THESIS

TARGETING PROTEIN KINASE C IN AN AUTOIMMUNE DIABETES MOUSE MODEL

Submitted by

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ABSTRACT

TARGETING PROTEIN KINASE C IN AN AUTOIMMUNE DIABETES MOUSE MODEL

Protein kinase C, a family of intracellular signal transducing proteins, are critical for a myriad of cellular actions with implications in both diabetes and its complications. The isoform PKCθ is enriched in T lymphocytes and functions to mediate signal transduction during T cell receptor stimulation. PKCθ has been shown to play a role in T cell mediated autoimmunity, whereby its activation increases autoimmune T cell function and suppresses that of regulatory T cells. We hypothesize that specific inhibition of PKCθ in autoimmune diabetes will enhance islet tolerance, increase regulatory T cell phenotypes, and delay the onset of overt diabetes in the NOD mouse model. A cell permeable, pseudo-substrate peptide inhibitor was shown to inhibit T cell receptor activation, in both *in vitro* T cell stimulations and *ex vivo* isolated T cells from treated mice. Pre-diabetic NOD mice treated daily with the peptide inhibitor for 4 weeks exhibited increased glucose tolerance at 15 weeks of age as well as increases in regulatory T cell phenotypes and reduced insulitis at 17 weeks of age in comparison to controls. Through long term diabetes onset studies, the peptide inhibitor slightly reduced the incident rate and delayed the onset of diabetes in a subset of animals. The moderate effects observed in preventing disease may be attributed to low efficacy or stability of the peptide inhibitor *in vivo*, which might be remedied by either genetic or pharmacological ablation of PKCθ activity. However, our data demonstrate the potential for targeting PKCθ in autoimmune diabetes as an immunotherapy to prevent or delay disease by increasing tolerance through the expansion of protective regulatory T cells.

TABLE OF CONTENTS

ABSTRACT ……..... ii
INTRODUCTION AND LITERATURE REVIEW 1
HYPOTHESIS AND SPECIFIC AIMS..... 12
MATERIALS AND METHODS 13
RESULTS 19
DISCUSSION.....32
PROJECT SUMMARY AND FUTURE DIRECTIONS 37
REFERENCES 38

INTRODUCTION AND LITERATURE REVIEW

I. Type 1 diabetes mellitus, overview and disease history

Type 1 diabetes (T1D) is a chronic disease characterized symptomatically by insulin deficiency and eventual hyperglycemia, thought to be instigated by immune-associated destruction of pancreatic β cells [1]. Traditionally, clinicians attributed the disease as only affecting children and adolescents but this opinion has changed to include individuals that don't present with disease symptoms until well into adulthood, a disease now termed latent autoimmune diabetes in adults (LADA) [2]. There are a trio of reported symptoms from patients including polydipsia, polyphagia, and polyuria though T1D seems heterogeneous in both etiology and manifestation. Overt hyperglycemia and more recently, glycated hemoglobin ($HBA1_c$) are used as acute and chronic measures, respectively, to determine the severity of disease at onset and track response to therapy. Insulin replacement therapy, though with significant innovation in delivery and analogues, remains the sole effective treatment and is required for life. T1D has been extensively studied in the last three decades, however no successful immunotherapy, transplantation, or β cell regeneration has been shown to prevent or cure disease long term.

The distinction between the two opposing forms of diabetes, type 1 and type 2, was observed in the late 19^{th} century by the French physician Lancereaux, stating there is a "fat" and "thin" form of diabetes, *diabete gras and diabete maigre*[3]. About 15 years before, a British scientist had made a similar distinction on how diabetes had "various forms" requiring "opposing forms of treatments"[4]. This distinction wasn't entirely clear until the use of insulin therapy. They found the "thin" form responded well to insulin therapy and while the "fat" form did not respond

well but could use diet manipulations alone[5]. This culminated in the experiments of Himsworth when he developed a test, a crude version of a glucose/insulin clamp, where oral glucose was given along with IV insulin. Based on the test results of many diabetics he stated that "in insulin-sensitive diabetics the disease is due to deficiency of insulin, whilst in insulin-insensitive patients diabetes mellitus results, not from lack of insulin, but from lack of an unknown factor which renders the body insensitive to insulin."[6] We now know that type 2 diabetes is more complex than simply insulin-resistance in skeletal muscle alone, but these observations roughly define clinical characterization of these two diseases to this day.

In the mid 1970's a paradigm shift occurred in immunology to finally accept the evidence for autoimmunity, helping understand first, Hashimoto's and Addison's disease. These two diseases were in part classified by the hallmark production of autoreactive antibodies, interestingly some diabetics also had high levels of autoantibodies to both thyroid and gastric tissue[5]. Clinicians found these patients were often receiving insulin therapy, leading to the speculation by Ungar that there is an autoimmune basis to insulin-dependent cases of diabetes[7]. After some technical struggles in the field, novel experimentation implicated cell-mediated[8,9] and humoral[10,11] autoimmune activation in insulin-dependent diabetes. Yet, islet cells autoantibodies are not always detectable as a biomarker of disease in patients[12], exemplifying the temporal and heterogeneity of this chronic, immune mediated disease.

There is ample evidence for a viral linkage in the cause of diabetes autoimmunity[13]. Originally thought to be secondary to mumps[14], now many viruses are implicated in triggering disease[13,15]. It is thought the immune response to the virus generates a cross reactivity of antigens in virus and islet that instigate immune infiltration of the pancreatic islets (insulitis) and β cell destruction.

There are also many implications for a genetic cause of T1D revolving around certain HLA haplotypes[16,17]. Once again there is marked heterogeneity among HLA haplotypes in affected humans, though the majority fall within two district groups. Interestingly, through human data analysis, some haplotypes are protective in a dominate fashion, potentially implying the role of HLA in conferring protection instead of predisposing to disease[18]. Recent GWAS analysis has uncovered a large portion of the genes associated with type 1 diabetes are linked to immune regulation and specifically T cells[19,20]. Environmental factors such as level of sun exposure[21] and the microbiome[22] have been implicated in disease-modulating immune activation. Animal models were vital to further understand the pathogenesis of type 1 diabetes and potential therapies.

In 1976, the first two animal models of auto immune diabetes were defined. The streptozotocin induced diabetic mouse generated autoimmune disease after multiple treatments with the selective β cell toxin streptozotocin, which induced inflammation and eventual autoimmune activation to islet antigens[23]. The second model was the Bio Breeding (BB) rat that had a genetic predisposition for insulin deficiency and the histological presence of cellular infiltrate in the islets, often referred to as insulitis[24]. Yet, the most widely used model, the non-obese diabetic (NOD) mouse, would not be described until 1980. This was another animal or mouse model of autoimmune diabetes sharing many genetic similarities with diabetic humans [25], similar autoantibody repertoire[26], and exhibit a predominately T cell driven pathogenesis [1,27]. The studies described in this thesis use the NOD mouse model to further investigate the T cell-mediated aspects of T1D, disruption of self-tolerance, and a cellular target for the following proposed therapy.

II. Cellular contributors to autoimmune diabetes and potential interventions

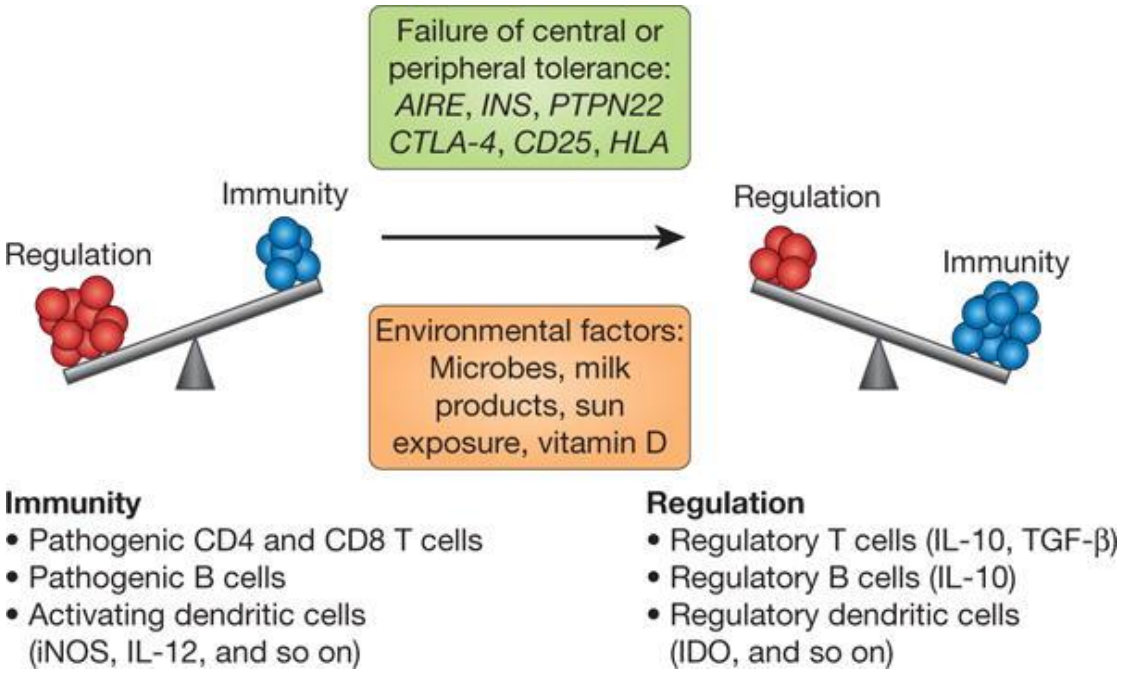


Figure 1.1 Balance of immune activation and regulation in autoimmune diabetes from Bluestone et al. A complex balance of immune cells mediate the break of tolerance in autoimmune diabetes, with both genetic and environmental etiologies. *Nature* **464**, 1293-1300, doi:10.1038/nature08933 (2010).

The manifestation of autoimmunity into full-blown islet destruction is likely driven by an amalgam of cellular players including (**FIG 1**); B cells producing islet specific antibodies, and macrophages and dendritic cells presenting auto antigens to eventually cause the activation and proliferation of autoreactive T cells[1]. This cellular autoimmune activation and loss of tolerance leads to eventual immune infiltration of the islets and the destruction of insulin producing β cells.

Autoantibodies can usually be detected in the serum of patients before they develop clinical diabetes[28]. The main antigens targeted by autoantibodies in T1D are glutamic acid decarboxylase (GAD), islet tyrosine phosphatase (IA-2), and insulin itself. The presence of a singular, specific antibody does not indicate the likelihood of disease progression, yet the

number of autoantibodies in a patient is a strong predictor that they will develop T1D[28]. For example, someone with only autoantibodies to GAD is less likely to progress to overt disease compared to an individual with autoantibodies against GAD, IA-2, and insulin. Auto-antibody. Antibody testing is accomplished using A/G radiobinding assays to detect titers of autoantibodies exceeding control thresholds[29]. Detecting islet autoantibodies has been adapted as an important diagnostic approach for individuals who have a relative with diabetes or a known genetic predisposition. However, in mouse adoptive transfer studies[30], disease can be efficiently transferred by T cells alone. Diabetes also develops in NOD mice expressing a transgene rendering B cells incapable of secreting immunoglobulin[31]. Interestingly, these mice had a greater incident rate of diabetes compared to NOD mice deficient in B cells, yet less than normal NOD mice. This implicates the importance of B cells in presenting antigen to T cells. Though auto antibodies are thought to contribute little to the overall disease, the presence of islet antibody-specific B cells in pancreatic lymph node could assist indirectly through antigen presentation and generally increasing the inflammatory environment by cytokine production in the islet milieu.

Macrophages and dendritic cells are the first cell types to enter the islet in NOD mice. They are thought to be important in early disease as antigen presenting cells[32], and partially mediating β cell destruction[33] during disease progression. Small levels of β cell antigens presented in draining lymph nodes do not incite immune activation, is due in part to tolerogenic dendritic cells as well as lymph node stromal cells[34]. If there is a predisposing HLA haplotype or autoreactive T cell, these antigens break the threshold of self-tolerance and allow for immune activation and proliferation to islet antigens. The dendritic cell has become a target for immune therapy by promoting the function or expansion of tolerogenic dendritic cells[35]. This may be

successful if early intervention can disrupt the critical antigen presentation steps to T cells, the true driver of β cell destruction.

Autoreactive effector T cells break the balance between their counterpart, tolerogenic regulatory T cells, and drive disease through both cytotoxic[35] and helper functions[36]. There is much evidence that these effector T cells can arise from a variety of sources. Expression of insulin in the thymus is controlled by *AIRE* genes and mutations here may generate defective negative selection and cause these cells to enter the circulation[37]. As mentioned, there might be cross-reactivity between viral antigens and those found in the pancreas. Autoreactive effector T cells break the balance between tolerogenic regulatory T cells and drive disease through both cytotoxic[36] and helper functions[37]. There is much evidence that these effector T cells can arise from a variety of sources. Expression of insulin in the thymus is controlled by *AIRE* genes and mutations here may generate defective negative selection and cause these cells to enter circulation[38]. As mentioned, there might be cross-reactivity between viral antigens and those found in the pancreas[13]. This cross-reactivity is then thought to generate an autoimmune reaction that destroys β cells. The majority of evidence surround genetic mutation in T cell regulatory and activation genes such as *IL2RA*, *CTLA4*, and the target of this work, *PRKCQ*[39]. This gene encodes a core protein in T cell receptor activation, protein kinase C isoform θ (PKCθ). Regardless of mutation, phenotypic manifestations of disease seems to be driven by autoreactive effector cells overriding the tolerance afforded by regulatory T cells.

This dynamic has become a major focus of therapeutic intervention[39]. While many therapies directly or indirectly targeting T cells have been successful in the NOD model[40], none have effectively translated into human treatment[41]. This has resulted in criticism of the NOD mouse model[42], likely due to the controlled environmental and defined genetic background,

associated with inbred laboratory animals. However, the model still is of great value, replicating almost all aspect of human T1DM including populations of multiple autoreactive T cell clones. Also, a robust and broad therapy if shown to prevent or even reverse disease in the NOD model may indeed translate to heterogenic human populations with the proper consideration (of what?).

The dynamic between effector and regulatory T cells has become a major focus of therapeutic intervention[40]. While many therapies directly or indirectly targeting T cells have been successful in the NOD model[41], none have effectively translated into human treatment[42]. This has become a large criticism of the NOD animal model[43], likely due to the environmental and genetic control afforded by laboratory inbred animals. However, the model still is of great value replicating almost all aspects of human disease including populations of multiple autoreactive T cell clones. Also, a robust and broad therapy that could prevent or even reverse disease in the NOD model may indeed translate to heterogenic human populations.

The aforementioned islet-antigen specific effector T cells, both CD4+ and CD8+, often express high levels of γ interferon and are usually classified as Th1 T cells, yet Th2 also play a role[43] in T1DM pathogenesis[44]. CD4+ effector T cells enter the islet and enhance inflammation in the microenvironment through cytokine production driving tissue destruction by macrophages and CD8+ T cells[45]. CD8+ T cells also play an important role in disease pathogenesis. Transferring both CD8+ and CD4+ T cells in adoptive transfer studies in NOD mice increases the incident rate of disease when compared to CD4+ T cells alone, while transferring CD8+ cells alone is ineffective[46]. The impact of CD8 T cells on disease is mostly mediated by their cytolytic action on β cells. Broad immunosuppressants are a viable therapy in preventing overt diabetes[47] by suppressing T cell effector activity but this treatment renders the patient highly susceptible to secondary infections. An ideal therapy targeting effector T cells would selectively induce anergy

or apoptosis in islet-antigen-restricted T cells, rendering these cells incapable of mediating their cellular and microenvironment effects, while at the same time maintaining overall immunocompetence.

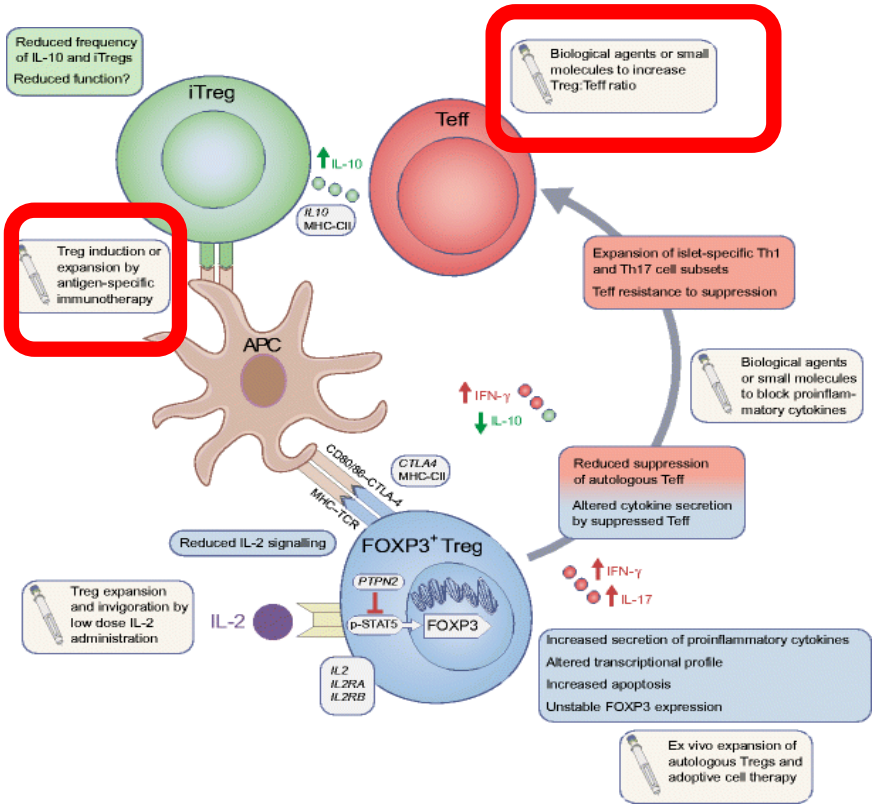


Figure 1.2 Potential interventions for effector and regulatory T cells in autoimmune diabetes. From Hull et al. Highlighting the multiple routes of intervention by manipulating Treg function or activation; red boxes represent approaches used in this project. Diabetologia (2017) 60: 1839. https://doi.org/10.1007/s00125-017-4377-1

Regulatory T cells (Tregs) are on the opposing side of this balance of tolerance, an enticing cell type for potential therapeutic targeting (**FIG 1.2**). These CD4+ T cells are often defined by their expression of the transcription factor FOXP3 and high levels of CD25. They exert their tolerogenic effect through antigen-antigen-specific and non-specific production of anti-inflammatory cytokines IL-10 and TGFβ[48]. Islet antigen specific Tregs are found in both humans and NOD mice and their action, or lack thereof, is thought to influence T1DM disease

progression[49]. Originally thought to only be derived in the thymus (natural Tregs), mounting evidence demonstrates the ability of naive effector T cells to be converted into Treg (induced Tregs). This concept may allow the conversion of islet-antigen specific effector T cells to regulatory T cells as a therapy but has yet to be fully explored. Low dose IL-2 treatment has also prevented disease onset by stimulating Treg activity[50]. The addition of exogenous cytokines such as IL-10 also show promise but and lacks antigen specificity and would result in generalized immunosuppression. Autologous *ex vivo* expansion and adoptive transfer of Treg shows much promise in the NOD mouse[51] and could be a potential treatment option in humans[52], especially if the Tregs are islet-antigen specific. A treatment that can both block effector T cell activity while expanding Tregs may be necessary to generate antigen specific tolerance, either before complete β cell destruction or after transplantation, in patients with T1DM.

III. PKCθ in T cells and autoimmunity

Protein kinase C isoform θ (PKCθ) is one member of a larger family of signal transducing protein kinase. It is highly expressed in T cells and skeletal muscle. T cell receptor ligation and CD28 costimulation is transduced in part by PKCθ, which then activates NF-AT, AP-1, and importantly NFκ-B, proteins key in T cell activation and proliferation. There are many ascribed implications of this PKC isoform in disease of muscle cells, such as insulin mediated vasoreactivity[53], lipid-induced insulin resistance[54], and satellite and myoblast function[55]. Negative regulation of this protein is often beneficial, as hypothesize in these studies. Using a model of enteric colitis, inhibiting PKCθ in T cells has been shown to function in reducing autoimmune T cell activation while expanding regulatory T cell pollutions[56], a mechanism applied to T1D that is explored in these studies.

Protein kinase C isoforms are normally cytosolic and upon activation translocate to the plasma membrane. Here, they are activated by DAG and IP_3, hydrolysis products of the membrane phospholipid PIP_2 by phospholipase C[57]. They aid in transducing many signals mediated by phospholipase C. After activation, PKCs phosphorylate serine and threonine residues to activate downstream molecules. The PKC family is divided into two group, the Ca_+ dependent, and independent "novel" isoforms[58]. PKCθ falls into the latter group. It functions in T cells by translocating to the immunological synapse where the TCR, along with CD28 engage an antigen presenting cell[59]. Importantly PKCθ requires both TCR (CD3) and costimulation (CD28) to become activated[59]. An [60]. PKCθ has an important activation loop that when phosphorylated (T528), induces protein activation leading to enhanced T cell function and cell proliferation. This is accomplished mainly though the transcriptional activators NFκ-B, NF-AT, and AP1[61,62]. The T528 site has been targeted with phospho-specific antibody as an immunomodulatory therapy[63]. PKCθ can bind an endogenous pseudo-substrate peptide that induces a conformational change, effectively inactivating the protein[63]. PKCθ signaling to activate the aforementioned transcription factors is in part mediated through effector proteins, the first of which is CARMA1[65]. In some cases, PKCθ translocates directly to the nucleus to exert its transcriptional effects[59,62].

$PKC\theta^{-/-}$ knock-out mice have an expanded population of regulatory T cells[66]. In $PKC\theta^{-/-}$ mice, this isoform seems dispensable for most bacterial and viral immune responses[67], yet necessary in Th2 and Th17 T cell activation response to parasitic infections and autoimmune antigens, respectively. This is an important distinction from broad immune suppressant targets

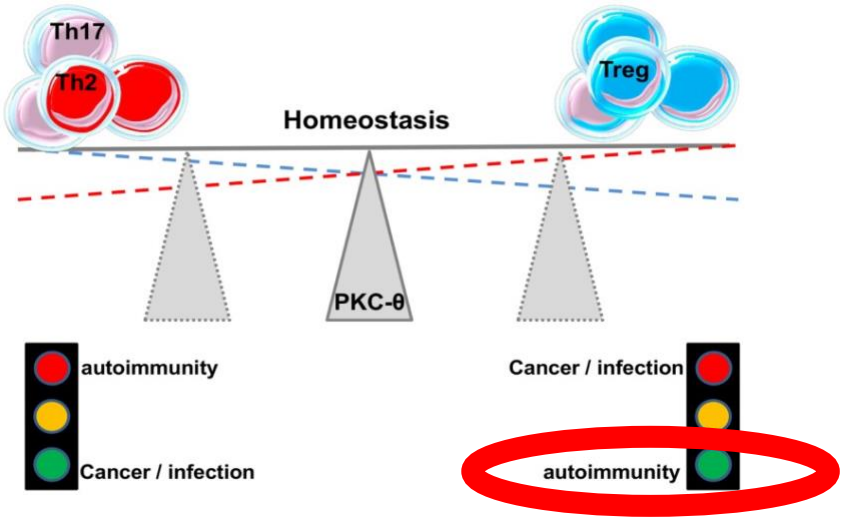


Figure 1.3 The balance of T cell immune tolerance is in part regulated by PKCθ. From Brezar et al, demonstrates PKCθ function in balancing T cell homeostasis between effector and regulatory cells. Frontiers in immunology, 6, 530. doi:10.3389/fimmu.2015.00530

that reduce responses to common pathogens. The θ isoform was also shown to be dispensable in regulatory T cell function[68]. Experiments have also shown that PKCθ is sequestered away from the immunological synapse in Tregs[69], the opposite of its function in effector T cells. Inhibition of PKCθ has been successful in alleviating autoimmunity in animal models of autoimmune encephalitis[70] and enteric colitis[71]. The majority of evidence in these studies points towards PKCθ functioning as a negative regulator of Treg function, whereby inhibition allows these regulatory cells to exert their effect uninhibited[72]. Therefore, it is thought that PKCθ may provide a delicate balance between tolerance and autoimmunity (**FIG 1.3**). There is also the possibility that inhibition of PKCθ during antigen recognition of effector T cells promotes anergy or deletion, both beneficial outcomes in the context of autoreactive T cells. Based on these studies, and increasing evidence for the use of PKCθ as a broad autoimmune inhibitor[73], PKCθ inhibition to enhance T cell mediated tolerance was pursued as a novel immunotherapy in the type 1 diabetes NOD mouse model.

HYPOTHESIS AND SPECIFIC AIMS

Hypothesis

Inhibition of PKCθ in pre diabetic NOD mice will reduces autoimmune T cell activation and expands regulatory T cells, preventing total β cell destruction and eventual onset of type 1 diabetes.

This hypothesis will be tested via the following aims:

Aim 1

Determine an effective inhibitor of PKCθ and validate the treatment in murine splenocytes and isolated T cells.

Aim 2

Determine if pre-diabetic treatment with PKCθ inhibitory peptide prevents or delays the onset of type 1 diabetes over time.

Aim 3

Determine the phenotype of, and potential for T cells from peptide treated NOD mice to prevent the onset of diabetes when adoptively transferred to NOD/SCID mice.

MATERIALS AND METHODS

Animals and peptide treatment

All mice were handled in a specific-pathogen free facility according to University policy under IACUC approval (CSU 18-7773A). NOD/ShiLtJ and NOD/SCID mice were obtained from Jackson Laboratories and fed the standard mouse diet *ad libitum*. Peptides were synthesized by GenScript at 95% purity and tested for endotoxin contamination. To increase cell permeability, an N-terminal myristoylation was added to both the control peptide (ICAKKKVGRHLHQRVHAQ) and PKCθ inhibiting peptide (LHQRRGAIKQAKVHHVKC). The original pseudo-substrate peptide sequence was scrambled based on bioinformatic tool "Shuffle Protein" on bioinformatics.org to generate a peptide containing motifs with no known interactions. Peptides were suspended in sterile PBS and intraperitonially injected daily for 4 or 5 weeks at either 50ug or 100ug per dose. The same peptide solution was used for *in vitro* assays.

Adoptive transfer of diabetes into NOD/SCID

CD3+ T cells were isolated by negative selection from NOD mouse pancreas at 8-10 weeks of age with an antibody based CD3 negative selection kit (Biolegend). After isolation, T cells were suspended in a 0.9% saline solution with 6-10x10_6 cells in 100ul and loaded slowly into a 28G insulin syringe. Mice were restrained and the tail briefly heated with a lamp to vasodilate tail veins. Either lateral tail vein on the NOD/SCID was injected with 100ul of cell suspension and monitored for immediate adverse effects.

Splenocyte and T cell isolation

Mice were euthanized by CO_2 asphyxiation per institutional guidelines. The spleen was immediately removed and placed in RMPI-1640 media containing penicillin and streptomycin.

The spleen was then pushed through a 70μm cell strainer to generate a single cell suspension of splenocytes. For isolation of whole splenocytes, the suspension was centrifuged, and pelleted cells suspended in a red blood cell lysis solution (Biolegend) for 2 minutes then quenched with PBS and centrifuged again. The resulting lysed pellet was resuspended in media to be used in downstream assays.

T cells were isolated in a similar manner as splenocytes. After the spleen was pushed through the cell strainer, total nucleated cells are enumerated and sorted by negative selection for either CD4+ or CD3+ T cells with murine specific Mojo sorting kits (Biolegend). Briefly, splenocyte suspensions were incubated with a biotinylated antibody cocktail labeling all non-T cell subsets in the spleen (CD11b, CD11c, CD19, CD24, CD45R/B220, CD49b, CD105, I-A/I-E (MHC II), TER-119/Erythroid, TCR-γδ, and CD8a when only isolating CD4+ T cells). Following antibody labeling, the suspension was incubated with streptavidin labeled iron beads. This mixture was placed in a magnet where antibody labeled cells are pulled to the walls of the tube and unlabeled CD4+ or CD3+ cells are decanted into a fresh tube. The cells are then washed, resuspended in media and the purity of T cells (90-98% pure) quantified by flow cytometry staining.

Flow cytometry staining and analysis

All cells were stained in 100ul of FACS buffer (PBS, 0.01% Na azide, 1% FBS) with 1mg/ml unlabeled mouse IgG (Jackson labs) as blocking antibodies. For extra-cellular staining $1x10_6$ cells were blocked for 15 minutes, incubated with fluorescent-conjugated antibodies for 30 minutes at 4°C then stained for viability with Zombie dyes for 10 minutes (Biolegend). Intracellular staining followed the same blocking protocol for extra-cellular staining. After the Zombie viability dye, cells were fixed and permeabilized, then stained for intracellular targets.

To stain for tetramers 5x10$_6$ cells were incubated in FACS buffer with both insulin specific (inspG8, NIH tetramer core) and hybrid insulin peptide (HIP 2.5 provided by Dr. Haskins from NIH tetramer core) fluorescent-conjugated MHC class II tetramers at 37°C for 1 hour prior to surface and intracellular staining. The list of antibody targets and conjugates can be found in Table 1. All samples were analyzed on either a three laser Beckman Coulter Gallios or a four laser Cytek Aurora. FlowJo software was used to process data and generate graphs. Figure 1.4 demonstrates the gating strategy used throughout the study.

Table (1) Antibodies and tetramers in immunophenotyping of T cells

Target	**Fluorochrome label**	**Vendor/Clone**
CD4	AF700	Biolegend/RM4-5
CD8a	BV510	Biolegend/53-6.1
CD25	PE-Cy7	Biolegend/PC61
CD272(BTLA)	AF-594	Biolegend/6A6
CD152(CTLA4)	BV605	Biolegend/UC10 4B9
LAG3	BV650	Biolegend/C9B7W
PD1	BV421	Biolegend/29F.1A12
FOXP3	FITC	Biolegend/150D
Insp-Ig8 tetramer I-Ag7	APC	NIH tetramer core/-
HIP2.5 tetramer I-Ag7	PE	NIH tetramer core/-
Viability dye	Zombie NIR	Biolegend/-

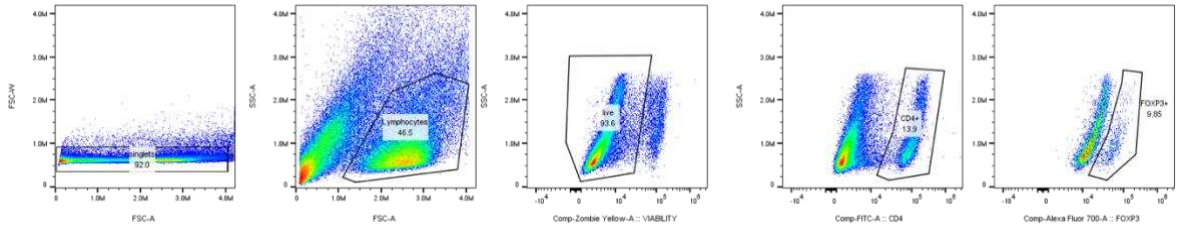


Figure 1.4 Basic flow cytometry gating strategy. Cells were gated for singlets (FSC-A, FSC-W), then for lymphocytes (FSC-A, SSC-A), live cells (negative for zombie yellow), and finally for specific markers.

***In vitro* stimulation, proliferation, and IL-2 production assay**

Isolated splenocytes or T cells were plated at 1-2x10_6 cells per well in a 48 well plate in 1ml mouse T cell media (Opti-MEM media, 1X pen/strep, 5% heat inactivated mouse serum, 1X NEAA, 10mM HEPES, 2uM 2-ME, 4mM L-glutamine). Cells were treated with inhibitors for specified time then stimulated with 5ug/ml anti-CD3 and 1ug/ml anti-CD28 functional grade antibodies (Biolegend), to mimic T cell receptor (TCR) ligation and co-stimulation, then incubated for 24, 48, or 72 hours. In identical experiments, cell cytoplasm was labeled with 2μm cell trace violet (ThermoFisher) to determine the amount of proliferation based on dilution of fluorescent signal after each subsequent round of division. Supernatants were collected after incubations for cytokine bead array (Biolegend) or IL-2 ELISA (ebioscience). Cells were collected and stained by flow cytometry.

Fluorescent-labeled peptide uptake assay

Both scrambled control and PKC peptide were labeled with amine-linked protein labeling kits (Lighting Link, Novus Biologicals) to conjugate peptide to Cyanine 5 (Cy5) fluorophore. Splenocytes or T cells were incubated with labeled peptides for 2 hours. The cells were washed and split, half the cells were quenched with TCEP, a cell impermeable quencher that reduces extracellular florescent signal of Cy5. Cells were then analyzed on a Cytek Aurora and gated by forward scatter and side scatter for lymphocytes.

IL-10, IL-2, and IFNγ ELISpot

Precoated IL-10, IL-2, and IFNγ 96 well ELISpot plates (R&D Systems) were blocked at RT for 2 hours in ELISpot media (Click's media, 1X pen/strep, 5% heat inactivated mouse serum, 1X NEAA, 10mM HEPES, 2uM 2-ME, 4mM L-glutamine). Splenocytes and T cells from pancreas of treated NOD mice pancreas were isolated and 5x10_5 cells incubated in 200ul of

ELISpot media per well. Cells were either stimulated with 10ug of HIP 2.5 peptide, 5ug of whole insulin, or anti-CD3 anti-CD28 antibodies (10 and 5 ug/ml). Cells and antigens or antibodies were incubated for 72 hours then plates developed per manufacturer's instructions.

T cell metabolic response assay

A Seahorse extracellular flux analyzer was used to assess T cells responses to stimulus in real time. T cells were purified as before and loaded into a seahorse plate that had been previously coated with Cell-Tak (Corning). Oxygen consumption rate (OCAR) and extra-cellular acidification rate (ECAR) were measured every 5 minutes, at least three times before and after injections, with mixing cycles between measurements. The first injection was either peptide or control. The second injection was a 2:1 ratio of anti-CD3/CD28 conjugated Dynabeads (ThermoFisher) to T cells in order to stimulate the TCR. The third injection was either peptide or control.

Glucose tolerance test and random glucose level measurements

To collect blood for glucose measurements the lateral tail vein was pricked to generate a small droplet of blood and glucose readings collected with an AlphaTRAK glucometer and Freestyle Lite test strips. For the glucose tolerance test, animals were fasted for 6-8 hours then an initial glucose reading is taken. Mice are then given a 2g/kg bolus of a sterile 45% glucose solution. Glucose readings are taken every 20 minutes until an hour after the initial reading. Random blood glucose levels were collected once or twice a week on non-fasting animals in the early afternoon with the same blood collections methods as used in the tolerance test.

Tissue preparation and islet insulitis scoring

After euthanasia, the pancreas and pancreatic lymph nodes were fixed in 4% paraformaldehyde in PBS for 48 hours. The tissue was then paraffin embedded and cut into 5µm sections and mounted on glass slides. The slides were stained for H&E and visualized at 40X.

All islets were counted to determine total number of islets per section. Islets were scored 0-4 based on methods previously described in Current Protocol in Immunology[74]. Islets with no infiltration were assigned a score of 0, peri-insulitis a score of 1, less than half infiltrated a score of 2, more than half a score of 3, and if 20% or less remained, a score of 4. All scores for a section were divided by the total number of islets in a section and all sections from animals in a group were averaged to determine final islet score proportions. Examples of an islet from each scoring category can be seen below (**FIG 1.5**).

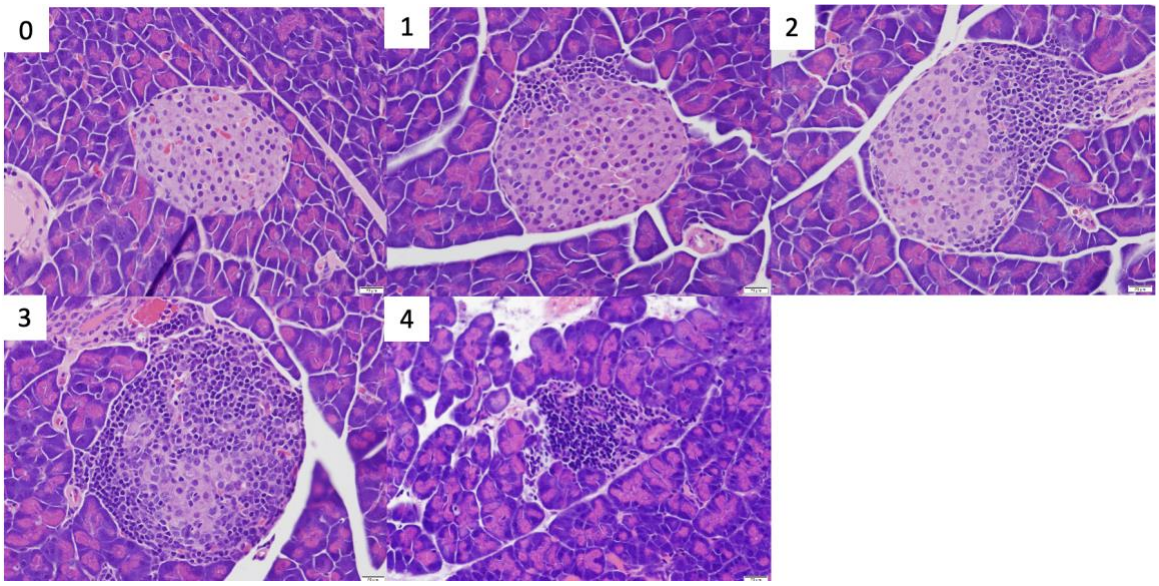
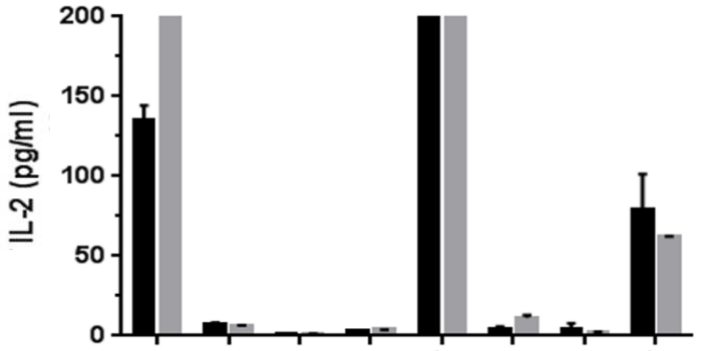


Figure 1.5 Example islets for insulitis scoring in NOD mice. The islets are scored based on the level of insulitis; no insulitis (0), peri-insulitis (1), <50% infiltrated (2), 50-80% infiltrated (3), and >80% infiltrated (4).

RESULTS

Aim 1: *Determine an effective inhibitor of PKCθ and validate the treatment in murine splenocytes and isolated T cells.*

To our knowledge at the time, there were two commercially available inhibitors of PKCθ: rottlerin[75], a natural extract from the Asian Tree *Mallotus philippensis*, and a cell permeable, pseudo-substrate peptide inhibitor[64] identical in sequence to an endogenous intracellular inhibitory peptide and highly specific for the θ isoform of PKC. IL-2 from T cells is produced in high levels after their activation to stimulate adjacent immune cells through paracrine and autocrine pathways [76,77]. After pretreatment with either inhibitor, IL-2 production in supernatants was entirely abrogated in response to TCR stimulation (**FIG 2.1**).



CD3/28	+	+	+	-	+	+	-	+
Peptide	6	15	30	-	-	-	-	-
Rotterlin	-	-	-	-	-	+	-	-
PBS	-	-	-	+	+	-	-	-
DMSO	-	-	-	-	-	-	+	+

Figure 2.1 Inhibition of T cell stimulated IL-2 production by both a myristoylated PKCθpseudo-substrate peptide inhibitor and rottlerin. Isolated splenocytes were treated for 24 hours with either the peptide at three concentration (6,15,30 ug/ml) or rotterlin (10ug/ml) and then stimulated for 8 (black bars) and 24 hours (grey bars) through TCR ligation by anti-CD3/CD28 antibodies. Resulting IL-2 was measured as a proxy of activation.

Whole splenocytes were isolated and incubated for 24 hours with three concentrations of peptide inhibitor, rottlerin, PBS vehicle control (for peptide), or DMSO vehicle control (for rottlerin). The cells were then stimulated with the functional grade antibodies and supernatants collected at 8- and 24-hours post stimulation. At both 15 and 30ug/ml of peptide and 10ug/ml rottlerin there was no detectable IL-2 production (**FIG 2.1**) above basal levels. DMSO vehicle control significantly reduced IL-2 production at both 8 and 24 hours compared to the PBS control (**FIG 2.1**).

Due to pleotropic pharmacodynamics[78], low isoform specificity[79], and observed vehicle toxicity we opted to no longer use rottlerin. Based on the preliminary *in vitro* results we continued the study with the pseudo-substrate peptide inhibitor for both *in vitro* and *in vivo* experiments.

To ensure the myristoylated peptide was cell permeable and reached the intracellular compartment, the peptide was fluorescently labeled and incubated with cells (**FIG 2.2**).

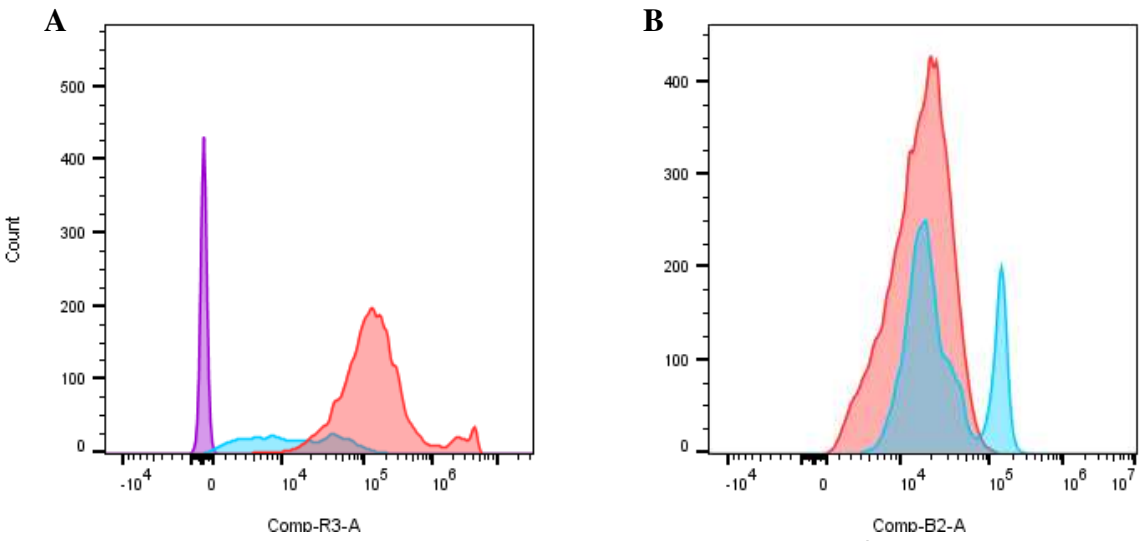


Figure 2.2 Myristoylated and fluorescently-labeled (Cy5) PKCθ peptide inhibitor is cell permeable and can be detected within lymphocytes. Histograms of flow cytometry data showing (**A**) fluorescence of lymphocytes incubated with peptide (blue) greater than untreated control (purple) and still exhibiting high fluorescence after quenching with TCEP (red). (B) Anti-CD4 antibody staining (blue) was used as a control of extracellular fluorescent quenching (red).

Tris(2 carboxyethyl)phosphine (TCEP) was used to quench extracellular fluorescence[80] of the peptide. After quenching, the peptide treated cells still exhibited high levels of fluorescence. Quenching activity of TCEP was confirmed by the quenching of a fluorescently-labeled, extracellular antibody targeting CD4. There was an increase in overall fluorescence intensity after TCEP treatment, something we are not sure how to explain.

A scrambled peptide, consisting of the same amino acids, was generated to ensure the inhibiting peptide's observed effects are indeed specific and not generated by the addition of exogenous, myristoylated peptide alone. Both PKCθ specific and scrambled control peptides were incubated with splenocytes prior to TCR stimulation to determine if the control peptide had any impact on IL-2 production and cellular proliferation (**FIG 2.3**). This data is from one experiment that is representative of 5 independent experiments. The scrambled control peptide has a slight reduction on the total amount of IL-2 produced when compared to the untreated control (**FIG 2.3 A**), but still produced a large amount of IL-2. The peptide inhibitor once again completely abrogates IL-2 production into supernatant to basal unstimulated levels (**FIG 2.3 A**). In regard to cellular proliferation response to TCR ligation, the peptide inhibitor almost entirely prevented T cell proliferation (**FIG 2.3 B**) with the majority of cells viable but undivided after 48 hours of stimulation. The scrambled control had less cells in the peaks denoting 2 and 3 rounds of division when compared to untreated controls but still allowed robust proliferation of T cells (**FIG 2.3 B**).

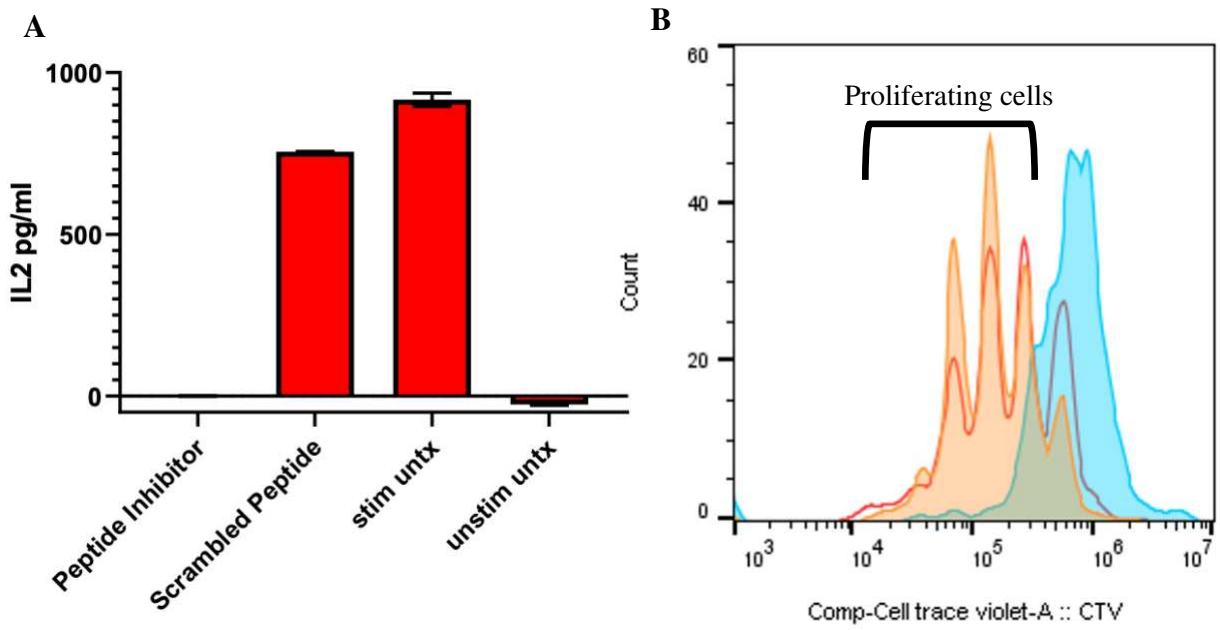


Figure 2.3 Inhibited IL-2 production and cellular proliferation of splenocytes after treatment with PKCθ peptide inhibitor and but not a scrambled control peptide. Supernatant production of IL-2 after TCR stimulation (A). Proliferation of CD4+ cells labeled with cell trace violet (B). Peptide treated cells (blue) showed limited proliferation in comparison to the scrambled peptide (orange) and untreated control (red line).

An increase in T cell glycolysis is a metabolic marker of TCR ligation and therefore activation[81]. This was used to assess the impact of the peptide inhibitor in real time on T cells isolated from spleen. The peptide was injected both before and after anti-CD3, CD28 ligation with Dynabeads. When the inhibiting peptide is injected prior to stimulation, the basal glycolytic rate is reduced (**FIG 2.4**) and the increase in glycolysis after stimulation is blocked entirely. Interestingly, if the peptide isis injected after stimulation, the glycolytic rate began to drop, signifying the peptide may be able to partially inhibit T cell activation even after TCR ligation.

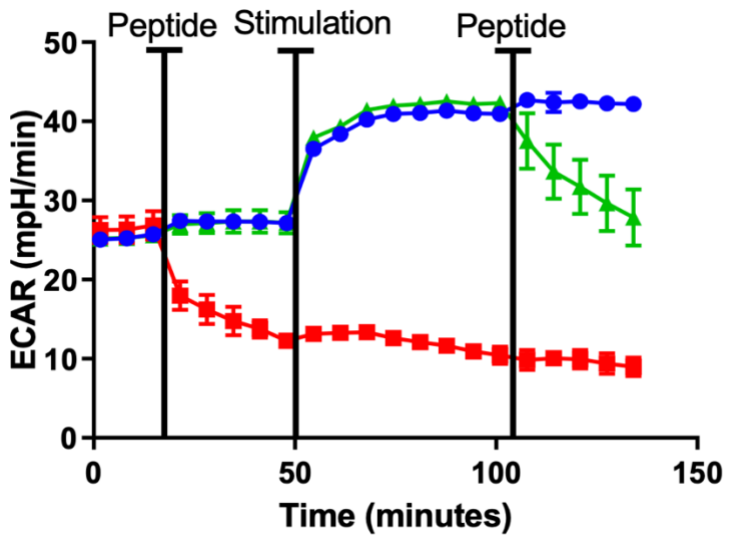


Figure 2.4 Metabolic inhibition of T cell activation by PKCθ peptide inhibitor. Peptide was injected either before (red square) or after (green triangle) anti-CD3, CD28 dynabeads to determine the inhibitors impact on glycolysis induced by T cell stimulation, in comparison to untreated control cells (blue circle).

To determine an effective dose for peptide inhibition of T cell stimulation, mice were treated for 7 days with IP injections of either 25 or 50 μg of the peptide inhibitor then euthanized on the final day of treatment and isolated splenocytes stimulated by anti-CD3, CD28 antibodies (**FIG 2.5**). After 24 hours of stimulation, the 50ug dose of peptide generated a significant decrease in IL-2 production.

The observed peptide inhibition of IL-2 production, cellular proliferation, glycolytic induction, and *in vivo* T cell inhibition provided strong evidence that the myristoylated peptide could be an effective therapy in the NOD mouse model.

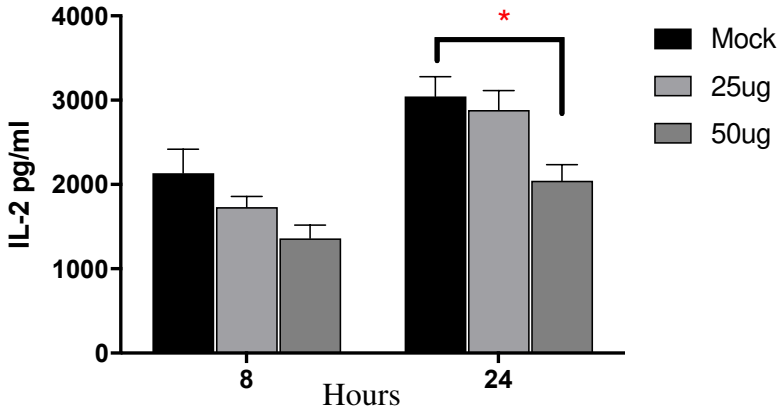


Figure 2.5 *In vivo* inhibition of T cell stimulated IL-2 production of *ex vivo* isolated splenocytes. Animal treated 7 days IP with 50ug of peptide inhibitor exhibited reduced IL-2 production in response to TCR ligation 8 or 24 hours post stimulus. *=P≤ 0.05

Aim 2: *Determine if pre-diabetic treatment with PKCθ inhibitory peptide prevents or delays the onset of type 1 diabetes over time.*

The initial animal study was designed to determine the optimal time course of treatment that altered immune phenotype and glucose tolerance at 17 weeks of age. The pre-insulitis period, from 4-8 weeks old, was chosen as a prophylactic therapeutic intervention to determine if overt disease onset could be delayed or prevented. A post-insulitis regimen was also tested, from 12-16 weeks old, to determine if disease progression could be halted before detectable hyperglycemia. Animals (n=10) were treated daily for 4 weeks; weeks 4-8 in the pre-insulitis group and weeks 12-16 in the post-insulitis group. All animals were euthanized at 17-weeks of age.

At the 17-week termination point, the post-insulitis treatment group was no different than control, untreated animals in evaluated glucose tolerance and immunophenotype (data not shown). However, the pre-insulitis group exhibited significant differences in glucose tolerance,

frequency and severity of insulitis, and immunophenotype in comparison to control mice (**FIG 2.6**).

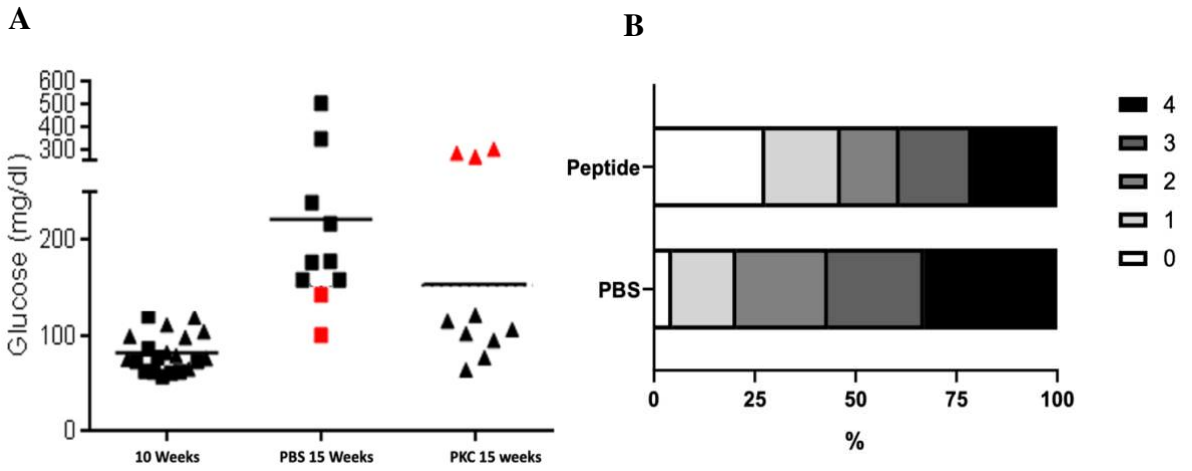


Figure 2.6 Pre-insulitis peptide treated mice have greater glucose tolerance and reduced insulitis. Comparison of glucose levels 20 minutes after the bolus (A) at 10 weeks of age and following 5 weeks of treatment, and terminal insulitis (B) scoring at 17 weeks of age. Red data points mark animals that are non -iabetic in the PBS group and diabetic in the peptide treatment group. This is used in the following figure (2.7) to highlight the corresponding immunophenotype of animals to their blood glucose phenotype. n=10

At ten weeks of age (**FIG 2.6 A**) both pre-insulitis peptide treated mice and PBS control mice rapidly returned to normoglycemia 20 minutes after receiving an IP bolus of glucose. At 15 weeks of age, 7 of the 10 peptide treated mice retained the ability to rapidly clear glucose, while only two of the 10 control mice could do so (**FIG 2.6 A**). Nonresponding Non-responsive animals, in their respective groups, are labeled red to track their immunophenotype. There were more destroyed (grade 4) islets in control mice than peptide treated (**FIG 2.6 B**). Peptide treated mice also had a greater proportion of intact, non-infiltrated islets compared to control mice (**FIG 2.6 B**). These data indicate that pre-insulitis peptide treatment has generated reduced islet inflammation while preserving β cell function in a glucose tolerance test, at 17 weeks of age.

Regulatory immunophenotype of isolated splenocytes was determined through antibody surface phenotyping and ELISpot of isolated splenocytes (**FIG 2.7**).

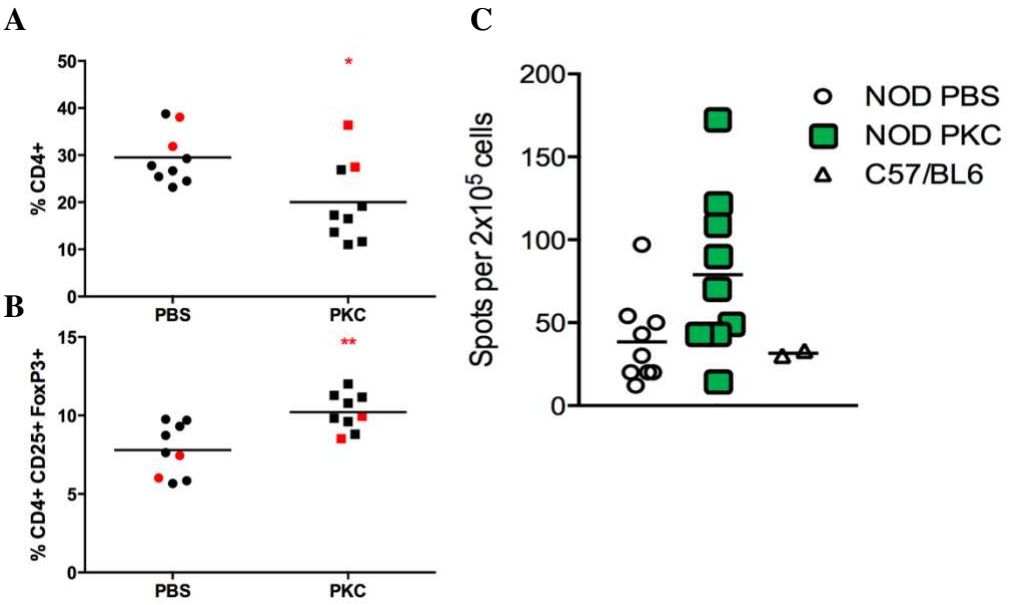


Figure 2.7 Regulatory immunophenotype induced by peptide treatment. Regulatory immunophenotype of CD4+ T cells (A) isolated at 17 weeks of age by CD25, FOXP3 expression (B) and IL-10 production (C). *=P≤ 0.05

There was a lower proportion of CD4+ T cells in peptide treated mice (**FIG 2.7 A**). Of those CD4+ T cells, treated mice had a significant increase in FOXP3+ regulatory T cells (**FIG 2.7 B**). This regulatory phenotype was again observed in an IL-10 ELISpot detection assay (**FIG 2.7 C**) with a greater number of IL-10 producing cells in treated mice. This phenotype aligned with that of an active population of regulatory T cells. These data indicate that selective inhibition of PKCθ may drive a regulatory phenotype of T cells, delaying or preventing overt diabetes.

To address the long-term benefit afforded by the peptide inhibitor, a diabetes onset study was accomplished (**FIG 2.8**). The scrambled control peptide was used to assure the observed effects were on target and not simply induced by a foreign peptide. Mice (n=12) were treated with the pre-insulitis regimen from 4-8 weeks of age. Starting at 12 weeks of age, animals were

monitored weekly for onset of overt diabetes by random blood glucose measurements. An animal was determined to be diabetic after two consecutive random glucose readings above 250mg/dl.

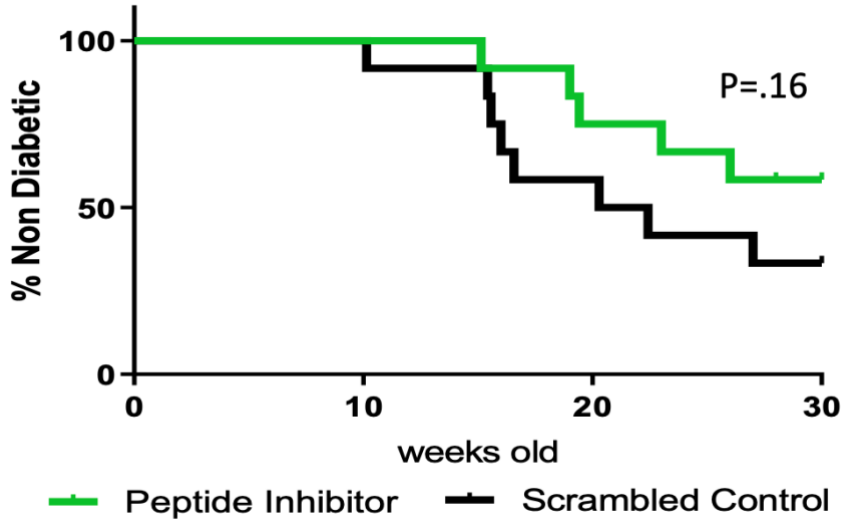


Figure 2.8 Reduced incidence rate and delayed onset of diabetes in 50ug daily PKCθ peptide treated mice. Kaplan-Meier survival curve based on two consecutive random glucose levels exceeding 250mg/dL.

Fewer peptide treated mice were overtly diabetic by the end of the study and the overall onset of disease was moderately delayed, though not statistically significant (**FIG 2.8**). Interestingly, at the 17-week time point used in the previous study as a termination point, 1 of 12 peptide treated mice was considered diabetic compared to 6 of 12 in the scrambled control group. These differences were not statically significant, likely due to too low of a dose or a lack of power from a low number of animals per group (n=12).

In order to address these potential pitfalls, the experiment was repeated with a larger group size (n=24) and twice the original dose (100ug) for 5 weeks of daily dosing compared to the previous 4 weeks (**FIG 2.9**).

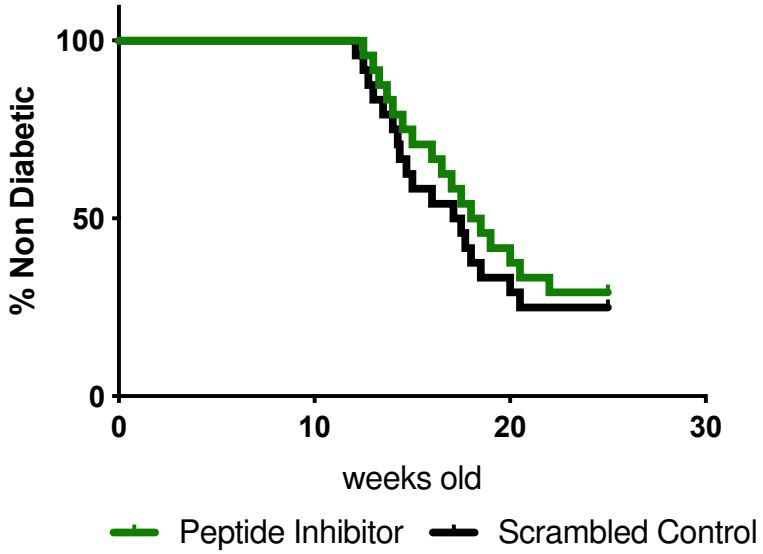


Figure 2.9 100ug PKCθ peptide treatment moderately delayed onset of diabetes. Kaplan-Meier survival curve based on two consecutive random glucose levels exceeding 250mg/dL.

Aim 3: *Determine the phenotype of, and potential for T cells from peptide treated NOD mice to prevent the onset of diabetes when adoptively transferred to NOD/SCID mice.*

Concurrent with the diabetes onset study, a separate group of animals (n=5) were treated with the same 100ug regimen of peptide or scrambled control for 5 weeks. Immediately after the 5-week treatment, animals were euthanized. The resultant splenocytes were assayed to determine the status of the T cell immunophenotype directly after treatment but before disease onset. A tetramer loaded with a hybrid insulin peptide (HIP 2.5+, kindly provided by Dr. Haskins), an immunodominant peptide in the NOD mouse model, was used in conjunction with antibody staining to phenotype islet-antigen specific T cells (**FIG 2.10**).

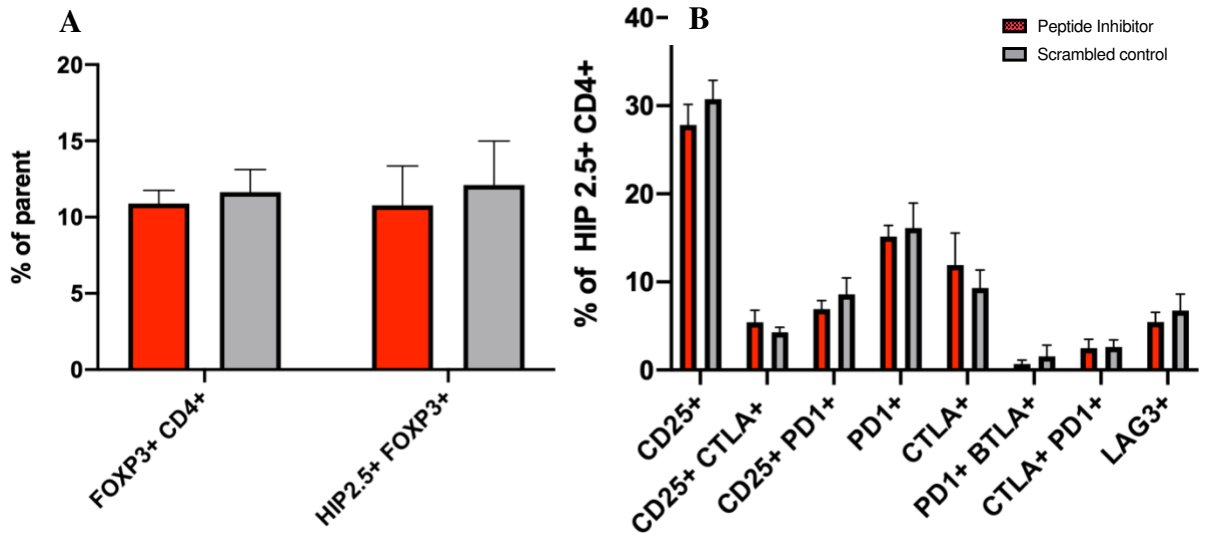


Figure 2.10 Peptide-treated and scrambled control animals exhibit similar immunophenotype of HIP 2.5+ T cells directly post treatment. Intracellular FOXP3 expression (A) of peptide treated mice (red) and scrambled control (grey) as well as the anergic surface phenotype (B) of splenocytes isolated directly after peptide treatment at 8 weeks of age. n=7

Little difference was noted in T cell phenotype between peptide and scrambled treated animals (**FIG 2.10**). A slight trend of increased CTLA4 (p=0.19) and decreased CD25 (p=0.09) expression on HIP 2.5+ T cells from peptide treated animals was observed (**FIG 2.10 B**). When analyzing total populations of CD4+ and CD8+ T cells, the surface phenotype between groups was not different. FOXP3 expression in both total CD4+ and HIP 2.5+ T cells was not statically different (**FIG 2.10 A**).

The HIP 2.5 peptide, along with whole insulin, was used as an antigen stimulus in ELISpot assays to detect secreted cytokines IL-2, IL-10 and IFNγ (**FIG 2.11**) from splenocytes. Greater IL-2 and IFNγ producing cells were detected with the HIP 2.5 peptide than whole insulin (**FIG 2.11 A,B**), yet neither plate showed a difference between treatments. Though trending higher in peptide treated animals, the IL-10 spot count was not significantly different between group (**FIG 2.11 C**). Taken together, both flow antibody staining and cytokine production, there were no detectable changes in immunophenotype immediately following treatment.

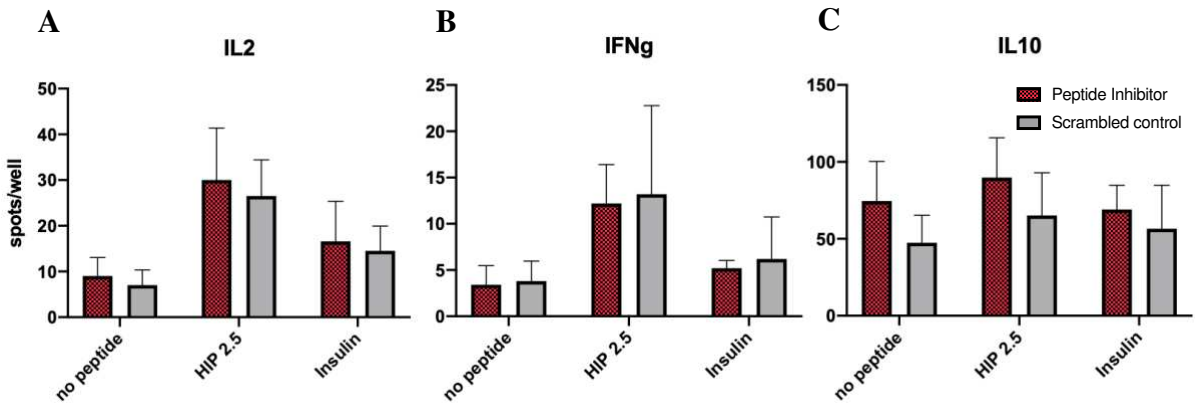


Figure 2.11 Comparable cytokine ELISpot phenotypes between peptide treated and scrambled control animals. Splenocyte cytokine production for IL-2, IFNγ, and IL-10 measured by ELISpot spot generation from HIP 2.5 peptide and whole insulin antigen stimulation of peptide treated (red) and scrambled control treated (grey) animals.

To determine the peptide inhibitor ability to reduce the diabetogenic potential of specifically T cells, purified (>95%) T cells from NOD mice donors were transferred into SCID recipients. CD3+ T cells (6-10x10_6 cells) were isolated from treated animals and adoptively transferred into NOD/SCID mice, blood glucose was monitored weekly for overt diabetes. Isolated T cells? (10x10_6) were adoptively transferred into aged matched SCID mice (n=5) from NOD mice treated with 50ug of peptide daily (**FIG 2.12 A**). There was a moderate delay in diabetes and 1 of 5 animals never developed hyperglycemia. The study was repeated with T cells (6x10_6 cell due to low recovery) from NOD mice treated with 100ug of peptide daily (**FIG 2.12 B**) and transferred into SCIDs (n=7). These results showed little difference in time of disease onset and number of animals with overt diabetes. Whether the peptide's effect observed in aim 2 is transient or rely on multiple cells types is not known.

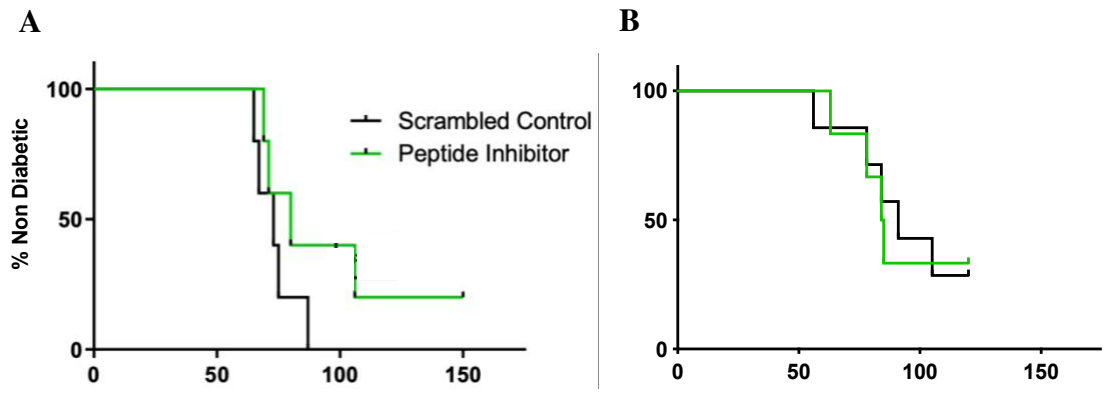


Figure 2.12 Adoptive transfer of diabetes from peptide and scramble control treated mice. Diabetes onset studies in SCID mice that received T cells from NOD mice donors treated with the peptide inhibitor.

DICUSSION

Autoimmune diabetes affects roughly 25 million Americans, with incident rates increasing over time[82]. Modern Type 1 diabetes therapy has evolved far in technology, yet little in concept; treating not the cause of disease but the hyperglycemic symptom. Autoimmune researchers have toiled over the past three decades to further elucidate immune mechanisms mediating β cell destruction and points of potential intervention. Though highly heterogeneous in genetic and environmental etiology, the tipping point of disease is thought to push the delicate balance of regulatory and effector T cells away from tolerance. Here we explored the selective inhibition of PKCθ, a key protein in autoimmune T cell activation, as immunotherapy for type 1 diabetes in the NOD mouse model. Based on previous interventions in autoimmune models and expansion of regulatory T cells in PKCθ$_{(-/-)}$ null mice, a cell permeable pseudo-substrate peptide inhibitor was implemented as a potential immunomodulatory therapy by inhibiting PKCθ in T cells.[82], with incident rates increasing over time[83]. Modern Type 1 diabetes therapy has evolved far in technology, yet little in concept; treating not the cause of disease, but the hyperglycemic symptom. Autoimmune researchers have toiled over the past three decades to further elucidate immune mechanisms mediating β cell destruction and points of potential intervention. Though highly heterogeneous in genetic and environmental etiology, the tipping point of disease is thought to push the delicate balance of regulatory and effector T cells away from tolerance. Here we explored the selective inhibition of PKCθ, a key protein in autoimmune T cell activation, as immunotherapy for type 1 diabetes in the NOD mouse model. Based on previous interventions in autoimmune models and expansion of regulatory T cells in PKCθ$_{(-/-)}$ null mice, a cell permeable pseudo-substrate peptide inhibitor was implemented as a potential immunomodulatory therapy by inhibiting PKCθ in T cells.

In the first aim of this study, the *in vitro* efficacy of the peptide inhibitor was examined to determine if TCR stimulated activation and proliferation could be abrogated through negative regulation of PKCθ. In both splenocytes and isolated T cells, the peptide inhibitor prevented IL-2 production, cellular proliferation, and glycolytic flux in response to TCR stimulation. The strength of T cell inhibition *in vitro* at low concentration (<15ug/ml) provided confidence in the peptides ability to translate *in vivo*. Splenocytes from mice treated for 7 days IP also had significantly lower IL-2 production in response to TCR ligation and costimulation. It is important to note that none of the *in vitro* work was performed on islet-antigen specific T cells, therefore it is unclear if they will also be inhibited by the peptide as the polyclonal isolated cells behaved, though it was reasonably to hypothesize that they would. After validating the apparent efficacy in inhibiting T cell stimulation, the peptide was used to therapeutically treat NOD animals.

In the second aim, the effect of peptide inhibition on hyperglycemia and immunophenotype was dissected. Initially, NOD mice were treated before and after insulitis with a defined termination point to determine which window of intervention was most effective. The earlier, pre-insulitis regimen was more effective at both enhancing glucose tolerance and generating increased proportions of regulatory T cells. This early treatment regimen aligned with previous studies targeting immune tolerance, such as IL-10 therapy and adoptive transfer of Tregs, where pre-insulitis intervention is often necessary to prevent or delay disease. To assess the peptides ability to prevent or delay disease, long-term diabetes survival studies were accomplished. The peptide had a moderate effect on the incident rate and time of disease onset, yet the data in these studies was not statistically significant. This trend aligns with the increased glucose tolerance and regulatory immune phenotype afforded by the peptide in the previous terminal study.

In the third and final aim, the immunophenotype of isolated T cells directly post therapy was characterized. These isolated T cells were also adoptively transferred to NOD/SCID mice to assess the direct impact of the peptide inhibitor on the diabetogenicity of T cells alone. To our surprise, there was little detectable difference in surface phenotype nor cytokine production of T cells post treatment. This was noted in both polyclonal T cells and islet antigen specific T cells. It is distinctly possible the immunomodulatory effects of peptide inhibition do not manifest until later in disease, possibly after the T cell is re-stimulated by the same autoantigen. The resulting onset of diabetes in the SCID adoptive transfer study generated similar moderate effects in reducing incident rate and delaying disease onset without statistical significance. The lack of strong disease intervention provided by the peptide is likely a product of ineffective therapy and not of the target itself. This work provides support for the continued evaluation of PKCθ as valid target to promote T cell tolerance in autoimmune type 1 diabetes.

Limitations

Though generating strong *in vitro* evidence of functional T cell inhibition, the impact of peptide inhibition during *in vivo* studies was less than expected. A core limitation of this project is the lack of an effective way to determine the relative activity of the protein target, PKCθ. This is in part due to the fact that its phosphorylation seems to be constitutive *in vitro*[62], while isolating *in vivo* samples in such a time frame to preserve phosphorylation states is hard to assure. One would go down the pathway to the cytosolic target of activated PKCθ, CARMA1, yet phospho-specific antibodies directed at CARMA1 have yet to be generated. Therefore, we are unable to definitively say if the peptide treatment was effectively and specifically targeting PKCθ *in vivo*, though literature strongly supports the latter[64,84]. Another weakness was the use of a systemic therapy, lacking cell type and autoantigen specificity. If the peptide could be

delivered directly to islet-infiltrating cells and pancreatic lymph node, any off targets effects or broad immune suppression could be avoided. Yet without surgery or organ specific liposome delivery systems[85,86], treating the delicate pancreatic tissue directly was beyond the scope of this work.

That being said, there is a distinct possibility that the peptide was unstable *in vivo* and degraded by peptidases rapidly, thereby preventing a high degree of inhibition of PKCθ. The translatable strength of replicating an endogenous peptide for therapy could also be a weakness in its lack of stability. There have not been PK/PD studies involving this peptide, but without stabilization, its half-life is hypothetically around 4 hours, without considering exposure to *in vivo* protease and peptidases. If the peptide could be stabilized through a synthetic formulation[87] or stabilizing agent[88] its half-life could be greatly increased and possibly its efficacy in treatment. This also provides the caveat that additions to a pseudo-substrate peptide may alter the peptides ability to efficiently bind and inhibit its target, though this has yet to be explored. It is important to note that there are pharmacological compounds, such as C20[89] and sotrastaurin[90], with somewhat selective inhibition of PKCθ. They have been used in various studies to effectively suppress T cell mediated autoimmunity but to our knowledge, are not commercially available.

The peptide treatment may have needed to be continued throughout disease to truly prevent onset. In our studies, the effect may have been transient, and lacked durability to produce effect months after treatment. To address this, animals could be treated twice daily for the duration of the study to determine if the peptide inhibitor can keep immune destruction at bay when administered for life. It is also possible that inhibition of auto reactive T cells by the peptide inhibitor alone is insufficient. The treatment of regulatory polarizing cytokines, such as

IL-10 and TGF-β, along with the peptide inhibitor may sway naïve or activated T cells into anergy or even reprogrammed to iTregs.

To truly address the impact of PKCθ on diabetes in the NOD model, genetic modification is required. We attempted to knock down PKCθ with a translation blocking morpholino with no success. PKCθ RNA interference was not explored. Another option would be to generate a PKCθ knock out mouse on the NOD background. The genetic loci required for disease transmission in the NOD mouse has been well mapped[17,18,91]. This allows for a relativity quick back crossing of knock out mice, such as the existing PKCθ$^{(-/-)}$ mouse, to NOD by ensuring the polygenic disease is transmitted in offspring. They could also be generated by CRISPR knock out. Following these mice and determining the incident rate of diabetes as well as immunophenotype would definitively answer whether PKCθ is required for disease and a truly viable immunotherapy target.

Finally, it is important to mention the limitation of the NOD mouse itself. Being an inbred mouse strain, housed in a controlled laboratory setting; many factors important in human disease are ignored such as the heterogeneity of human mating and environmental impacts of disease. Yet to undertake such in depth immunotherapy studies, the NOD mouse model remains a strong option, replicating T cell mediated autoimmune β cell destruction.

PROJECT SUMMARY AND FUTURE DIRECTION

The purpose of this work was to target the inhibition of PKCθ as a potential immunotherapy in type 1 diabetes. The rationale behind this idea stemmed from the evidence that PKCθ plays a key role in determine the balance of self-tolerance in T cells. A pseudo-substrate, myristoylated peptide inhibitor was used to; inhibit T cell activation and proliferation *in vitro* and *in vivo*, moderately reduce the incident rate and delay the onset of T1D in NOD mice, and finally directly assess the impact of PKCθ on T cells in a NOD/SCID adoptive transfers systems.

In summary, targeting PKCθ in type 1 diabetes seems to be a valid and potentially fruitful course of action. However, the efficacy of our chosen peptide treatment and the regimen likely reduced the observed effects in preventing overt diabetes. I would argue the increased presence of regulatory T cell populations and reduced insulitis at 17 weeks demonstrates the tolerogenic impact PKCθ inhibition may have in type 1 diabetes.

To truly address the impact of PKCθ in the NOD mouse model, a NOD/PKCθ$_{(-/-)}$ mouse should be generated either by backcrossing or CRISPR, and monitored for T cell driven autoimmunity and overt diabetes. Exactly how PKCθ inhibition enhances tolerance also needs to be further explored, whether it expands Tregs, induces anergy in T effectors, or a combination of both. This work should function to open further exploration into inhibiting PKCθ in type 1 diabetes, both alone and in combination with regulatory T cell polarizing treatments. Like other diseases, a multi-therapy approach may be necessary in preventing life-long T cell autoimmunity.

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