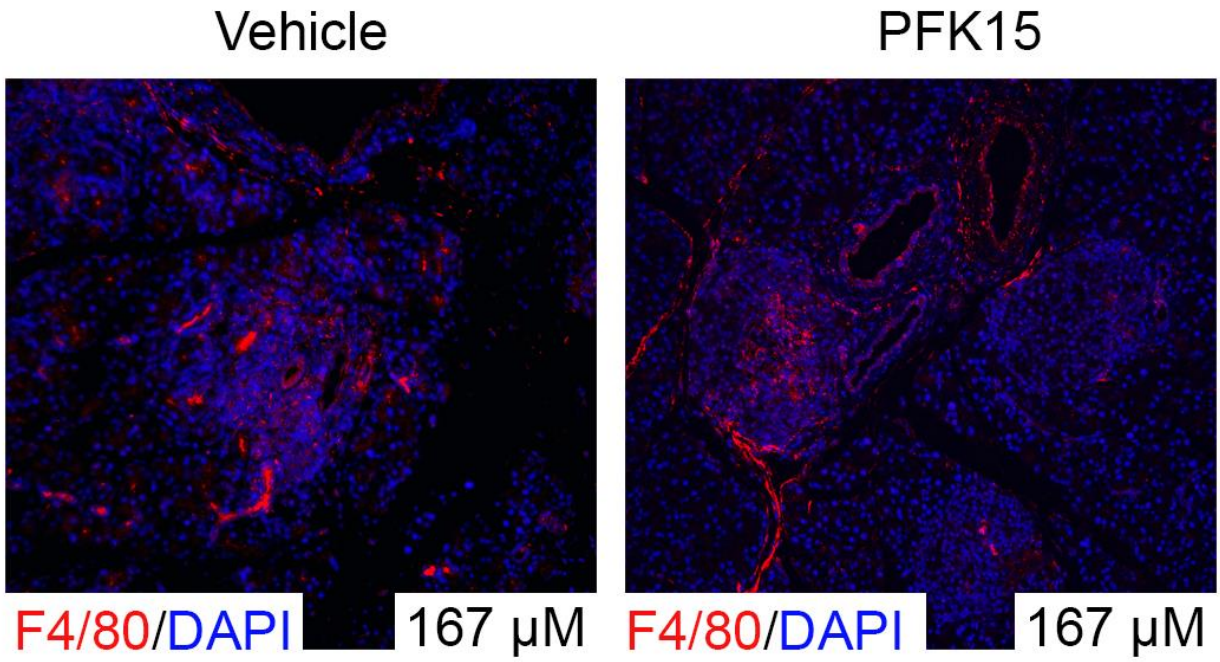


**Figure 25. PFK15 does not alter macrophage infiltration of pancreatic islets in *vivo*.**

Representative immunofluorescence staining of macrophage infiltration by F4/80 staining in pancreatic sections from control and PFK15 treated animals from adoptive transfer studies outlined in chapter 3.

## 4.5 DISCUSSION

Macrophages and DCs are important mediators of immunity, and are crucial for initiating autoreactive T cell responses against β cells during the pathogenesis of T1D [1, 33-35, 45, 201]. Recently, interest in the field of immunometabolism has demonstrated that bioenergetic pathways are important for proper immune cell function, both during the initiation and resolution of inflammation [59, 60]. Specifically, macrophages and dendritic cells metabolically transition to the less efficient aerobic glycolysis during their activation, similarly to both T cells and cancer cells. Notably, while T cells and cancer cells shared reliance on the glycolysis pathway occurs as a means to generate nucleotides and biosynthetic intermediates required for proliferation and in the case of T cells, effector functions, macrophages and DCs resort to this pathway as a survival mechanism [75, 76, 78, 188, 190, 191]. Upon stimulation, APCs increase the expression of inducible nitric oxide synthase (iNOS), which produces nitric oxide (NO) from arginine, a toxic gas with microbicidal, antiviral, and antitumoral properties [202]. NO inhibits mitochondrial electron transport and reduces oxygen consumption and coupled ATP production, ultimately leading to a collapse in mitochondrial respiration [59]

Adoption of glycolysis, however, is not the only reason APCs rely on this pathway, as it's required for initiating activation early following stimulation. Evidence of this is supported by studies treating BMDC and BMDM macrophage cultures with the glycolysis inhibitor 2-DG prevented their activation, costimulatory molecule expression, and ability to secrete proinflammatory cytokines [75, 76, 78, 188]. In contrast to previously published literature, glycolysis inhibition via PFK15 treatment of BMDM and BMDCs from the autoimmune prone NOD mouse did not alter their activation or effector function. Further, this was not due to the inherent defects associated with APCs in the NOD mouse and T1D patients, as BMDMs from

control C57BL/6J were also unaffected by PFK15 treatment. The differences in the observed outcomes of our study with previously reported literature may be due to differences in the glycolysis inhibitor used in each study. As discussed in chapter 3, 2-DG is often the glycolysis inhibitor of choice for immunometabolism based studies. However, 2-DG is not a specific inhibitor of a glycolysis enzyme like PFK15 is, but rather is a non- metabolizable glucose analog that must be administered in often toxic doses to outcompete endogenous glucose levels in cell culture supernatants or in *in vivo* models [155]. As we demonstrated in chapter 3 with the differential effects of 2-DG and PFK15 on T cell responses, it appears this is also true with BMDC and BMDM responses to stimulation. These data provide further evidence to support that treatment with PFK15 selectively targets autoreactive T cells, while leading other mediators of T1D unaffected.

Another possibility contributing to the inability for PFK15 to dampen APC function could be due to an ability for macrophages and DCs to be metabolically adaptable. While T cells appear unable to utilize other forms of energy to fuel effector functions, APCs demonstrate metabolic plasticity to fulfill their ability to fend off foreign invaders. In a study by Raulien et al., monocytes stimulated with LPS under conditions of glucose starvation were capable of shifting their metabolism toward OXPHOS fueled by FAO, thus allowing them to maintain activation and proinflammatory cytokine secretion [80]. However, the expression of iNOS and the production of NO was not tested in this model, and would be important to measure given its role in collapse of the ETC and oxidative metabolism. It would be interesting to know whether glycolysis is required for NO production, and what specific pathways mediate the various aspects of APC biology and function. It is also important to consider that use of BMDCs and BMDMs differentiated from bone marrow progenitors, while useful model systems, are not entirely representative of tissue resident APCs. Further studies assessing immunometabolism of APCs isolated from within the pancreas

and pancreatic draining lymph nodes during the course of T1D would provide diabetes relevant data, and ensure the phenotypes observed were specific to macrophages and DCs resident in the pancreatic microenvironment. Cumulatively, our data demonstrate that macrophages and DC function are unaltered by PFK15 treatment *in vitro* and *in vivo*, and suggest that the therapeutic benefits associated with PFK15 treatment are due to a direct effect on $CD4^+$ T cells (chapter 3). Due to their importance in the immunopathology of T1D, use of PFK15 in conjunction with therapeutics that also suppress macrophage and DC functions may prove to be beneficial. However, it will be important to know whether functional APCs are required for PFK15 mediated induction of T cell exhaustion, as the expression of PD-1 and LAG-3 on $CD4^+$ T cells occurs as a result of activation, and is dependent on APC interaction with T cells.

# 5.0 FUTURE DIRECTIONS

## 5.1 IMMUNOMETABOLISM IN TYPE 1 DIABETES

### 5.1.1 Metabolic characterization of β cell reactive $CD4^+$ T cells

While the data presented in chapters 2 and 3 demonstrate that T cells from the NOD mouse are susceptible to modulation with the glycolysis inhibitor PFK15, the metabolic profile of autoreactive T cells in T1D remains unknown. There is mounting evidence to suggest that autoreactive T cells do not play by the same rules metabolically [86, 115, 155]. As described in great detail in the introduction, studies in SLE, MS, and RA have provided robust evidence that autoreactive T cells display altered metabolic dependencies and programs [55, 81, 82]. Generally speaking, autoreactive T cells demonstrated enhanced bioenergetics characterized elevated glycolysis and mitochondrial oxidative metabolism, as well as mTOR activity compared to control animals [84, 86, 101].

In T1D, although specific evidence is lacking in regard to the metabolic preferences of diabetogenic T cells, recent studies have provided insight into the bioenergetic profiles of β cell reactive T cells. Firstly, Chen et al. observed that T cells from human T1D patients displayed increased mitochondrial hyperpolarization (MHP) compared to control subjects, which correlated with increased activation induced IFNγ secretion and ROS production [117]. Typically, enhanced MHP is associated with an activated phenotype characterized by increased bioenergetics [110]. In line with these data, work by our laboratory demonstrated that ROS are required for metabolic reprogramming to glycolysis during the activation of diabetogenic $CD4^+$ T cells [123]. As glycolysis is required for ROS and IFNγ production, and with T cells from T1D patients displaying

increased MHP, it's likely that autoreactive T cells in T1D are more metabolically active than control T cells, however further exploration is required to validate these hypotheses. Interrogating the metabolic preferences of autoreactive T cells would allow for an ability to potentially utilize multiple metabolic inhibitors as a means to control their activation and effector functions during the course of T1D. Combinatorial approaches have been tested in other autoimmune disorders, where treatment of lupus prone mice with 2-DG and Metformin (inhibitor of mitochondrial metabolism) was proven beneficial in reducing autoreactive T cell effector functions [15]. However, use of Metformin in T1D is somewhat controversial due to its anti- hyperglycemic properties, with a high risk of the development of hypoglycemia in T1D patients [203].

To begin to explore these questions, we are planning future experiments to assess the metabolic phenotypes of autoreactive T cells from the NOD mouse. As female NOD mice spontaneously go diabetic, we would be able to follow the progression of T1D and take T cells at different timepoints to assess metabolic characteristics via Seahorse Extracellular Flux Analysis to assess glycolytic and oxidative metabolisms. We will also activate these T cells ex vivo and determine whether bioenergetics relay autoreactive T cells enhanced effector functions. The MHC-II matched non-obese diabetes resistant (NOR), and non- autoimmune prone C57BL/6J animals will be used to isolate T cells from control animals. One caveat of this study is the use of bulk T cell populations, as only a small percentage of the T cell repertoire are autoreactive, with an even smaller frequency in control strains. However, studies in other autoimmune diseases used bulk T cell populations and were still able to capture differences metabolically between control and diseases animals [86, 115, 155]. If needed, we can specifically isolate insulin or BDC2.5 reactive T cells via tetramer staining to assess the metabolic preferences of autoreactive T cells specifically. Another limitation of these studies is that autoreactive T cells in the NOD mouse may be inherently more "active" even in

younger mice prior to onset of disease, as they develop in and have likely encountered their antigen (pancreatic β cells). Moreover, increased activation phenotypes of autoreactive T cells encounter their antigen in the thymus during negative selection, as insulin is expressed there. However, in T1D, autoreactive T cells are able to escape deletion through unknown mechanisms. Therefore, it's possible that β cell reactive T cells leave the thymus already in an activated state, thus allowing for their persistence and resistance to tolerance inducing mechanisms in the periphery [15].

A major focus of our study, and the future directions proposed here remained focused on interrogating the metabolic requirements and vulnerabilities of $CD4^+$ T cells in T1D. However, translating our work to human studies would be another future direction of this project. As has been done in SLE, it would be interesting to determine the metabolic profile of T cells from T1D patients [84-87, 115]. We currently have collaborations with Drs. Eddie James and Sally Kent, two experts in the isolation of autoreactive T cells from the PBMCs, as well as in the isolation of infiltrating T cells from the pancreata of T1D patients. In fact, a major focus of a recent R21 submission from our laboratory is focused on understanding whether PFK15 can induce T cell exhaustion in primary human T cell clones from type 1 diabetics, and would allow for translational potential of PFK15 to reduce human $CD4^+$ T cell effector functions in those genetically predisposed to autoimmunity.

### 5.1.2 Effects of metabolic modulation on $CD8^+$ T cells, macrophages, and dendritic cells

Our work presented here was primarily focused on modulating $CD4^+$ T cell metabolism as a means to prevent autoimmune targeting of pancreatic β cells, due to their importance in mediating the pathogenesis of T1D. However, as mentioned previously, a number of immune cells target pancreatic β cells for destruction, including APCs and $CD8^+$ T cells.

In chapter 4, we performed preliminary *in vitro* studies to determine whether APCs were affected by PFK15 treatment, as they too transition to aerobic glycolysis upon activation [75, 78]. In contrast to what has been reported in the literature, glycolysis inhibition had no impact on BMDC and BMDM activation or effector functions. This is in line with the data in chapter 3, where PFK15 treatment did not alter expression of early activation marker CD69, indicating an ability to become activated. One reason for the stark results obtained in our study versus others may be due to the choice of inhibitor. As we demonstrated in chapter 2 when treating BDC2.5 splenocytes with 2-DG, PFK15, YN1, and PFK158 to confirm the phenotypes we observed were due to targeting glycolysis, we observed vastly different outcomes on the T cell responses when treating with 2-DG versus specific inhibitors of the glycolysis enzyme PFKFB3. In the literature, 2-DG has been the glycolysis inhibitor of choice in studies examining the immunometabolism of APCs. Therefore, future studies should be aimed at expanding the studies we performed with PFK15 using other metabolic inhibitors like 2-DG to compare the therapeutic benefits associated with each inhibitor. Other parameters associated with APC function, such as antigen processing and presentation should also be tested to fully appreciate the impact glycolysis inhibition has on APC function. Another reason for the different outcomes we observed in modulating glycolysis utilization by APCs beyond use of different inhibitors could be due to potential metabolic adaptability. Raulien et al. demonstrated that monocytes are sufficiently metabolically flexible, as LPS stimulation of monocytes under conditions of glucose deprivation did not alter their migration and phagocytosis abilities, nor their ability to secrete effector cytokines like TNFα, IL-6, or IL-1β [80]. Instead, metabolic assessment revealed that glucose deprived monocytes shifted their metabolism towards OXPHOS fueled in large part by FAO [80]. Although $CD4^+$ T cells were unable to metabolically transition to a different energy source upon PFK15 treatment, metabolic

plasticity of APCs allows them to utilize alternative fuel sources to maintain important effector functions. It would be interesting to know whether similarly to the study by Raulien et al., if PFK15 treated APCs stimulated with LPS are able to metabolically revert back to OXPHOS to generate ATP for effector capabilities. Lastly, the NOD mouse and human patients with T1D suffer from severe maturation defects in their APCs, likely due to associated MHC-II haplotypes (I-Ag7 in the NOD mouse, or HLA class II alleles DR4, DQ8, and DQ2, which confer the highest genetic risk of T1D in human patients) [124, 197-200, 204]. As maturation of APC responses relies on the metabolic transition to glycolysis upon activation, its likely macrophages and DCs in mouse model and humans with T1D may have metabolic defects that correlate with their dysfunctionality. Further studies fully characterizing the metabolic profile of APCs in the NOD mouse and human patients would be interesting to pursue to have a better understanding of the mechanisms mediating immaturity and dysfunction of APCs that may contribute to the pathogenesis of T1D.

$CD8^+$ T cells typically attract the most attention in the pathogenesis of T1D, as they are able to directly cause β cell death through their cytolytic capabilities [45]. However, their ability to target pancreatic β cells relies on $CD4^+$ T cell help, as secretion of IFNγ by autoreactive $CD4^+$ T cells aids in the activation and maturation of CTL responses [45, 121, 155, 189]. Further, in mouse models of autoimmune diabetes $CD4^+$ T cells are able to cause diabetes on their own, while adoptive transfer of $CD8^+$ T cells alone fail to cause diabetes when transferred into immunodeficient recipients, with the exception of the A14 $CD8^+$ T cell clone [205]. Studies in T1D have demonstrated that $CD8^+$ T cells require glycolysis for their cytolytic activity, and are susceptible to regulation by 2-DG [151, 206]. Therefore, it's likely that PFK15 too would have similar effects on the activation and effector functions of diabetogenic $CD8^+$ T cells. However, even if PFK15 did not directly affect $CD8^+$ T cell function, we would hypothesize that PFK15

induced exhaustion of $CD4^+$ T cells would have downstream consequences on $CD8^+$ T cells, as they would not be as able to provide help to efficiently activate CTL responses. We are currently in the process of acquiring the diabetogenic A14 $CD8^+$ T cell clone to perform *in vitro* studies testing the ability of PFK15 treatment to induce exhaustion in the $CD8^+$ T cell compartment similarly to $CD4^+$ T cells.

Ultimately, while the isolation and characterization of the metabolic profiles of specific immune cell subsets would be interesting, these studies are unrealistic. T1D is a result of the innate and adaptive immune systems working cooperatively to mediate β cell death. Therefore, studies taking this into account would provide the most beneficial results with translational potential. To do this, we propose performing prevention studies in female NOD mice. Prior to the onset of diabetes, NOD mice would be treated with PFK15 to determine the impact glycolysis inhibition has on the pathogenesis of T1D over the natural course of disease. Then, we could look at the phenotypes of $CD4^+$ and $CD8^+$ T cells, as well as APCs both in the periphery and within the pancreatic islets to characterize changes in islet infiltration. Differences in the kinetics of disease onset in NOD mice may be difficulty in assessing the best time to initiate treatment. In those instances, synchronization with cyclophosphamide or checkpoint blockade, as has been previously reported, could be used to circumvent this [30].

### 5.1.3 Resisting Tolerance: Linking metabolism, checkpoint molecules, and TCR signal strength to autoreactive T cell persistence

As discussed in great detail in chapter 3, treatment of $CD4^+$ T cells with PFK15 *in vitro* and *in vivo* induced the expression of the checkpoint molecules PD-1 and LAG-3 and led to downstream functional and metabolic exhaustion. While T cell exhaustion is associated with negative consequences in the immune response to cancer, this phenotype is protective in settings of

autoimmunity. This is especially true in T1D, as T cells from human patients display a reduced ability to upregulate PD-1 compared to T cells from healthy subjects [134], suggesting an inability to be regulated by IR signaling in the periphery due to reduced expression of checkpoint molecules. Interestingly in the NOD mouse, expression of PD-1 on $CD4^+$ T cells has been implicated in suppressing islet infiltration during autoimmune diabetes, indicating a protective role for PD-1 in mediating protection of β cells [135]. Further, polymorphisms in the PD-1 have been identified in T1D patients, and are associated with susceptibility to disease [185]. However, IR expression alone is not sufficient to induce T cell exhaustion, as activated T cells upregulate both PD-1 and LAG-3 upon activation and TCR signaling. In fact, exhaustion can occur even in the absence of checkpoint molecules, further questioning the exact role these molecules play in enforcing or maintaining functional exhaustion [177].

While IRs may play a lesser role in driving exhaustion in cancer, they appear to be pivotal in restoring tolerance against autoimmune responses. For example, in the absence of PD-1 or LAG-3, and during PD-1 or LAG-3 blockade, NOD mice demonstrate accelerated diabetes [30, 32, 131]. In contrast, PD-1 or LAG-3 deletion in non- autoimmune prone mouse strains results in minor phenotypes without spontaneous disease development [155]. This divergence in the phenotypes of PD-1 and LAG-3 KO animals on the C57BL/6 versus NOD backgrounds could come down to faulty antigen presentation by APCs in T1D. Piganelli et al. demonstrated that splenic macrophages in the NOD mouse are defective in their ability to present antigen, leading to less-than-optimal T cell activation [124]. This reduced strength of signal during autoreactive T cell activation, while enough to promote differentiation to effector subsets, may be insufficient to upregulate IRs, leading to uncontrolled and persistent pathogenic responses to self-antigen. Finally, decreased TCR:MHC signal strength may explain why autoreactive T cells escape deletion

in the thymus, as binding affinity to self- peptides controls what T cell clones are positively selected for or deleted.

Reduced TCR signal strength could also explain why Tregs fail to control diabetogenic effector T cell subsets during T1D, as strong TCR signals are required for selection of self- reactive Tregs in the thymus [207]. Regarding the role of signal strength and Treg suppressive function, stimulation of Tregs by peptide- MHC complexes determines strength of Treg mediated suppression capabilities [208]. Therefore, weak engagement of self- reactive Tregs with peptide-MHC complexes may permanently imprint reduced suppressive capabilities, rendering them unable to control effector T cells in the periphery.

Tregs utilize a number of methods to suppress conventional T cells, including cell contact dependent or independent mechanisms. One cell contact dependent mechanism of Treg suppression is the expression of immune inhibitory receptors and their ligands by Tregs, with evidence supporting a critical role of IRs in mediating Treg function [209]. Specifically, the PD-1/PD-L1 axis is vital to both Treg development and function [209]. Francisco et al. reported that PD-L1 signaling can induce FoxP3 expression and enhance their immunosuppressive function [210]. PD-L1 signaling was also capable of converting naïve Th1 T cells into Tregs via downregulation of the AKT and mTOR pathways [210, 211]. Importantly, reduced expression of IRs would be detrimental, as an inability for IRs to engage their ligands could lead to a breakdown in self- tolerance. Similarly to as has been described in conventional T cells in T1D where IR expression is dysregulated [134], Tregs from T1D patients are defective in their ability to upregulate PD-1 as well [212, 213]. Cumulatively, these data provide evidence that defective IR expression affects the ability of effector T cells to be suppressed, and also leads to dysfunctional Treg suppressive function, therefore contributing to the ability of autoreactive T cells to become

activated and mediate T1D pathogenesis. Garnering a better understanding of the mechanisms leading to IR defects associated with T1D will allow for targeted therapeutic approaches.

The expression, or lack thereof, of IRs on T cells ultimately have the ability to influence metabolism. Especially given the role bioenergetics and IRs have in modulating T cell function. IRs themselves can control T cell metabolic programs. For example, PD-1 has been implicated in suppressing glycolysis and promoting lipolysis and FAO, thereby maintaining tolerance and inhibiting effector T cell differentiation [83, 109]. LAG-3 has also been implicated in controlling T cell metabolic programs, and has been a major focus within out laboratory. Previte et al. demonstrated that LAG-3 deficiency enhanced the metabolic profile of $CD4^+$ T cells, characterized by increased aerobic glycolysis and effector functions following activation [110]. Although a characterization of LAG-3 expression has not been performed in T1D patients to, we would hypothesize that dysfunctional IR expression encompasses LAG-3 as well, especially due to the synergistic nature of PD-1 and LAG-3, as well as the redundant nature that exists amongst IRs [110]. Based on this assumption, we would expect that autoreactive T cells would have metabolic profiles similar to LAG-$3^{-/-}$ T cells, characterized by increased glycolysis and oxidative metabolisms. Further work is required to understand the role LAG-3 plays in the pathogenesis of T1D.

Regarding Tregs, who primarily utilize FAO and OXPHOS via the mitochondria, reduced expression of IRs in setting of autoimmunity may promote Tregs to commit to a glycolytic program. Although Tregs do utilize glycolysis to fuel their proliferation, with some evidence supporting use of this pathway by human Tregs as well [67, 74], increased glycolysis may allow Tregs to adopt a proinflammatory phenotype that has been documented in T1D patients, where Tregs lose their suppressive capabilities and begin to secrete IFNγ [9, 10].

In sum, a plethora of defects affecting patients with T1D ultimately lead to the escape and activation of self- reactive T cells that mediate attack of β cells. These defects, including aberrant T cell activation and excessive effector cytokine production, Treg dysfunction, and reduced IR expression, likely contribute to metabolic abnormalities that deserve and require further exploration [15]. The work presented here, however, demonstrates an ability to modulate the glycolysis pathway as a means to induce T cell exhaustion, a protective phenotype with the ability to prevent or significantly delay the onset of autoimmunity in T1D. Our work demonstrates a novel ability to correct a number of defects associated with autoreactive T cells in T1D, and should be expanded to interrogate how metabolic modulation impacts all mediators involved in the pathogenesis of autoimmune diabetes.

## 5.2 THERAPEUTIC OPPORTUNITIES FOR MODULATING IMMUNE CELL METABOLISM IN TYPE 1 DIABETES

Insulin replacement has been the front- line therapy for type 1 diabetics since the early 1920's, with a number of adverse complications associated with its long- term use [34]. Although the administration of exogenous insulin is vital to the health and wellness of T1D patients, it acts as somewhat of a band aid, covering up the problem rather than managing the underlying problem: self- reactivity. A number of immune mechanisms and pathways have been targeted in T1D [214], however a large portion of these studies have been performed in patients with new- onset or established T1D. As only 10-20% of β cell mass remains at the time of diagnosis, immunomodulatory therapies would be better suited if they could be administered to patients prior to the onset of clinical disease. As discussed in chapter 3, predicting patients most at risk for

progressing to T1D is possible, as the presence of 2 or more autoantibodies predicts a lifetime risk of almost 100% [187]. Proof of principle of this concept is highlighted by the Teplizumab prevention study, where use of an anti- CD3 antibody delayed T1D onset by 2 years, and was associated with partial exhaustion of $CD8^+$ T cells in responding patients [146, 215]. Importantly, while prevention could be possible, determining the time of intervention can be difficult, as it can be challenging to pinpoint exactly when autoreactive T cells are activated. On the other hand, reversal strategies for T1D, including islet transplantation and gene therapy approaches to restore endogenous β cell mass have been tested as well. However again, these therapies while beneficial initially ultimately fail due to a reoccurrence of autoimmunity [143]. These studies beg the question, when should metabolic inhibitors like PFK15 be administered in a clinical setting?

While prevention may be the ideal way of handling autoimmunity in T1D, it's certainly not the easiest to determine the exact kinetics of a patient's path to T1D onset. Even the presence of autoantibodies, while effective at determining lifetime risk of disease development does not provide a specific timeframe for disease onset to occur. One benefit to targeting the glycolysis pathway that is largely lacking from other immunotherapies, including Teplizumab, is lack of specificity. Although PFK15 is not specific for autoreactive T cell populations itself, it's inherently specific for activated T cells, due to their reliance on glycolysis to fuel effector functions, and theoretically would not have an effect on other immune cells such as Tregs or established memory T cells. This specificity would allow for specific targeting of effector T cells, as long as treatment was administered at a time when these cells were activated. Second, there have been reports indicating that metabolic inhibitors display cellular selectivity based on demand, with only the most activated cells being susceptible to modulation, and therefore leaving less metabolically active cells unscathed [17, 29, 154, 155, 168]. Further, although there's a possibility that an

individual experiencing autoimmunity could also be fighting off an infection and have active immune responses against multiple targets, there is evidence to support that autoreactive T cells are more metabolically active than non- autoreactive counterparts. This has been demonstrated in one study, where lupus prone mice receiving metabolic modulators were still capable of mounting a response to nominal antigen immunization., suggesting global immunity remained intact during this type of treatment [84]. Although our studies did not test the ability of PFK15 treated animals to respond to foreign antigen immunization, there is precedence in the literature that metabolic inhibitors could have a therapeutic benefit without global immune consequences, and therefore deserve further exploration. While we have demonstrated prevention is possible in mouse models of autoimmune diabetes with PFK15, we would need to ensure an active immune response was occurring for these types of therapies to be initiated in a clinical setting. Testing for autoantibodies is a start, as their presence is heavily suggestive of an ongoing immune response, however it would also be helpful to phenotype T cells from patients at risk to determine their activation status. Further assessment of biomarkers for at risk patients would greatly advance the ability to predict T1D onset, and provide better therapeutic windows for modulation of the glycolysis pathway to obtain beneficial outcomes.

Use of PFK15 in conjunction with therapeutic approaches to reverse disease and restore β cell mass could be even more promising, and would likely benefit a larger percentage of patients. One study by Xiao et al. demonstrated an ability for an AAV viral vector gene therapy approach to reprogram α cells within pancreatic islets into functional, insulin secreting β cells, capable of restoring euglycemia in diabetic animals durably for 4 months [143]. This delay in the reoccurrence of autoimmunity and diabetes onset was significant, however again, this type of therapy failed to cure self- reactivity. However, this could be a setting where PFK15 would be

beneficial as a combinatorial approach to 1) restore β cell mass endogenously in the patient and 2) induce exhaustion of autoreactive T cells that will eventually target the neogenic β cells. We are currently in collaboration with Dr. Xiangwei Xiao of Dr. George Gittes's group here at the University of Pittsburgh to determine the ability of PFK15 to maintain protection of these neogenic β cells by inducing exhaustion of autoreactive T cells that ultimately target them without further intervention. These studies are ongoing and are a future direction of the concepts and ideas demonstrated here. Ultimately, we believe modulating T cell metabolism is a viable therapeutic opportunity for the clinical prevention or reversal of T1D, and deserves further exploration in both aspects with the goal of finding a cure for those affected. Especially given the terminal exhaustion phenotype we observed when treating diabetogenic T cells, as this suggests only a short- term treatment would be required to provide lasting tolerance to β cells.

### 5.2.1 Optimization of Controlled Release Microparticle Systems for Delivery of Metabolic Modulators in conjunction with Antigen- Specific approaches

In chapter 2, we explored use of PLGA microparticles encapsulated with PFK15 to specifically target autoreactive T cells by administering them in close proximity to the pancreas. While PFK15 MPs were able to inhibit T cell responses to ConA stimulation *in vitro* similarly to soluble drug, we did not observe a statistically significant delay in diabetes onset in adoptive transfer prevention studies. One future direction for this project would be to further optimize the dose and delivery kinetics required to obtain therapeutically beneficial results, as we had observed with systemic PFK15 treatment. It is possible that a high systemic dose of PFK15 treatment is required for the induction of T cell exhaustion that correlated with disease prevention in our studies, therefore it may not be possible to efficiently deliver an equivalent dose locally to obtain the desired outcome. In one study (data not shown), we treated recipient animals in adoptive transfer studies with 12.5

mg/kg and 25 mg/kg of PFK15. Animals receiving the lower dose all succumbed to diabetes, with the higher 25 mg/kg required for therapeutic benefit, suggesting that optimally deriving microparticles able to deliver a lower therapeutic amount locally could be difficult to achieve. Further, delivery near the pancreas is challenging. Although the pancreas is in close proximity to the spleen, exact delivery at the site is difficult and requires further optimization. Further, delivery near the pancreatic draining lymph node would be more beneficial, however its lack of accessibility for subcutaneous delivery of MPs would be another hurdle to overcome.

An alternative approach to use of PFK15 MPs alone would be to utilize them in conjunction with antigen- specific approaches. One idea being pursued to expand this project currently is the generation of multiple antigenic peptides, or MAPs [216]. MAPs are peptides that are branched artificially to a lysine backbone able to support up to 8 different peptide branches. These systems can be used to present antigens to the immune system, and are immunogenic [216]. This approach would allow for the presentation of multiple autoantigens, that could be used to attract autoreactive T cells responding to a panel of antigenic peptides. These MAPs could then be encapsulated into MPs to allow for local delivery, and could act as "bait" to attract autoreactive T cells to the site administered. At the same time, PFK15 MPs could be administered at the sight, released in the local area, and therefore specifically target the autoreactive T cells responding to the antigenic peptides presented on the MAPs. Often, antigen specific solutions do not take into account the breadth of the autoreactive T cell repertoire and focus on one of multiple known autoantigens, with insulin being an ideal candidate in T1D. However, autoreactive T cells respond to a number of different β cell specific proteins, not just insulin, therefore highlighting the need to provide solutions to target a broader range of autoreactive T cell clones. Ultimately, we know that PFK15 is capable of suppressing T cell responses when delivered systemically *in vivo*. However, we

believe that optimization of controlled release systems to better modulate T cell responses locally without potential toxic effects could be beneficial when translating these therapies into the clinic. We plan to continue our collaboration with Dr. Abhinav Acharya and Dr. Steven Little to continue our investigation to optimize our drug delivery systems with the ultimate goal of delivering PFK15 therapeutically without global consequences.

# 6.0 SIGNIFICANCE TO PUBLIC HEALTH

A portion of this section was adapted from the author's version of a review paper. The definitive version of this work was published in *Immunometabolism*.

**"Targeting T cell Metabolism to Combat Autoimmunity: Implications for the Future of Type 1 Diabetes Therapeutics"**

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In 2020, diabetes was the 8$^{th}$ leading cause of death in the United States, with a health care cost estimated to be approximately $327 billion USD [217]. While a majority of diabetes cases are related to Type 2 Diabetes (T2D), T1D patients make up approximately 10% of total cases. Regarding T1D specifically, 1.25 million Americans are living with T1D, including 200,000 youth and over a million adults [217]. If current trends continue, 5 million people in the U.S. are expected to have T1D by 2050, including 600,000 people under the age of 20 years old [217]. Since the 1920's, patients with T1D have been treated by the daily administration of exogenous insulin in order to maintain euglycemia; however, insulin is not a cure for these individuals [40, 51]. Complications still arise from exogenous insulin use, as a number of patients experience low blood sugar unawareness and increased susceptibility to infections, further impacting their day-to-day activities [33-35, 217]. As a result, less than one-third of people with T1D are achieving target blood glucose levels. Furthermore, while the life expectancy of patients with T1D continues to improve, it remains reduced by approximately 20 years compared to the general population [33-35, 217]. For these reasons, new and innovative preventative measures are needed to combat T1D.

As is the case in several autoimmune diseases, the activation of autoreactive CD4$^{+}$ T cells that escape central tolerance and bypass tolerogenic mechanisms in the periphery drive T1D pathogenesis [40, 51]. Therefore, understanding the mechanisms governing T cell activation and aberrant immune- mediated responses are vital for finding druggable therapeutic targets for disease prevention or treatment. An emerging concept in immunology is that metabolic reprogramming and lymphocyte activation are intricately linked, as both T cell fate and function are dependent on cellular metabolism [54, 56, 58-62, 64, 66]. Consequently, cellular metabolism has the ability to influence the final outcome of the innate and adaptive immune responses, further impacting health and disease outcome [54]. Studies interrogating T cell activation and function in animal models

and human patients with T1D have indicated autoreactive T cells may have a heightened activation status [55, 81, 82]. This altered status, along with functionally defective Tregs, leads to T cell activation, enhanced effector capabilities, and excessive proinflammatory cytokine secretion, especially interferon-γ (IFNγ).

In the studies presented here, we took advantage of the metabolic changes that occur over the course of the T cell lifecycle, and selectively targeted the glycolysis pathway as a means to suppress the activation and effector function of autoreactive $CD4^+$ T cells in preclinical models of T1D. Not only did treatment with the glycolysis inhibitor PFK15 significantly delay the onset of autoimmune diabetes in adoptive transfer models, but inhibition of glycolysis led to the induction of terminal exhaustion of diabetogenic T cell clones characterized by increased expression PD-1 and LAG-3. This phenotype was permanent, as treatment with checkpoint blockade immunotherapy failed to reverse T cell exhaustion phenotypes *in vitro* and *in vivo*, indicating formation of durable tolerance.

While T cell exhaustion is detrimental in cancer and chronic infection, as this phenotype leads to an inability to efficiently clear tumors or pathogens, induction of T cell exhaustion in settings of autoimmunity has protective benefits and thus safe host tissues from autoimmune targeting [15, 155]. Exhausted T cell subsets not only have functional defects, but also suffer from metabolic insufficiency and a reduced capacity to generate ATP. We too observed this phenotype in PFK15 induced exhausted T cells. Metabolic dysfunction has been implicated in the formation of terminally exhausted cells, which are refractory to reinvigoration by restimulation or checkpoint blockade [15, 155]. This has broad implications in chronic infection and cancer, as restoring metabolic sufficiency to exhausted T cell subsets is vital to reinvigorating exhausted T cell responses. Ultimately, the link we have established between immunometabolism and T cell

exhaustion are vastly applicable to a number of areas including autoimmunity, cancer immunology, and chronic infection. Lastly, the lessons learned here offer valuable insight into both the promotion and reversal of T cell hyporesponsiveness, with the glycolysis pathway at the center of proper T cell function.

**Appendix A Supplementary Data for "Glycolysis Inhibition induces Functional and Metabolic Exhaustion of CD4 T cell in Type 1 Diabetes"**

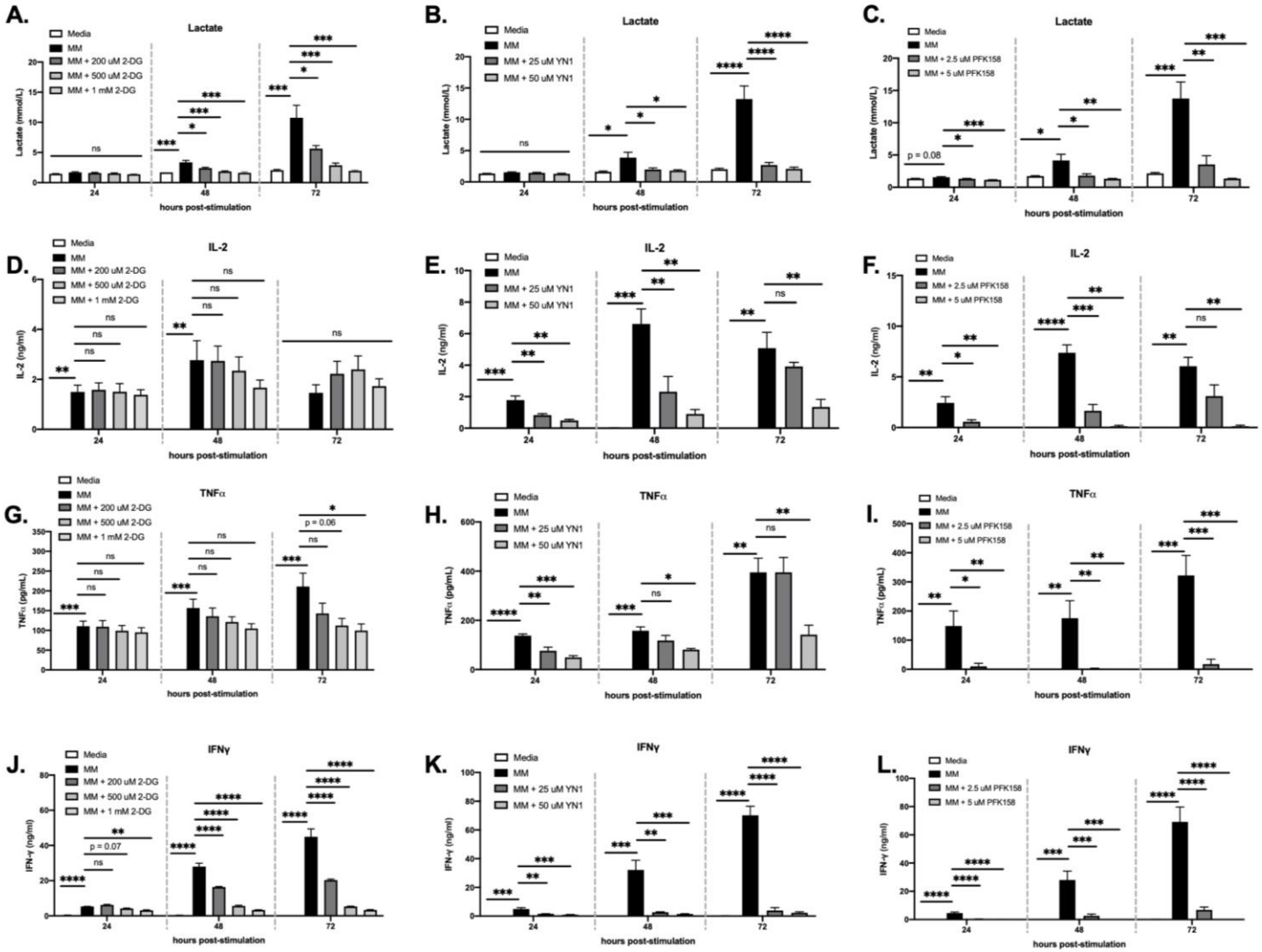


**Figure 26. Specific targeting of PFKFB3, not glycolysis inhibition alone, reduces BDC2.5 T cell effector functions *in vitro*.**
Comparing the impact of glycolysis inhibition with 2-DG or PFKFB3 inhibition (via administration of YN1 or PFK158) on diabetogenic $CD4^+$ T cell responses *in vitro*. NOD.BDC2.5 splenocytes were stimulated with their cognate peptide MM ± 200 μM, 500 μM, and 1 mM 2-DG; 25-50 μM YN1, or

2.5-5 μM PFK158 for 24-72 hrs. **A-C.** Lactate measurements in 24- 72 hr cell culture supernatants from 2-DG (**A**), YN1 (**B**), and PFK158 (**C**) treated cultures. **D-F.** ELISA analysis of IL-2 in 2-DG (**D**), YN1 (**E**), and PFK158 (**F**) treated T cell culture supernatants 24-72 hrs post stimulation. **G-I.** ELISA analysis of TNFα in 2-DG (**G**), YN1 (**H**), and PFK158 (**I**) treated T cell culture supernatants 24-72 hrs post stimulation. **J-L.** ELISA analysis of IFNγ in 2-DG (**J**), YN1 (**K**), and PFK158 (**L**) treated T cell culture supernatants 24-72 hrs post stimulation. All data are presented as the mean ± SEM. (n = 3-5; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.005$, **** = $p < 0.0001$).

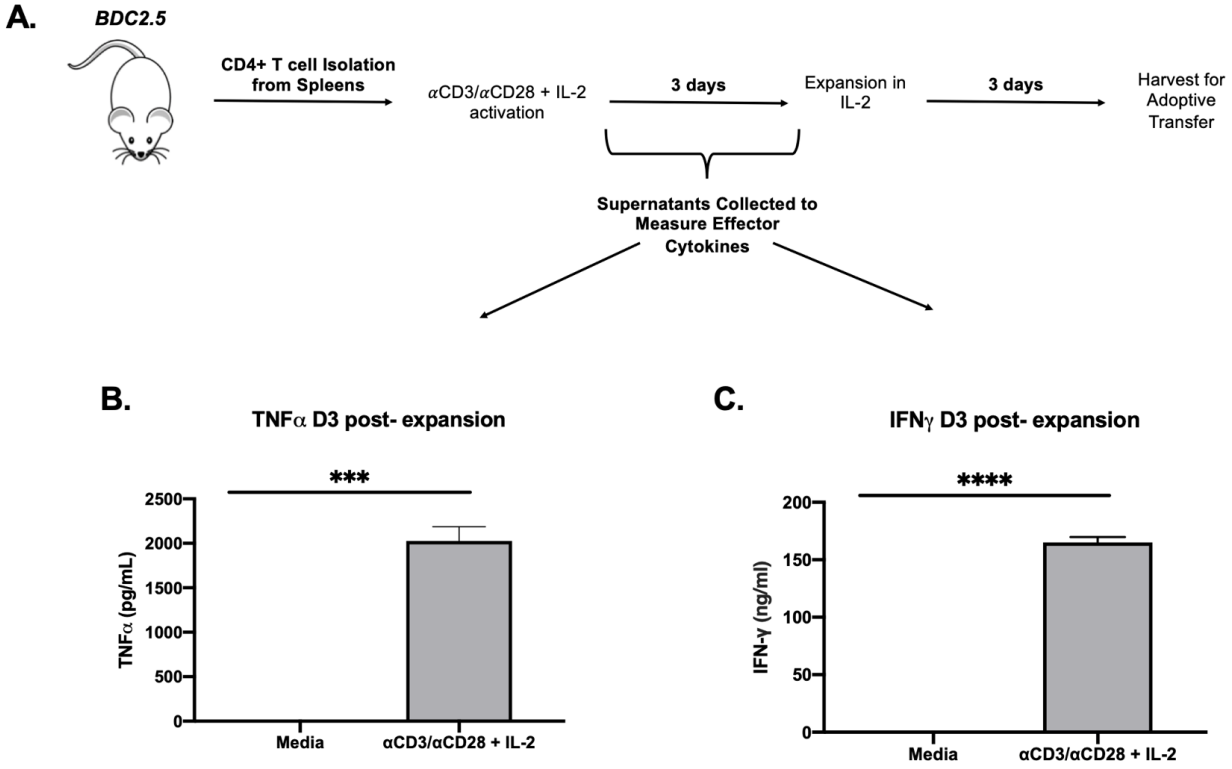


**Figure 27. ex vivo activated BDC2.5 T cells produce proinflammatory cytokines TNFα and IFNγ prior to adoptive transfer.**
Confirming the effector phenotype of *ex vivo* activated BDC2.5 T cells prior to adoptive transfer studies. Isolated $CD4^+$ T cells from the spleens of NOD.BDC2.5.TCR.Tg animals were activated and expanded *ex vivo* with plate- bound αCD3/αCD28 and EL-4 supernatant as a source of IL-2. **A.** Schematic diagram of experimental design and timepoints for analysis. Cells in media alone served as controls. **B.** ELISA analysis of TNFα in BDC2.5 T cell culture supernatants 3 days post stimulation. **C.** ELISA analysis of IFNγ in BDC2.5 T cell culture supernatants 3 days post stimulation. All data are presented as the mean ± SEM. (n = 3; *** = $p < 0.005$, **** = $p < 0.0001$).

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